Inflammatory response to viral airway infections and secondary bacterial complications
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Involvement of the platelet activating factor receptor in host defense against \textit{Streptococcus pneumoniae} during postinfluenza pneumonia
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Abstract

Although influenza infection alone may lead to pneumonia, secondary bacterial infections are a much more common cause of pneumonia. *Streptococcus (S.) pneumoniae* is the most frequently isolated causative pathogen during postinfluenza pneumonia. Considering that *S. pneumoniae* utilizes the platelet-activating factor receptor (PAFR) to invade respiratory epithelium and that the PAFR is upregulated during viral infection, we here used PAFR gene deficient (PAFR<sup>−/−</sup>) mice to determine the role of this receptor during postinfluenza pneumococcal pneumonia. Viral clearance was similar in wildtype and PAFR<sup>−/−</sup> mice and influenza virus was completely removed from the lungs at the time mice were inoculated with *S. pneumoniae* (day 14 after influenza infection). PAFR<sup>−/−</sup> mice displayed a significantly reduced bacterial outgrowth in their lungs, a diminished dissemination of the infection and a prolonged survival. Pulmonary levels of IL-10 and KC were significantly lower in PAFR<sup>−/−</sup> mice, whereas IL-6 and TNF-α were only trendwise lower. These data indicate that the pneumococcus uses the PAFR to accomplish severe pneumonia in a host previously exposed to influenza A.
Role of the PAFR during post-influenza pneumonia

Introduction

Although influenza A infection alone may lead to pneumonia, secondary bacterial infections during and shortly after recovery from influenza are much more common causes of pneumonia [1, 2]. Bacteria such as *Staphylococcus aureus* and *Haemophilus influenzae* are known to cause post-influenza pneumonia, but *Streptococcus pneumoniae* is the most prominent pathogen causing secondary bacterial pneumonia in recent decades [1]. Primary infection with this pathogen is usually less severe than secondary infection [3]. Influenza is known to increase adherence of and subsequent colonization with bacterial respiratory pathogens. Bacteria may adhere to the basal membrane after disruption of the airway epithelial layer by the cytopathic effect of the virus [4], but may also bind to specific receptors in the airway epithelium expressed during influenza virus infection [5, 6]. Since the platelet-activating factor receptor [PAFR] has been described to be upregulated during viral infections [7] and since the PAFR is able to bind phosphorylcholine, a cell-wall component of *S. pneumoniae* [8-10], it has been suggested that the PAFR may play a critical role during secondary bacterial pneumonia [5].

The PAFR, a G-protein-coupled receptor, is mainly expressed on macrophages, monocytes, neutrophils and epithelial cells [11-14]. Activation of epithelial cells leads to upregulation of the PAFR at the cell-surface, which facilitates colonization and invasion of *S. pneumoniae* [8, 14]. A recent study by McCullers et al. investigated the potential role of the PAFR in pneumococcal pneumonia following influenza A infection [5]. These authors showed that PAFR blockade during secondary pneumococcal pneumonia does not prevent lethal synergism between influenza virus and *Streptococcus pneumoniae* [5]. Moreover, administration of the PAFR antagonist CV-6209 resulted in enhanced bacterial outgrowth, even in mice with primary pneumococcal pneumonia [5]. These findings contrast with earlier studies reporting that administration of PAFR antagonists reduced pneumococcal outgrowth in rabbits [8,15]. In line, our laboratory recently demonstrated that PAFR gene deficient (PAFR<sup>−/−</sup>) mice display a diminished bacterial outgrowth and a reduced lethality after intranasal infection with *S. pneumoniae* [16]. To obtain further insight in the role of the PAFR during secondary bacterial pneumonia, we inoculated PAFR<sup>−/−</sup> mice and wildtype mice with *S. pneumoniae* on day 14 after influenza virus infection and studied host defense against primary influenza virus infection and secondary *S. pneumoniae* infection.
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Materials and methods

Mice
PAFR<sup>−/−</sup> mice were generated as described [18], and backcrossed 7 times to a C57BL/6 background. Wildtype C57BL/6 mice were obtained from Harlan Sprague Dawley. Pathogen-free 8 week-old female C57Bl/6 mice and PAFR<sup>−/−</sup> mice were maintained at biosafety-level 2 during the experiments. All experiments were approved by the Institutional Animal Care and Use Committee of the Academic Medical Center.

