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Flagellin Stimulates Protective Lung Mucosal Immunity: Role of Cathelicidin-Related Antimicrobial Peptide

Fu-shin Yu,*†‡ Michael W. Newstead,*§ Melissa A. Kovach,*§ Michael W. Newstead,*§ Xianying Zeng,*§ Ashok Kumar,*†‡ Nan Gao,*†‡ Sang Gi Yoon,*†‡ Richard L. Gallo,* and Theodore J. Standiford§

TLRs are required for generation of protective lung mucosal immune responses against microbial pathogens. In this study, we evaluated the effect of the TLR5 ligand flagellin on stimulation of antibacterial mucosal immunity in a lethal murine Pseudomonas aeruginosa pneumonia model. The intranasal pretreatment of mice with purified P. aeruginosa flagellin induced strong protection against intratracheal P. aeruginosa-induced lethality, which was attributable to markedly improved bacterial clearance, reduced dissemination, and decreased alveolar permeability. The protective effects of flagellin on survival required TLR5 and were observed even in the absence of neutrophils. Flagellin induced strong induction of innate genes, most notably the antimicrobial peptide cathelicidin-related antimicrobial peptide. Finally, flagellin-induced protection was partially abrogated in cathelicidin-related antimicrobial peptide-deficient mice. Our findings illustrate the profound stimulatory effect of flagellin on lung mucosal innate immunity, a response that might be exploited therapeutically to prevent the development of Gram-negative bacterial infection of the respiratory tract. The Journal of Immunology, 2010, 185: 000–000.

Pathogens stimulate the innate immune system via Toll-like receptors (TLRs). TLRs are a family of type I transmembrane receptors that respond to pathogen-associated molecular patterns expressed by a diverse group of infectious microorganisms, resulting in activation of the host’s immune system (6–8). Most P. aeruginosa strains express flagellin, which primarily consists of the protein flagellin (9). Flagellin is recognized by and activates several pathogen recognition receptors, including TLR5, TLR2, and Ipaf, a component of the NOD/infamasosme pathway (10–16). In the lungs, flagellin can induce neutrophil accumulation, an effect that is dependent on TLR5 expression by lung structural cells rather than bone marrow-derived cells (17). In addition to mediating neutrophil influx, flagellin can activate a broad array of protective innate responses. For instance, the i.p. administration of purified flagellin protected mice from lethal intestinal Salmonella infection, rotavirus-induced colitis, and bacterial corneal infection (18–20). Recently, the repeated intranasal (i.n.) administration of flagellin has been shown to rescue TLR2/4 double-deficient mice challenged with nonflagellated P. aeruginosa (21). Mechanism of protection in these models has not been defined, but is believed to be partially due to stimulation of chemokines that facilitated the recruitment of inflammatory cells (18). Flagellin has also been shown to be protective in several noninfectious models, including chemical-induced colitis and radiation pneumonitis (18, 22).

An important component of innate immunity of the respiratory tract is the release of molecules with antimicrobial activity at the mucusal surface. The two best characterized families of cationic antimicrobial peptides are defensins and cathelicidins (23, 24). Cathelicidins are proteins that contain a highly conserved prepro region at the N terminus, referred to as the cathelin domain, and substantial heterogeneity at the C-terminal domain (24–26). These peptides are stored intracellularly as inactive propeptide precursors that are proteolytically cleaved to active peptides on stimulation (27). The single known human cathelicidin, hCAP-18, is cleaved by proteinase 3 to form the active peptide LL-37. The murine homolog, cathelicidin-related antimicrobial peptide (CRAMP), is encoded by the gene Cnlp (28). Cathelicidins are constitutively expressed in high levels by neutrophils (29). They are also inducibly expressed in response to infection and injury by epithelial cells at mucosal surfaces (30–32). Cathelicidin peptides exert bactericidal activity against a broad range of both Gram-negative and Gram-positive organisms, including P. aeruginosa. As compared with wild-type (WT) controls, Cnlp−/− mice display increased susceptibility to several Gram-positive and Gram-negative bacterial infections (33–35). The in vivo contribution of cathelicidins to lung mucosal immunity is not well characterized. However, the forced transgenic expression of LL-37 restored the killing of P. aeruginosa and Staphylococcus aureus by bronchial

