Redundant and Cooperative Interactions between TLR5 and NLR4 in Protective Lung Mucosal Immunity against *Pseudomonas aeruginosa*

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Key Words

Mucosal immunity · Toll-like receptors · Nod-like receptors

Abstract

Flagellin is the major structural component of flagella expressed by *Pseudomonas aeruginosa* (PA) and other bacteria. This protein has been shown to activate the Toll-like receptor TLR5 and the Nod-like receptor Nlr4/Ipf, culminating in the expression of innate cytokines and antimicrobial molecules. In this study, we tested the hypothesis that TLR5 and Nlr4 in combination are required for maximal protective lung innate mucosal immunity against PA. To test this hypothesis, we compared innate immune responses in wild-type (WT) C57B6 mice challenged with PA intratracheally to those observed in mice genetically deficient in TLR5 (TLR5^{-/-}) or Nlr4 (Nlr4^{-/-}) alone or in combination (TLR5/Nlr4^{-/-}). As compared to WT, TLR5^{-/-} and Nlr4^{-/-} mice, we observed a significant increase in mortality in TLR5/Nlr4^{-/-} mice, which was associated with a >5,000-fold increase in lung PA colony-forming units and systemic bacterial dissemination. The increased mortality observed in double-de-

ficient mice was not attributable to differences in lung leukocyte influx or lung injury responses. Levels of biologically active IL-1 β and IL-18 were reduced in the bronchoalveolar lavage fluid from PA-infected Nlr4^{-/-} and TLR5/Nlr4^{-/-} but not TLR5^{-/-} mice, indicating the requirement for Nlr4-dependent caspase-1 activation. Similarly, decreased production of biologically active IL-1 β and activation of caspase-1 was observed in PA-stimulated pulmonary macrophages isolated from Nlr4^{-/-} and TLR5/Nlr4^{-/-} but not TLR5^{-/-} mice, whereas the expression of iNOS and the production of NO were significantly reduced in cells from double-mutant but not single-mutant mice. Collectively, our findings indicate that TLR5 and Nlr4 have both unique and redundant roles in lung antibacterial mucosal immunity, and the absence of both pathogen recognition receptors results in an increase in susceptibility to invasive lung infection.

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Introduction

Healthcare-associated pneumonia is the most lethal infection amongst all nosocomial infections and is associated with increased morbidity and longer length of hospital stay when compared to patients with community-acquired pneumonia [1–6]. *Pseudomonas aeruginosa* (PA) is a flagellated aerobic Gram-negative bacterium that rarely causes pulmonary disease in immunocompetent subjects, but is the second most common nosocomial infection, and pneumonia due to PA is associated with the highest mortality amongst nosocomial infections [7, 8]. PA expresses several virulence factors, such as flagellin and the type III secretion system, molecules that activate pathogen recognition receptors (PRRs) and promote nuanced innate responses that can be protective or at times deleterious to the host [7, 9–11].

Toll-like receptors (TLRs) are a family of type I transmembrane PRRs that are activated by pathogen-associated molecular patterns expressed by a diverse group of microorganisms, resulting in activation of host antimicrobial responses [12–15]. Flagellin, expressed by most strains of PA [16], is the sole activator of TLR5 [17–21]. TLR5 is a MyD88-dependent TLR that is expressed by both myeloid and structural cells, most abundantly in the gut, liver and lung [21]. In the lung, airway and alveolar epithelial cells and alveolar macrophages express TLR5, and the cell-surface expression of TLR5 is upregulated in response to bacteria [22–24]. Upon binding to TLR5, flagellin activates the MyD88 pathway, resulting in NF- κ B nuclear translocation and ultimately increased expression of antimicrobial genes [25–28].

Nod-like receptors (NLRs) are a family of PRRs that recognize intracellular danger signals. During infection, NLRs are activated by a diverse group of pathogen-associated molecular patterns, including the bacterial cell wall components peptidoglycan and muramyl dipeptide, bacterial flagellin and several bacterial toxins [29–33]. Nlrc4 (Ipaf) is a specific NLR that upon binding its ligand activates the inflammasome containing the costimulatory protein ASC and caspase-1 [34]. Interleukin (IL)-1 family members, including IL-1 β and IL-18, are translated as inactive precursor zymogens which are cleaved by caspase-1 to their active forms, resulting in the generation of host inflammatory responses [25–28]. Nlrc4 is activated by intracellular flagellin. In addition, Nlrc4 binds to and is activated by type III secretion system proteins in a flagellin-independent manner, resulting in caspases-1-mediated IL-1 β and IL-18 [35–38].

