Mechanisms of immune activation during infection
Branger, J.

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Platelet activating factor receptor deficient mice have an unaltered host defense against non-typeable *Haemophilus influenzae* pneumonia
Abstract

Platelet-activating factor (PAF), a glycerophospholipid with proinflammatory properties, exerts its biological effects by interacting with the PAF receptor (PAFR) expressed on many different cell types. The PAFR specifically binds phosphorylcholine (ChoP), the biologically active component of PAF. ChoP however is also a component of the cell wall of non-typeable Haemophilus influenzae (NTHi). In in vitro experiments, the invasion of respiratory epithelial cells by NTHi was mediated by the PAFR. To determine the role of the PAFR in host defense against pneumonia induced by NTHi, PAFR deficient (PAFR-/-) and normal wild type mice were intranasally inoculated with NTHi. The absence of a functional PAFR was associated with a normal innate immune response as indicated by similar bacterial counts, myeloperoxidase activity, cytokine and chemokine production and inflammation within the pulmonary compartment of PAFR-/- and wild type mice. These data indicate that the PAFR is not important for host defense in NTHi induced pneumonia.
Role of PAFR in non-typeable *H. influenzae* pneumonia

**Introduction**

Platelet-activating factor (PAF) is a glycerophospholipid produced by several cell types including platelets, endothelial cells, macrophages and neutrophils, and plays an important role in the regulation of different inflammatory reactions (1-3). The biological activity of PAF is mediated through a specific G-protein-linked receptor, the PAF receptor (PAFR), which is expressed on different cell types including neutrophils, monocytes, macrophages, endothelial and epithelial cells. By binding to the PAFR, PAF induces several proinflammatory responses 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).

Phosphorylcholine (ChoP), the biological active component of PAF, binds specifically to the PAFR (1-3). ChoP is also a prominent part of the cell wall of several bacteria, including *Streptococcus pneumoniae* (4), and non-typeable *Haemophilus influenzae* (NTHi) (5, 6). In vitro experiments, bacterial cell wall ChoP has been shown to increase the adherence of NTHi to bronchial epithelial cells by binding to the PAFR (7). Furthermore, the PAFR facilitated the invasion of epithelial cells by NTHi, while both adhesion and invasion of epithelial cells by NTHi could be blocked by a PAFR antagonist (7-9). In a chinchilla model of otitis media, the expression of ChoP in NTHi was associated with an increased virulence (10).

PAFR has also been implicated in cell invasion by other bacteria including *Streptococcus pneumoniae* (11-13) and *Actinobacillus actinomycetemcomitans* (14). The relevance of the PAFR was evident in several in vivo models of pneumococcal infection showing an attenuated inflammatory response, reduced bacterial numbers and an improved outcome using PAFR gene deficient (PAFR−/−) mice or PAFR antagonists (15-17).

NTHi is a Gram-negative bacterium that lacks a polysaccharide capsule (‘non-typeable’) in contrast to other *H. influenzae* isolates. NTHi is a commensal organism in the human respiratory tract and an important cause of localized infections such as middle ear infection, sinusitis and conjunctivitis. Furthermore, it is frequently implicated in exacerbations of underlying chronic obstructive pulmonary disease and an important cause of community acquired pneumonia (18-21). The role of the PAFR in pulmonary infections induced by NTHi is unknown. Therefore, in the present study, we sought to determine the role of the PAFR in pneumonia induced by NTHi. PAFR−/− and PAFR+/+ wild type (WT) mice were intranasally
inoculated with NTHi, and bacterial outgrowth and host inflammatory responses during the
course of the infection were assessed.

Materials and Methods

Mice
PAFR-/- mice were generated as described previously (22), backcrossed seven times to a
C57BL/6 background, and bred in the animal facility of the AMC. WT C57BL/6 mice were
obtained from Harlan Sprague Dawley Inc (Horst, the Netherlands). All experiments were
conducted with 9-10 week old female mice. All experiments were approved by the
Institutional Animal Care and Use Committee of the Academic Medical Center, Amsterdam,
the Netherlands.

