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Platelet-Activating Factor Receptor Contributes to Host Defense against Pseudomonas aeruginosa Pneumonia but Is Not Essential for the Accompanying Inflammatory and Procoagulant Response

Marieke A. D. van Zoelen,2*† Sandrine Florquin,‡ Joost C. M. Meijers,§ Regina de Beer,*† Alex F. de Vos,*† Onno J. de Boer,‡ and Tom van der Poll*†

Pseudomonas aeruginosa is a major cause of nosocomial pneumonia, which is associated with high morbidity and mortality. Because of its ubiquitous nature and its ability to develop resistance to antibiotics, it is a problematic pathogen from a treatment perspective. Platelet-activating factor receptor (PAFR) is involved in phagocytosis of several pathogens. To determine the role of PAFR in the innate immune response to P. aeruginosa pneumonia, pafr gene-deficient (PAFR−/−) mice and normal wild-type (Wt) mice were intranasally inoculated with P. aeruginosa. PAFR deficiency impaired host defense as reflected by increased bacterial outgrowth and dissemination in mice with a targeted deletion of the PAFR gene. PAFR−/− neutrophils showed a diminished phagocytosing capacity of P. aeruginosa in vitro. Relative to Wt mice, PAFR−/− mice demonstrated increased lung inflammation and injury as reflected by histopathology, relative lung weights and total protein concentrations in bronchoalveolar lavage fluid, which was accompanied by higher levels of proinflammatory cytokines in lung homogenates and plasma. In addition, PAFR deficiency was associated with exaggerated local and systemic activation of coagulation as determined by fibrin staining of lung tissue and pulmonary and plasma concentrations of thrombin-antithrombin complexes and D-dimer. These data suggest that PAFR is an essential component of an effective host response to P. aeruginosa pneumonia, at least partly via its contribution to the phagocytic properties of professional granulocytes. Additionally, our results indicate that PAFR signaling is not essential for the induction of a local and systemic inflammatory and procoagulant response to Pseudomonas pneumonia. The Journal of Immunology, 2008, 180: 3357–3365.

Pseudomonas aeruginosa is a Gram-negative bacterium that is the leading cause of nosocomial pneumonia in the United States and Europe (1). Pseudomonas pneumonia often results in sepsis and death (2, 3) and, as a cause of ventilator-associated pneumonia, P. aeruginosa has a high mortality compared with other pathogens (3). From a treatment perspective, P. aeruginosa is regarded as a problem pathogen due to its remarkable ability to resist antibiotics (4). Therefore, insight in factors involved in the host defense during P. aeruginosa pneumonia and sepsis can help identify possible new therapeutic targets in this severe infection.

Platelet-activating factor (PAF)3 is a phospholipid that exerts various immunomodulatory actions during host defense against bacterial infections, including stimulation and degranulation of granulocytes, monocytes, and macrophages, the release of cytokines and toxic oxygen metabolites and facilitation of phagocytosis, killing, and cell adhesion (5, 6). PAF is produced mainly by neutrophils, macrophages, platelets, and endothelial cells (5, 6) and its biological activity is mediated through a G protein-linked receptor (PAF receptor, PAFR) that is expressed on different cell types, including neutrophils, macrophages, monocytes, and epithelial cells (7).

Previous studies have suggested a possible dual role of the PAFR during bacterial pneumonia that at least in part may depend on the causing pathogen. On one hand, PAF and its receptor may improve the ability of the host to deal with infections by facilitating phagocytosis and killing of engulfed microorganisms (8–10). In contrast, PAFR activation may underlie the tissue injury in the lungs (11–13). In addition, the PAFR can bind phosphorylcholine, which is not only a biologically important component of its natural ligand PAF but also part of the cell wall of a number of respiratory pathogens, including Streptococcus pneumoniae (14, 15) and Haemophilus influenzae (16); in the case of pneumococcal pneumonia, the interaction between phosphorylcholine and the PAFR facilitates bacterial invasion (17, 18), whereas it does not impact on host defense during H. influenzae pneumonia (19). Lung injury associated with P. aeruginosa infection results from both the direct destructive effects of the bacterium on the lung parenchyma as well as from excessive host immune responses (2). Despite its potential impact on the host response, the role of endogenous PAFR in the pathogenesis of Pseudomonas pneumonia and the ensuing sepsis syndrome has not...
been investigated so far. By using PAFR−/− deficient mice, we here show for the first time that PAFR contributes to host defense against *P. aeruginosa* pneumonia and sepsis.

