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Lung epithelial MyD88 drives early pulmonary clearance of *Pseudomonas aeruginosa* by a flagellin dependent mechanism

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Submitted 23 February 2016; accepted in final form 8 June 2016

Anas AA, van Lieshout MH, Claushuis TA, de Vos AF, Florquin S, de Boer OJ, Hou B, van’t Veer C, van der Poll T. Lung epithelial MyD88 drives early pulmonary clearance of *Pseudomonas aeruginosa* by a flagellin dependent mechanism. *Am J Physiol Lung Cell Mol Physiol* 311: L219–L228, 2016. First published June 10, 2016; doi:10.1152/ajplung.00078.2016.—*Pseudomonas aeruginosa* is a flagellated pathogen frequently causing pneumonia in hospitalized patients and sufferers of chronic lung disease. Here we investigated the role of the common Toll-like receptor (TLR) adaptor myeloid differentiation factor (MyD)88 in myeloid vs. lung epithelial cells in clearance of *P. aeruginosa* from the airways. Mice deficient for MyD88 in lung epithelial cells (Sftpccre-MyD88-lox mice) or myeloid cells (LysMcre-MyD88-lox mice) and bone marrow chimeric mice deficient for TLR5 (the receptor recognizing *Pseudomonas* flagellin) in either parenchymal or hematopoietic cells were infected with *P. aeruginosa* via the airways. Sftpccre-MyD88-lox mice demonstrated a reduced influx of neutrophils into the bronchoalveolar space and an impaired early antibacterial defense after infection with *P. aeruginosa*, whereas the response of LysMcre-MyD88-lox mice did not differ from control mice. The immune-enhancing role of epithelial MyD88 was dependent on recognition of pathogen-derived flagellin by epithelial TLR5, as demonstrated by an unaltered clearance of mutant *P. aeruginosa* lacking flagellin from the lungs of Sftpccre-MyD88-lox mice and an impaired bacterial clearance in bone marrow chimeric mice lacking TLR5 in parenchymal cells. These data indicate that early clearance of *P. aeruginosa* from the airways is dependent on flagellin-TLR5-MyD88-dependent signaling in respiratory epithelial cells.

MyD88; *Pseudomonas*; lung epithelial; TLR; pneumonia

*Pseudomonas aeruginosa* pneumonia frequently occurs in hospitalized patients and is associated with high mortality rates and substantial financial costs (3, 12). In addition, *Pseudomonas* often colonizes the airways of patients suffering from chronic lung diseases such as cystic fibrosis, chronic obstructive pulmonary disease, and bronchiectasis. Colonization by *Pseudomonas* induces chronic inflammation and contributes to a further decline in lung function (14, 22). Moreover, antibiotic multiresistance of *Pseudomonas* is an increasing problem (17, 30). Hence, studies on induction of host defense during airway infection by *Pseudomonas* and mechanisms by which this pathogen initiates inflammation are of great importance.

Toll-like receptors (TLRs) occupy a prominent position in the innate immune system by virtue of their capacity to recognize bacterial components (2, 13). *Pseudomonas* possesses ligands for several TLRs, including TLR2 (lipoprotein), TLR4 (lipopolysaccharide, LPS), TLR5 (flagellin), and TLR9 (cytosine-phosphate-guanosine DNA) (16), all of which rely on the common adapter myeloid differentiation factor (MyD)88 for intracellular signaling (2, 13). The importance of TLR-dependent signaling for clearance of this pathogen was illustrated by the strongly impaired defense of MyD88-deficient (MyD88−/−) mice during *Pseudomonas* pneumonia (24, 33, 34). The interplay between TLR2, TLR4, and TLR5 and the redundancy of these receptors during *Pseudomonas* infection have been elegantly demonstrated by experiments in which Tlr2−/−, Tlr4−/−, and Tlr2−/−/Tlr4−/− mice were infected with wild-type (WT) or a flagellin-deficient strain of *P. aeruginosa* (8, 27). In addition, a recent study in TLR5−/− mice demonstrated that TLR5 contributes to the early antibacterial response and the recruitment of neutrophils during *Pseudomonas* pneumonia (19).