While TLR5 has previously been shown to be dispensable for protective lung mucosal immunity against PA, cooperative interactions between TLR5 and TLR4 are necessary for optimal clearance of this organism [23]. By comparison, Nlrc4 is necessary for effective innate lung responses against the Gram-negative organism *Klebsiella pneumoniae*. However, this NLR can mediate deleterious inflammation in the setting of PA challenge [32, 39, 40]. Physiological interaction between these two PRRs during bacterial infection has not been investigated. In this study, we sought to identify possible interactions and mechanisms involved during experimental pneumonia due to PA. Our findings indicate that TLR5 and Nlrc4 have both redundant and unique roles in antibacterial mucosal immunity, and the absence of both PRRs results in a substantial increase in susceptibility to invasive lung infection.

Materials and Methods

Animals

Specific pathogen-free wild-type (WT) C57BL/6 mice (age and sex matched) were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA). The TLR5^{-/-} breeding pairs were originally purchased from A. Aldeman (Institute of Systems Biology, Seattle, Wash., USA). The Nlrc4^{-/-} mice were generated at the University of Michigan (Gabriel Nunez), whereas the TLR5/ Nlrc4^{-/-} mice were obtained from Andrew Gewirtz (Emory University). All mice were bred >5 generations on a C57BL/6 background and housed in specific pathogen-free conditions within the animal care facility (Unit for Laboratory Animal Medicine, University of Michigan, Ann Arbor, Mich., USA) 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).

Bacterial Preparation

Flagellated *P. aeruginosa* strain 19660 (American Type Culture Collection, Manassas, Va., USA) was used in our studies. Strains were grown overnight in Difco nutrient broth (BD Biosciences, Franklin Lakes, N.J., USA) at 37°C with constant shaking. Bacterial concentrations were then determined by measuring the amount of absorbance at 600 nm and compared to a predetermined standard curve based on known colony-forming unit (CFU) values. Bacterial cultures were then diluted to the desired concentration for intratracheal (i.t.) inoculation or in vitro stimulation. An aliquot of the inoculated PA suspension serially diluted onto blood agar plates determines the actual dose of bacterial CFU.

i.t. Inoculation

For i.t. inoculation, mice were anesthetized with an intraperitoneal (i.p.) mixture of ketamine and xylazine. Under sterile conditions, the trachea was exposed using blunt dissection and a 30- μ l inoculum was administered using a 26-gauge needle. The skin incision was then closed using surgical staples.

Pulmonary Macrophage Isolation

Pulmonary macrophages (PM) consisting of both alveolar and interstitial lung macrophages were isolated from dispersed lung digest cells by adherence purification as previously described [41]. The purity of these macrophages was >90% as determined by forward- and side-scatter characteristics, and F480 and CD11b staining.

Whole Lung and Spleen CFU Determination

Mice were euthanized by CO₂ asphyxiation at specified time points and the thoracic cavity was opened under sterile technique. In order to perfuse the cardiopulmonary vasculature, 1 ml of sterile PBS containing 5 mM EDTA was infused through the right ventricle. Whole lungs and spleens were then removed, taking care to dissect away lymph nodes. Organs were then homogenized separately in 1 ml of sterile PBS with protease inhibitor (Boehringer Mannheim, Indianapolis, Ind., USA). Homogenates were serially diluted 1:5 in sterile PBS and plated on nutrient agar (BD Biosciences) plates to determine CFU.

Bronchoalveolar Lavage

Bronchoalveolar lavage (BAL) was performed for the collection of BAL fluid (BALF) as previously described. Briefly, the trachea was exposed and intubated using 26-gauge polyethylene tubing. Three 1-ml aliquots of PBS containing 5 mM EDTA were instilled and aspirated using syringe suctioning. More than 80% of BALF was retrieved [41].