### Materials and Methods

#### Animals

Ten- to 12-wk-old male mice with a targeted deletion in the gene for paf, resulting in a complete deficiency of PAFR (PAFR−/− mice), were generated as previously described (20) and backcrossed seven times to a C57Bl/6 background. Wild-type (WT) C57Bl/6 mice were obtained from Harlan Sprague Dawley. The Institutional Animal Care and Use Committee of the Academic Medical Center, University of Amsterdam, approved all experiments.

#### Induction of pneumonia

*P. aeruginosa* pneumonia was induced as described previously (21). In brief, *P. aeruginosa* (strain PA01), grown to mid-logarithmic phase in Luria-Bertani, was harvested by centrifugation at 3400 × g for 15 min. After washing in pyrogen-free 0.9% NaCl (2×) and suspension of the bacteria in 10 ml of 0.9% NaCl, the number of bacteria was determined by serial dilution in sterile isotonic saline and cultured on blood agar plates. Mice were lightly anesthetized by inhalation of isoflurane (Abbott Laboratories), after which 50 μl of the bacterial solution, containing 10^9 (or in one experiment 10^10) CFUs was administered intranasally. After 6 h, mice were anesthetized with ketamine (Eurovet Animal Health) and medetomidine (Pfizer Animal Health). The 6-h time point was chosen because we and others have previously shown that it provides representative information about the growth of *Pseudomonas* in murine lungs and the ensuing inflammatory response (21–24). In one experiment, the 24-h time point was used. Additionally, in a separate survival study mice were infected with 10^7 CFUs.

#### Preparation of blood samples and organ homogenates

Blood was collected from the vena cava inferior in heparin-containing tubes and centrifuged at 1500 × g for 10 min, after which plasma was collected and frozen at −20°C until assayed. The lungs were removed and processed as described previously (25–27). Lung weight was expressed as milligram of lung per gram of mouse to obtain relative organ weight. Lungs, liver, and spleen were harvested and homogenized at 4°C in 4 vol of sterile isotonic saline with a tissue homogenizer (Biospect Products) that was carefully cleaned and disinfected with 70% ethanol after each homogenization. Serial 10-fold dilutions in sterile saline were made from these homogenates, blood, and bronchoalveolar lavage fluid (BALF) (see below) and 50–μl volumes were plated onto sheep blood agar plates. Mice were killed by cervical dislocation. Lung homogenates, blood, and bronchoalveolar lavage fluid (BALF) were prepared as described (31). Blood was collected from the vena cava inferior in heparin-containing tubes and centrifuged at 1500 × g for 10 min, after which plasma was collected and frozen at −20°C until assayed. The lungs were removed and processed as described previously (25–27). Lung weight was expressed as milligram of lung per gram of mouse to obtain relative organ weight. Lungs, liver, and spleen were harvested and homogenized at 4°C in 4 vol of sterile isotonic saline with a tissue homogenizer (Biospect Products) that was carefully cleaned and disinfected with 70% ethanol after each homogenization. Serial 10-fold dilutions in sterile saline were made from these homogenates, blood, and bronchoalveolar lavage fluid (BALF) (see below) and 50–μl volumes were plated onto sheep blood agar plates. Mice were killed by cervical dislocation. Lung homogenates, blood, and bronchoalveolar lavage fluid (BALF) were prepared as described (31). Blood was collected from the vena cava inferior in heparin-containing tubes and centrifuged at 1500 × g for 10 min, after which plasma was collected and frozen at −20°C until assayed. The lungs were removed and processed as described previously (25–27).