Several cell types express TLRs in human and murine lung tissue, most notably airway epithelial cells, neutrophils, and alveolar macrophages (16). Respiratory epithelial cells are assumed to play an important role in the initiation of the host response and the attraction of inflammatory cells when they first encounter a pathogen (23). The importance of MyD88-dependent signaling in nonhematopoietic cells for the induction of an effective innate host response against *Pseudomonas* was demonstrated in a mouse bone marrow (BM) chimera model (9). In accordance, selective expression of MyD88 in lung epithelial cells in otherwise MyD88 deficient mice was sufficient to control bacterial growth, although this effect was largely dependent on MyD88 mediated in IL-1β receptor signaling (18). Additional studies making use of TLR5 BM
chimeras revealed that the expression of TLR5 on residential cells is crucial for the induction of a proinflammatory response to purified flagellin in the lungs (7, 11). However, at present the relative contribution of TLR5-dependent signaling in resident and hematopoietic cells to the innate immune response during infection with a flagellated pathogen is unknown. Therefore, in the present study we aimed to investigate the cell-type specific role of MyD88 in myeloid vs. lung epithelial cells and the role of the interaction between TLR5 and flagellin herein. To this end we performed experiments in myeloid and epithelial cell-specific MyD88-deficient mice by using WT and flagellin-deficient P. aeruginosa, as well as TLR5 BM chimeras.

**METHODS**

*Animals.* Homozygous MyD88*fl/fl* mice (10) were crossed with LysMcre (4) (Jackson Laboratory, Bar Harbor, ME) or Stfpccre mice (20, 21) to generate myeloid (LysMcre-MyD88-lox) and type II lung alveolar epithelial (Stfpccre-MyD88-lox) specific MyD88-deficient mice, respectively. MyD88*fl/fl* Cre-negative littermates were used as controls in all experiments. In studies using TLR5*−/−* mice, generated as described (7), WT C57Bl/6 mice were obtained from Harlan (Horst, The Netherlands) as controls. All genetically modified mice were backcrossed at least eight times to a C57Bl/6 background and age and sex matched when used in experiments. Mice were infected at 9–12 wk of age. The Animal Care and Use Committee of the University of Amsterdam approved all experiments.

*Induction of pneumonia and sampling of organs.* Pneumonia was induced by intranasal inoculation with 5×10⁷ colony-forming units (CFU) of *P. aeruginosa* PA01 or PA01Δflic (8) as described (29, 38). This infectious dose is nonlethal, causing serious discomfort several hours after infection, with a gradual recovery thereafter. After 6 or 24 h of infection, mice were euthanized after injection anesthesia with ketamine/medetomidine and heart puncture as described before (35). For bronchoalveolar lavage (BAL) the trachea was exposed through a midline incision; after cannulation of the trachea and occlusion of the left main bronchus with suture thread, lavage of the right lung was performed by instilling 2×0.3 ml of sterile phosphate-buffered saline; the left lung was preserved for histopathology after fixation in 10% formalin. Lung was homogenized in sterile saline after 6 or 24 h of infection with *P. aeruginosa* strain PA01 via the airways, and lung bacterial loads were compared with those measured in Cre-negative MyD88*fl/fl* littermates at 6 or 24 h thereafter. At 6 h after infection, Stfpccre-MyD88-lox mice had 10- to 100-fold higher bacterial loads in Stfpccre-MyD88-lox mice (Fig. 1, A and B) compared with those measured in Cre-negative MyD88*fl/fl* littermates at 6 or 24 h thereafter. At 6 h after infection, Stfpccre-MyD88-lox mice had 10- to 100-fold higher bacterial burdens in lungs (Fig. 1A) and BALF (Fig. 1C) when compared with littermate controls (*P* < 0.001); the impaired antibacterial defense in Stfpccre-MyD88-lox mice was further illustrated by the fact that 50% (4/8) of these animals had a positive blood culture for *Pseudomonas* vs. none of eight control mice (*P* < 0.05). In contrast, bacterial loads in lung and BALF of LysMcre-MyD88-lox and control mice were similar at this early time point (Fig. 1, A and D), and neither group had positive blood cultures. At 24 h postinfection, lung bacterial loads in Stfpccre-MyD88-lox and LysMcre-MyD88-lox mice were similar to those in their respective littermate control mice (Fig. 1, A and B). These data suggest that epithelial MyD88, but not myeloid MyD88, contributes to an effective early clearance of *Pseudomonas* from the airways.

