Improved host defense against pneumococcal pneumonia in platelet-activating factor receptor-deficient mice

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Improved Host Defense against Pneumococcal Pneumonia in Platelet-Activating Factor Receptor–Deficient Mice

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Platelet-activating factor (PAF) is a phospholipid with proinflammatory properties that binds to a specific receptor (PAF receptor [PAFR]) that is expressed on many different cell types. PAFR is able to bind phosphorylcholine, which is present in both PAF and the pneumococcal cell wall. Activation of respiratory epithelial cells in vitro results in up-regulation of PAFR, which, in turn, facilitates invasion of Streptococcus pneumoniae. To determine the role of PAFR in host defense against pneumococcal pneumonia, PAFR-deficient (PAFR−/−) and wild-type (wt) mice were inoculated intranasally with S. pneumoniae. PAFR−/− mice were relatively resistant to pneumococcal pneumonia, as indicated by delayed and reduced mortality, diminished outgrowth of pneumococci in lungs, and reduced dissemination of the infection (all P < .05, vs. wt mice). PAFR−/− mice also had less pulmonary inflammation. These data provide evidence that PAFR is used by S. pneumoniae to induce lethal pneumonia.

Platelet-activating factor (PAF) is a glycerophospholipid produced mainly by platelets, endothelial cells, macrophages, and neutrophils that plays an important role in the orchestration of different inflammatory reactions [1–3]. The biological activity of PAF is mediated through a specific G-protein–linked receptor (PAF receptor [PAFRI]) that is expressed on different cell types, including neutrophils, monocytes, macrophages, and epithelial cells. Via PAFR, PAF exerts several immunomodulatory actions involved in host defense against bacterial infections, including stimulation of migration and degranulation of granulocytes, monocytes, and macrophages and the release of cytokines and toxic oxygen metabolites [1–3].

PAF has been thought to play a crucial role in the pathogenesis of pneumococcal disease [4]. The biological activity of PAF is mainly determined by phosphorylcholine (PC), which binds specifically to PAFR [1–3]; PC is also a prominent part of the cell wall of Streptococcus pneumoniae [5]. Activation of endothelial or epithelial cells results in up-regulation of PAFR at their surface, which, in turn, facilitates invasion by S. pneumoniae via an interaction between PAFR and the PC component of the pneumococcal cell wall [6–8]. The relevance in vivo of the interaction between pneumococcal PC and PAFR is supported by several findings. First, administration of either a PAFR antagonist or an anti-PC antibody reduced leukocytosis and protein concentrations in the cerebrospinal fluid of rabbits injected intracisternally with S. pneumoniae [9]. Second, administration of a PAFR antagonist also reduced the recruitment of leukocytes and the increase in protein concentrations in bronchoalveolar lavage (BAL) fluid (BALF) of rabbits challenged intratracheally (int) with killed S. pneumoniae [9]. Third, the combination

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of int administration of live *S. pneumoniae* and a PAFR antagonist to rabbits resulted in reduced bacteria loads in BALF obtained up to 48 h after inoculation, compared with BALF from animals given pneumococci only [6]. A recent study, however, reported enhanced bacterial outgrowth after intravenous treatment with a PAFR antagonist in a mouse model of pneumococcal pneumonia [10].

The objective of the present study was to obtain more insight into the role of PAFR in the pathogenesis of pneumococcal pneumonia. For this purpose, we compared host responses in PAFR-deficient (PAFR<sup>−/−</sup>) and normal wild-type (wt) mice after intranasal (inl) inoculation with live *S. pneumoniae*.

**MATERIALS AND METHODS**

**Animals.** PAFR<sup>−/−</sup> mice were generated in Japan, as described elsewhere [11], and were shipped to the animal facility of the Academic Medical Center in Amsterdam in 1999 (i.e., 3 years before the experiments were conducted). Hence, all PAFR<sup>−/−</sup> mice used in the present study were born in Amsterdam. PAFR<sup>−/−</sup> mice were backcrossed 7 times to a C57BL/6 background, making them 99.6% pure C57BL/6. wt C57BL/6 mice were obtained from Harlan Sprague Dawley. Both PAFR<sup>−/−</sup> and wt mice were specific pathogen free. All experiments were conducted with 10–12-week-old male mice. Fighting between mice did not occur during the studies described. All experiments were approved by the Institutional Animal Care and Use Committee of the Academic Medical Center.