Experimental infection protocol
Influenza A/PR/8/34 (ATCC VR-95; Rockville, MD) was grown on LLC-MK2 cells (RIVM, Bilthoven, Netherlands). Virus was harvested by a freeze/thaw cycle, followed by centrifugation at 680g for 10 minutes. Supernatants were stored in aliquots at -80°C. Titration was performed in LLC-MK2 cells to calculate the median tissue culture infective dose (TCID<sub>50</sub>) of the viral stock [19]. A non-infected cell culture was used for preparation of the control inoculum. None of the stocks were contaminated by other respiratory viruses, i.e. influenza B, human parainfluenza type 1, 2, 3, 4A and 4B, Sendai virus, RSV A and B, rhinovirus, enterovirus, corona virus and adenovirus, as determined by PCR or cell culture. Viral stock and control stock were diluted just before use in phosphate-buffered saline (PBS, pH 7.4). Primary influenza infection and secondary pneumococcal pneumonia were induced according to previously described methods [20, 21]. In brief, mice were anesthetized by inhalation of isoflurane (Abbott Laboratories, Kent, UK) and intranasally inoculated with 10 TCID<sub>50</sub> influenza (1400 viral copies) or control inoculum in a final volume of 50 µl PBS. Pneumococcal pneumonia was induced 14 days after inoculation with influenza or control suspension. <i>S. pneumoniae</i> serotype 3 (ATCC 6303; Rockville, MD) was cultured for 16 hours at 37°C in 5% CO<sub>2</sub> in Todd Hewitt broth. This suspension was diluted 100 times in fresh medium and grown for 5 hours to midlogarithmic phase. Bacteria were harvested by centrifugation at 2750 g for 10 minutes at 4°C and washed twice with ice-cold saline. After the second wash, the bacteria were resuspended in saline and diluted to a concentration of 2 x 10<sup>5</sup> colony forming units (CFU) per ml, which was verified by plating out 10-fold dilutions onto blood-agar plates. Mice were anesthetized by inhalation with isoflurane and were inoculated with 50 µl of the bacterial suspension (10<sup>4</sup> CFU <i>S. pneumoniae</i>).
Determination of viral outgrowth

Viral load was determined on day 8 and 14 after viral infection and 48 hours after pneumococcal infection (i.e. 16 days after viral infection) using real-time quantitative PCR as described [20-22]. Mice (8 per time-point) were anesthetized using 0.3 ml FFM (fentanyl citrate 0.079 mg/ml, fluanisone 2.5 mg/ml, midazolam 1.25 mg/ml in H2O; of this mixture 7.0 ml/kg intraperitoneally) and sacrificed by bleeding out the vena cava inferior. Lungs were harvested and homogenized at 4°C in 4 volumes of sterile saline using a tissue homogenizer (Biospec Products, Bartlesville, OK). Hundred μl of lung homogenates were treated with 1 ml Trizol reagent to extract RNA. RNA was resuspended in 10 μl DEPC-treated water. cDNA synthesis was performed using 1 μl of the RNA-suspension and a random hexamer cDNA synthesis kit (Applera, Foster City, CA). 5 μl out of 25 μl cDNA-suspension was used for amplification in a quantitative real-time PCR reaction (ABI PRISM 7700 Sequence Detector System). The viral load present in each sample was calculated using a standard curve of particle-counted influenza virus (virus particles were counted by electron microscopy), included in every assay. The following primers were used: 5'-GGACTGCAGCTGAGACGCT-3' (forward); 5'-CATCCTGTTGTATATGAGGCCCAT-3' (reverse) and 5'-CTCAGTTATTCTGTGGTGCACTTGCC-3' (5'-FAM labelled probe).

Determination of bacterial outgrowth

Serial 10-fold dilutions of the lung homogenates in sterile saline and 10 μl volumes were plated out onto blood-agar plates. Plates were incubated at 37°C at 5% CO2 and CFUs were counted after 16 hours.

Histopathological analysis

Lungs for histological examination were harvested 48 hours after pneumococcal infection, fixed in 10% formalin and embedded in paraffin. Four μm sections were stained with hematoxylin and eosin and analyzed by a pathologist who was blinded for the groups.

Bronchoalveolar lavage (BAL)

The trachea was exposed through a midline incision and cannulated with a sterile 22-gauge Abbocath-T catheter (Abbott, Sligo, Ireland). BAL was performed by instillation of two 0.5-mL aliquots of sterile saline into the right lung. The retrieved BAL fluid (approximately 0.8 mL) was spun at 260g for 10 min at 4°C and the pellet was resuspended in 0.5 mL sterile PBS. Total cell numbers were counted using a Z2 Coulter Particle Count and Size Analyzer.
(Beckman-Coulter Inc., Miami, FL). Differential cell counts were done on cytospin preparations stained with modified Giemsa stain (Diff-Quick; Baxter, UK).