Stfpccre-MyD88-lox mice have an impaired early pulmonary inflammatory response during *Pseudomonas* infection. The impaired early bacterial clearance in Stfpccre-MyD88-lox
mice at 6 h postinfection coincided with a markedly diminished influx of neutrophils into the bronchoalveolar space of these animals at this early time point, as demonstrated by reduced neutrophil counts in BALF (Fig. 2A, \( P < 0.01 \) vs. control mice). The number of neutrophils in lung tissue did not differ between Sftpccre\textsuperscript{-}\textit{MyD88}\textsuperscript{lox} and control mice, as reflected by similar MPO concentrations in whole lung homogenates (Fig. 2C) and equal numbers of Ly6+ neutrophils in lung tissue slides, quantified by digital image analysis (Fig. 3A, representative pictures in Fig. 3C). In LysMcre\textsuperscript{-}\textit{MyD88}\textsuperscript{lox} mice, BALF neutrophil counts, lung MPO levels, and the number of Ly6+ neutrophils in lung tissue were not altered relative to control animals (Fig. 2, B and D, and Fig. 3, B and D, respectively). These results indicate that epithelial MyD88 is important for a swift influx of neutrophils into the alveolar space during \textit{Pseudomonas} pneumonia, whereas myeloid MyD88 has a limited role herein. The extent of lung pathology, quantified at 6 and 24 h after infection, according to the scoring system described previously (39), was similar in Sftpccre\textsuperscript{-}\textit{MyD88}\textsuperscript{lox} and LysMcre\textsuperscript{-}\textit{MyD88}\textsuperscript{lox} mice when compared with their respective controls (Fig. 4, A–D, and Table 1). Total lung inflammatory scores within the groups were significantly higher after 24 h compared with the scores after 6 h. To obtain insight into the contribution of MyD88 dependent signaling in mice at 6 h postinfection.
myeloid and respiratory epithelial cells to the early release of inflammatory mediators in the lungs, we measured the concentrations of proinflammatory cytokines and chemokines in whole lung homogenates harvested from Sftpc-MyD88-lox, LysMcre-MyD88-lox and control mice 6 h after infection with *Pseudomonas* (Table 2). Sftpc-MyD88-lox mice displayed higher (CXCL1 and IL-6) or unaltered (CXCL2, TNF-α, IL-1β, and G-CSF) lung levels of neutrophil attracting mediators; of all mediators measured, only CCL20 levels were significantly lower in lungs of Sftpc-cre-MyD88-lox mice (*P* < 0.001 compared with controls, Table 2). Remarkably, while mediator levels in lungs of LysMcre-MyD88-lox and control mice were largely similar, the former mouse strain showed reduced TNF-α and IL-1β concentrations (*P* < 0.05 relative to controls). Together these data suggest that epithelial and myeloid MyD88 differentially contribute to proinflammatory mediator release in the lungs, wherein epithelial MyD88 in particular mediates CCL20 release, whereas myeloid MyD88 is important for TNF-α and IL-1β production. Considering the attenuated neutrophil influx into the bronchoalveolar space of Sftpc-MyD88-lox mice, we also measured neutrophil attracting chemokines in BALF of these animals (Table 3); CXC chemokines were either higher (CXCL1) in Sftpc-cre-MyD88-lox mice or similar (CXCL2 and CXCL5) when compared with controls.