*Department of Ophthalmology, †Department of Microbiology, and ‡Department of Immunology, Wayne State University, Detroit, MI 48201; §Division of Pulmonary and Critical Care Medicine, Department of Medicine, University of Michigan Medical Center, Ann Arbor, MI 48109; and †Division of Dermatology, Department of Medicine, University of California San Diego, San Diego, CA 92612

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Address correspondence and reprint requests to Dr. Theodore J. Standiford, Division of Pulmonary and Critical Care Medicine, University of Michigan Medical Center, 109 Zina Pitcher Place, 4065 BSRB, Ann Arbor, MI 48109-2200. E-mail address: tsstandif@umich.edu

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Abbreviations used in this paper: AEC, alveolar epithelial cell; BAL, bronchoalveolar lavage; BALF, BAL fluid; CRAMP, cathelicidin-related antimicrobial peptide; i.n., intranasal; i.t., intratracheal; PMN, polymorphonuclear neutrophil; WT, wild-type.

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epithelial cells isolated from patients with cystic fibrosis, and the in vivo pulmonary transgenic expression of LL-37 in mice challenged with \textit{P. aeruginosa} simultaneously reduced lung bacterial burden and reduced inflammation (32, 36). In addition to direct bactericidal properties, cathelicidins exert unique immunomodulatory effects, including binding to anionic molecules, such as LPS, resulting in reduced endotoxin immunotoxicity (37–39).

In this study, we evaluated the effect of flagellin on protective lung mucosal immune responses in a lethal murine \textit{P. aeruginosa} pneumonia model. The intranasal (i.n.) delivery of purified \textit{P. aeruginosa} flagellin induced strong protective immunity against \textit{P. aeruginosa}, which required, in part, the antimicrobial peptide CRAMP. Flagellin also prevented lung injury, which was associated with reduced expression of caspase-3 in lung during pneumonia.

Materials and Methods

Reagents

Anti-CRAMP Abs used in Western immunoblotting were purchased from Genzyme (Cambridge, MA). Purified recombinant murine CRAMP was obtained from Mary O’Riordan at the University of Michigan (Ann Arbor, MI). For neutrophil depletion, we treated mice with RB6-8C5 mAb. RB6-8C5 is a rat anti-mouse mAb directed against Ly-6G (Gr-1). The Ab was produced by TSD Bioservices (Germantown, NY) by the i.p. injection of hybridoma RB6-8C5 into nude mice and collection of acities. Administration of 200 μg i.p. per mouse results in peripheral blood neutropenia (<50 polymorphonuclear neutrophils [PMNs]/μl) by days 1 and 3 postadministration, with return of peripheral counts to pretreatment levels by day 5 (40).

Animals

SPF C57BL/6 mice (age- and sex-matched) were purchased from The Jackson Laboratory. 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.

Isolation of \textit{P. aeruginosa} flagellin

Purified flagellin was isolated as described previously (19, 20). Briefly, the \textit{P. aeruginosa} strain \textit{P. aeruginosa} 01 suspension was blended to remove flagellin from the cells. The homogenate was then centrifuged at 12,000 \textit{g} at 4˚C for 30 min to remove the insoluble materials from solution. The 15 and 20% insoluble fractions that contained the greatest amount of flagellin with the least amount of contaminants were dissolved in 50 mM sodium phosphate buffer (pH 8.0) and dialyzed against the same buffer (28). The flagellin-containing sample was applied to Hitrap DEAETrap FF column, washed with 50 mM sodium phosphate buffer (pH 8.0) and then eluted with 0.6 M NaCl and 50 mM sodium phosphate buffer (pH 8.0) with AKTA prime chromatography. The protein-containing fractions were collected, concentrated on filters (Amicon Centriplus YM-3; Millipore, Bedford, MA), and applied to 1 ml prepacked gel affinity columns (Detoxi-Gel Affinity Pak; Pierce Biotechnology, Rockford, IL) to remove LPS. The amount of LPS was determined using a quantitative limulus amebocyte lysate kit (QCL-1000; BioWhittaker, Walkersville, MD). The amount of LPS in the flagellin samples after the two steps of chromatography was 2.7 endotoxin unit/mg protein (0.00027 endotoxin unit/μg protein). Identity of flagellin was confirmed by immunoblot analysis with rabbit anti-\textit{P. aeruginosa} flagellum B antiserum.

Bacterial preparation

\textit{P. aeruginosa} strain 19660 (ATCC) was used in our studies. Bacteria were grown overnight in Difco nutrient broth (BD) at 37˚C while constantly shaken. The concentration of bacteria in broth was determined by measuring the absorbance at 600 nm, and then plotting the OD on a standard curve generated by known CFU values. The bacterium culture was then diluted to the desired concentration.

