Pneumonia: an investigation of host defence mechanisms
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Platelet activating factor receptor deficient mice have an improved host defense against pneumococcal pneumonia.
Platelet activating factor (PAF) is a phospholipid with pro-inflammatory properties, and binds to a specific receptor (PAFR) expressed on many different cell types. The PAFR is able to bind phosphorylcholine, which is present in PAF but also in the pneumococcal cell wall. Activation of respiratory epithelial cells in vitro results in upregulation of PAFR, which in turn facilitates invasion of *S. pneumoniae*. To determine the role of the PAFR in host defense against pneumococcal pneumonia, PAFR deficient (PAFR−/−) and wild type (Wt) mice were intranasally inoculated with *S. pneumoniae*. PAFR−/− mice were relatively resistant against pneumococcal pneumonia, as indicated by a delayed and reduced mortality, a diminished outgrowth of pneumococci in lungs and a reduced dissemination of the infection (all *P*<0.05 vs. Wt). PAFR+/− mice also displayed less pulmonary inflammation. These data provide evidence that the PAFR is used by *S. pneumoniae* to induce lethal pneumonia.

**Introduction**

Platelet-activating factor is a glycerophospholipid, mainly produced by platelets, endothelial cells, macrophages and neutrophils, that plays an important role in the orchestration of different inflammatory reactions.  

The biological activity of PAF is mediated through a specific G-protein-linked receptor expressed on different cell types, including neutrophils, monocytes, macrophages and epithelial cells. Via this receptor, PAF exerts several immunomodulatory actions involved in host defense against bacterial infections, among which stimulation of migration and degranulation of granulocytes, monocytes and macrophages, and the release of cytokines and toxic oxygen metabolites.

The PAFR has been implicated to play a crucial role in the pathogenesis of pneumococcal disease. The biological activity of PAF is mainly determined by phosphorylcholine (PC) that binds specifically to the PAFR; PC is also a prominent part of the cell wall of *S. pneumoniae*. Activation of endothelial or epithelial cells results in upregulation of the PAFR at their surface, which in turn facilitates invasion by *S. pneumoniae* via an interaction between the PAFR and the PC component of the pneumococcal cell wall. The *in vivo* relevance of the pneumococcal PC-PAFR interaction is supported by several findings. First, administration of either a PAFR antagonist or an anti-PC antibody reduced the leukocytosis and protein concentrations in the cerebrospinal fluid of rabbits intracisternally injected with *S. pneumoniae*. Second, administration of a PAFR antagonist also reduced the recruitment of leukocytes and the rise in protein concentrations in bronchoalveolar lavage fluid (BALF) of rabbits challenged with killed *S. pneumoniae* intratracheally. Third, the combined intratracheal administration of live *S. pneumoniae* and a PAFR antagonist to rabbits resulted in reduced bacterial loads in BALF obtained up to 48h post-infection, when compared to BALF from animals given pneumococci only. A recent study, however, reported enhanced bacterial outgrowth after intravenous treatment with a PAFR antagonist in a mouse model of pneumococcal pneumonia.

The objective of the present study was to obtain more insight into the role of the PAFR in...
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the pathogenesis of pneumococcal pneumonia. For this purpose, we compared host responses in PAFR gene deficient (PAFR\textsuperscript{−/−}) and normal wild type (Wt) mice after intranasal infection with live \textit{S. pneumoniae}.

Material and Methods

\textit{Animals.} Ten to twelve week old male mice with a targeted deletion in the gene for PAFR, resulting in a complete deficiency of PAFR (PAFR\textsuperscript{−/−}), were generated as previously described,\textsuperscript{11} and backcrossed seven times to a C57BL/6 background. Wt type C57BL/6 mice were obtained from Harlan Sprague Dawley Inc. (Horst, the Netherlands). All experiments were approved by the Institutional Animal Care and Use Committee of the Academic Medical Center, the Netherlands.

\textit{Induction of pneumonia.} Pneumococcal pneumonia was induced as described previously\textsuperscript{12,13} In brief, \textit{S. pneumoniae}, serotype 3, obtained from American Type Culture Collection (ATCC 6303; Rockville, MD), were grown for 6 hours to midlogarithmic phase at 37°C using Todd-Hewitt broth (Difco, Detroit, MI), harvested by centrifugation at 1500 x g for 15 minutes, and washed twice in sterile isotonic saline. Bacteria were then resuspended in sterile isotonic saline at approximately 1 x 10\textsuperscript{7} colony forming units (CFU)/ml, as determined by plating serial 10-fold dilutions on sheep-blood agar plates. Mice were lightly anesthetized by inhalation of isoflurane (Abott, Queensborough, Kent, UK), and 50 μl of bacterial suspension was inoculated intranasally, corresponding with 5 x 10\textsuperscript{5} CFU \textit{S. pneumoniae}.