*Sftpc-MyD88-lox mice have unremarkable antibacterial response and neutrophil recruitment after infection with flagellin-deficient *Pseudomonas*. We hypothesized that epithelial cell MyD88 might initiate a protective immune response during *Pseudomonas* airway infection by recognition of bacterial flagellin via TLR5. To test this possibility, we infected Sftpc-cre-MyD88-lox and control mice with flagellin-deficient *Pseudomonas* (PAO1ΔfliC) (8). We expected that if flagellin drives TLR-dependent MyD88 activation in respiratory epithelial cells, the impaired host defense of Sftpc-cre-MyD88-lox mice seen after infection with WT *Pseudomonas* PAO1 would not be demonstrable after infection with PAO1ΔfliC. Indeed, bacterial loads were similar in lungs and BALF of Sftpc-cre-MyD88-lox and control mice at 6 h after infection with PAO1ΔfliC (Fig. 5, A and B). In addition, neutrophil numbers in BALF (which were reduced in Sftpc-cre-MyD88-lox mice after infection with WT PAO1, Fig. 2A) did not differ between mouse strains after infection with PAO1ΔfliC (Fig. 5C). Lung MPO concentrations (Fig. 5D) did not differ between Sftpc-cre-MyD88-lox and control mice. Similar to our findings in Sftpc-cre-MyD89-lox mice after infection with WT PAO1, lung cytokine and chemokine levels were similar in the two mouse strains after infection with PAO1ΔfliC, with the exception of CCL20 (Table 4).

**TLR5 expressed by parenchymal cells drives early clearance of *Pseudomonas* from the lungs.** Previous studies have indicated that TLR5 facilitates clearance of *Pseudomonas* from the airways (19). To establish that an interaction between flagellin and epithelial TLR5 drives the clearance of *Pseudomonas* from the airways, we continued with experiments using *TLR5<sup>−/−</sup>* mice. First, we confirmed a beneficial role for TLR5 in antibacterial defense during *Pseudomonas* pneumonia by showing higher bacterial loads in lungs and BALF of *TLR5<sup>−/−</sup>* mice 6
h after infection, when compared with WT mice ($P < 0.01$, Fig. 6, A and B). The number of neutrophils in BALF was similar in both groups (Fig. 6 C). To dissect the contribution of parenchymal (P) and hematopoietic (H) TLR5 in the TLR5-mediated clearance of *Pseudomonas* from the airways, we created BM chimeras for TLR5 according to previously described methods (35, 36). To this end, irradiated WT recipient mice were infused with $^{TLR5}$-/- BM and vice versa, thereby creating WT mice reconstituted with $^{TLR5}$-/- BM (P+/H-) and $^{TLR5}$-/- mice reconstituted with WT BM (P-/H+), as well as two groups transplanted with autologous BM as controls for the BM transfer procedure: WT mice transplanted with...
WT BM (P+/H+) and TLR5−/− mice transplanted with TLR5−/− BM (P−/H−). All mice were infected with P. aeruginosa PA01 and euthanized 6 h later for analyses. The impaired antibacterial defense found in TLR5−/− mice was reproduced: TLR5 P−/H− mice had significantly higher bacterial loads in their lungs and BALF compared with TLR5 P+/H+ mice (P < 0.05 to < 0.01) (Fig. 6, D and E). Clearly, TLR5 expression on parenchymal cells was more important for clearance of Pseudomonas from the respiratory tract than TLR5 expression on hematopoietic cells: TLR5 P−/H− mice had significantly higher bacterial loads in lungs and BALF than TLR5 P+/H+ mice (P < 0.01 and P < 0.05), whereas median lung and BALF CFU counts did not differ between TLR5 P+/H− and TLR5 P+/H+ mice, hinting to an insignificant role for hematopoietic TLR5 in antibacterial defense during P. aeruginosa pneumonia.

DISCUSSION

P. aeruginosa is an important cause of pneumonia in hospitalized patients and those who suffer from chronic lung disease (3, 12). Previous studies have documented the importance of MyD88- and TLR-dependent signaling for the early induction of bacterial clearance during Pseudomonas airway infection (24, 27, 33, 34). In the present study we aimed to identify the role of MyD88-dependent signaling in myeloid cells vs. type II alveolar lung epithelial cells using the Cre-lox system in a model of acute P. aeruginosa pneumonia. We demonstrate that mice with a selective deficiency of MyD88 in lung epithelial cells (but not mice with myeloid specific MyD88 deficiency) have an impaired clearance of P. aeruginosa from the airways. Additional studies provided evidence that epithelial MyD88 drives pulmonary host defense during Pseudomonas pneumonia by TLR5-mediated recognition of flagellin. Indeed, MyD88 expression in lung epithelial cells was dispensable for an adequate immune response during infection with a mutant Pseudomonas strain lacking flagellin, and BM chimeric mice deficient for TLR5 in parenchymal (including epithelial) cells showed a similarly impaired bacterial clearance as epithelial cell MyD88-deficient mice after infection with WT Pseudomonas. Together these data suggest that early MyD88-dependent signaling in lung epithelial cells mediates clearance of Pseudomonas from the airways by a mechanism that depends on the presence of flagellin (expressed by the pathogen) and TLR5 (expressed by the host).

