Cell-specific pattern recognition receptor signaling in antibacterial defense
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Lung epithelial MyD88 drives early pulmonary clearance of Pseudomonas aeruginosa by a flagellin dependent mechanism

Submitted

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Abstract

Pseudomonas (P.) aeruginosa is a flagellated pathogen frequently causing pneumonia in hospitalized patients and sufferers of chronic lung disease. Toll-like receptors (TLRs) comprise a family of pattern recognition receptors crucial for induction of innate immunity, including during Pseudomonas infections of the lower respiratory tract. Here we investigated the role of the common TLR adaptor myeloid-differentiation factor (MyD)88 in myeloid versus lung epithelial cells in clearance of P. aeruginosa from the airways. Mice deficient for MyD88 in lung epithelial cells (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, while the response of mice deficient for MyD88 in myeloid cells (LysMcre-Myd88-lox mice) was unremarkable. The immune enhancing role of epithelial MyD88 was dependent on a 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. Together these data indicate that clearance of P. aeruginosa from the airways is dependent on flagellin-TLR5-MyD88 dependent signaling in respiratory epithelial cells.
Lung epithelial MyD88 drives early pulmonary clearance of Pseudomonas aeruginosa by a flagellin dependent mechanism

Introduction

*Pseudomonas (P.) aeruginosa* pneumonia frequently occurs in hospitalized patients and is associated with high mortality rates and substantial financial costs (1, 2). 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 (3, 4). Moreover, antibiotic multiresistance of *Pseudomonas* is an increasing problem (5, 6). 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 (7, 8). *Pseudomonas* possesses ligands for several TLRs, including TLR2 (lipoprotein), TLR4 (lipopolysaccharide, LPS), TLR5 (flagellin) and TLR9 (cytosine-phosphate-guanosine DNA) (9), all of which rely on the common adapter myeloid-differentiation factor (MyD)88 for intracellular signaling (7, 8). 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 (10-12). 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* (13, 14). 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 (15).

Several cell types express TLRs in human and murine lung tissue, most notably airway epithelial cells, neutrophils, and alveolar macrophages (9). 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 (16). The importance of MyD88 dependent signaling in non-hematopoietic cells for the induction of an effective innate host response against *Pseudomonas* was demonstrated in a mouse bone marrow (BM) chimera model (17). 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 (19, 20). 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 versus 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 using WT and flagellin-deficient *P. aeruginosa*, as well as TLR5 BM chimeras.
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Results

Sftpccre-Myd88-lox mice have impaired early bacterial clearance after infection with Pseudomonas via the airways

To investigate the relative contribution of MyD88 dependent signaling in myeloid and respiratory epithelial cells in host defense during Pseudomonas pneumonia, we crossed mice homozygous for the conditional Myd88 flox allele (Myd88\(^{fl/fl}\) mice) (21) with mice expressing Cre under control of the myeloid cell lysozyme M (LysM) promoter (22) (to generate LysMcre-Myd88-lox mice) (23) or the surfactant protein C (SftpC) promoter (24, 25) (to generate Sftpccre-Myd88-lox mice). In two separate experiments Sftpccre-Myd88-lox mice and LysMcre-Myd88-lox were infected with P. aeruginosa (strain PA01, 5 x 10\(^6\) colony forming units (CFU) via the airways, and lung bacterial loads were compared with those measured in Cre negative Myd88\(^{fl/fl}\) littermates at 6 or 24 hours thereafter. At 6 hours after infection Sftpccre-Myd88-lox mice had 10-100 fold higher bacterial burdens in lungs (Figure 1A) and bronchoalveolar lavage fluid (BALF) (Figure 1C) when compared with littermate controls (\(p < 0.001\)); the impaired antibacterial defense in Sftpccre-Myd88-lox mice was further illustrated by the fact that 50% (4/8) of these animals had a positive blood culture for Pseudomonas, versus none of 8 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 (Figure 1B,D) and neither group had positive blood cultures. At 24 hours post infection, lung bacterial loads in Sftpccre-Myd88-lox and LysMcre-Myd88-lox mice were similar to those in their respective littermate control mice (Figure 1A,B). These data suggest that epithelial MyD88, but not myeloid MyD88, contributes to an effective early clearance of Pseudomonas from the airways.