The i.n. or intratracheal inoculation

Mice were anesthetized with an i.p. ketamine and xylazine mixture. For i.n. administration of flagellin or vehicle, 10 μl were administered to each nostril. For intratracheal (i.t.) inoculation of \textit{P. aeruginosa}, the trachea was exposed, and 30 μl inoculum was administered via a sterile 26-gauge needle. The skin incision was closed using surgical staples.

Murine alveolar epithelial cell isolation

Primary alveolar epithelial cells (AECs) from WT and mutant mice were isolated as previously published (42). Briefly, after mice were heparinized and euthanized, they were exsanguinated and lungs 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 24˚C for 45 min and then lung tissue was teased away 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 then incubated with anti-CD32 and anti-CD45 Abs. Cells are then incubated with streptavidin-coated magnetic particles and positive bone marrow-derived cells were collected 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 (41).

Lung macrophage isolation

Lung macrophages (consisting of both alveolar and interstitial macrophages) were isolated from dispersed lung digest cells by adherence purification as previously described (43).

Whole lung homogenization for CFU determination

At designated time points, the mice were euthanized by CO₂ inhalation. Before lung removal, the pulmonary vasculature was perfused by infusing 1 ml PBS containing 5 mM EDTA into the right ventricle. Whole lungs were removed, taking care to dissect away lymph nodes. The lungs were then homogenized in 1 ml PBS with protease inhibitor (Boehringer Mannheim, Indianapolis, IN). Homogenates were then serially diluted 1:5 in PBS and plated on blood agar to determine lung CFUs.

Peripheral blood CFU determination

Blood was collected in a heparinized syringe from the right ventricle at designated time points, serially diluted 1:2 with PBS, and plated on blood agar to determine blood CFUs.

Bronchoalveolar lavage

Brochoalveolar lavage (BAL) was performed for collection of BAL fluid (BALF) as previously described (43). Briefly, the trachea was exposed and intubated using a 1.7-mm outer diameter polyethylene catheter. BAL was performed by instilling PBS containing 5 mM EDTA in 1 ml aliquots. A total of 3 ml PBS was instilled per mouse, with 90% of BALF retrieved.

Total lung leukocyte preparation by lung digestion

Lungs were removed from euthanized animals and leukocytes prepared as previously described (43). Briefly, lungs were minced with scissors to fine slurry in 15 ml digestion buffer (RPMI 1640/10% FCS/1 mg/ml collagenase [Boehringer Mannheim Biochemical]30 μg/ml DNase, [Sigma-Aldrich]) per lung. Lung slurries were enzymatically digested for 30 min at 37˚C. Any undigested fragments were further dispersed by drawing the solution up and down through the bore of a 10-ml syringe. The total lung cell suspension was pelleted, resuspended, and spun through a 40% Percoll gradient to enrich for leukocytes. Cell counts and viability were determined using Trypan blue exclusion counting on a hemacytometer. Cytospin slides were prepared and stained with a modified Wright-Giemsa stain.

Real-time quantitative RT-PCR

Measurement of gene expression was performed utilizing the ABI Prism 7000 Sequence Detection System (Applied Biosystem, Foster City, CA) as previously described (43). Briefly, total cellular RNA from the frozen lungs were isolated, reverse transcribed into cDNA, and then amplified using specific primers for \textit{mTNF-α}, MIP-2, IL-17, IL-22, β-defensin 3, CRAMP, and caspase-3 with β-actin serving as a control. Specific thermal cycling parameters used with the TaqMan One-Step RT-PCR Master Mix Reagents kit included 30 min at 48˚C, 10 min at 95˚C, and 40 cycles involving denaturation at 95˚C for 15 s, annealing/extension at 60˚C for 1 min. Relative quantitation of cytokine mRNA levels was plotted as fold-change compared
with untreated control cells or whole lung. All experiments were performed in duplicate.

**Marine ELISA for albumin measurement**

Albumin (Albumin Quantification Kit; Bethyl Laboratories, Montgomery, TX) for lung permeability assessment were quantified using a modified double ligand method.