BALF Leukocyte Analysis

BALF was centrifuged at 1,500 rpm at 4°C for 10 min. Cell-free BALF was separated and aliquoted for murine cytokine, chemokine and albumin ELISA measurements. One milliliter of sterile deionized water was added to each cell pellet and thoroughly mixed for 20 s in order to lyse red blood cells. To prevent leukocyte lysis, 10 ml of Life Technologies RPMI Medium (Invitrogen, Carlsbad, Calif., USA) was added. Samples were centrifuged again at 1,500 rpm at 4°C for 10 min. Supernatant was aspirated off and the cell pellet was resuspended in 250 µl of RPMI (Invitrogen). Cell counts and viability were determined using trypan blue exclusion counting on a hemacytometer. Cytospin slides were prepared to a concentration of 2×10^4 cells/slide and stained with modified Wright-Giemsa stain.

Murine ELISAs for Cytokine/Chemokine Measurement

Cell-free BALF was analyzed for IL-1 β , TNF- α and KC/CXCL1 using mouse DuoSet ELISA kits (R&D Systems, Minneapolis, Minn., USA) employing a modified double-ligand method. The IL-18 ELISA was purchased from Affymetrix eBioscience (San Diego, Calif., USA). The IL-1 β and IL-18 ELISAs measured the active forms of these cytokines and not the propeptide forms [42].

Murine ELISA for Albumin Measurement

BALF albumin (albumin quantification kit; Bethyl Laboratories, Montgomery, Tex., USA) for lung permeability assessment was quantified using a modified double-ligand method.

Nitric Oxide Analysis

Nitric oxide levels in PM supernatants were analyzed using a standard Griess assay [42].

Fig. 1. Effect of TLR5, Nlrc4 or TLR5/Nlrc4 gene deletion on survival after i.t. PA challenge. Mice were administered 7×10^5 CFU i.t., and then survival was assessed up to 6 days. Results shown are combined from two separate experiments; $n = 8$ –10 mice per group. * $p < 0.05$ as compared to the other groups.

Real-Time Quantitative RT-PCR

Measurements of gene expression were performed utilizing the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, Calif., USA) as previously described. Briefly, total cellular RNA from frozen lung samples was isolated, reverse transcribed into cDNA, and then amplified using specific primers for IL-1 β and iNOS (NOS2) with β -actin serving as an internal control. Specific thermal cycling parameters used with the TaqMan one-step RT-PCR master mix reagents kit are as follows: 30 min at 48°C, 10 min at 95°C, and 40 cycles involving denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. The relative quantification of cytokine mRNA levels was plotted as the fold change compared with untreated control cells or whole lung [41].

Statistical Analysis

Survival curves were compared using log-rank (Mantel-Cox) tests. Statistical significance was determined by using one-way ANOVA. All calculations were performed using GraphPad Prism 6.0 software for Windows (GraphPad Software, La Jolla, Calif., USA).

Results

Genetic Deletion of TLR5 and Nlrc4 in Combination Results in Reduced Survival and Impaired Bacterial Clearance after i.t. Administration of PA

To determine the role that TLR5 and Nlrc4 alone or in combination play in the innate immune response to flagellated Gram-negative bacteria, WT mice and mice lacking the genes for TLR5 (TLR5^{-/-}, Nlrc4 (Nlrc4^{-/-}), or mice

Fig. 2. Effect of TLR5, Nlrc4 or TLR5/Nlrc4 gene deletion on lung and spleen PA CFU after i.t. PA challenge. Mice were administered 5×10^5 CFU i.t., and then PA CFU were quantitated in lung (a) and spleen (b) 24 h later. Data are expressed as $\log_{10}$, combined from two separate experiments; $n = 6-8$ per group. * $p < 0.0001$ as compared to the other groups.

deficient in both (TLR5/Nlrc4^{-/-}) were i.t. administered PA strain 19660 (PA) at a dose of 7×10^5 CFU/30 μ l and monitored for 6 days. As shown in figure 1, survival was >85% in WT or single-mutant mice. By comparison, survival was <40% in similarly challenged TLR5/Nlrc4^{-/-} mice ($p < 0.05$). No deaths were observed after 6 days.