**Cytokine and chemokine measurements**

Lung homogenates were lysed with an equal volume of lysisbuffer (300 mM NaCl, 30 mM Tris, 2 mM MgCl₂, 2 mM CaCl₂, 1% (v/v) Triton X-100, 20 ng/ml Pepstatin A, 20 ng/ml Leupeptin, 20 ng/ml Aprotinin, pH 7.4) and incubated for 30 minutes on ice, followed by centrifugation at 680 g for 10 minutes. Supernatants were stored at -80°C until further use. Cytokines and chemokines in total lung lysates were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol. Reagents for interleukin (IL)-6, IL-10, cytokine-induced neutrophil chemoattractant (KC) and tumor necrosis factor (TNF)-α measurements were obtained from R&D systems (Abingdon, UK); interferon (IFN)-γ reagents were obtained from Pharmingen (San Diego, CA).

**Statistical analysis**

All data are expressed as mean ± SE, unless stated otherwise. Differences between groups were analysed by Mann-Whitney U test. Survival was analysed with Kaplan-Meier using a log-rank test; p < 0.05 was considered to represent a statistically significant difference.

**Results**

**Primary influenza virus infection**

Viral load in the lungs was measured on day 8 and day 14 after infection to determine the role of the PAFR during primary influenza A infection. Viral load was similar in PAFR⁺/- and wildtype mice on day 8 after infection (Figure 1).

![Figure 1: Viral load in the lungs on day 8 after influenza infection. Viral load is determined in total lung homogenates of wildtype (filled bars) mice and PAFR⁻/⁻ mice (open bars) by real-time PCR (5-8 mice per group) and expressed as RNA copies per gram lungtissue (mean ± SE) as Both wildtype mice and PAFR⁻/⁻ mice had cleared the virus on day 14 after infection (data not shown).](image-url)
On day 14 after infection, both wildtype and PAFR<sup>−/−</sup> mice had cleared the virus completely. These data indicate that PAFR deficiency does not hamper the clearance of influenza A in vivo.

**Figure 2:** Prolonged survival in PAFR<sup>−/−</sup> mice after secondary bacterial pneumonia. Survival after pneumococcal infection in influenza-recovered PAFR<sup>−/−</sup> mice (triangles) versus wildtype mice (squares). All mice (11 mice per group) received 10<sup>4</sup> CFU <i>S. pneumoniae</i> on day 14 after viral infection and were monitored at least twice a day after pneumococcal infection.

**Prolonged survival during secondary pneumococcal infection in PAFR<sup>−/−</sup> mice**

To investigate the role of the PAFR during secondary bacterial pneumonia we inoculated mice with <i>S. pneumoniae</i> on day 14 after influenza infection. Lethality was monitored in PAFR<sup>−/−</sup> and wildtype mice (11 mice per group) during secondary pneumococcal pneumonia at least twice daily (Figure 2). Influenza-recovered PAFR<sup>−/−</sup> mice displayed a prolonged survival after pneumococcal infection (p = 0.015 vs wildtype mice).

**Figure 3:** Reduced bacterial outgrowth in the lungs and blood of PAFR<sup>−/−</sup> mice. Bacterial outgrowth in the lungs (left graph) and blood (right graph) after pneumococcal infection in wildtype mice (squares) and PAFR<sup>−/−</sup> mice (triangles). All mice (7 mice per group) received 10<sup>4</sup> CFU <i>S. pneumoniae</i> on day 14 after viral infection and were sacrificed 48 hours later. Horizontal lines represent medians for each group. Note that 3 PAFR<sup>−/−</sup> mice had no bacteria in their blood 48 hours after infection.
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Bacterial outgrowth

To obtain insight into the role of the PA FR in the outgrowth of pneumococci in lungs previously exposed to influenza A and in the dissemination of bacteria, lungs and blood were cultured 48 hours after infection with *S. pneumoniae*. The number of *S. pneumoniae* CFUs was 3-fold lower in PA FR/− than in wildtype mice (p = 0.05, Figure 3). Moreover, the PA FR played a role in the dissemination of *S. pneumoniae* into the circulation: only 57% of PA FR/− mice had positive blood cultures versus 100% of wild type, and the number of *S. pneumoniae* CFUs in blood of PA FR/− mice was lower than in wildtype mice (p = 0.05, Figure 3).

![Figure 4: Cytokine levels in the lungs after secondary bacterial infection: TNF-α (A), IL-6 (B), IL-10 (C) and KC (D) in wildtype (filled bars) and PA FR/+ mice (open bars) at 48 hours after secondary bacterial pneumonia. Data are expressed in pg/g lung tissue (mean ± SE, 7 mice per group). * p < 0.05 vs wildtype mice.]