Previously, several reports pointed to a prominent role for parenchymal cells in host defense during acute P. aeruginosa pneumonia, first illustrated in a model of MyD88 BM chimeras (9). MyD88−/− mice transplanted with WT BM showed impaired neutrophil attraction and a delayed bacterial clearance during early stage infection, reproducing the phenotype of WT BM. However, the present study suggests that TLR5 expression on epithelial cells is critical for immediate bacterial clearance and epithelial MyD88 is essential for effective clearance of Pseudomonas from the airways.

Table 1. Total lung inflammation score parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Sftpcre-MyD88-lox</th>
<th>LysMcre-MyD88-lox</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interstitial damage</td>
<td>3 (1)</td>
<td>4 (1)</td>
<td>3 (0.5)</td>
</tr>
<tr>
<td>Endothelialitis</td>
<td>3 (0)</td>
<td>3 (1)</td>
<td>2 (0.5)</td>
</tr>
<tr>
<td>Peribronchitis</td>
<td>2.5 (1)</td>
<td>2.5 (1)</td>
<td>1 (0.5)</td>
</tr>
<tr>
<td>Edema</td>
<td>3 (1)</td>
<td>4 (1)</td>
<td>2 (0.5)</td>
</tr>
<tr>
<td>Thrombus formation</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Pleuritis</td>
<td>1 (0.5)</td>
<td>1.5 (1)</td>
<td>0.5 (1.5)</td>
</tr>
<tr>
<td>Percentage of lung surface affected*</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Total lung inflammation score</td>
<td>13 (3.5)</td>
<td>14 (2.5)</td>
<td>9 (3)</td>
</tr>
</tbody>
</table>

Grading of histology was based on seven different parameters, including interstitial damage, endothelialitis, peribronchitis, edema, thrombus formation, pleuritis, and the percentage of lung surface affected. Each item was scored on a scale of 0–4 by an experienced pathologist blinded for the experimental groups. The scores presented here are the median scores of each group and their interquartile ranges are in parentheses. Scores of each individual item were added up to calculate the total lung inflammation scores. *Percentages of lung surface that was affected as inflammation seen through the microscope were graded as follows: 0% = 0, 1–25% = 1, 26–50% = 2, 51–75% = 3, and 76–100% = 4.

Table 2. Lung cytokine and chemokine levels after infection with P. aeruginosa via the airways of mice deficient for MyD88 in epithelial or myeloid cells

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Control</th>
<th>Sftpcre-MyD88-lox</th>
<th>LysMcre-MyD88-lox</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>2,939 (494)</td>
<td>2,499 (623)</td>
<td>1,951 (294)</td>
</tr>
<tr>
<td>IL-1β</td>
<td>8,200 (994)</td>
<td>6,978 (1,447)</td>
<td>6,902 (1,523)</td>
</tr>
<tr>
<td>IL-6</td>
<td>3,572 (689)</td>
<td>11,566 (1,043)**</td>
<td>4,070 (1,131)</td>
</tr>
<tr>
<td>CXCL1</td>
<td>16,211 (3,316)</td>
<td>49,598 (7,699)**</td>
<td>16,029 (5,692)</td>
</tr>
<tr>
<td>CXCL2</td>
<td>7,280 (716)</td>
<td>5,391 (560)</td>
<td>4,134 (1,208)</td>
</tr>
<tr>
<td>CCL2</td>
<td>4,452 (833)</td>
<td>7,199 (1,006)</td>
<td>3,839 (1,380)</td>
</tr>
<tr>
<td>CCL20</td>
<td>18,306 (1,431)</td>
<td>6,853 (581)**</td>
<td>12,276 (2,915)</td>
</tr>
<tr>
<td>G-CSF</td>
<td>12,496 (1,870)</td>
<td>9,224 (1,836)</td>
<td>8,873 (1,555)</td>
</tr>
</tbody>
</table>