Sftpccre-Myd88-lox mice have an impaired early pulmonary inflammatory response during Pseudomonas infection

The impaired early bacterial clearance in Sftpccre-Myd88-lox mice at 6 hours post infection 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 (Figure 2A, \(p < 0.01\) versus control mice). The number of neutrophils in lung tissue did not differ between Sftpccre-Myd88-lox and control mice, as reflected by similar myeloperoxidase (MPO) concentrations in whole lung homogenates (Figure 2C) and equal numbers of Ly6+ neutrophils in lung tissue slides, quantified by digital image analysis (Figure 2E; representative pictures in Figure 2G). In LysMcre-Myd88-lox mouse BALF neutrophil counts, lung MPO levels and the number of Ly6+ neutrophils in lung tissue were not altered relative to control animals (Figure 2B,D,F,H). These results indicate that epithelial MyD88 is important for a swift influx of neutrophils into the alveolar space during Pseudomonas pneumonia, while myeloid MyD88 has a limited role herein.
Lung epithelial MyD88 drives early pulmonary clearance of Pseudomonas {\em aeruginosa} by a flagellin dependent mechanism

The extent of lung pathology, quantified at 6 and 24 hours after infection according to the scoring system described previously (26) and in the Methods section, was similar in Stfpcre-\text{-}Myd88-lox and LysMcre-\text{-}Myd88-lox mice when compared with their respective controls (Supplementary Figure 1). To obtain insight into the contribution of Myd88 dependent signalling in 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 Stfpccre-\text{-}Myd88-lox, LysMcre-\text{-}Myd88-lox and control mice 6 hours after infection with \emph{Pseudomonas} (Table 1). Stfpccre-Myd88-lox mice displayed higher (CXCL1, IL-6) or unaltered (CXCL2, TNF-\(\alpha\), IL-1\(\beta\), G-CSF) lung levels of neutrophil attracting mediators; of all mediators measured, only CCL20 levels were significantly lower in lungs of Stfpccre-Myd88-lox mice (\(p < 0.001\) compared to controls, table 1). Remarkably, while mediator levels in lungs of LysMcre-\text{-}Myd88-lox and control mice were largely similar, the former mouse strain showed reduced TNF-\(\alpha\) and IL-1\(\beta\) 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-\(\alpha\) and IL-1\(\beta\) production. Considering the attenuated neutrophil influx into the bronchoalveolar space of Stfpccre-Myd88-lox mice we also measured neutrophil attracting chemokines in BALF of these animals (supplementary table 1); CXC chemokines were either higher (CXCL1) in Stfpccre-Myd88-lox mice or similar (CXCL2, CXCL5) when compared with controls.
Table 1: 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>Lung</th>
<th>Control</th>
<th>Stfpccre-Myd88-lox</th>
<th>Control</th>
<th>LysMcre-Myd88-lox</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>2939 (494)</td>
<td>2499 (623)</td>
<td>1951 (294)</td>
<td>489 (192)*</td>
</tr>
<tr>
<td>IL-1β</td>
<td>8200 (994)</td>
<td>6978 (1447)</td>
<td>6902 (1523)</td>
<td>1513 (351)*</td>
</tr>
<tr>
<td>IL-6</td>
<td>3572 (689)</td>
<td>11566 (1043)**</td>
<td>4070 (1131)</td>
<td>4277 (1764)</td>
</tr>
<tr>
<td>CXCL1</td>
<td>16211 (3316)</td>
<td>49598 (7699)**</td>
<td>16029 (5692)</td>
<td>9377 (3386)</td>
</tr>
<tr>
<td>CXCL2</td>
<td>7280 (716)</td>
<td>5391 (560)</td>
<td>6134 (753)</td>
<td>3839 (1380)</td>
</tr>
<tr>
<td>CCL2</td>
<td>4425 (833)</td>
<td>7199 (1006)</td>
<td>3166 (499)</td>
<td>4134 (1208)</td>
</tr>
<tr>
<td>CCL20</td>
<td>18306 (1431)</td>
<td>6853 (581)***</td>
<td>12376 (2915)</td>
<td>8873 (1555)</td>
</tr>
<tr>
<td>G-CSF</td>
<td>12496 (1870)</td>
<td>9224 (1836)</td>
<td>3818 (438)</td>
<td>1749 (581)</td>
</tr>
</tbody>
</table>

Lung cytokine and chemokine levels in Stfpccre-Myd88-lox and LysMcre-Myd88-lox mice after *P. aeruginosa* airway infection. Mice were infected with 5x10^6 CFU *P. aeruginosa* and sacrificed after 6 hours. Homogenates were prepared from right lungs. Cytokine and chemokine levels are presented in pg/ml lung homogenate. Data are mean (SE) of 4-8 mice per group. *p < 0.05, **p < 0.01, ***p < 0.001 vs control mice.
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*Stfpcc-Myd88-lox mice have unremarkable antibacterial response and neutrophil recruitment after infection with flagellin-deficient Pseudomonas*

