Interaction between inflammation, coagulation and fibrinolysis during infection

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Chapter 7

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

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Abstract
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 *Streptococcus 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 < .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 (PAF) is a glycerophospholipid, mainly produced 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 (PAFR) 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 [1-3].

The PAFR has been implicated to play a crucial role in the pathogenesis of pneumococcal disease [4]. The biological activity of PAF is mainly determined by phosphorylcholine (PC) that binds specifically to the PAFR [1-3]; PC is also a prominent part of the cell wall of *Streptococcus (S.) pneumoniae* [5]. 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 [6-8]. 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* [9]. 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 [9]. 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 postinfection, when compared to 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 the PAFR in the pathogenesis of pneumococcal pneumonia. For this purpose, we compared host responses in PAFR gene deficient (PAFR-/-) and normal wild type (Wt) mice after intranasal infection with live *S. pneumoniae*.

**Material and methods**

**Animals**

Mice with a targeted deletion of the PAFR gene (PAFR-/- mice) were generated in Japan as described previously [11]. PAFR-/- mice 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-/- mice used in the current study were born in Amsterdam. PAFR-/- mice were backcrossed seven times to a C57BL/6 background, making them 99.6% pure C57BL/6. Wt C57BL/6 mice were obtained from Harlan Sprague Dawley Inc (Horst, the Netherlands). Both PAFR-/- and Wt mice were specific pathogen free. All experiments were conducted with 10-12 week old male mice. Fighting 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 previously [12, 13]. In brief, *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 10^7 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 10^5 CFU *S. pneumoniae*.

**Preparation of lung homogenates**

At 24 or 48h after inoculation mice were anesthetized by intraperitoneal injection with Hypnorm® (Janssen Pharmaceutica, Beerse, Belgium) and midazolam (Roche, Mijdrecht, the Netherlands), and blood was collected from the inferior caval venae. 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
50μl 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 (20ng/ml), pH 7.4) and spunned at 1500 x g at 4°C for 15 minutes; the supernatant was frozen at -20°C until cytokine measurement.

*Bronchoalveolar lavage*

The trachea was exposed through a midline incision and cannulated with a sterile 22-gauge Abbocath-T catheter (Abbott, 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 cytospin preparations stained with modified Giemsa stain (Diff-Quick; Baxter, McGraw Park, Ill).

*Histologic 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). Detection limits were 150pg/ml (TNF and IL-1β), 75pg/ml (IL-6), 47pg/ml (MIP-2), 12pg/ml (KC). Protein concentrations were measured in BALF by using a commercially available assay (Micro Bicinchoninic Acid Protein Assay; Pierce Biotechnology, Rockford, IL) according to the recommendations of the manufacturer.

*Statistical analysis*

Data are expressed as means ± SEM, unless indicated otherwise. Comparisons between groups were conducted using the Mann Whitney U test. Survival curves were compared by log-rank test. P < .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 survival until the end of the 10-day observation period (P < .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. N=14 per group. * indicates P<0.05 vs. Wt mice.](image)

**Figure 2 Decreased S. pneumoniae CFU’s in lungs of PAFR-/- mice.** Pneumococci in lungs of Wt (open bars) and PAFR-/- mice (closed bars) 24 (A) and 42h (B) after i.n. inoculation with S. pneumoniae. Data are mean ± SEM. N=7 per group per timepoint. * indicates P<0.05 versus Wt mice.

**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 CFU’s recovered from the lungs of PAFR-/- mice were significantly lower than those from Wt mice (P < .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 blood cultures in Wt mice and 50% of the PAFR-/- mice were positive (nonsignificant).
Unaltered neutrophil numbers and protein concentrations in BALF of PAFR−/− mice

Neutrophils play a prominent role in host defense against bacterial pneumonia [14, 15]. Since inhibition of PAFR function has been shown to reduce leukocyte influx into the lungs in response to intrapulmonary delivery of killed pneumoccoci [9], 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−/− mice at 42h after inoculation with S. pneumoniae (Figure 3). Moreover, protein concentrations measured in BALF at this time point did not differ between PAFR−/− and Wt mice.

Histology

At 42 hours 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−/− mice (Figure 4).
Table I

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Cytokine and chemokine concentrations in lung homogenates Data are mean ± SEM of 8 mice per group, 48 h after inoculation with *S. pneumoniae* CFU. *P < 0.05 vs. Wt mice.

**Lung cytokine and chemokine concentrations**

Cytokines and chemokines are pivotal mediators of an adequate host response to bacterial infection of the respiratory tract [Schultz, 2001 #1837; Moore, 2001 #693]. Therefore, we investigated whether the improved outcome of PAFR⁻⁻ 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 < .05), whereas at 42h all levels were similar in both strains (Table 1).

**Discussion**

*S. pneumoniae* is the most frequently isolated pathogen in community-acquired pneumonia [16]. 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 [17]. 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 [18]. 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 human respiratory epithelial cells, which facilitates bacterial entry into these cells [6]. In addition, the capacity of pneumococci to transcytose to the basal surface of rat and human endothelial cells is dependent on the PAFR [7]. Although to our knowledge an interaction between pneumococci and the murine PAFR has not been formally demonstrated, we here provide compelling evidence that this mechanism is important for the virulence of pneumococci during murine 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 signaling 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 [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 the PAFR, and/or the absence of endogenous PAF activity, and/or the presence of lower bacterial 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 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 [9].

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 [6], a PAFR antagonist administered intratracheally together with *S. pneumoniae* reduced the number of CFU’s recovered from BALF obtained up to 48h postinfection in rabbits, when compared to BALF from animals given bacteria only. In the second study [10], mice receiving another PAFR antagonist intravenously had higher bacterial loads in comparison with control animals. The two types of data indicate differences that remain to be explained, although specific properties of the 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 the 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 lung after systemic injection of LPS in rats [19], and in the broncho-alveolar lavage fluid (BALF) from septic patients [20]. Inhalation of aerosolized PAF provoked inflammatory cell influx in the interstitium and alveoli [21, 22]. 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 [23-26]. 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 [27]. Theoretically, these proinflammatory properties would make PAF a potentially protective mediator during pneumonia [Schultz, 2001 #1837; Moore, 2001 #693]. 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 PAFR-/- mice as used here [28]. 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. These data may also apply to other pathogens expressing PC, although clearly this needs to be investigated in future studies.

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

References