**Western immunoblotting**

Whole cell lysates were obtained by treating cells with RIPA buffer (1% w/v NP-40, 1% w/v sodium deoxycholate, 0.1% w/v SDS, 0.15 M NaCl, 0.01 M sodium phosphate, 2 mM EDTA, and 50 mM sodium fluoride) plus protease and phosphatase inhibitors. Protein concentrations were determined by the Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA). Samples were electrophoresed in 4–12% gradient SDS-PAGE gels, transferred to nitrocellulose and blocked with 5% skim milk in PBS. After incubation with primary Abs, blots were incubated with secondary Ab linked to HRP and bands visualized using ECL (SuperSignal West Pico Substrate, Pierce Biotechnology).

**Statistical analysis**

Survival curves were compared using the log-rank test. For other data, statistical significance was determined using the unpaired t test. All calculations were performed using the Prism 3.0 software program for Windows (GraphPad Software, San Diego, CA).

**Results**

The i.n. administration of flagellin markedly improves survival in mice challenged with *P. aeruginosa* i.t.

Previous studies suggest that flagellin can stimulate protective immunity in nonpulmonary infection models, although the mechanism of protection is unknown (18–21). To define the effect of flagellin in lung bacterial infection, we first pretreated C57BL/6 mice with graded concentrations of endotoxin-free flagellin (250 ng–1 μg) purified from *P. aeruginosa* strain *P. aeruginosa* 01 in 10 μl saline solution or vehicle i.n., then administered a lethal dose of *P. aeruginosa* strain 19660 (7–8 × 10^{5} CFU) 48 h later. As shown in Fig. 1A, pretreatment with flagellin resulted in a striking dose-dependent increase in survival, with nearly 90% of animals pretreated with 1 μg flagellin i.n. surviving, whereas, no survival was observed in animals pretreated with vehicle, followed by *P. aeruginosa* (*p* < 0.05). To define the temporal window of protection, mice were treated with flagellin (1 μg) 48 or 24 h prior to *P. aeruginosa*, or concomitant with the i.t. administration of *P. aeruginosa*. Pretreatment with flagellin at 48 or 24 h prior to *P. aeruginosa* stimulated equally strong protection (Fig. 1B). Importantly, administration of flagellin concomitant with *P. aeruginosa* induced partial protection (45% survival), albeit less than that observed with pretreatment. The protective effects of flagellin were not attributable to LPS contamination, as flagellin induced a high degree of protection in mice with defective TLR4 signaling (*TLR4^{−/−}*, as compared with saline solution-pretreated animals (Fig. 1C). To define whether the protection afforded by flagellin required TLR5, WT mice and TLR5 mutants (*TLR5^{−/−}*) were pretreated with 1 μg flagellin i.n., followed 24 h later by the i.t. administration of *P. aeruginosa*. Flagellin pretreatment provided significant survival benefit in the *P. aeruginosa*-infected WT mice but not in TLR5^{−/−} mice (Fig. 1D).

The i.n. administration of flagellin improves lung bacterial clearance and decreases dissemination in mice challenged with *P. aeruginosa* i.t.

To determine the mechanism by which flagellin induces protection, we first examined the effect of flagellin administration on bacterial clearance in the lung and dissemination of bacteria to the bloodstream and distant organs. Mice were pretreated with flagellin or vehicle i.n., *P. aeruginosa* administered 24 h later, then *P. aeruginosa* CFUs in lung, blood, and spleen were determined 8 and 24 h after *P. aeruginosa*. As shown in Fig. 2A, challenge with *P. aeruginosa* at a dose of 8 × 10^{5} CFU resulted in substantial numbers of bacteria in lung by 8 h, with the development of high-grade bacteremia and seeding of spleen by 24 h. Impressively, flagellin administration dramatically reduced bacterial burden in lung at both 8 and 24 h post-*P. aeruginosa* administration (>100- and >1000-fold reduction in CFUs, respectively, *p* < 0.01). Moreover, pretreatment with flagellin decreased blood *P. aeruginosa* CFUs by >1000-fold and completely eliminated splenic seeding.