We next determined if the differences in mortality observed were attributable to alterations in bacterial clearance. For these experiments, PA was administered at a

Fig. 3. Effect of TLR5/Nlrc4 gene deletion on total BALF cells (a) and BALF PMN (b) after PA challenge. Mice were administered 5×10^5 CFU i.t., and then total cells and PMN were quantitated at 6 and 18 h postchallenge; $n = 4-6$ per group.

dose of 5×10^5 CFU to WT, TLR5^{-/-}, Nlrc4^{-/-} and TLR5/Nlrc4^{-/-} mice, then lungs and spleen were harvested 6 and 24 h after instillation and CFU quantified. We observed no differences in lung CFU in the 4 groups at 6 h post-challenge, and no animals were bacteremic at that time point (fig. 2a, b). At 24 h, lung CFU were low in WT and single-deficient mice (<50 CFU), and no differences between groups were noted. In contrast, there was persistence of bacteria in the infected TLR5/Nlrc4^{-/-} mice, rep-

representing a >3.5 log increase, as compared to WT mice ($p < 0.0001$; fig. 2a). Likewise, there was a substantial increase in spleen CFU in TLR5/Nlrc4^{-/-} mice (fig. 2b), whereas no splenic dissemination was observed in either WT or single-mutant mice. Importantly, as compared to WT mice, there were no significant differences in the number of BAL total cells or neutrophils in the double-deficient mice at either 6 or 18 h after PA, indicating that the impairment in bacterial clearance observed in TLR5/Nlrc4^{-/-} mice was not attributable to differences in lung inflammatory cell influx (fig. 3).

Differences in Bacterial Clearance and Survival of TLR5/Nlrc4^{-/-} Mice after i.t. PA Challenge were not Attributable to Lung Injury

Bacterial pneumonia is a common cause of acute lung injury, which contributes to morbidity and mortality associated with pneumonia due to PA. As a surrogate for capillary leak, cell-free BALF albumin was measured 6 and 18 h after i.t. administration of PA (5×10^5 CFU/30 μ l) in WT, single- and double-mutant mice. As shown in figure 4, i.t. challenge with PA resulted in an increase in BAL albumin levels by 6 h, which was maximally increased by 18 h after PA administration. As compared to WT animals, we noted no difference in BALF albumin at 6 or 18 h postinfection in TLR5^{-/-} or TLR5/Nlrc4^{-/-} mice. Interestingly, BAL albumin levels were significantly reduced in Nlrc4^{-/-} mice at both 6 and 18 h compared to other groups ($p < 0.01$ at both time points).

Selective Reduction in Inflammatory Cytokines and Chemokines in Single- and Double-Mutant Mice following i.t. Administration of PA

Both TLR5 and Nlrc4 pathways have been shown to regulate the expression of cytokines and chemokines in the setting of bacterial infection. Nlrc4-mediated inflammasome activation also regulates the posttranslational conversion of pro-IL-1 β and pro-IL-18 to their active metabolites in a caspase-1-dependent fashion [28]. For that reason, we measured TNF- α , CXCL1/KC, IL-1 β and IL-18 levels in cell-free BALF collected from WT and mutant mice at 18 h after PA administration. We chose this time point as cytokine/chemokine levels peaked at 18 h. We observed no differences in BALF TNF levels of WT, single- or double-mutant mice after PA challenge (fig. 5a). Conversely, KC/CXCL1 levels in BALF were significantly reduced in TLR5^{-/-}, Nlrc4^{-/-} and TLR5/Nlrc4^{-/-} mice as compared to infected WT mice. Levels of IL-1 β were low at baseline in all groups, with a large increase in BALF IL-1 β by 18 h after PA in the WT and TLR5^{-/-} single-mutant

Fig. 4. Effect of TLR5, Nlrc4 or TLR5/Nlrc4 gene deletion on BALF albumin levels. Mice were administered 5×10^5 CFU i.t., and then BALF albumin levels were quantitated at 6 and 18 h postchallenge; $n = 4$ –6 per group. * $p < 0.01$ as compared to other groups.

mice. However, we observed no increase in BALF IL-1 β levels in Nlrc4^{-/-} and TLR5/Nlrc4^{-/-} double-deficient mice after PA administration (fig. 5a). Similarly, we observed a marked increase in BALF IL-18 levels in infected WT and TLR5^{-/-} mice, but only a minimal increase in IL-18 levels was noted in Nlrc4^{-/-} and TLR5/Nlrc4^{-/-} double-deficient mice 18 h after PA challenge.

