Interaction between inflammation, coagulation and fibrinolysis during infection

Weijer, S.

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: https://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.
Chapter 6

Unimpaired Host Response of Platelet Activating Factor Receptor Deficient Mice during Pulmonary Tuberculosis

Sebastiaan Weijer*, Jaklien C. Leemans*, Sandrine Florquin§, Takao Shimizu†, Satoshi Ishii‡, Tom van der Poll†

Laboratory of Experimental Internal Medicine*, Department of Infectious Diseass, Tropical Medicine and AIDS†, Department of Pathology§, Academic Medical Centre, University of Amsterdam, the Netherlands. Department of Biochemistry and Molecular Biology, Faculty of Medicine, The University of Tokyo, and CREST of Japan Science and Technology Corporation, Tokyo, Japan‡

Immunology. 2003 Aug;109(4):552-6
Abstract

Platelet activating factor (PAF) is a phospholipid with potent, diverse actions, that has been implicated as an important mediator in host defence against several intracellular pathogens. To determine the role of PAF in host defence in pulmonary tuberculosis, PAF receptor deficient (PAFR-/−) and wild-type (PAFR+/-) mice were intranasally infected with a virulent strain of *Mycobacterium tuberculosis*. Mycobacterial outgrowth in lungs and liver did not differ between PAFR-/− and PAFR+/- mice at 2 and 6 weeks post-infection. After 28 weeks, 86% of PAFR-/− mice and 79% of PAFR+/- mice had died (non-significant). In addition, both mouse strains were indistinguishable with respect to histopathology, the recruitment and activation of lymphocytes and cytokine concentrations in the lung. These data suggest that PAF is not involved in the protective immune response to tuberculosis.

Introduction

Tuberculosis (TB) is a re-emerging disease, affecting patients in both developing and industrialized countries. The increasing incidence of antibiotic resistance, together with synergism between HIV and TB, has increased our interest in this important infectious disease and in mechanisms contributing to anti-microbial host defence. Resistance to mycobacterial infections is mediated mainly by macrophages and T cells and requires the formation of granulomas, characterized by lymphocytes, macrophages, and granulocytes. Their interaction is dependent on the interplay of cytokines and chemokines produced by different inflammatory cell types.

Platelet-activating factor (PAF) is a potent phospholipid mediator that plays an important role in inflammatory and immune responses. PAF is produced by a large number of cells, including platelets, endothelial cells, stromal cells, lymphoid tissue and neutrophils. The biological activity of PAF is mediated through a specific G-protein-coupled receptor (PAFR) on the membrane of responsive cells, which has been identified on many haemopoietic cells, including neutrophils, dendritic cells, macrophages and monocytes. Recent studies have suggested that endogenous PAF may play an important role in an adequate immune response to intracellular microorganisms, such as *Leishmania amazonensis* and *Trypanosoma cruzi*. Indeed, treatment with PAF antagonists increased the outgrowth of microorganisms and mortality in murine models of these infections. In accordance, PAF reduced the intracellular growth of *Leishmania* and *Trypanosoma* in macrophages. Notably, *M. tuberculosis* is an intracellular microorganism that uses macrophages as its natural environment in the host, and many of the host defence mechanisms known to be important for the protection against *M. tuberculosis* are also involved in the protective immune response to other intracellular pathogens, including *Leishmania* and *Trypanosoma*. These findings led us to hypothesize that PAF may also be important for host defence against *M. tuberculosis*. Therefore, in the present study we sought to determine the role of PAF in the immunopathology of TB.
Material and methods

Mice

PAFR gene deficient (PAFR-/-) mice were generated as described previously. For the experiments described here, female PAFR-/ mice, backcrossed seven times to a C57BL/6 background, and female wild type C57BL/6 (PAFR+/+) mice (Harlan Sprague Dawley Inc., Horst, the Netherlands) were used at age 6-8 weeks. The Animal Care and Use Committee of the University of Amsterdam (Amsterdam, the Netherlands) approved all experiments.

Experimental infection

Pulmonary TB was induced exactly as described previously. Briefly, a virulent laboratory strain of M. tuberculosis H37Rv was grown in liquid Dubois medium containing 0.01% Tween 80 for 4 days. A replicate culture was incubated at 37°C, harvested at mid-log phase, and stored in aliquots at -70°C. For each experiment, a vial was thawed and washed twice with sterile 0.9% NaCl. Mice were anaesthetized by inhalation with isoflurane (Abbott Laboratories, Kent, U.K.) and infected with 1 x 10^5 live bacilli in 50 µl saline, as determined by viable counts on 7H11 Middlebrook agar plates. Bacterial administration was performed intranasally as described previously. Survival was monitored for 200 days in 14 PAFR-/- and 14 PAFR+/+ mice. In addition, groups of eight mice per time point were killed 2 or 6 wk post infection, and lungs and one lobe of the liver were removed aseptically. Organs were homogenized with a tissue homogeniser (Biospec Products, Bartlesville, OK) in 5 volumes of sterile 0.9% NaCl, and 10-fold serial dilutions were plated on Middlebrook 7H11 agar plates to determine bacterial loads. Colonies were counted after 21-day incubation at 37°C. Numbers of colony forming units (CFU) are provided as total in the lungs or as total per gram liver. For cytokine measurements, lung homogenates were diluted 1:1 in lysis buffer (150 mM NaCl, 15 mM Tris, 1 mM MgCl₂·H₂O, 1 mM CaCl₂, 1% Triton X-100, 100 µg/ml pepstatin A, leupeptin, and aprotinin), and incubated on ice for 30 min. Supernatants were sterilized using a 0.22-µm filter (Corning, Corning, NY) and frozen at -20°C until assays were performed.