We argued 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 Stfpccre-Myd88-lox and control mice with flagellin-deficient *Pseudomonas* (PAO1Δ*fliC*) (14). We expected that if flagellin drives TLR dependent MyD88 activation in respiratory epithelial cells, the impaired host defense of Stfpccre-Myd88-lox mice seen after infection with WT *Pseudomonas* PAO1 would not be demonstrable after infection with PAO1Δ*fliC*. Indeed, bacterial loads were similar in lungs and BALF of Stfpccre-Myd88-lox and control mice at 6 hours after infection with PAO1Δ*fliC* (Figure 3A,B). In addition, neutrophil numbers in BALF (which were reduced in Stfpccre-Myd88-lox mice after infection with WT PAO1; Figure 2A) did not differ between mouse strains after infection with PAO1Δ*fliC* (Figure 3C). Lung MPO concentrations (Figure 3D) did not differ between Stfpccre-Myd88-lox and control mice. Similar to our findings in Stfpccre-Myd88-lox mice after infection with WT PAO1, lung cytokine and chemokine levels were similar in the two mouse strains after infection with PAO1Δ*fliC*, with the exception of CCL20 (Table 2).

**Table 2: 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>Lung</th>
<th>Control</th>
<th>Stfpccre-Myd88-lox</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>2691 (475)</td>
<td>2583 (581)</td>
</tr>
<tr>
<td>IL-1β</td>
<td>9894 (1454)</td>
<td>7752 (1539)</td>
</tr>
<tr>
<td>IL-6</td>
<td>21990 (4709)</td>
<td>27528 (6660)</td>
</tr>
<tr>
<td>CXCL1</td>
<td>44694 (7782)</td>
<td>108085 (35964)</td>
</tr>
<tr>
<td>CXCL2</td>
<td>8349 (1141)</td>
<td>6729 (581)</td>
</tr>
<tr>
<td>CCL2</td>
<td>8137 (1710)</td>
<td>8902 (1271)</td>
</tr>
<tr>
<td>CCL20</td>
<td>9625 (827)</td>
<td>5349 (238)**</td>
</tr>
<tr>
<td>G-CSF</td>
<td>23557 (2480)</td>
<td>19552 (2045)</td>
</tr>
</tbody>
</table>

Lung cytokine and chemokine levels in Stfpccre-Myd88-lox mice after airway infection with a flagellin deficient *P. aeruginosa* strain. Stfpccre-Myd88-lox mice and control mice were infected with 5x10⁶ CFU *P. aeruginosa* PAO1Δ*fliC* and sacrificed after 6 hours. Homogenates were prepared from right lungs. Cytokine and chemokine levels are presented in pg/ml lung homogenate. Data are mean (SE) of 4-8 mice per group. *** p < 0.001 vs control mice.
Lung epithelial MyD88 drives early pulmonary clearance of Pseudomonas aeruginosa by a flagellin dependent mechanism

**Figure 3:** Sftpc-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 5x10^6 CFU of the unflagellated *P. aeruginosa* strain PAO1ΔFliC. Bacterial loads in lung (A) and BALF (B), neutrophil numbers in BALF (C) and myeloperoxidase (MPO) levels in lung (D) 6 hours 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.

**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 (15), most likely through recognition of flagellin (27). To further establish that an interaction between flagellin and epithelial TLR5 drives the clearance of *Pseudomonas* from the airways, we continued with experiments using *Tlr5*-/- 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*-/- mice 6 hours after infection, when compared with WT mice (p < 0.01, Figure 4A, B). 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 (23, 28). 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 autologus 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* PAO1 and euthanized 6 hours later for analyses.
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The impaired antibacterial defense found in Tlr5<sup>−/−</sup> mice was reproduced: Tlr5 P-/H- mice had significantly higher bacterial loads in their lungs and BALF compared to Tlr5 P+/H+ mice (p < 0.05 to < 0.01)(Figure 4C,D). 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), while 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.

Figure 4: Parenchymal TLR5 mediates bacterial clearance after infection with Pseudomonas via the airways. Tlr5<sup>−/−</sup> and WT mice were intranasally infected with 5x10<sup>6</sup> CFU P. aeruginosa. Bacterial loads in lung (A) and BALF (B) 6 hours after infection of WT (grey bars) and Tlr5<sup>−/−</sup> mice (white bars). WT (P+) and Tlr5<sup>−/−</sup> (P−) mice were irradiated and injected with WT (H+) or Tlr5<sup>−/−</sup> (H−) bone marrow cells. Six weeks after transplantation, mice were infected with 5x10<sup>6</sup> CFU P. aeruginosa. Bacterial loads in lung (C) and BALF (D) of TLR5 chimeras 6 hours after infection (n = 11–12 per group). Data are expressed as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile, and largest observation. ** p < 0.01 compared to WT mice with Mann-Whitney U test in panels A,B. *p < 0.05, **p < 0.01 vs P+/H+ mice determined with Mann–Whitney U test as a follow-up test on Kruskall–Wallis test in panels C,D.
Discussion