#### Bronchoalveolar lavage

In separate mice not used for pathology or preparation of lung homogenates, the trachea was exposed through a midline incision and cannulated with a sterile 22-gauge Abbocath-T catheter (Abbott Laboratories). Bilateral bronchoalveolar lavage was performed by instilling two 0.5-ml aliquots of sterile isotonic saline into each lung. Fifty microliters of whole blood was incubated with 10 μl of heat-killed FITC-labeled *P. aeruginosa* (equivalent to 1 × 10^7 CFUs). After incubation, 0.3, 30, and 60 min at 37°C, phagocytosis was stopped by immediate transfer of the cells to 4°C and washing with ice-cold FACS buffer (PBS supplemented with 0.01% NaN_3, 0.5% BSA, and 0.35 mM EDTA). Cells were treated with vital blue stain (Orpegen) to quench extracellular fluorescence, labeled with Gr1-PE (BD Pharmingen), washed with FACS buffer, and analyzed using a flow cytometer (FACSCalibur; BD Biosciences). Neutrophils were gated based on forward scatter and Gr1 positivity. Results are expressed as phagocytosis index, defined as: (mean fluorescence × percent positive cells at 37°C) minus (mean fluorescence × percent positive cells at 4°C). To determine whether there is a difference in the adherence of heat-killed *P. aeruginosa* to the WT and PAFR−/− neutrophils, we repeated this assay with and without quenching in one separate experiment.

### Bacterial killing of P. aeruginosa

Bacterial killing was determined according to a protocol published recently (30, 31). Peritoneal lavage was performed in WT and PAFR−/− mice (n = 7) using 5 ml of sterile saline. Lavage fluid was collected in sterile tubes and put on ice. Peritoneal macrophages were washed, counted, and resuspended in RPMI 1640 with l-glutamine at a final concentration of 0.2 × 10^5/ml. Cells were then allowed to adhere in 12-well microtiter plates (Greiner) overnight at 37°C. Adherent monolayer cells were washed thoroughly with RPMI 1640 with l-glutamine. *P. aeruginosa* were added at a multiplicity of infection of 500 and spun onto cells at 2000 rpm for 5 min, after which plates were placed at 37°C for 10 min. Each well was then washed five times with ice-cold PBS to remove extracellular bacteria. To determine bacterial uptake after 10 min., wells were lysed with sterile H_2O and designated as t = 0. Prewarmed RPMI 1640 with l-glutamine was added to the remaining cells and plates were placed at 37°C for 5 and 30 min, after which the cells were again washed five times with ice-cold PBS and lysed as described before. Cell lysates were plated in serial-fold dilutions on blood agar plates and bacterial counts were enumerated after 16 h. Bacterial killing was expressed as the percentage of killed bacteria in relation to t = 0 (percent killing = 100 − [(No. of CFUs at time × No. of CFUs at time 0) × 100]).

### Cell counts and differentials

Cell counts were determined in BALF using an automated counter (Coulter ZF; Beckman Coulter). Subsequently, BALF was centrifuged and supernatant was stored at −20°C until further assayed; the pellet was suspended in PBS until a final concentration of 10^3 cells/ml and differential cell counts were performed on cytospin Giemsa stain (Diff-Quick; Dade Behring).

### Histological examination

Lungs, livers, and spleens for histologic examination were harvested after 6 h, fixed in 4% formaldehyde, and embedded in paraffin. Four-micrometer-thick sections were stained with H&E and analyzed by a pathologist who had no knowledge of the genotype of the mice. To score lung inflammation and damage, the lung samples were screened for the following parameters: interstitial inflammation, endothelialitis, bronchitis, edema, and pleuritis. Each parameter was graded on a scale of 0–5 (0, absent; 1, very mild; 2, mild; 3, moderate; 4, severe; and 5, very severe). The total histology score was expressed as the (mean) sum of the score for all parameters. Neutrophil stainings were performed as described previously (32). Neutrophil infiltration was analyzed (blinded) in four nonoverlapping areas in each specimen and graded on a scale of 0–5 as described above. The mean neutrophil staining score was expressed as the mean score of four areas. Fibrinogen stainings were performed as earlier described (25, 33). Surface areas immunostained with fibrinogen were analyzed in a blinded fashion using image analysis software. Of each specimen, digital images were captured of three nonoverlapping areas in each specimen and graded on a scale of 0–5 as described above.