Induction of pneumonia
Haemophilus influenzae strain 12 (kindly donated by S.J. Barenkamp, St. Louis, MO) is a
clinical isolate that was originally retrieved from the middle ear fluid of a patient with acute
otitis media. The strain was classified as nontypeable based on the absence of agglutination
with typing antisera for H. influenzae types a-f (Burroughs Wellcome) and the failure to
hybridize with pUO38, a plasmid that contains the entire cap b locus (23, 24). The NTHi
strain was stored at -80°C in brain heart infusion (BHI) broth with 20% glycerol. For
preparation of the inoculum, bacteria were streaked from frozen aliquots onto a chocolate
agar plate and incubated overnight at 37°C in a 5% CO₂ incubator. Next, bacteria obtained
from the chocolate agar plate, were grown for 3 hours to midlogarithmic phase in BHI broth
supplemented with 10 μg/ml hemin and 3.5 μg/ml NAD at 37°C (all reagents from Difco,
Detroit, MI). Bacteria were harvested by centrifugation at 1500 x g for 15 minutes and
resuspended in a buffer containing 10 mM Na₂HPO₄, 10 mM KCl, 100 mM NaCl, 2 % w/v
casamino acids (Becton Dickinson, Sparks, MD) and 1.75 mM glucose (pH 7.4) at a
concentration of approximately 2 x 10⁷ colony forming units (CFU) per ml, as determined by
plating serial 10-fold dilutions on chocolate agar plates. Mice were lightly anesthetized by
inhalation of isoflurane (Upjohn, Ede, the Netherlands), and pneumonia was induced by
intranasal inoculation of 50 μl of the bacterial suspension corresponding with 10⁷ CFU of
non-typeable H. influenzae.
Determination of bacterial outgrowth

At 24 or 48 h after infection, mice were anesthetized by FFM (fentanyl citrate 0.079 mg/ml, flunisolone 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. Blood was collected in EDTA containing microtubes (Becton Dickinson, Meylan, France). Both lungs were harvested and the right lung was homogenized at 4°C in 4 volumes of sterile saline using a tissue homogenizer (Biospec Products, Bartlesville, OK). Serial 10-fold dilutions were made in sterile saline and 10 µl volumes were plated on chocolate agar plates. In addition, 20 µl volumes of blood were plated. Plates were incubated at 37°C in 5% CO2, and CFU were counted after 20 h.

Myeloperoxidase (MPO) assay

MPO activity was measured as described previously (25, 26). Briefly, lung tissue was homogenized in potassium phosphate buffer, pH 7.4. After centrifugation (4500 x g for 20 minutes at 4°C), pelleted cells were lysed in potassium phosphate buffer pH 6.0 containing 0.5% hexadecyltrimethyl ammoniumbromide (HETAB) and 10 mM EDTA. MPO activity was determined by measuring the H2O2 dependent oxidation of 3,3’5,5’tetramethylbensidine (TMB). The reaction was stopped with Glacial Acetic Acid followed by reading the absorbance at 655 nm using a spectrophotometer. MPO activity was expressed as units of MPO activity per gram lung tissue per reaction time. All reagents for the MPO assay were purchased from Sigma (St. Louis, MO).

Histologic examination

Lungs for histologic examination were harvested at 24 h and 48 h after inoculation, fixed in 10% formaline and embedded in paraffin. Four µm sections were stained with haematoxylin and eosin, and analyzed by a pathologist who was blinded for groups. To score lung inflammation and damage, the entire lung surface was analyzed with respect to the following parameters: interstitial inflammation, edema, endothelialitis, bronchitis, pleuritis and thrombus formation. Each parameter was graded on a scale of 0 to 3, with 0: absent, 1: mild, 2: moderate and 3: severe. The total “lung inflammation score” was expressed as the sum of the scores for each parameter, the maximum being 21.
Cytokine and chemokine measurements

For cytokine measurements, lung homogenates were diluted 1:2 in lysis buffer (150 mM NaCl, 15 mM Tris, 1mM MgCl₂, 1 mM CaCl₂, 1% Triton X-100, 100 µg/ml Pepstatin A, Leupeptin and Aprotinin, pH 7.4) and incubated at 4°C for 30 min. Homogenates were centrifuged at 1500 x g for 15 min after which the supernatants were stored at -20°C until further use. Cytokine and chemokine levels in lung homogenates were measured by ELISA according to the manufacturer’s instructions: TNFα, IL-6, MIP-2 and KC assays were all obtained from R&D (Minneapolis, MN).

Statistical analysis

All data are expressed as mean ± SEM. Differences between groups were analyzed by Mann-Whitney U test. P < 0.05 was considered to represent a statistically significant difference.