Histological analysis

The right lungs of 6 PAFR-/ and wild-type PAFR+/+ were removed 2 or 6 wk after intranasal inoculation with M. tuberculosis and fixed in 4% Paraformaldehyde in PBS for 24h. After embedding in paraffin wax, 4-µm-thick sections were stained with haematoxylin-eosin or the Ziehl-Neelsen (ZN) stain for acid-fast bacilli. All slides were coded and semi quantitatively scored for the total area of inflammation (percentage of surface of the slide) and granuloma format by a pathologist. In separate experiments, organs of 6 uninfected PAFR-/ and wild-type PAFR+/+ were harvested and examined as described above.
FF ACS analysis

For FACS analysis pulmonary cell suspensions were obtained using an automated disaggregation device (Medimachine System; Dako, Glostrup, Denmark) and processed as described previously. Cells from two mice per group (n = 10) were pooled for each time point (yielding five samples for FACS analysis per group) and were brought to a concentration of $4 \times 10^6$ cells/ml FACS buffer (PBS supplement with 0.5% BSA, 0.01% NaN$_3$, and 100 mM EDTA). Immuno-staining for cell surface molecules was performed for 30 min at 4°C using directly labelled Abs against CD3 (anti-CD3 PE), CD4 (anti-CD4 CyChrome), CD8 (anti-CD8 FITC, anti-CD8 PerCP), CD25 (anti-CD25 FITC), and CD69 (anti-CD69 FITC). All Abs were used in concentrations recommended by the manufacturer (PharMingen, San Diego, CA). To correct for aspecific staining, an appropriate control Ab (rat IgG2; PharMingen) was used. The number of positive cells was obtained by setting a quadrant marker for non-specific staining.

Cytokine measurements

Interferon (IFN)-γ and interleukin (IL)-4 concentrations were measured using commercially available ELISA reagents according to the instructions of the manufacturer (R&D Systems, Abingdon, United Kingdom).

Statistical analysis

All values are expressed as mean ± SEM. Comparisons were done with Mann-Whitney U tests. For comparison of survival curves, Kaplan-Meier analysis with a log rank test was used. Values of $p \leq 0.05$ were considered statistically significant.

Results

Survival

PAFR−/− and PAFR+/+ mice were intranasally inoculated with $10^5$ live bacilli M. tuberculosis and their survival was monitored during 200 days (Figure 1). Although PAFR−/− mice tended to succumb to TB earlier than PAFR+/+ mice, the difference between the two strains was not significant. Overall survival was 14% of PAFR−/− and 21% of PAFR+/+ mice (not significant).
PAF receptor deficiency does not influence survival during murine lung tuberculosis.

Survival of PAFR-/- and PAFR+/+ mice intranasally infected with $10^5$ M. tuberculosis CFU (n=14 per group). No significant difference was found in lethality between the two strains of mice.

**Mycobacterial outgrowth**

Next, the numbers of M. tuberculosis CFU were determined in lungs and livers of PAFR-/- and PAFR+/+ mice at 2 and 6 weeks after intranasal infection. Both organs contained a similar number of M. tuberculosis CFU in PAFR-/- and PAFR+/+ mice at each time point (Figure 2).

**Cellular recruitment to lungs**

Histology of parenchymatous organs of PAFR-/- and PAFR+/+ mice (age 8–10 wk) without M. tuberculosis infection was similar and displayed no signs of abnormalities (data not shown). Histopathological examination of lungs from PAFR-/- and
PAFR+/+ mice 2 or 6 weeks after intranasal infection with *M. tuberculosis* revealed no differences between the two mouse strains. Figure 3 shows representative slides of lungs from mice killed after 6 weeks. At that time, dense and diffuse infiltrates were found in the lungs of both mouse strains; the percentage of inflamed parenchyma was similar in both groups (data not shown). To obtain further insight in the cellular composition of the pulmonary infiltrates, we analysed whole lung cell suspension by FACS analysis. The percentages of CD4 and CD8 positive lymphocytes did not differ between PAFR-/- and PAFR+/+ mice; furthermore, the surface expression of CD25 and CD69 on T cells was similar in both mouse strains (shown for 6 weeks in table 1).

![Figure 3](image)

**Figure 3** No differences in histopathology between PAFR +/+ and PAFR -/- mice. Representative slides of lung tissue of PAFR+/+ mice (A) 6 weeks after intranasal infection with $10^5$ *M. tuberculosis* CFU showing a diffuse inflammatory infiltrate which is almost confluent. Macrophages were the most predominant cell type observed together with small collection of lymphocytes (H&E staining, original magnification x25). A comparable picture was observed in PAFR-/- mice (B) 6 weeks post-infection. (H&E staining, original magnification x25). Slides are representative for 6 mice per strain.