\textit{Preparation of lung homogenates.} At 24 or 42h after inoculation mice were anesthetized by intraperitoneal injection with Hypnorm\textsuperscript{®} (Janssen Pharmaceutica, Beerse, Belgium) and midazolam (Roche, Mijdrecht, the Netherlands), and blood was collected from the inferior caval vene. Whole lungs were harvested and homogenized at 4°C in 5 volumes of sterile isotonic saline with a tissue homogenizer (Biospect Products, Bartlesville, OK) which was carefully cleaned and disinfected with 70% ethanol after each homogenization. Serial 10-fold dilutions in sterile saline were made from these homogenates (and blood), and 50 μl volumes were plated onto sheep-blood agar plates and incubated at 37°C. CFU were counted after 16 hours. For cytokine measurements lung homogenates were lysed in lysisbuffer (300 mM NaCl, 15 mM Tris, 2 mM MgCl, 2 mM Triton (X-100), Pepstatin A, Leupeptin, Aprotinin (20 ng/ml), pH 7.4) and spun at 1500 x g at 4°C for 15 minutes; the supernatant was frozen at -20°C until cytokine measurement.

\textit{Bronchoalveolar lavage.} The trachea was exposed through a midline incision and cannulated with a sterile 22-gauge Abbcath-T catheter (Abzott, Sligo, Ireland). Bronchoalveolar lavage (BAL) was performed by instilling two 0.5 ml aliquots of sterile isotonic saline. 0.9-1 ml of lavage fluid was retrieved per mouse, and total cell numbers were counted from each sample in a hemocytometer. BALF differential cell counts were determined on cytopsin preparations stained with modified Giemsa stain (Diff-Quick; Baxter, McGraw Park, Ill).
Histological examination. After 24h fixation of lungs in 10% formaline and embedding in paraffin, 4 μm thick sections were stained with hematoxylin and eosin. All slides were coded and scored by a pathologist without knowledge of the genotype of the mice.

Assays. Cytokine and chemokine levels were measured by using commercially available ELISAs, in accordance with the manufacturers recommendations: tumor necrosis factor-α (TNF), interleukin (IL)-6 (Pharmingen, San Diego, CA), IL-1β, macrophage inflammatory protein 2 (MIP-2) and KC (all R&D systems, Abingdon, United Kingdom). Lowest detection limits were 150 pg/ml for TNF and IL-1β, 75 pg/ml for IL-6, 47 pg/ml for MIP-2 and 12 pg/ml for KC.

Statistical analysis. Data are expressed as mean ± SEM, unless indicated otherwise. Comparisons between groups were conducted using the Mann Whitney U test. Survival curves were compared by log-rank test. P-value <0.05 was considered to represent a statistically significant difference.

Results

PAFR−/− mice are protected against pneumococcal pneumonia. To investigate the involvement of the PAFR in the outcome of pneumococcal pneumonia, PAFR−/− and Wt mice were intranasally infected with 5 x 10^5 CFU S. pneumoniae and followed for 10 days. All Wt mice died within 85h after induction of pneumonia. Mortality was delayed and reduced among PAFR−/− mice, of which 21% survived until the end of the 10-day observation period (P<0.0001 for the difference between both mouse strains; Figure 1).

Figure 1. Enhanced survival in PAFR−/− mice.
Survival after intranasal inoculation with S. pneumoniae in Wt (open circles) and PAFR−/− mice (closed squares). Mortality was assessed twice daily for 10 days. *P<0.05 vs. Wt mice. N = 14 per group.

PAFR−/− mice display a reduced outgrowth of pneumococci. To obtain insight into the role of the PAFR in early antibacterial defense during pneumococcal pneumonia, we assessed the number of viable bacteria in the lungs 24 and 42h after infection, i.e. at time points prior to the occurrence of the first deaths. At both time points, the numbers of CFUs recovered from the lungs of PAFR−/− mice were significantly lower than those from Wt mice (P<0.05; Figure 2). Blood cultures were positive in 71% of the Wt mice and in 14% of the PAFR−/− mice 24h after inoculation (P=0.03). After 42h 83% of the bloodcultures in Wt mice and 50% of the PAFR−/− mice were positive (not significant).
Figure 2. Decreased *S. pneumoniae* CFUs in lungs of PAFR<sup>−/−</sup> mice. Pneumococci in lungs of Wt (open bars) and PAFR<sup>−/−</sup> mice (closed bars) 24 (A) and 42h (B) after inoculation with *S. pneumoniae*. Data are mean ± SEM. N = 7 per group. *P<0.05 vs. Wt mice.