In addition to the aforementioned cytokines, we also measured mRNA and protein expression of other important innate effector molecules in infected WT, TLR5^{-/-}, Nlrc4^{-/-} and TLR5/Nlrc4^{-/-} mice. Specifically, we assessed the mRNA expression of IL-17 and IL-22 in whole lungs of WT and mutant mice at both 6 and 18 h after PA administration and found no differences in any of the groups (data not shown). We also measured IL-17 in cell-free BALF at 18 h after PA administration and found no significant differences. Finally, we measured CRAMP mRNA expression (RT-PCR) and protein (by Western blotting) at both 6 and 18 h in WT, single- and double-mutant mice and detected no differences (data not shown).

PM Produce IL-1 β in an Nlrc4-Dependent Fashion, whereas Maximal Expression of iNOS and NO Requires Cooperative Effects of TLR5 and Nlrc4

PM are a major early cellular source of inflammatory cytokines and the antimicrobial molecule NO in response to bacterial invasion. To assess the impact of TLR5 and/

Fig. 5. Effect of TLR5, Nlrc4 or TLR5/Nlrc4 gene deletion on BALF cytokine/chemokine levels. Mice were administered 5×10^5 CFU i.t., and then BALF TNF- α (a), KC/CXCL1 (b), IL-1 β (c) and IL-18

(d) levels were quantitated by ELISA at 18 h postchallenge; $n = 4-6$ per group. * $p < 0.05$ as compared to infected WT control mice.

or Nlrc4 deficiency on the expression of IL-1 β and NO from PM in vitro, we isolated PM from uninfected WT and mutant mice, and then stimulated cells with live PA (MOI 5:1). Cells were isolated for RNA and cell-free conditioned media collected after 6 or 18 h in culture, then assayed for IL-1 β or iNOS mRNA levels (fig. 6a) and cytokine protein or NO levels (fig. 6b). Maximal induction of IL-1 β mRNA expression in PM from WT mice was noted 6 h after PA. We observed no significant reduction in IL-1 β mRNA levels in PM from single- or double-mutant mice. In contrast, there was a substantial reduction in active IL-1 β release by PA-stimulated PM from Nlrc4^{-/-} and TLR5/Nlrc4^{-/-} double-deficient mice, as

compared to WT and TLR5^{-/-} PM. The expression of iNOS mRNA was maximal at 18 h after PA in WT PM, and significant reductions were observed in cells from TLR5/Nlrc4^{-/-} mice and a trend toward reduced expression in PM from TLR5^{-/-} mice ($p = 0.06$). Release of NO from stimulated cells was strongly induced by PA (>40-fold increase), but only significantly reduced in PM from double-mutant mice.

To further explore mechanisms of impaired IL-1 β release in single- and/or double-mutant mice, we examined the activation of caspases-1, the protease responsible for cleavage of IL-1 β and IL-18 propeptide to active forms. Stimulation of PM with PA resulted in abundant accumu-

Fig. 6. Effect of TLR5, Nlr4 or TLR5/Nlr4 gene deletion on the expression of IL-1 β , NO and caspase-1 activation in PA-stimulated PM in vitro. Expression of IL-1 β and iNOS mRNA (**a**), levels of IL-1 β and NO in PM-conditioned media (**b**), and amount of total

and active (p10) forms of caspase-1 by Western blotting (**c**). $n = 4-6$ per group for mRNA and protein studies, whereas Western blotting is representative of 2 separate experiments. * $p < 0.05$ as compared to PM from WT mice.

lation of the cleaved 10 kDa form of caspase-1 in WT and TLR5^{-/-} macrophages (fig. 6c). Interestingly, no active caspase-1 was detected in PM from either Nlrc4^{-/-} or TLR5/Nlrc4^{-/-} double-mutant mice.

Discussion

Our study demonstrates that effective antibacterial responses to i.t. administration of PA require the presence of either TLR5 or Nlrc4, and the presence of one of these PRRs can compensate for the absence of the other. However, mice that are deficient in both TLR5 and Nlrc4 display substantially reduced survival and impaired bacterial clearance as compared to their WT or single-mutant counterparts. The differences in bacterial clearance could not be explained based on differences in inflammatory cell recruitment or the expression of key innate cytokines, including TNF- α , chemokines and IL-1 family cytokines.