Results

Bacterial outgrowth

Intranasal inoculation of 10⁷ CFU NTHi did not result in lethality, and bacteria were cleared from the pulmonary compartment of WT mice within 8-10 days (data not shown). Therefore, time-points early in infection were chosen for all experiments described. To gain insight into the role of the PAFR in early host defense against pneumonia induced by NTHi, bacterial outgrowth in the lungs of PAFR-/- and WT mice was compared. Bacteria were counted at 24 and 48 hours after infection with NTHi. At 24 and 48 h post-infection, lungs of PAFR-/- and WT mice contained similar numbers of bacteria. At 48 h, the bacterial load in the lungs had decreased significantly in both mouse strains (Figure 1). None of the mice had positive blood cultures at either time-point.

![Figure 1. PAFR deficiency does not influence the outgrowth of non-typeable H. influenzae. Bacterial outgrowth in lungs in PAFR-/- and WT mice at 24 and 48 h after intranasal inoculation with 10⁷ CFU non-typeable H. influenzae. Data are mean ± SEM of 7-8 mice.](image-url)
Role of PAFR in non-typeable *H. influenzae* pneumonia

**MPO activity**

The rapid influx of neutrophilic granulocytes to the site of infection is regarded as an essential host defense mechanism during bacterial pneumonia (27). We therefore determined MPO activity as an indicator of granulocyte numbers in lung tissue 24 and 48 h after bacterial challenge. At both time-points, MPO activity was elevated in comparison with saline controls (data not shown). No difference in MPO activity in lungs of PAFR-/- and WT mice could be detected at either 24 or 48 h post-infection (Figure 2).

![Figure 2. Similar myeloperoxidase activity in lungs of PAFR-/- and WT mice 24 and 48 h after bacterial challenge. Data are mean ± SEM of 7-8 mice.](image)

**Histopathology**

At 24 h after induction of NTHi infection, lungs of both WT (Figure 3A) and PAFR-/- (Figure 3B) mice displayed dense interstitial infiltrates, pleuritis and infiltrates around vessels and bronchi. The infiltrates consisted predominantly of granulocytes. The distribution and intensity of the inflammatory infiltrates were comparable in WT and PAFR-/- mice: the lung inflammation score was 7.6 and 7.7 in WT and PAFR-/- mice respectively. Although bacterial numbers in the lungs had diminished after 48 h, inflammation in the lungs had increased at this time-point in comparison with the 24 h time-point while the presence of granulocytes was less prominent and more lymphocytes were seen. No difference in the degree of inflammation or inflammatory cell types involved between WT (Figure 3C) and PAFR-/- mice (Figure 3D) was seen as shown by similar inflammation scores (9.1 and 9.0 respectively).
Cytokine and chemokine response

In pulmonary infections, local cytokine and chemokine production is an important factor in the host immune response (27, 28). We determined the role of the PAFR in pulmonary cytokine and chemokine production in NTHi pneumonia. Cytokine (TNFα, IL-6) and chemokine (MIP-2, KC) levels in lung tissue measured 24 h after induction of pneumonia did not differ between PAFR-/− and WT mice. After 48 h, all cytokine and chemokine concentrations had declined except for TNFα in PAFR-/− mice (*P < 0.05 vs. WT mice at the same time-point; Table 1).

Discussion

NTHi is a commensal inhabitant of the human nasopharynx and is a frequent cause of infections including otitis media and pneumonia (18-21). In vitro experiments have elucidated an important mechanism by which NTHi interacts with respiratory epithelial cells. ChoP, an important epitope of the NTHi cell wall, specifically binds to the PAFR expressed
on respiratory epithelial cells facilitating bacterial adherence and invasion into these cells (7, 9). This finding raised the question as to whether the absence of the PAFR would alter the course of infection caused by NTHi. Using mice with a targeted deletion of the PAFR gene, we here demonstrate that the PAFR is not important for host defense against pulmonary infection with NTHi, as reflected by similar lung bacterial counts, MPO activity and histopathology.