**PAFR<sup>−/−</sup> mice have an unaltered influx of neutrophils into the lungs during pneumococcal pneumonia.** Neutrophils play a prominent role in host defense against bacterial pneumonia. Since inhibition of PAFR function has been shown to reduce leukocyte influx into the lungs in response to intrapulmonary delivery of killed pneumococci, we assessed the number of neutrophils recruited to the alveoli. No difference was seen in the number of neutrophils in BALF from Wt and PAFR<sup>−/−</sup> mice at 42h after inoculation with *S. pneumoniae* (Figure 3).

Figure 3. PAFR<sup>−/−</sup> does not influence neutrophil recruitment into alveoli during pneumococcal pneumonia. Mean and SEM granulocyte influx in BALF 42h after intranasal inoculation of *S. pneumoniae* in Wt and PAFR<sup>−/−</sup> mice. *P<0.05 vs. Wt mice. N = 8 per group.

**Histology.** At 42h after infection, lungs of Wt mice displayed heavy inflammatory infiltrates characterized by endothelialitis, peribronchial inflammation, and pleuritis. Lung inflammation was clearly less pronounced in PAFR<sup>−/−</sup> mice (Figure 4).

**Lung cytokine and chemokine concentrations during pneumococcal pneumonia.** Cytokines and chemokines are pivotal mediators of an adequate host response to bacterial infection of the respiratory tract. Therefore, we investigated whether the improved outcome of PAFR<sup>−/−</sup> mice was associated with a favourable shift in cytokine or chemokine production by measuring the concentrations of TNF, IL-1β, IL-6, KC and MIP-2 in lung homogenates. However, at 24h after the induction of pneumonia, the pulmonary levels of
these protective mediators were lower in PAFR−/− mice than in Wt mice (all P<0.05), whereas at 42h all levels were similar in both strains (Table 1).

![Figure 4. Histopathology.](image)

Lungs demonstrating heavy inflammatory infiltrates characterized by endothelialitis, peribronchial inflammation, and pleuritis (42h post-infection). Lung inflammation was clearly less pronounced in PAFR−/− mice (B) compared to Wt mice (A). Representative slides are shown. HE staining magnification x 33.

**Table 1. Cytokine and chemokine concentrations in lung homogenates**

<table>
<thead>
<tr>
<th>Cytokine/chemokine (ng/ml lung homogenates)</th>
<th>24h after inoculation</th>
<th>42h after inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wt</td>
<td>PAFR−/−</td>
</tr>
<tr>
<td>TNF</td>
<td>2.1 ± 0.4</td>
<td>0.9 ± 0.2*</td>
</tr>
<tr>
<td>IL-1β</td>
<td>8 ± 0.8</td>
<td>3.3 ± 1.1*</td>
</tr>
<tr>
<td>IL-6</td>
<td>5.3 ± 0.6</td>
<td>1.7 ± 0.7*</td>
</tr>
<tr>
<td>KC</td>
<td>8.8 ± 0.5</td>
<td>5.8 ± 0.6*</td>
</tr>
<tr>
<td>MIP-2</td>
<td>7.0 ± 1.5</td>
<td>4.3 ± 0.6*</td>
</tr>
</tbody>
</table>

Data are mean ± SEM of 8 mice per group, 24 and 42h after inoculation with *S. pneumoniae* CFU. *P<0.05 vs. Wt mice.

**Discussion**

*S. pneumoniae* is the most frequently isolated pathogen in community acquired pneumonia. In the United States alone, more than half a million cases of pneumococcal pneumonia are reported each year, with a fatality rate of 5-7%. In recent sepsis trials, *S. pneumoniae* emerged as important causative pathogen especially in the context of pneumonia. The mortality rate of 40,000 per year caused by *S. pneumoniae* in the United States is larger than the mortality rate caused by any other bacterial pathogen. Together with the fact that infections caused by...
S. pneumoniae are increasingly difficult to treat due to the emergence of antibiotic resistant strains, it is clear that respiratory tract infection by S. pneumoniae represents a major health care problem. Fundamental research has elucidated an important mechanism by which the pneumococcus interacts with cells lining the respiratory tract to cause tissue invasion. In particular, the PC component that prominently features in the pneumococcal cell wall, specifically binds to the PAFR expressed on respiratory epithelial cells, which facilitates bacterial entry into these cells. We here provide compelling evidence that this mechanism is important for the virulence of pneumococci during respiratory tract infection in vivo. Using mice with a targeted deletion of the PAFR gene, we demonstrated that the PAFR is used by S. pneumoniae to induce lethal pneumonia, as reflected by a strongly reduced mortality, an attenuated bacterial outgrowth in the lungs and a diminished dissemination of the infection in PAFR−/− mice.