Data are represented as means ± SE of 4–8 mice per group. Lung cytokine and chemokine levels in Sftpcre-MyD88-lox and LysMcre-MyD88-lox mice after P. aeruginosa airway infection. Mice were infected with 5 × 10^6 CFU P. aeruginosa and killed after 6 h. Homogenates were prepared from right lungs. Cytokine and chemokine levels are presented in picograms per milliliter lung homogenate. MyD88, myeloid-differentiation factor 88; P. aeruginosa, Pseudomonas aeruginosa; CFU, colony forming unit; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; IL-6, interleukin-6; CXCL1, chemokine (C-X-C motif) ligand 1; CXCL2, chemokine (C-X-C motif) ligand 2; CCL2, chemokine (C-C motif) ligand 2; CCL20, chemokine (C-C motif) ligand 20; G-CSF, granulocyte colony-stimulating factor; CFU, colony-forming units. *P < 0.05, **P < 0.01, ***P < 0.001 vs. matching control mice.

AJP-Lung Cell Mol Physiol • doi:10.1152/ajplung.00078.2016 • www.ajplung.org
Sftpccre-MyD88-lox mice described here, whereas WT mice transplanted with MyD88 deficient BM were as capable to reduce bacterial loads as control WT chimeras (9). While our experiments were performed using mice with selective deficiency in alveolar type II cells, our results are in accordance with an earlier report in which a different approach was used to study the effect of epithelial MyD88 in response to *P. aeruginosa*; herein MyD88 was overexpressed in CC10 positive (Clara) epithelial cells in otherwise MyD88-deficient mice and Clara epithelial cell-selective MyD88 transgenic mice showed enhanced bacterial clearance and an increased number of migrating neutrophils into the lung after infection with *P. aeruginosa* when compared with complete MyD88−/− mice (18). This protective effect of selective MyD88 overexpression in lung epithelium was due to its role in IL-1 receptor signaling since it could be partially blocked by an IL-1 receptor antagonist (18). However, in this study an unflagellated *P. aeruginosa* strain was used (18), which is of importance since mice deficient for the IL-1 receptor had an improved bacterial clearance and an increased number of neutrophils x 10⁶/ml BALF after infection with *P. aeruginosa* via the airways of epithelial cell MyD88 deficient mice.

### Table 3. CXC chemokine levels in bronchoalveolar lavage fluid of mice deficient for MyD88 in epithelial cells after infection with *P. aeruginosa* via the airways.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>BALF Control</th>
<th>Sftpccre-MyD88-lox</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCL1</td>
<td>2626 (256)</td>
<td>4448 (509)**</td>
</tr>
<tr>
<td>CXCL2</td>
<td>1969 (257)</td>
<td>1905 (184)</td>
</tr>
<tr>
<td>CXCL5</td>
<td>9158 (1128)</td>
<td>7791 (623)</td>
</tr>
</tbody>
</table>

Data are presented in picograms per milliliter BALF as means ± SE; n = 8 mice per group. Control and Sftpccre-MyD88-lox mice were intranasally infected with 5 × 10⁶ CFU *P. aeruginosa*. Six hours after infection, mice were sacrificed, the right lung was lavaged, and cytokine levels were determined in BALF supernatant. MyD88, myeloid-differentiation factor 88; *P. aeruginosa*, *Pseudomonas aeruginosa*; BALF, bronchoalveolar lavage fluid; CFU, colony forming unit; CXCL1, chemokine (C-X-C motif) ligand 1; CXCL2, chemokine (C-X-C motif) ligand 2; CXCL5, chemokine (C-X-C motif) ligand 5. **P < 0.01.