**FIGURE 1.** Effect of i.n. flagellin administration on survival post-*P. aeruginosa* challenge. A. Dose-dependent effect of flagellin on survival (*n* = 10–15 per group, combined from two separate experiments). Animals were pretreated with vehicle or flagellin 24 h prior to *P. aeruginosa* administration. B. Effect of varying times of flagellin (1 μg) administration on survival (*n* = 10–15 per group, combined from two separate experiments). C. Effect of flagellin pretreatment (24 h prior to *P. aeruginosa*) on survival in WT and TLR4 mutant mice (*TLR4^{−/−}, n* = 5 per group). D. Effect of i.n. administration of flagellin or vehicle 24 h pre-*P. aeruginosa* challenge on survival in WT and TLR5^{−/−} mice, *n* = 5 per group. *p* < 0.05 as compared with infected animals pretreated with vehicle.
Flagellin has been shown to promote protective effects on non-respiratory epithelium (22, 37). To determine whether pretreatment with flagellin reduced lung injury in response to *P. aeruginosa* administration, animals were administered vehicle or flagellin (1 μg) i.n., followed 24 h later by the i.t. administration of *P. aeruginosa* (5 × 10⁵ CFU). BAL was performed at 6 and 24 h after *P. aeruginosa* challenge. Whole lung caspase-3 mRNA was determined by real-time PCR at 6 and 12 h post-*P. aeruginosa*. Each value for albumin and mRNA represents mean ± SEM of five mice per data point. *p* < 0.05 as compared with vehicle/P. aeruginosa group.

The i.n. administration of flagellin does not substantially alter lung leukocyte influx in mice challenged with *P. aeruginosa* i.t., and flagellin-induced protection did not require PMNs

A possible mechanism of improved bacterial clearance in flagellin-treated mice is by altering the recruitment of innate phagocytic cells. To address this, we quantitated the number of lung PMNs, macrophages, and total leukocytes postadministration (Fig. 3A). However, no differences in numbers of lung PMNs, macrophages, or total leukocytes were observed between flagellin- and vehicle-pretreated groups at 12 or 24 h post-*P. aeruginosa* administration. In fact, the number of lung PMNs tended to be lower in the flagellin-pretreated group 24 h post-*P. aeruginosa*. These studies suggest that flagellin-mediated protection occurred in the absence of large changes in numbers of infiltrating phagocytic cells. These results were confirmed histologically, as flagellin administration resulted in a modest influx of inflammatory cells at 24 h (Fig. 3B, upper right panel). Lung inflammation was most prominent in *P. aeruginosa*-infected mice pretreated with vehicle (Fig. 3B, lower left panel), especially when compared with that observed in flagellin-pretreated mice 24 h after *P. aeruginosa* (Fig. 3B, lower right panel).

To definitively exclude a role for neutrophils in flagellin-induced protection, mice were depleted of PMNs by the i.p. administration of rat anti-mouse anti-Ly-6G Ab (200 μg/animal), given concomitant with either i.n. flagellin or vehicle, followed 24 h later by the i.t. administration of *P. aeruginosa* (5 × 10⁴ CFU). The inoculum of *P. aeruginosa* in this experiment was reduced due to enhanced susceptibility to bacterial pneumonia in neutrophil-depleted animals. As shown in Fig. 3C, pretreatment of neutrophil-depleted mice with flagellin resulted in a >100-, >1000-, and >400-fold reduction in lung, blood, and spleen CFUs at 24 h postbacterial administration, as compared with vehicle-pretreated mice challenged with *P. aeruginosa*.

The i.n. administration of flagellin induces the expression of innate host defense genes in lung

To further explore the mechanism of protection in flagellin-treated animals, we assessed the expression of innate genes that participate in lung antibacterial host defense by quantitative PCR. As shown in Fig. 4, the i.n. administration of flagellin resulted in an early induction of TNF-α mRNA (23-fold increase) and IL-17 message (14-fold increase for both) in whole lung by 6 h, with a decline in expression by 24 h, whereas, peak mRNA expression of the neutrophil active chemokine MIP-2/CXCL1 and IL-22 occurred at 24 h postflagellin. Flagellin also strongly induced expression of genes encoding antimicrobial peptides. In particular, CRAMP was the most abundantly expressed gene, with a >40-fold induction at 6 h, and persistently high expression out to 24 h. β-defensin 3 was also induced in response to flagellin, albeit to a considerably lesser degree and in a delayed fashion relative to CRAMP.