TLR5 and Nlrc4 have previously been shown to be important PRRs recognizing bacterial flagellin [43, 44]. While TLR5 exclusively binds extracellular flagellin, Nlrc4 has been shown to be activated by intracellular flagellin. In addition, Nlrc4 is activated by components of the type III secretion system, leading to downstream inflammasome activation [36]. Our data suggest that TLR5 and Nlrc4 can independently regulate the expression of the CXC chemokine KC/CXCL1, whereas the generation of biologically active IL-1 β and IL-18 is solely Nlrc4 dependent and completely independent of TLR5. Impaired production of chemokines, including KC/CXCL1, has been observed in Nlrc4 but not TLR5 single-deficient mice during Gram-negative bacterial pneumonia [23, 40, 45]. The finding of unaltered mRNA expression but substantially reduced IL-1 β release and caspase-1 activation in in vitro stimulated PM from Nlrc4- and TLR5/Nlrc4-deficient mice strongly suggests that the reduction in IL-1 β and IL-18 in BALF is entirely mediated by Nlrc4-dependent propeptide processing. By comparison, it does appear that the full induction of iNOS and production of NO by PA-stimulated PM requires the cooperative effects of both TLR5 and Nlrc4. These PRRs have been independently shown to contribute to the induction of iNOS, and recent data indicate that this induction requires caspase-1 [46].

The differences in mortality observed were not attributable to differential lung injury responses, as we observed similar increases in BAL albumins at both 6 and 18 h. An interesting observation was that reduced lung injury was observed in Nlrc4-deficient mice after PA ad-

ministration, as compared to WT, TLR5^{-/-} or TLR5/Nlrc4^{-/-} mice. Indeed, Nlrc4^{-/-} mice were protected from the lethal effects of PA when challenged with an LD₈₀ inoculum of PA [unpublished observation]. These findings are in line with recent observations by Cohen and Prince [39], who found protection from lung injury and reduced mortality in Nlrc4-deficient mice after i.t. challenge with a cytotoxic PA strain, an effect which was attributed to reduced secretion of biologically active IL-1 β and IL-18. Conversely, activation of TLR5 has been shown to prevent alveolar epithelial cell apoptosis and high permeability edema in models of both infectious and noninfectious lung injury [47, 48]. The lung injury response found in our double-mutant mice mimics that observed in TLR5^{-/-} single-mutant mice, suggesting the phenotype observed is dictated by the TLR5-mediated effects, presumably on alveolar epithelium.

The mechanism accounting for impaired lung bacterial clearance in TLR5/Nlrc4^{-/-} mice has not been precisely defined. There was a substantial reduction in IL-1 β and IL-18 in double-deficient mice. However, the defect in these cytokines was of similar magnitude in Nlrc4^{-/-} mice, which demonstrated no defect in clearance responses. Moreover, we were unable to restore immunity in TLR5/Nlrc4^{-/-} double-deficient mice by the reconstitution of IL-1 β or IL-18 alone or in combination (500 ng i.t. or i.p.; data not shown). We also observed reduced KC/CXCL1 expression in mutant mice, but this defect was not greater in double-mutant mice as compared to their single-mutant counterparts, and for unclear reasons reduced chemokine production was not associated with impaired polymorphonuclear neutrophils (PMN) or total cell influx at either 6 or 18 h after PA (fig. 3). The one effector molecule that was more substantially reduced in PM from TLR5/Nlrc4^{-/-} mice was NO, which was due, in part, to reduced expression of iNOS. Nitric oxide is a well-described antimicrobial molecule against bacterial pathogens, including Gram-negative organisms, and we have shown that inhibition of NO production can worsen lung bacterial clearance in experimental murine *K. pneumoniae* pneumonia in vivo [42]. While a defect in NO production in PM-conditioned media from double-knockout mice was noted in vitro, we were unable to confirm this in vivo, as NO could not be detected in BALF from either control or infected WT or mutant mice (data not shown).

Our finding of unaltered bacterial clearance in Nlrc4^{-/-} mice infected with PA is consistent with the observation of others [39]. In contrast, mice deficient in Nlrc4 are more susceptible to another Gram-negative organism, *K. pneumoniae* [40]. The pathogen-specific

differences are likely accounted for by the more virulent nature of *K. pneumoniae*, which is a heavily encapsulated organism that requires a fully intact innate immune response, including both PMN, alveolar and recruited macrophages for optimal clearance. Conversely, PA is an unencapsulated, less virulent organism that is generally cleared by resident lung macrophages unless multiple defects in immune mechanisms are disrupted. Regardless, our findings indicate a largely redundant role for TLR5

and Nlrc4 in lung mucosal innate immunity against PA, and only in the absence of both does one observe a defect in host immunity.

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