### Assays

TNF-α, IL-6, MCP-1, and IL-10 levels were determined using a cytometric beads array (CBA) multiplex assay (BD Biosciences) in accordance with the manufacturer’s recommendations. Keratinocyte-derived chemokine (KC) and monocyte chemotactic protein-2 levels were measured by ELISA (R&D Systems) according to the manufacturer’s instructions. Myeloperoxidase (MPO) measurements were performed on cytospin Giemsa stain (Diff-Quick; Dade Behring).

As a Hittachi analyzer (Boehringer Mannheim). Thrombin-antithrombin complexes (TAT) were measured using the bicinchoninic acid protein kit (Pierce) with BSA as a standard. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined with commercially available kits (Sigma-Aldrich) according to the manufacturer’s instructions. Myeloperoxidase (MPO) measurements were performed on cytospin Giemsa stain (Diff-Quick; Dade Behring).
and B PAFR determined the pulmonary bacterial loads 6 h after inoculation. p lung homogenates and BALF than Wt mice (vested from PAFR/H11002 tranasal inoculation with 10^8 CFUs of compared with the corresponding body samples from Wt mice (all were collected and stored at 20°C until assayed for TNF-α, KC.

Results

Ex vivo alveolar macrophages stimulation

Alveolar macrophages were harvested from Wt and PAFR^-/- mice by bronchoalveolar lavage as previously described (26, 27). Total cell numbers were counted from each BALF sample using a hemocytometer. Cells were washed and resuspended in RPMI 1640 containing 1 mM pyruvate, 2 mM l-glutamine, 10% FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen Life Technologies) in a final concentration of 1 x 10^6 cells/ml. Cells were then cultured in 96-well microtiter plates (Greiner) for 24 h and washed with RPMI 1640 to remove nonadherent cells. Adherent monolayer cells were stimulated with LPS from P. aeruginosa, 0.1 or 1 µg/ml (Sigma-Aldrich), or RPMI 1640 (control) for 24 h. Supernatants were collected and stored at -20°C until assayed for TNF-α and KC.

Statistical analysis

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

PAFR deficiency enhances local bacterial outgrowth and dissemination to distant organs

To obtain insight in the role of PAFR in host defense against respiratory tract infection by P. aeruginosa, we intranasally infected PAFR^-/- and Wt mice with Pseudomonas (10^8 CFUs) and determined the pulmonary bacterial loads 6 h after inoculation. PAFR^-/- mice had significantly higher bacterial loads in their lung homogenates and BALF than Wt mice (p < 0.05; Fig. 1, A and B). In addition, blood, liver, and spleen homogenates harvested from PAFR^-/- mice also contained more bacteria when compared with the corresponding body samples from Wt mice (all p < 0.05; Fig. 1, C–E). Thus, PAFR^-/- mice showed a clearly increased outgrowth of P. aeruginosa at the primary site of infection which was accompanied by an enhanced dissemination of the bacteria. To obtain further proof of a beneficial role for PAFR in host defense against Pseudomonas pneumonia, we inoculated Wt and PAFR^-/- mice with a 10-fold lower inoculum (10^7 CFUs). This bacterial dose caused a relatively mild pneumonia without bacterial dissemination to blood or distant organs (data not shown).

In accordance with the results obtained with the higher inoculum, PAFR^-/- mice displayed significantly higher bacterial loads in their lungs than Wt mice (p < 0.05, Fig. 2).

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PAFR deficiency results in a decreased phagocytosis and killing capacity of P. aeruginosa

The increased bacterial load in PAFR$^{-/-}$ mice could be caused by an intrinsic defect of PAFR$^{-/-}$ cells to phagocytose P. aeruginosa. To investigate this possibility, we harvested whole blood from uninfected Wt and PAFR$^{-/-}$ mice and compared the capacity of neutrophils to phagocytose FITC-labeled P. aeruginosa. PAFR$^{-/-}$ neutrophils displayed a decreased ability to phagocytose P. aeruginosa (Fig. 3A). Quenching, which removes Pseudomonas from the cell surface, had little if any effect on the FACS signal in either Wt or PAFR$^{-/-}$ cells, suggesting that the vast majority of bacteria were intracellular (data not shown). To investigate whether this reduced phagocytosis capacity could explain the observed enhanced bacterial outgrowth in the PAFR$^{-/-}$ mice, we performed a killing assay using live P. aeruginosa. In line with the phagocytosis data, killing of viable P. aeruginosa by PAFR$^{-/-}$ cells was diminished (Fig. 3B).