The i.n. administration of flagellin ± *P. aeruginosa* induces the expression of CRAMP protein in lung

Given that CRAMP was the most robustly expressed gene in response to purified flagellin, we next sought to determine the induction of CRAMP protein in response to flagellin alone and in *P. aeruginosa*-infected mice pretreated with flagellin i.n. As determined by Western blot analysis, minimal CRAMP was detected in lung at baseline (Fig. 5A). Flagellin administration resulted in a >5-fold increase in lung levels of the propeptide form of CRAMP (20 kDa). Challenge with *P. aeruginosa* in vehicle-pretreated mice also resulted in an increase lung CRAMP expression, although the induction of CRAMP was greatest in *P. aeruginosa*-infected mice pretreated with flagellin. Importantly, flagellin-induced expression of CRAMP was abrogated in TLR5−/− mice compared with that observed in WT animals (Fig. 5B).

Flagellin induces expression of CRAMP by MLE-12 AECs, primary AECs, and lung macrophages

In preliminary studies, immunohistochemical analysis indicated that PMNs, alveolar macrophages, and AECs expressed CRAMP post-*P. aeruginosa* administration, and expression by these cells was greatest in animals pretreated i.n. with flagellin (data not shown). To more definitely establish the cellular sources of CRAMP in...
response to flagellin, we isolated primary mouse AECs and pulmonary macrophages recovered from whole lung digests as previously described (42). In addition, we used MLE-12 cells, a transformed murine AEC line that retains many features of type II AECs (44). Treatment with flagellin strongly stimulated the time-dependent expression of CRAMP mRNA, with an 8-, 50-, and 35-fold increase in message observed in AECs, MLE-12, and lung macrophages at 24 h, respectively, compared with untreated cells (Fig. 6A).

We next performed Western blot analysis to detect the presence of CRAMP in cell lysates from flagellin-stimulated MLE-12 and primary murine AECs. Minimal CRAMP was detected at baseline in unstimulated MLE-12 or primary AECs. Treatment of cells with purified flagellin resulted in an increase in CRAMP by 4 h in stimulated MLE-12, and 8 h in primary AECs (Fig. 6B).

Contribution of CRAMP to flagellin-induced protection in P. aeruginosa pneumonia

Our earlier studies clearly identified flagellin as a major inducer of CRAMP, raising the possibility that CRAMP might mediate, in part, the protective effects of flagellin on antibacterial host responses. In preliminary studies, we found that incubation of murine recombinant CRAMP with P. aeruginosa resulted in suppression of bacterial growth, with bacteriostatic activity observed at concentrations of 1 μM and above (Supplemental Fig. 1). To determine the contribution of CRAMP to flagellin protective effects, we used mice deficient in the gene encoding CRAMP (Cnlp<sup>−/−</sup> mice). Cnlp<sup>−/−</sup> and WT C57BL/6 mice were pretreated with flagellin (1 μg) or vehicle i.n., then administered P. aeruginosa at a dose of 2–3 × 10<sup>5</sup> CFU, and P. aeruginosa CFUs quantitated in lung and blood 24 h later (Fig. 7A). A lower P. aeruginosa inoculum was chosen in these experiments due to concerns for excessive mortality in CRAMP-deficient mice. As compared with vehicle-treated WT infected mice, bacterial CFUs in lung were ∼6.5-fold greater in lungs of Cnlp<sup>−/−</sup> mutant mice (p = 0.05). More impressively, there was markedly increased dissemination of P. aeruginosa in Cnlp<sup>−/−</sup> mice, with >20-fold higher bacterial counts in blood of knockout mice (p < 0.05). As compared with WT mice pretreated with vehicle, flagellin-pretreated mice had a significant reduction in lung CFUs and no dissemination to blood. In contrast, no reduction in lung CFUs was observed in Cnlp<sup>−/−</sup> mice pretreated with flagellin, as compared with Cnlp<sup>−/−</sup> mice not receiving flagellin, although a trend toward reduction in blood CFUs was observed in flagellin/P. aeruginosa-infected Cnlp<sup>−/−</sup> mice.

To further determine the relative contribution of CRAMP to the protective effects of flagellin, we pretreated WT and Cnlp<sup>−/−</sup> mice with flagellin (1 μg) i.n., followed by administration of P. aeruginosa.
previously, no mortality was observed in P. aeruginosa-Cnlp of WT mice pretreated with flagellin. By comparison, only one third reduced bacterial dissemination, and protection against is associated with enhanced bacterial clearance from the lung, Moreover, protection occurred in the absence of significant changes indicates stimulation of innate, rather than adaptive immunity. Detection induced by flagellin (within 24 h postadministration) responses has not been completely characterized. The early pro-