Dependent signaling in lung epithelial cells in early neutrophil recruitment and bacterial clearance during infection with *P. aeruginosa* by a TLR5-flagellin-dependent mechanism. In accordance, airway instillation of *P. aeruginosa* resulted in rapid NF-κB activation in the lungs that was primarily localized to the bronchial epithelium, and NF-κB inhibition reduced neutrophil influx and impaired bacterial clearance (31). Interestingly, while neutrophil numbers in BALF were reduced in Sftpccre-MyD88-lox mice upon infection with *Pseudomonas*, neutrophil counts in lung tissue, determined by MPO levels in whole lung homogenates and quantitative Ly6 staining of lung tissue slides, were not altered, suggesting that epithelial MyD88 contributes to transmigration of neutrophils from the interstitium into the bronchoalveolar space. The reduced neu-

### Table 4. Lung cytokine and chemokine levels after infection with flagellin deficient *P. aeruginosa* via the airways of epithelial cell MyD88 deficient mice.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Lung Control</th>
<th>Sftpccre-MyD88-lox</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>2,691 (475)</td>
<td>2,583 (581)</td>
</tr>
<tr>
<td>IL-1β</td>
<td>9,894 (1,454)</td>
<td>7,752 (1,539)</td>
</tr>
<tr>
<td>IL-6</td>
<td>21,990 (4,709)</td>
<td>27,528 (6,660)</td>
</tr>
<tr>
<td>CCL20</td>
<td>44,694 (7,782)</td>
<td>108,085 (35,964)</td>
</tr>
<tr>
<td>CCL2</td>
<td>8,349 (1,141)</td>
<td>6,729 (581)</td>
</tr>
<tr>
<td>CCL20</td>
<td>8,137 (1,710)</td>
<td>8,902 (1,271)</td>
</tr>
<tr>
<td>CCL21</td>
<td>9,625 (827)</td>
<td>5,349 (238)***</td>
</tr>
<tr>
<td>G-CSF</td>
<td>23,557 (2,480)</td>
<td>19,552 (2,045)</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE of 4–8 mice per group. Lung cytokine and chemokine levels in Sftpccre-MyD88-lox mice after airway infection with a flagellin deficient *P. aeruginosa* strain. Sftpccre-MyD88-lox mice and control mice were infected with 5 × 10⁶ CFU *P. aeruginosa* PA01Δflic and sacrificed after 6 h. Homogenates were prepared from right lungs. Cytokine and chemokine levels are presented in picograms per milliliter lung homogenate. MyD88, myeloid-differentiation factor 88; *P. aeruginosa*, *Pseudomonas aeruginosa*; CFU, colony forming unit; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; IL-6, interleukin-6; CXCL1, chemokine (C-X-C motif) ligand 1; CXCL2, chemokine (C-X-C motif) ligand 2; CXCL5, chemokine (C-X-C motif) ligand 5. ***P < 0.001 vs. matching control mice.

![Fig. 5. Sftpccre-MyD88-lox mice have unremarkable antibacterial response and neutrophil recruitment after infection with flagellin-deficient *Pseudomonas*. Control and Sftpccre-MyD88-lox mice were intranasally infected with 5 × 10⁶ colony-forming units of the unflagellated *Pseudomonas aeruginosa* strain PAO1Δflic. Bacterial loads in lung (A) and bronchoalveolar lavage fluid (B), neutrophil numbers in bronchoalveolar (C), and myeloperoxidase levels in lung (D) 6 h after infection of control (grey bars, n = 8) and Sftpccre-MyD88-lox mice (white bars, n = 8). Data are expressed as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile, and largest observation. Differences between groups were not significant.](http://ajplung.physiology.org/doi/10.1152/ajplung.00078.2016)
phages and epithelial cells in vitro (28) and in vivo (19). Our data confirmed this beneficial role of TLR5 since this strain is also less motile and therefore less virulent (1, 27). Later, the importance of TLR5 for bacterial clearance was shown in the first time, using TLR5 BM chimeras, that parenchymal (most sensitive cells (9)).