**Induction of pneumonia.** Pneumococcal pneumonia was induced as described elsewhere [12, 13]. In brief, *S. pneumoniae* serotype 3 (ATCC 6303) were grown in Todd-Hewitt broth (Difco) for 6 h to mid-logarithmic phase at 37°C, harvested by centrifugation at 1500 g for 15 min, and washed twice in sterile isotonic saline. Bacteria were then resuspended in sterile isotonic saline at a concentration of ∼1 × 10<sup>7</sup> cfu/mL, as determined by plating serial 10-fold dilutions on sheep’s blood agar plates. Mice were lightly anesthetized by inhalation of isoflurane (Abbott), and 50 μL of bacterial suspension was inoculated inl, corresponding with 5 × 10<sup>5</sup> cfu of *S. pneumoniae*.

**Preparation of lung homogenates.** At 24 or 48 h after inoculation, mice were anesthetized by intraperitoneal injection with Hypnorm (Janssen Pharmaceutica) and midazolam (Roche), and blood was obtained from the inferior caval vein. Whole lungs were harvested and homogenized at 4°C in 5 volumes of sterile isotonic saline by use of a tissue homogenizer (Biospect Products), which was carefully cleaned and disinfected with 70% ethanol after each homogenization. Serial 10-fold dilutions in sterile saline were made from these lung homogenates and from blood, and 50-μL volumes were plated onto sheep’s blood agar plates and incubated at 37°C. Colony-forming units were counted after 16 h. For cytokine measurement, lung homogenates were lysed in lysis buffer (300 mmol/L NaCl, 15 mmol/L Tris, 2 mmol/L MgCl<sub>2</sub>, 2 mmol/L Triton X-100, and 20 ng/mL pepstatin A, leupeptin, and aprotinin [pH 7.4]) and spun at 1500 g at 4°C for 15 min; the supernatant was frozen at −20°C until cytokine measurement.

**BAL.** The trachea was exposed through a midline incision and was cannulated with a sterile 22-gauge Abbocath-T catheter (Abbott). BAL was performed by instilling two 0.5-mL aliquots of sterile isotonic saline; 0.9–1 mL of BALF was retrieved from each mouse, and total cell numbers were counted from each sample in a hemocytometer (Emergo). BALF differential cell counts were determined on cytospin preparations stained with modified Giemsa stain (Diff-Quick).

**Histologic examination.** After lungs were fixed in 10% formaline and embedded in paraffin for 24 h, 4-μm-thick sections were stained with hematoxylin-eosin. All slides were coded and scored by a pathologist who did not know the genotype of the mice.

**Assays.** Levels of the following cytokines and chemokines were measured by use of commercially available ELISAs, in accordance with the manufacturers’ recommendations: tumor necrosis factor (TNF)–α and interleukin (IL)–6 (Pharmingen) and IL-1β, macrophage inflammatory protein (MIP)–2, and KC (R&D systems). Limits of detection were 150 pg/mL for TNF–α and IL-1β, 75 pg/mL for IL-6, 47 pg/mL for MIP-2, and 12 pg/mL for KC. Protein concentrations were measured in BALF by use of a commercially available assay (Micro Bicinchoninic Acid Protein Assay; Pierce Biotechnology), according to the recommendations of the manufacturer.

**Statistical analysis.** Data are shown as means ± SEM, unless otherwise indicated. Comparisons between groups were conducted by use of the Mann-Whitney U test. Survival curves were compared by log-rank test. *P < .05* was considered to be statistically significant.

**RESULTS**

**Protection against pneumococcal pneumonia in PAFR<sup>−/−</sup> mice.** To investigate the involvement of PAFR in the outcome of pneumococcal pneumonia, PAFR<sup>−/−</sup> and wt mice were infected inl with 5 × 10<sup>5</sup> cfu of *S. pneumoniae* and monitored for 10 days. All wt mice died within 85 h after induction of pneumonia. Mortality was delayed and reduced among PAFR<sup>−/−</sup> mice; 21% survived until the end of the 10-day observation period (*P < .0001*, wt vs. PAFR<sup>−/−</sup> mice; figure 1).