PAFR$^{-/-}$ mice show more severe lung damage during P. aeruginosa pneumonia

To evaluate the role of endogenous PAFR in lung inflammation and injury during P. aeruginosa pneumonia, we analyzed lung tissue slides obtained from PAFR$^{-/-}$ and Wt mice 6 h after inoculation. Upon histopathologic examination, the lungs of Wt mice showed interstitial inflammation along with endothelialitis, bronchitis, edema, and pleuritis (Fig. 4A). Lung inflammation (e.g., interstitial inflammation, edema) in PAFR$^{-/-}$ mice (Fig. 4B) was more profound compared with that in Wt mice. The mean total histology score of the lungs (determined using the scoring system described in Materials and Methods) was significantly higher in PAFR$^{-/-}$ mice than in Wt mice ($p < 0.01$; Fig. 4C). In line, PAFR$^{-/-}$ mice had an increased relative lung weight ($p < 0.05$; Fig. 4D). Furthermore, the extent of lung injury was determined by total protein concentrations in BALF (reflecting lung leakage and/or exudate formation), which were elevated in PAFR$^{-/-}$ mice compared with Wt mice ($p < 0.05$; Fig. 4E). In conclusion, PAFR$^{-/-}$ mice showed more lung inflammation and damage.

FIGURE 4. PAFR$^{-/-}$ mice have more severe lung inflammation and damage. Wt and PAFR$^{-/-}$ mice were inoculated intranasally with $10^8$ CFUs of P. aeruginosa. Representative H&E stainings of lung tissue at 6 h after inoculation in Wt (A) and PAFR$^{-/-}$ (B) mice. Original magnification, $\times 200$. Graphic representation of the degree of lung inflammation (C), determined according to the scoring system described in Materials and Methods. Mean histology scores of healthy, uninfected Wt and PAFR$^{-/-}$ strain), respectively, and total protein levels in BALF (E; levels of healthy, uninfected Wt and PAFR$^{-/-}$ mice were 467.4 $\pm$ 23.2 and 448.5 $\pm$ 25.2 $\mu $g/ml (means $\pm$ SEM of four mice per mouse strain), respectively) at 6 h after inoculation. Data are means $\pm$ SEM of 8–11 mice/genotype. *, $p < 0.05$ vs Wt mice; **, $p < 0.01$ vs Wt mice.

FIGURE 5. PAFR$^{-/-}$ mice display enhanced neutrophil recruitment to lung tissue. Wt and PAFR$^{-/-}$ mice were inoculated intranasally with $10^8$ CFUs of P. aeruginosa and sacrificed after 6 h. Representative neutrophil stainings of lung tissue of Wt (A) and PAFR$^{-/-}$ (B) mice. Original magnification, $\times 200$. Graphic representation of the number of neutrophils (C) according to the scoring system described in Materials and Methods. MPO levels in lung tissues (D; levels of healthy, uninfected Wt and PAFR$^{-/-}$ mice were 356.8 $\pm$ 119.8 and 441.0 $\pm$ 51.4 ng/ml (means $\pm$ SEM of four mice per mouse strain), respectively). Leukocyte counts in BALF (E) and influx of neutrophils to the bronchoalveolar space (F). Data are means $\pm$ SEM of 8–11 mice/genotype. *, $p < 0.05$ vs Wt mice; **, $p < 0.01$ vs Wt mice.
PAFR<sup>−/−</sup> mice demonstrate increased neutrophil recruitment to lung interstitium