Earlier reports have suggested that TLR2, TLR4, and TLR5 have redundant roles in the detection of P. aeruginosa by demonstrating that either the presence of flagellin or LPS is sufficient for efficient bacterial clearance (26, 27). However, in these studies the use of a flagellin-deficient Pseudomonas strain probably concealed the role of TLR5 since this strain is also less motile and therefore less virulent (1, 27). Later, the importance of TLR5 for bacterial clearance was shown in TLR5−/− mice infected with the PAK strain of P. aeruginosa (19). Our data confirmed this beneficial role of TLR5 in airway infection by P. aeruginosa PA01 and further revealed for the first time, using TLR5 BM chimeras, that parenchymal (most likely epithelial) cells mediate this effect. In accordance, lung inflammation induced by purified flagellin relied on TLR5 expression by parenchymal cells (7, 37), and mice pretreated with purified flagellin were protected from infection by 10.220.33.3 on October 13, 2017 http://ajplung.physiology.org/ Downloaded from
Pseudomonas aeruginosa from the airways (5). Together these data suggest that flagellin-triggered induction of NLRC4 inflammasome signaling (ascrbed primarily to the alveolar macrophage) (5) and TLR5 signaling (in epithelial cells) have opposite effects on P. aeruginosa clearance during pneumonia.

The extent of inflammatory responses showed some variation between experiments. This can potentially be explained by modest differences in the genetic background of mouse strains (Sftpccre-Myd88-lox and LysMcre-Myd88-lox mice, while both backcrossed on a C57BL/6 background were bred separately) and/or modest differences in the exact bacterial inoculum. Importantly, however, in all instances littermate controls were used and genetically modified and control mice were always infected at the same time with the exact same bacterial inoculum, allowing for adequate comparison between groups. The bacterial dose used does not cause lethality. In our hands, only very high doses of Pseudomonas are associated with mortality after infection via the airways (10- to 100-fold higher as used here). Considering that our main interest lay in regulation of the early host response to Pseudomonas and its impact on bacterial clearance, and considering restrictions on mortality studies in mice in our country, we did not study the influence of cell-specific Myd88 deficiency on lethality. Of note, an earlier report demonstrated dose-dependent responses in bacterial clearance in TLR5-deficient mice infected with aerosolized P. aeruginosa, wherein TLR5 deficiency was associated with impaired clearance of low but not high bacterial doses (19). As the method of administration differed with that used here (aerosolized vs. intranasal inoculation, respectively), the total amounts of CFUs are difficult to compare.

Previous experiments using human epithelial cells demonstrated that recognition and induction of inflammation of Pseudomonas flagellin is TLR5-Myd88 mediated (43), which is in line with our findings. A regulatory role for inhibitory proteins such as Tollip was also found. Our study is limited in that we did not test whether the lack of response to flagellin-deficient Pseudomonas is due to possible inhibitory effects on TLR5.

In conclusion, we demonstrate here in vivo that early clearance of P. aeruginosa from the airways is dependent on flagellin-TLR5-Myd88-deficient signaling in respiratory epithelial cells. The current results further elaborate insight in the pathophysiology of Pseudomonas pneumonia and may be helpful for the development of therapeutics aimed at specific cell types as an adjunctive therapy to antibiotics, for which this pathogen is increasingly resistant.

ACKNOWLEDGMENTS
We express our gratitude to Anthony L. DeFranco (Department of Microbiology & Immunology, University of California, San Francisco, CA) for providing us with the MyD88<sup>−/−</sup> mice, to Dr. Richard A, Flavell (Yale University School of Medicine, New Haven, CT) for the TLR5<sup>−/−</sup> mice, and to Dr. Brigid Hogan, (Duke University School of Medicine, Durham, NC) for the Sftpccre mice. We thank Dr. Reuben Ramphal (University of Florida, Gainesville, FL) for the P. aeruginosa PAO1ΔflhC and the PAO1 parent strain and Marieke ten Brink and Joost Daalhuisen for expert technical assistance.

GRANTS
A. Anas and M. van Lieshout were supported by a grant from the AMC Graduate School of Medical Sciences.

DISCLOSURES
No conflicts of interest, financial or otherwise are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES
ROLE OF MyD88 IN PSEUDOMONAS PNEUMONIA