The favourable outcome of PAFR−/− mice can not be explained by an enhanced innate immune response to S. pneumoniae. Indeed, the local levels of protective cytokines and chemokines even were lower in PAFR−/− mice early after the infection, suggesting that the initiation of the production of these mediators at least in part depends on the early interaction between the pneumococcus and the PAFR. Alternatively, the absence of PAF signalling itself may have contributed to this finding, since PAF inhibition has been found to attenuate the production of cytokines, especially of TNF, induced by LPS. Similarly, the attenuated inflammatory response in lung tissue of PAFR−/− mice can be explained by either the absence of an interaction between pneumococcal PC and the PAFR, and/or the absence of endogenous PAF activity. In addition, the absence of an enhanced innate immune response in PAFR−/− mice was further supported by the fact that the number of neutrophils in BALF tended to be lower in BALF of these animals. These data are in line with earlier investigations, demonstrating that the local administration of a PAFR antagonist diminished the leukocytosis and increased protein concentrations in the cerebrospinal fluid and BALF of rabbits given S. pneumoniae intracisternally or intratracheally respectively.

To our knowledge, two earlier studies investigated the effect of PAFR antagonists on the outgrowth of pneumococci in models of pneumonia. In the first study, a PAFR antagonist administered intratracheally together with S. pneumoniae reduced the number of CFUs recovered from BALF obtained up to 48h postinfection in rabbits, when compared to BALF from animals given bacteria only. In the second study, mice receiving another PAFR antagonist intravenously had higher bacterial loads in comparison with control animals. The paradoxical results of this letter study remain unexplained, although specific properties of the PAFR antagonist used may have played a role. Nonetheless, the present data obtained with PAFR−/− mice together with earlier data strongly support the hypothesis that the PAFR is used by S. pneumoniae in vivo to cause full-blown pneumonia.

PAF functions as a proinflammatory mediator in models of severe bacterial infection. Indeed, during clinical sepsis circulating levels of PAF were elevated compared to healthy volunteers or non-septic ventilated patients. Inhibition of endogenous PAF activity by
PAFR antagonists attenuated systemic inflammation induced by heat-killed bacteria or bacterial products like lipopolysaccharide (LPS) and lipoteichoic acid. Moreover, intravenous administration of PAF elicited symptoms of shock and organ failure in animals. Interestingly, PAF seems to be of particular importance for the initiation of pulmonary inflammatory responses. High PAF levels were detected in the lung after systemic injection of LPS in rats, and in the broncho-alveolar lavage fluid (BALF) from septic patients. Inhalation of aerosolized PAF provoked inflammatory cell influx in the interstitium and alveoli. Finally, pretreatment with PAFR antagonists strongly diminished the pulmonary changes elicited by systemic or intrapulmonary administration of LPS, including increased pulmonary vascular leak and edema formation. Together, these data suggest that PAF promotes inflammatory responses to bacteria, in particular in the lung. A proinflammatory role for PAF in the pulmonary compartment is further supported by recent findings in PAFR−/− mice, revealing strongly reduced lung injury and respiratory failure induced by acid aspiration. Theoretically, these proinflammatory properties would make PAF a potentially protective mediator during pneumonia. Such a protective role of PAF in host defense against respiratory tract infection indeed was found in a model of pneumonia caused by *K. pneumoniae*, a bacterium that does not express PC, using the same PAFR−/− mice as used here. The current investigation clearly establishes that the absence of the PAFR overshadows this potential PAF mediated increase in antibacterial defense, most likely through a function that is unrelated to its interaction with PAF, i.e. through its interaction with pneumococcal PC.

It has been shown that *S. pneumoniae* needs the PAFR to enter epithelial cells. Indeed, our study confirms this by showing that PAFR−/− mice demonstrate a decreased invasive capacity and improved host defense during the pneumococcal infection. Thus, PAFR antagonism appears to be useful. However, the blockage of the proinflammatory properties of PAF by this strategy, might be detrimental for an acute inflammation.

References