Having shown that PAFR contributes to containment of the infection within the lungs, we then asked ourselves which factors might be involved in the early spread of <i>P. aeruginosa</i>. Given that leukocyte recruitment to the site of infection is an important part of host defense during pneumonia (34, 35), we next investigated neutrophil influx in the lungs of Wt and PAFR<sup>−/−</sup> mice 6 h after inoculation. PAFR<sup>−/−</sup> mice had an increased influx of neutrophils in the lungs compared with the Wt mice, as reflected by neutrophil stainings of lung tissue (Fig. 5, A and B). The granulocyte Ly-6 scores of the lungs (semiquantified according to the scoring system described in Materials and Methods) were significantly higher in PAFR<sup>−/−</sup> mice than in Wt mice (<i>p</i> < 0.05; Fig. 5C). In line, PAFR<sup>−/−</sup> mice had higher MPO levels in their lung homogenates (<i>p</i> < 0.01; Fig. 5D). Remarkably, cellular influx in the bronchoalveolar space was unaffected, reflected by similar total leukocyte counts (Fig. 5E) and similar neutrophil influx (Fig. 5F) in BALF of Wt and PAFR<sup>−/−</sup> mice. These data indicate that PAFR deficiency is associated with enhanced neutrophil recruitment to the lung interstitium.

Effect of PAFR deficiency on CXC chemokine release

CXC chemokines have been implicated in the attraction of neutrophils to the site of an infection (36, 37). To investigate whether a difference in local chemokine levels could have influenced the neutrophil influx in the interstitial lung tissue and bronchoalveolar space, we determined KC and MIP-2 levels in lung homogenates and BALF 6 h after <i>P. aeruginosa</i> inoculation in Wt and PAFR<sup>−/−</sup> knockout mice. PAFR<sup>−/−</sup> mice showed significantly lower levels of KC in their lung homogenates and BALF than Wt mice (both <i>p</i> < 0.05; Fig. 6, A and B), suggesting that elevated levels of the neutrophil-attracting chemokine KC is a less probable explanation of the increased neutrophil influx in the lung interstitium in PAFR<sup>−/−</sup> mice. MIP-2 levels were increased in lung homogenates of the PAFR<sup>−/−</sup> mice compared with Wt mice (Fig. 6C), while MIP-2 BALF concentrations were similar in both genotypes (Fig. 6D).

Increased cytokine responses in PAFR<sup>−/−</sup> mice

To investigate whether PAFR deficiency influences the local or systemic cytokine response to <i>P. aeruginosa</i>, pro- and anti-inflammatory cytokines were measured in lung homogenates and plasma at 6 h after inoculation (Table I). In lung homogenates, IL-6, IL-10, and TNF-α levels were elevated in PAFR<sup>−/−</sup> mice (IL-6, IL-10; <i>p</i> < 0.01; TNF-α; <i>p</i> = 0.08 vs Wt mice). In addition, in plasma all cytokine levels were increased in the PAFR<sup>−/−</sup> mice (IL-6, MCP-1, and IL-10; <i>p</i> < 0.05; TNF-α; <i>p</i> = 0.08 vs Wt mice).

<i>PAFR</i>−/−<i> alveolar macrophages show more TNF-α and less KC in their supernatants upon stimulation with <i>P. aeruginosa</i> LPS in vitro</i>

Remarkably, PAFR<sup>−/−</sup> mice displayed lower KC and higher TNF-α levels in their lungs after infection with <i>Pseudomonas</i>. To determine the capacity of PAFR<sup>−/−</sup> alveolar macrophages to release these mediators, we harvested primary alveolar macrophages from PAFR<sup>−/−</sup> and Wt mice and stimulated these with LPS from <i>P. aeruginosa</i>. Supernatants from PAFR<sup>−/−</sup> macrophages contained more TNF-α (<i>p</i> < 0.05; Fig. 7A) and less KC (<i>p</i> < 0.005; Fig. 7B) upon stimulation with <i>Pseudomonas</i> LPS than those of Wt alveolar macrophages.