In the recruitment of PMNs or exudate macrophages. The effects of flagellin appear to be primarily mediated by TLR5, as we observed substantial loss of flagellin-mediated protection in TLR5<sup>−/−</sup> mice. This observation is consistent with the observations of others (10–14) and suggests that other pathogen recognition receptors, including TLR2 and the Ipaf/NOD pathway are less relevant in the generation of protective immunity in responses to exogenous P. aeruginosa flagellin (15, 16). Importantly, our data indicate that flagellin stimulatory effects in our model are maintained in mice with defective TLR4 signaling (TLR4<sup>−/−</sup>), confirming that the effects of flagellin are not mediated by LPS contamination. Flagellin induced potent protection against P. aeruginosa strain 19660, which is a cytotoxic strain expressing type III secreted exotoxins and flagellin. In addition, flagellin-induced broad protection against both flagellated and nonflagellated isogenic mutant P. aeruginosa strains (PAK and PAKΔflgC, provided by A. Prince, Columbia University, New York City, NY) and the more virulent encapsulated Gram-negative bacteria Klebsiella pneumoniae (data not shown). Our findings are consistent with immunostimulatory properties of flagellin against diverse microbial pathogens, including lethal intestinal Salmonella infection, rotavirus-induced colitis, and bacterial corneal infection (33–35), and provide further support for induction of innate rather than specific immunity.

There are several possible mechanisms accounting for enhanced lung P. aeruginosa clearance. First, flagellin induced an influx of PMNs into the lung airspace by 24 h after administration. However, the influx of PMNs was relatively modest at the time of bacterial challenge, and flagellin pretreatment induced strong protection even in animals depleted of PMNs. Collectively, these data indicate that flagellin effects on PMN influx is unlikely to be a major contributor to enhancement of bacterial clearance. An early induction of innate cytokine and chemokine genes, including TNF-α, MIP-2, IL-17, and IL-22, was also observed in flagellin-pretreated animals. Finally, flagellin is a potent inducer of cationic antimicrobial peptides, including β-defensin 2 and especially CRAMP. CRAMP exerts direct bactericidal effects against both Gram-positive and Gram-negative organisms, including P. aeruginosa (45). We found reduced lung bacterial clearance and increased dissemination in P. aeruginosa-infected CRAMP-deficient mice. Importantly, flagellin-induced protective effects on bacterial clearance and survival were significantly diminished in Cnlp<sup>−/−</sup> mice relative to WT animals, indicating that CRAMP was a dominant mediator of protection in response to flagellin. We cannot exclude

(7–8 × 10<sup>5</sup> CFU), then assessed survival. As shown in Fig. 7B, all control P. aeruginosa-infected WT and Cnlp<sup>−/−</sup> mice died after challenge with this inoculum of P. aeruginosa. As observed previously, no mortality was observed in P. aeruginosa-infected WT mice pretreated with flagellin. By comparison, only one third of Cnlp<sup>−/−</sup> survived even when pretreated with flagellin.

Discussion

Protective immunity against bacterial pathogens of the lung requires the generation of robust but tightly controlled innate immune responses, which is mediated, in part, by TLRs. Our studies indicate that the compartmentalized administration of flagellin can augment host immunity against P. aeruginosa, which is associated with enhanced bacterial clearance from the lung, reduced bacterial dissemination, and protection against P. aeruginosa-induced lung injury.

The mechanism by which flagellin activates protective immune responses has not been completely characterized. The early protection induced by flagellin (within 24 h postadministration) indicates stimulation of innate, rather than adaptive immunity. Moreover, protection occurred in the absence of significant changes

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![FIGURE 4](http://www.jimmunol.org/)

Levels of murine TNF-α, MIP-2, IL-17, IL-22, β-defensin 3, and CRAMP mRNA in lung after i.n. flagellin administration. Lungs were harvested at times indicated postflagellin (1 µg), homogenates prepared, and cytokine mRNA levels determined by real-time PCR. Fold-decrease represents change from vehicle control. Each value represents mean of four mice per data point.