**Lung IFN-γ and IL-4 concentrations**

Cytokine concentrations in lung homogenates of PAFR-/- and PAFR+/+ mice (age 8–10 wk) without *M. tuberculosis* infection were either low or undetectable, with no differences between groups. IFN-γ and IL-4 concentrations in lung homogenates obtained at 2 and 6 weeks post-infection were similar in PAFR-/- and PAFR+/+ mice (shown for 6 weeks in table 1).
<table>
<thead>
<tr>
<th></th>
<th>PAFR+/+</th>
<th>PAFR-/−</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total cells (10⁶/ml)</strong></td>
<td>290 ± 31.1</td>
<td>278 ± 45</td>
</tr>
<tr>
<td><strong>Cell subsets (percentage of total)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4⁺</td>
<td>67.2 ± 0.8</td>
<td>71.3 ± 1.2</td>
</tr>
<tr>
<td>CD8⁺</td>
<td>27.1 ± 1.1</td>
<td>22 ± 0.9</td>
</tr>
<tr>
<td>CD4⁺/CD69⁺</td>
<td>9.9 ± 0.9</td>
<td>11.5 ± 2.4</td>
</tr>
<tr>
<td>CD4⁺/CD25⁺</td>
<td>8.6 ± 0.7</td>
<td>11.1 ± 1.6</td>
</tr>
<tr>
<td>CD8⁺/CD69⁺</td>
<td>16.7 ± 2.2</td>
<td>15.2 ± 0.6</td>
</tr>
<tr>
<td>CD8⁺/CD25⁺</td>
<td>1.7 ± 0.2</td>
<td>2 ± 0.4</td>
</tr>
<tr>
<td><strong>Cytokines (ng/ml)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td>7.75 ± 0.64</td>
<td>7.68 ± 0.64</td>
</tr>
<tr>
<td>IL-4</td>
<td>5.96 ± 0.75</td>
<td>6.67 ± 0.64</td>
</tr>
</tbody>
</table>

### Cellular composition and cytokine concentrations in lungs
Total cell counts and lymphocyte typing were performed on pulmonary cell suspensions 6 weeks post-infection as described in the Methods. FACS analysis was performed on pooled cells from two mice for each analysis from a total of ten mice per group (i.e. yielding 5 samples per mouse strain). FACS results are expressed as the percentage of CD4⁺, CD8⁺, CD25⁺, and CD69⁺ within the CD3⁺ population (i.e. for each of the 5 samples per mouse strain the percentage of positive cells relative to the total number of CD3+ cells was determined, and from these data means ± SE were calculated). Cytokine data are obtained from 8 mice per group and data are expressed as mean ± SE.

### Discussion
PAF has been implicated as a protective mediator in the host response to several intracellular pathogens. The data presented here argue against such a protective role of PAF in pulmonary TB. Indeed, intranasal infection with live *M. tuberculosis* was associated with similar mortality rates in PAFR−/− and PAFR+/+ mice, and mycobacterial loads in lungs and liver, determined during the early phase of the infection when all animals were still alive, did not differ between the two mouse strains.

Host defence against TB at least in part relies on CD4+ and CD8+ T cells. We therefore determined the number of T cells in whole lung cell suspensions and in addition obtained insight into their activation state by measuring the surface expression of CD25 and CD69. Theoretically, PAF can inhibit certain lymphocyte functions. Indeed, PAF has been found to reduce proliferation of CD4+ T cells induced by phytohemagglutinin, which was associated with a reduced expression of
CD25. PAF also suppressed the mitogen-stimulated production of IL-2 by human lymphocytes. However, to our knowledge little if anything is known about the effects of PAF on lymphocyte activation in vivo. We here demonstrate that deficiency of the PAFR does not influence the recruitment or activation of CD4+ and CD8+ lymphocytes during pulmonary TB.

The clinical outcome of pulmonary TB is considered to be dependent on a type 1 mediated host response. We therefore determined whether PAFR deficiency influences the type 1/type 2 balance by measuring type 1 cytokine IFN-γ and the type 2 cytokine IL-4 in lung homogenates of infected PAFR−/− and PAFR+/+ mice. However, no differences in the pulmonary concentrations of these cytokines were found between these two strains.

Our assumption that PAF could be involved in the protective immune response to TB was primarily based on its reported protective role in experimental infections of mice with Leishmania amazonensis, Trypanosoma cruzi and Candida albicans. From the present study it remains unclear why PAF does not contribute to protective immunity in TB. PAFR−/− mice are capable of producing PAF, yet PAF cannot exert any biological effect due to the absence of its receptor. Knowledge of the production of PAF in TB, either experimentally induced or in patients, is to the best of our knowledge not available. In this respect it is important to realize that PAF measurements do not necessarily provide insight into the production of this lipid mediator, since PAF that is synthesised predominantly remains in cell-associated form. Clearly, further research is warranted to dissect the distinct molecular mechanisms that contribute to an adequate immune response to different intracellular pathogens.

References