**Reduced outgrowth of pneumococci in PAFR<sup>−/−</sup> mice.** To obtain insight into the role of PAFR in early antibacterial defense during pneumococcal pneumonia, we assessed the number of viable bacteria in the lungs 24 and 42 h after inoculation (i.e., at time points before the occurrence of the first deaths). At both time points, the numbers of colony-forming units recovered from
Role of PAF Receptor in Pneumococcal Pneumonia

• JID 2004:189 (15 February) • 713

Figure 1. Enhanced survival in platelet-activating factor receptor–deficient (PAFR−/−) mice. Survival after intranasal inoculation with Streptococcus pneumoniae in wild-type (wt) (○) and PAFR−/− (■) mice was assessed twice daily for 10 days (n = 14 mice/group). *P < .05, vs. wt mice.

Figure 2. Decreased nos. Streptococcus pneumoniae organisms in lungs of platelet-activating factor receptor–deficient (PAFR−/−) mice. Pneumococci in lungs of wild-type (wt) (white bars) and PAFR−/− (black bars) mice were measured 24 (A) and 42 (B) h after inoculation with S. pneumoniae. Data are mean ± SEM (n = 7 mice/group/time point). *P < .05, vs. wt mice.

the lungs of PAFR−/− mice were significantly lower than those recovered from wt mice (P < .05; figure 2). At 24 h after inoculation, blood cultures were positive for 71% of the wt mice and for 14% of the PAFR−/− mice (P = .03). At 42 h after inoculation, blood cultures were positive for 83% of the wt mice and for 50% of the PAFR−/− mice (P, not significant).

Unaltered neutrophil numbers and protein concentrations in BALF of PAFR−/− mice. Neutrophils play a prominent role in host defense against bacterial pneumonia [14, 15]. Because inhibition of PAFR function has been shown to reduce leukocyte influx into the lungs in response to intrapulmonary delivery of killed pneumococci [9], we assessed the number of neutrophils recruited to the alveoli. At 42 h after inoculation, no difference was seen in the number of neutrophils in BALF from wt and PAFR−/− mice (figure 3). Moreover, protein concentrations measured in BALF at this time point did not differ between PAFR−/− and wt mice (234.3 ± 42.8 and 298.3 ± 68.4 µg/mL, respectively).

Histologic analysis. At 42 h after inoculation, lungs of wt mice displayed heavy inflammatory infiltrates characterized by endothelitis, peribronchial inflammation, and pleuritis. Lung inflammation was clearly less pronounced in PAFR−/− mice (figure 4).

Lung cytokine and chemokine concentrations. Cytokines and chemokines are pivotal mediators of an adequate host response to bacterial infection of the respiratory tract [14, 16]. Therefore, we investigated whether the improved outcome of PAFR−/− mice was associated with a favorable shift in cytokine or chemokine production by measuring the concentrations of TNF-α, IL-1β, IL-6, KC, and MIP-2 in lung homogenates. However, at 24 h after the induction of pneumonia, the pulmonary levels of these protective mediators were lower in PAFR−/− mice than in wt mice (all P < .05), whereas, at 42 h, all levels were similar in both mouse strains (table 1).

DISCUSSION

S. pneumoniae is the most frequently isolated pathogen in community-acquired pneumonia [17]. In the United States alone, >500,000 cases of pneumococcal pneumonia are reported each year, with a fatality rate of 5%–7%. In recent sepsis trials, S. pneumoniae emerged as an important causative pathogen, especially in the context of pneumonia [18]. In the United States, the mortality rate of 40,000 deaths/year caused by S. pneumoniae is higher than that caused by any other bacterial pathogen [19]. Because infections caused by S. pneumoniae are increasingly difficult to treat as a result of 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 PAFR expressed on human respiratory ep-
Figure 3. Mean ± SEM granulocyte influx in bronchoalveolar lavage fluid (BALF) 48 h after intranasal inoculation of *Streptococcus pneumoniae* in wild-type (wt) and platelet-activating factor receptor–deficient (PAFR−/−) mice (*n* = 8 mice/group). PAFR deficiency does not influence recruitment of polymorphonuclear leukocytes (PMNLs) into alveoli during pneumococcal pneumonia. *P* < .05, vs. wt mice.