*P. aeruginosa* is an important cause of pneumonia in hospitalized patients and sufferers from chronic lung disease (29, 30). Previous studies have documented the importance of MyD88 and TLR dependent signaling for the early induction of bacterial clearance during *Pseudomonas* airway infection (10-13). In the present study we aimed to identify the role of MyD88 dependent signaling in myeloid cells versus 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 (17). *Myd88^-/-* mice transplanted with WT BM showed impaired neutrophil attraction and a delayed bacterial clearance during early stage infection, reproducing the phenotype of *Stfpccre-Myd88-lox* mice described here, while WT mice transplanted with MyD88 deficient BM were as capable to reduce bacterial loads as control WT chimeras (17). Our results are also in accordance with an earlier report in which MyD88 was over-expressed in CC10 positive (Clara) epithelial cells in otherwise MyD88 deficient mice; these 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). Of note, 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 of a flagellated *Pseudomonas* strain (31, 32). These data signify our current data on the importance of MyD88 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 (33). Interestingly, while neutrophil numbers in BALF were
reduced in Stfpccre-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.

We observed a selective impairment in CCL20 production in Stfpccre-Myd88-lox mice early after infection with either WT or flagellin deficient *P. aeruginosa*. CCL20 was shown to be specifically produced by type II alveolar cells in response to LPS (a TLR4 ligand) (34), which may explain that Stfpccre-Myd88-lox mice still produced less CCL20 in response to a flagellin deficient mutant strain. Of note, CCL20 exerts bactericidal activity towards *Pseudomonas* (35), suggesting that impaired CCL20 production may contribute to the impaired bacterial clearance in Stfpccre-Myd88-lox mice. In contrast, levels of CXCL1 and IL-6 were higher in Stfpccre-Myd88-lox mice; although the production of these cytokines was found to be MyD88 dependent in both alveolar macrophages and epithelial cells in vitro (36) and in *MyD88−/−* mice in vivo (37), the presence of high bacterial loads most likely caused cells other than alveolar type II cells to produce high levels of these inflammatory mediators. Lung IL-1β and TNF-α levels were markedly reduced in LysMcre-Myd88-lox mice early after infection. In accordance, a previous study localized IL-1β production primarily to alveolar macrophages, not epithelial cells, after infection with *Pseudomonas* (38), and data from BM chimeras indicated that the production of IL-1β and TNF-α is induced in a MyD88 dependent way in radiosensitive cells (17).

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 (13, 37). 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 (13, 27). Later, the importance of TLR5 for bacterial clearance was shown in *Tlr5−/−* mice infected with the PAK strain of *P. aeruginosa* (15). 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 (19, 39).

Notably, *Pseudomonas* flagellin not only is sensed by TLR5, but can also activate the NLRC4 inflammasome (40). Unlike *Tlr5−/−* and *Tlr5 P-/H+* mice, *Nlrc4−/−* mice demonstrated enhanced clearance of *Pseudomonas* from the airways (38). Together these data suggest that flagellin triggered induction of NLRC4 inflammasome signaling (ascribed primarily to the alveolar macrophage) (38) and TLR5 signaling (in epithelial cells) have opposite effects on *P. aeruginosa* clearance during pneumonia.

In conclusion, we demonstrate here in vivo that clearance of *P. aeruginosa* from the airways is dependent on flagellin-TLR5-MyD88 dependent 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

Methods

Animals
Homozygous Myd88^{fl/fl} mice (21) were crossed with LysMcre (22) (Jackson Laboratory, Bar Harbor, Maine), or Stfpccre mice (24, 25), 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 (19), WT C57Bl/6 mice were obtained from Harlan (Horst, the Netherlands) as controls. All genetically modified mice were backcrossed at least 8 times to a C57Bl/6 background and age- and sex matched when used in experiments. Mice were infected at 9-12 weeks 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 5x10^6 CFU of P. aeruginosa PAO1 or PAO1ΔfliC (14), as described (41, 42). After 6 or 24 hours of infection mice were euthanized after injection anesthetia with ketamine/medotomidine and heart puncture as described before (28). For BAL the trachea was exposed through a midline incision; after cannulation of the trachea and occlusion of the left main bronchus with suture tread 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 (1:5, weight/vol) using a tissue homogenizer (Biospec Products, Bartlesville, Oklahoma), and CFUs were determined in lung homogenates, BALF and blood from serial dilutions plated on blood agar plates, incubated at 37°C for 16 hours before colonies were counted. Cell counts were determined for each BALF sample in a hemocytometer (Beckman Coulter, Fullerton, CA, USA) and differential cell counts were performed on cytospin preparations stained with Giemsa stain (Diff-Quick; Dade Behring AG, Düdingen, Switzerland). For cytokine and chemokine measurements lung homogenates were lysed in an equal volume of lysis buffer (150mM NaCl, 15mM Tris, 1mM MgCl, 1mM CaCl2, 1% Triton, pH 7.4) with protease inhibitors (Roche Complete Protease Inhibitor cocktail) on ice for 30 minutes and spun down. BALF and lung homogenate supernatants were stored at -20 °C until further analysis.