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![FIGURE 5](http://www.jimmunol.org/)

Expression of CRAMP protein in lung after flagellin ± P. aeruginosa administration. A is the Western blot analysis (upper panel) and corrected densitometry (lower panel) showing CRAMP induction 6 and 24 h after i.n. flagellin (top row) and 24 h after P. aeruginosa (5 × 10<sup>3</sup> CFU) in vehicle- or flagellin-pretreated animals (bottom row). Three animals were included in each group. Mean densitometry is corrected for β-actin. *p < 0.05 as compared with vehicle control. B represents expression of CRAMP in lung 24 h after administration of vehicle or flagellin in WT or TLR5<sup>−/−</sup> mice as assessed by Western blot analysis. Densitometry (lower panel) represents mean of three separate mice, corrected for β-actin. *p < 0.05 as compared with vehicle control.
the possibility that the induction of CRAMP in flagellin-treated mice is indirect due to induction of other regulatory genes, such as IL-22. However, because IL-22 has not been previously shown to be expressed by AECs, flagellin-induced CRAMP expression in these cells in vitro strongly argues in support of a direct stimulatory effect.

Multiple cells in the lung express TLR5 and are capable of responding in a TLR5-dependent fashion, including airway and AECs and alveolar macrophages (12, 15, 17). Interestingly, lung neutrophil accumulation that occurs after the i.n. administration of purified flagellin has been shown to be mediated by structural cells rather than TLR5-mediated myeloid cell responses (17). In vitro studies suggest that flagellin is a strong inducer of CRAMP in alveolar epithelium, including both AEC lines (MLE-12) and primary murine AECs. CRAMP has been shown to be expressed at several epithelial surfaces, such as skin, cornea, intestine, and urinary tract (31, 33–35, 46). Although the human homolog LL-37 is known to be expressed by airway epithelial cells and A549 AECs (32, 47), the production of CRAMP by primary AECs has not been previously reported. Epithelial cell-derived CRAMP contributes meaningfully to antibacterial host defense. For example, Cnlp−/− mice display impaired clearance of group A Streptococcus in a skin infection model, and this defect in clearance persisted in Cnlp−/− mice depleted of neutrophils (35). Moreover, keratinocytes isolated from Cnlp−/− mice displayed reduced intracellular killing of bacteria relative to WT keratinocytes. Our data showing protection in neutrophil-deficient mice implicate meaningful contributions from cells other than PMNs in response to flagellin.

These data indicate that pretreatment with flagellin protected against P. aeruginosa-induced lung injury. Although the reduction in lung injury observed could be attributable to improved lung bacterial clearance, a plausible alternative explanation is that flagellin might exert prosurvival effects on AECs, resulting in improved barrier function of the alveolar capillary membrane and reduced access of bacteria to the bloodstream. Flagellin has previously been shown to drive prosurvival responses in other epithelial cell populations, and can promote protective responses in a noninfectious radiation-induced lung injury model (18, 22). We have found that treatment of either murine primary AECs or epithelial cell lines promotes resistance to apoptosis and selective induction of antia apoptotic genes (T. Standiford, unpublished observations). These findings are consistent with effects observed in other epithelial cell populations. We cannot exclude that the effects of flagellin are indirect via induction of other molecules regulating apoptosis. For instance, CRAMP exerts multiple immunomodulatory effects that might influence epithelial integrity, including stimulation of epithelial proliferation and repair, angiogenesis, and cytoprotection via stimulation of IL-10 (30, 31, 37, 48).

Our findings indicate that pre-exposure to flagellin, as compared with concomitant administration, markedly enhanced protective mucosal immunity. A possible explanation for enhanced efficacy with pretreatment is a priming effect on the expression of innate genes such as TLR5, with concomitant administration, markedly enhanced protective mucosal immunity. A possible explanation for enhanced efficacy with pretreatment is a priming effect on the expression of innate genes such as TLR5, with concomitant administration, markedly enhanced protective mucosal immunity. A possible explanation for enhanced efficacy with pretreatment is a priming effect on the expression of innate genes such as TLR5, with concomitant administration, markedly enhanced protective mucosal immunity. A possible explanation for enhanced efficacy with pretreatment is a priming effect on the expression of innate genes such as TLR5, with concomitant administration, markedly enhanced protective mucosal immunity.
ulate lung mucosal immunity that can be exploited therapeutically to prevent serious bacterial infections of the respiratory tract.

Disclosures
The authors have no financial conflicts of interest.

References
Supplemental Figure 1. Inhibition of PA growth by mrCRAMP. PA (5x10^4 CFU/ml) was incubated in Difco nutrient broth at 37°C in the presence or absence of mrCRAMP (0.1-50 μM), then PA CFU quantitated 4 hrs later and expressed as percent of untreated control. Concentration of PA at 4 hrs in vehicle treated positive control was 4x10^5 CFU/ml. *p<0.05 as compared to untreated control, n = 4 per condition.