In addition, the capacity of pneumococci to transcytose to the basal surface of rat and human endothelial cells is dependent on PAFR [7]. Although, to our knowledge, an interaction between pneumococci and the murine PAFR has not been formally demonstrated, here we provide compelling evidence that this mechanism is important for the virulence of pneumococci during murine respiratory tract infection in vivo. Using PAFR−/− mice, we have demonstrated that PAFR is used by *S. pneumoniae* to induce lethal pneumonia, as reflected by greatly reduced mortality, attenuated bacterial outgrowth in the lungs, and diminished dissemination of the infection in PAFR−/− mice.

The favorable outcome of PAFR−/− mice can not be explained by an enhanced innate immune response to *S. pneumoniae*. Indeed, even the local levels of protective cytokines and chemokines were lower in PAFR−/− mice early after the inoculation, suggesting that the initiation of the production of these mediators depends, at least in part, on the early interaction between pneumococci and PAFR. Alternatively, the absence of PAF signaling itself may have contributed to this finding, because inhibition of PAF has been found to attenuate the production of cytokines, especially TNF-α, induced by lipopolysaccharide (LPS) [1–3]. 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 PAFR, the absence of endogenous PAF activity, or the presence of lower bacteria loads in the lungs of PAFR−/− mice, providing a less potent proinflammatory stimulus to the direct environment. Of note, neutrophil influx and protein concentrations in the BALF were similar in PAFR−/− and wt mice, which contradicts the results of earlier investigations, demonstrating that local administration of a PAFR antagonist diminished leukocytosis and increased protein concentrations in the cerebrospinal fluid and BALF of rabbits given *S. pneumoniae* intracisternally or int, respectively [9].

To our knowledge, 2 earlier studies investigated the effect of PAFR antagonists on the outgrowth of pneumococci in models of pneumonia. In the first study [6], a PAFR antagonist administered int together with *S. pneumoniae* reduced the number of colony-forming units recovered from BALF obtained up to 48 h after inoculation in rabbits, compared with BALF from rabbits administered bacteria only. In the second study [10], mice that received another PAFR antagonist intravenously had higher bacteria loads than did control mice. The 2 types of data indicate differences that remain to be explained, although spe-
PAFR antagonist may have played a role. Nonetheless, the present data obtained with PAFR−/− mice, together with earlier data [6, 9], are consistent with the hypothesis that PAFR is used by S. pneumoniae in vivo to cause severe pneumonia.

PAF functions as a proinflammatory mediator in models of severe bacterial infection. Indeed, high PAF levels were detected in the lungs of rats after systemic injection of LPS [20] and in the BALF of patients with sepsis [21]. Inhalation of aerosolized PAF provoked inflammatory cell influx in the interstitium and alveoli [22, 23]. 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 [24–27]. Together, these data suggest that PAF promotes inflammatory responses to bacteria, especially 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 aspiration of acid [28]. Theoretically, these proinflammatory properties would make PAF a potentially protective mediator during pneumonia [14, 16]. Such a protective role of PAF in host defense against respiratory tract infection indeed was found in a model of pneumonia caused by Klebsiella pneumoniae, a bacterium that does not express PC, using the same type of PAFR−/− mice that were used in the present study [29]. The present investigation clearly establishes that the absence of 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). These data may also apply to other pathogens that express PC, although this needs to be investigated in future studies.

It has been shown that S. pneumoniae needs PAFR to enter epithelial cells. Indeed, our study confirms this by showing that PAFR−/− mice are less likely to develop invasive disease and have improved host defense during pneumococcal infection. Thus, PAFR antagonism appears to be protective. However, the blockage of the proinflammatory properties of PAF by this strategy might be detrimental in acute inflammation.

### References


### Table 1. Cytokine and chemokine concentrations in lung homogenates of wild-type (wt) and platelet-activating factor receptor–deficient (PAFR−/−) mice inoculated with Strep- toccoccus pneumoniae.

<table>
<thead>
<tr>
<th>Cytokine/chemokine</th>
<th>24 h after inoculation</th>
<th>42 h after inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wt</td>
<td>PAFR−/−</td>
</tr>
<tr>
<td>TNF-α</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 ± 0.8</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>

**NOTE:** Data are mean ± SEM nanograms of each cytokine or chemokine per milliliter of lung homogenate (n = 8 mice/group). IL, interleukin; MIP, macrophage inflammatory protein; TNF-α, tumor necrosis factor-α.

* P < .05, vs. wt mice.