Assays
TNF-α, IL-6 and CCL2 were measured by using a cytometric bead array multiplex assay (BD Biosciences, San Jose, CA) or ELISA (R&D Systems, Minneapolis, MN). IL-1β, CXCL1, CXCL2, CXCL5, CCL20 and G-CSF were measured by ELISA’s (R&D Systems, Minneapolis, MN); MPO was measured by ELISA from Hycult Biotechnology (Uden, the Netherlands).
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**Bone marrow transplantation**
BM chimeric mice were generated exactly as described (26, 28). Briefly, recipient groups (6 weeks of age) received a lethal total body irradiation of two times 4.5 Gy with three hours between the two doses, using a 137Cs irradiator (CIS Bio International, Gif, France) at a dose rate of 0.5 Gy/min, followed by intravenous injection of 5x10^6 BM cells and 2x10^5 splenocytes (to protect the irradiated recipient mice from immediate infections) isolated from donor animals as described before (26). Engraftment was checked by flow cytometry based on differential expression of CD45.1 and CD45.2 by donor and recipient cells exactly as described (26). BM chimeras were infected at 12 weeks of age and euthanized 6 hours after infection for analyses.

**Histologic examination**
For histologic examination left lungs were harvested and instantly fixed in formalin. After paraffin embedding 5 μm sections were made and stained with hematoxylin and eosin. These lung tissues were scored by a pathologist blinded for experimental groups at a scale of 0 (absent) to 4 (very severe) with respect to the following parameters: interstitial damage, endothelialitis, peri-bronchitis, oedema, thrombus formation and pleuritis and the surface of the lung that was affected by confluent pneumonia, as described (26). Granulocyte immunohistochemical stainings were prepared using a FITC-labeled anti-mouse Ly6-C/G mAb (BD Biosciences, San Jose, CA) exactly as described before (23).

**Statistical analysis**
Data are expressed as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile, and largest observation or as means ± standard error of the mean (tables). For experiments with 2 groups, the Mann–Whitney U test was used to determine statistical significance. For comparisons between more than two groups Kruskall-Wallis test was used as a pretest, followed by Mann Whitney U tests where appropriate. All analyses were done using GraphPad Prism (San Diego, CA). p < 0.05 was considered statistically significant.

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Disclosure

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References


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


Supplementary appendix chapter 6

Supplementary Table 1: 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>BALF</th>
<th>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>

Control and Sftpccre-Myd88-Lox mice were intranasally infected with 5x10^6 CFU *P. aeruginosa*. Six hours after infection, mice were sacrificed, the right lung was lavaged and cytokine levels were determined in BALF supernatant. Data are presented in pg/ml BALF as mean ± SEM. N=8 mice per group. **p < 0.01,

Legend Supplementary figure 1 (see page 122): Lung inflammation in Sftpccre-Myd88-Lox and LysMcre-Myd88-Lox mice during *P. aeruginosa* infection. Control, Sftpccre-Myd88-Lox and LysMcre-Myd88-Lox mice were intranasally infected with 5x10^6 CFU *P. aeruginosa* and sacrificed after 6 or 24 hours. Total lung inflammation score as described in the methods (A, B) 6 and 24 hours after infection of control (grey bars, n =4- 8), Sftpccre-Myd88-Lox (white bars, n = 8) and LysMcre-Myd88-Lox mice (striped bars, n = 4-7 mice). Panels C and D show representative images of H&E staining on lung slides from control, Sftpccre-Myd88-Lox and LysMcre-Myd88-Lox mice 6 and 24 hours after infection, original magnification 20x. Data are expressed as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile, and largest observation.
Supplementary figure 1: Lung inflammation in Sftpccre-Myd88-lox and LysMcre-Myd88-lox mice during *P. aeruginosa* infection. See legend on page 121.