The influx of granulocytes, as measured by MPO activity, into the pulmonary compartment, as well as lung cytokine and chemokine concentrations were similar or even elevated in PAFR−/− mice compared to WT mice, suggesting that the initiation of an early inflammatory response in NTHi pneumonia does not require an interaction between NTHi ChoP and the PAFR. These results are in contrast with a recent study from our laboratory showing a favourable outcome of PAFR−/− mice in pneumonia caused by ChoP expressing - S. pneumoniae (15). In this study, infected PAFR−/− mice showed a reduced bacterial outgrowth and reduced cytokine and chemokine concentrations in the lungs as well as an increased survival compared to WT mice. Analogous results were obtained in a rabbit model of experimental meningitis where intracisternal administration of a specific PAFR antagonist attenuated protein influx into the CSF elicited by intracisternal inoculation of S. pneumoniae but had no effect on inflammation induced by H. influenzae (16).

Our findings suggest that in NTHi pneumonia the role of the PAFR is limited and that other receptors are responsible for the regulation of innate host responses. This hypothesis is supported by several studies (29-31). Toll-like receptors (TLR) function as important pattern-recognition receptors in recognizing pathogen-associated molecular patterns and are pivotal for the induction of host inflammatory responses in infection (32). To date, ten members of the TLR family have been identified of which two have been implicated in the regulation of the innate immune response against H. influenzae infection. TLR4 has emerged as the key

### Table 1. Cytokine and Chemokine Concentrations in lung tissue 24 and 48 h after induction of NTHi pneumonia in PAFR−/− and WT mice.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>PAFR−/− 24 h</th>
<th>WT 24 h</th>
<th>PAFR−/− 48 h</th>
<th>WT 48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFα</td>
<td>16.7 ± 2.0</td>
<td>15.9 ± 2.6</td>
<td>16.9 ± 2.0*</td>
<td>6.8 ± 1.8</td>
</tr>
<tr>
<td>IL-6</td>
<td>17.9 ± 1.1</td>
<td>14.0 ± 2.6</td>
<td>9.0 ± 0.8</td>
<td>6.4 ± 1.6</td>
</tr>
<tr>
<td>MIP-2</td>
<td>55.2 ± 3.8</td>
<td>48.7 ± 7.8</td>
<td>40.3 ± 3.1</td>
<td>28.0 ± 4.2</td>
</tr>
<tr>
<td>KC</td>
<td>30.8 ± 4.0</td>
<td>29.5 ± 5.5</td>
<td>18.4 ± 1.7</td>
<td>17.9 ± 3.7</td>
</tr>
</tbody>
</table>

Data are mean ± SEM of 7-8 mice per group at each time point. *P < 0.05 vs. WT mice.
receptor in infection caused by Gram-negative bacteria by virtue of its capacity to signal LPS-induced inflammatory responses (33-37). Wang et al. (29) evaluated the role of Toll-like receptor (TLR) 4 in pneumonia induced by *H. influenzae* type-b in mice. In *H. influenzae* type-b induced pneumonia, the absence of a functional TLR4 was associated with enhanced bacterial numbers, a reduced influx of neutrophils and attenuated cytokine and chemokine levels in the lung (29). These findings were supported by experiments conducted in our laboratory showing an impaired host response in TLR4 deficient mice after intranasal inoculation with NTHi (unpublished results). Furthermore, in in vitro experiments using epithelial cells, the expression of TLR2 was shown to stimulate the induction of inflammatory responses induced by NTHi (30, 31).

The relevance of endogenous PAF in bacterial pneumonia has been described in a model of murine pneumonia induced by *Klebsiella pneumoniae*, a bacterium that does not express ChoP (38). In this study, infected PAFR-/- mice or mice treated with a PAFR antagonist had reduced pulmonary TNF levels and increased bacterial counts compared with WT mice. In addition, survival was impaired in PAFR-/- and PAFR antagonist treated mice. These data underline the protective, proinflammatory effect of PAF in pneumonia and corroborate the findings of other studies using different models of lung injury (39-42). In vitro studies have established that the PAFR enhances adherence and invasion of NTHi by binding to the ChoP epitope on the bacterial cell wall (7, 9). The present study is the first to examine the role of the PAFR in NTHi pneumonia in vivo. Our data, showing an unaltered host defense in PAFR-/- mice compared with WT mice, argue against an important role of the PAFR in the pathogenesis of pneumonia caused by NTHi.

**Acknowledgements**

The authors thank J. Daalhuisen and I. Kop for excellent technical assistance.

**References**

Role of PAFR in non-typeable *H. influenzae* pneumonia