Pulmonary cytokine and chemokine concentrations

Cytokines and chemokines play an important role in host defense against bacterial pneumonia [23]. Therefore, to determine the effect of the PA FR on the induction of these mediators, the concentrations of TNF-α, IL-6, IL-10 and KC were measured in lung homogenates (Figure 4). IL-10 and KC levels were significantly lower in PA FR/− mice than in wildtype mice (p < 0.05). Lung levels of TNF-α and IL-6 tended to be lower in PA FR/− mice but the differences with wildtype mice were not statistically significant (p = 0.16 and p = 0.09 respectively).

<table>
<thead>
<tr>
<th>Cells (x 10^3)</th>
<th>Wildtype mice</th>
<th>PA FR+/− mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cell count</td>
<td>1398 ± 411</td>
<td>782 ± 335</td>
</tr>
<tr>
<td>Neutrophils (%)</td>
<td>1046 ± 325 (67.2%)</td>
<td>458 ± 184 (52.5%)</td>
</tr>
<tr>
<td>Macrophages (%)</td>
<td>336 ± 98 (31.5%)</td>
<td>321 ± 164 (46.8%)</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>16.2 ± 9.8 (1.3%)</td>
<td>2.9 ± 1.5 (0.7%)</td>
</tr>
</tbody>
</table>

Leukocyte counts (6 mice per group) are expressed as absolute numbers (x 10^3) and percentage of total cell count. All data are mean ± SE. No statistically significant differences were found between wildtype and PA FR+/− mice 48 hours after secondary pneumococcal pneumonia.
Cell influx in BALF

Neutrophils play a pivotal role in host defense against bacterial pneumonia [23]. Since inhibition of PAFR function has been shown to reduce leukocyte influx into the lungs in response to intrapulmonary delivery of killed pneumococci [15], we assessed the number of leukocytes recruited to the alveoli. Although total cell counts tended to be lower in BALF obtained from PAFR<sup>−/−</sup> mice than in BALF from wildtype mice, this difference was not statistically significant. The relative number of neutrophils was trendwise lower in PAFR<sup>−/−</sup> mice (p = 0.09 vs wildtype mice, Table 1), whereas the relative number of macrophages was trendwise higher in PAFR<sup>−/−</sup> mice (p = 0.09 vs wildtype mice, Table 1).

**Figure 5:** Histopathological analysis. Inflammatory response upon pneumococcal infection in wildtype mice (A) and PAFR<sup>−/−</sup> mice (B) after recovery from influenza virus. Mice received 10<sup>4</sup> CFU S. pneumoniae on day 14 after viral infection and were sacrificed 48 hours later. Lung slides were stained by hematoxylin and eosin (original magnification 33x). Representative slides of 6 mice per group are shown.

**Histopathology**

Forty-eight hours after pneumococcal infection, lungs were harvested to prepare H/E stained lung-slides for histopathological examination. Mice recovered from influenza infection with secondary pneumococcal pneumonia showed severe interstitial inflammation, bronchiolitis, endothelialitis and pleuritis in the lungs. No significant differences were observed between wild-type mice and PAFR<sup>−/−</sup> mice (Figure 5).

**Discussion**

Secondary pneumococcal pneumonia is a serious complication of influenza A infection. The increased susceptibility to secondary bacterial infections during and shortly after influenza is,
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at least in part, due to enhanced colonization and interaction of the respiratory epithelium with *S. pneumoniae* [1-3]. Since the PAFR has been described to be upregulated during viral infections [7] and since the PAFR facilitates invasion of the pneumococcus through epithelial cells through binding of phosphorylcholine in the cell-wall of *S. pneumoniae* [8], it has been suggested that the PAFR may play a critical role during postinfluenza pneumococcal pneumonia [5]. In the present study we show that targeted deletion of the PAFR improves host defense against *S. pneumoniae* in a mouse-model for secondary bacterial pneumonia, as reflected by prolonged survival and reduced bacterial loads in the lungs and the circulation.

Our data are contradictory to a previous study by McCullers et al. [5], who showed that influenza-infected mice treated with CV-6209, a PAFR-antagonist, displayed enhanced outgrowth of pneumococci (72 hours after infection). Although several differences between our investigation and that of McCullers et al [5] can be pointed out, including differences in the *S. pneumoniae* strains used (ATCC 6303, serotype 3 versus D39, serotype 2, respectively) and differences in the interval between influenza and secondary pneumococcal infection (14 versus 7 days respectively), the data reported by McCullers et al. are difficult to explain in the context of current knowledge on the role of the PAFR in pneumococcal infection. Indeed, these authors observed a reduced rather than an increased host defense after administration of a PAFR antagonist during primary pneumococcal pneumonia [5], which contrasts with at least three earlier investigations addressing this topic [8, 15, 16]. Our own laboratory found that PAFR*−/−* mice display enhanced survival and reduced bacterial outgrowth after primary pneumococcal pneumonia [16]. Similarly, intratracheal PAFR antagonist treatment during pneumococcal infection in rabbits resulted in reduced bacterial loads in the lung [8]. Conceivably, the specific properties of the PAFR antagonist used by McCullers et al [5] may have played a role. Hence, our current data seem to indicate that the pneumococcus uses the PAFR to invade the respiratory epithelium of the host previously exposed to influenza A. As such, the involvement of the PAFR in the pathogenesis of primary and postinfluenza pneumococcal pneumonia seems quite similar [16].

In our study an interval of 14 days between viral and bacterial infection was chosen to exclude a direct interaction between influenza virus and *S. pneumoniae*. Previous studies by our group have indicated that influenza virus is completely cleared from the lungs of wildtype mice on day 14 after infection [21], which was confirmed here. The present study establishes that clearance of influenza A is not altered in PAFR*−/−* mice: viral loads were similar in PAFR*−/−*
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and wildtype mice 8 days after infection and both strains had cleared the virus completely on day 14, the day on which pneumococcal pneumonia was induced. These findings not only revealed that the PAFR does not contribute to host defense against influenza A to a significant extent, but also allowed us to use PAFR<sup>−/−</sup> mice to study the role of the PAFR during postinfluenza pneumococcal pneumonia.

The improved outcome observed in PAFR<sup>−/−</sup> mice could also be explained by the release of protective mediators after pneumococcal infection. Pulmonary levels of KC were significantly lower in PAFR<sup>−/−</sup> mice than in wildtype mice in both mouse-strains after secondary bacterial challenge, whereas TNF-α and IL-6 levels were only trendwise lower. These reduced KC levels may at least in part account for the reduced neutrophil numbers in BALF [24, 25]. Alternatively, and not mutually exclusive, the tendency toward a reduced inflammatory response in lungs of PAFR<sup>−/−</sup> mice, which was also observed after primary pneumococcal pneumonia [16], could be the consequence of a lower bacterial load (providing a less potent proinflammatory stimulus) in the lungs [26].

We have previously shown that mice recovered from influenza produce high amounts of cytokines and excessive lung inflammation after induction of secondary pneumococcal pneumonia when compared with mice suffering from primary bacterial infection [21]. Since PAFR activation results in a pro-inflammatory stimulus, one could argue that the enhanced inflammatory reaction is partially due to PAFR expression in mice exposed to influenza. Indeed, the PAFR appears to be particularly important for the induction of pulmonary inflammation. Pretreatment with PAFR antagonists strongly diminished pulmonary vascular leakage and edema after systemic or intrapulmonary injection of lipopolysaccharide [27-29]. Studies by Nagase et al revealed a critical role for the PAFR during acid aspiration in mice [30]. Our current data argue against an important role for the PAFR in the exaggerated lung inflammation in mice with postinfluenza pneumonia, considering that PAFR<sup>−/−</sup> mice only showed a tendency toward a reduced inflammatory response as reflected by trendwise reduced neutrophils in their lungs. Besides, pulmonary levels of the anti-inflammatory cytokine IL-10 were modestly reduced as well. Of note, in theory the proinflammatory properties of PAF would make this phospholipid mediator a potential protective mediator during pneumonia [15]. Indeed, PAFR<sup>−/−</sup> mice displayed enhanced bacterial outgrowth in a mouse-model for *Klebsiella pneumoniae*, a bacterium that
Chapte rr  9
does not express phosphorylcholine [31]. This study supports the importance of phosphorylcholine binding by the PAFR during primary and secondary pneumococcal pneumonia.

*S. pneumoniae* is the main causative pathogen in postinfluenza pneumonia. *In vitro* studies have established that this bacterium can invade tissues by an interaction between phosphorylcholine present in its cell wall and the PAFR expressed by epithelial cells. We here demonstrate that the pneumococcus uses the PAFR to accomplish severe pneumonia in mice previously exposed to influenza. The fact that lethality also occurred in PAFR−/− mice indicates that the PAFR is not mandatory for tissue invasion, but rather that this receptor increased the potential virulence of *S. pneumoniae* in the respiratory tract. As such, the role of the PAFR in primary [16] and secondary (this study) pneumococcal pneumonia does not seem to differ significantly.

**Acknowledgment**

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**References**


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