<i>PAFR</i>−/−<i> mice demonstrate enhanced local and systemic coagulation activation</i>

Pulmonary coagulopathy is an important feature of pneumonia (38, 39). To establish whether this model is associated with activation of the coagulation system, we performed fibrin(ogen) stainings on lung tissue slides (semiquantified to the scoring system described in Materials and Methods) and measured TATc and D-dimer levels in BALF and plasma 6 h after intranasal inoculation of <i>P. aeruginosa</i>. Both PAFR<sup>−/−</sup> and Wt mice displayed strong evidence for local and systemic activation of coagulation: relative to uninfected mice, lungs of both mouse strains revealed markedly more fibrin(ogen) depositions (Fig. 8, A–D) and strongly elevated TATc and D-dimer levels (Fig. 8, E and G) and D-dimer (Fig. 8, F and H) concentrations in BALF and plasma. Importantly, coagulation activation was more profound in PAFR<sup>−/−</sup> mice, as reflected by enhanced fibrin(ogen) deposition and higher BALF and plasma TATc and D-dimer levels (all <i>p</i> ≤ 0.05 vs Wt mice, except for fibrin(ogen) staining, <i>p</i> = 0.06 and BALF D-dimer levels, <i>p</i> = 0.05 vs Wt mice).
mice; Fig. 8, B–H). Together, these data indicate that PAFR deficiency enhances the activation of coagulation and fibrinolysis both locally and systemically during *P. aeruginosa* pneumonia.

PAFR−/− mice show increased hepatocellular injury

To determine whether this model results in hepatocellular injury, we measured plasma levels of AST and ALT 6 h after *P. aeruginosa* inoculation. Plasma AST and ALT were significantly increased in both PAFR−/− and Wt mice when compared with healthy control mice (Fig. 9). Both AST and ALT concentrations were higher in PAFR−/− mice, although for ALT the difference between the two genotypes did not reach statistical significance. (AST, \( p < 0.05 \), Fig. 9A, and ALT, \( p = 0.08 \), Fig. 9B). Of note, the hepatic parenchyma was unremarkable upon histopathological examination (data not shown).

Impact of PAFR deficiency on the late host response to *Pseudomonas pneumonia*

Finally, to determine the impact of PAFR deficiency on the late host response to *P. aeruginosa* pneumonia, Wt and PAFR−/− mice were infected with 10⁸ CFUs and killed 24 h later. At this time point, pulmonary bacterial loads had decreased in both mouse strains and were not different between groups anymore (Fig. 10A). Nonetheless, the differences between Wt and PAFR−/− mice with regard to the inflammatory response were in line with the results obtained 6 h after infection. In particular, PAFR−/− mice demonstrated more extensive lung inflammation as determined by histopathology, neutrophil influx in lung interstitium, and relative lung weight (Fig. 10, B–F). In addition, like after 6 h, lung KC levels were lower in PAFR−/− mice, whereas MIP-2 lung concentrations were higher (both \( p < 0.05 \) vs Wt mice; data not shown). Pulmonary

![Figure 7](http://www.jimmunol.org/)

**Figure 7.** Supernatants of PAFR−/− alveolar macrophages contain more TNF-α and less KC upon stimulation with *P. aeruginosa* LPS. Alveolar macrophages from Wt and PAFR−/− mice were stimulated with *P. aeruginosa* LPS. After 24 h, TNF-α (A) and KC (B) were measured in the supernatants. Data are means ± SEM of 14 mice/genotype. *\( p < 0.05 \) vs Wt mice; **\( p < 0.005 \) vs Wt mice; ***\( p < 0.0005 \) vs Wt mice.

![Figure 8](http://www.jimmunol.org/)

**Figure 8.** PAFR−/− mice demonstrate enhanced local and systemic activation of coagulation. Wt and PAFR−/− mice were inoculated intranasally with 10⁶ CFUs of *P. aeruginosa*. Representative fibrin(ogen) immunostaining of lung tissue of uninfected Wt (A) and Wt and PAFR−/− mice 6 h after infection with *P. aeruginosa* (B and C, respectively). Original magnification, ×200. Graphic representation of the percentage of the total area with positive fibrin(ogen) staining (D) according to the scoring system described in Materials and Methods. TATc (E and G) and D-dimer (F and H) concentrations were measured locally (BALF; E and F) and systemically (plasma; G and H). Data are means ± SEM of 8–11 mice/genotype. *\( p < 0.05 \) vs Wt mice; **\( p < 0.01 \) vs Wt mice. Dotted lines represent the mean values obtained from normal (uninfected) mice.

![Figure 9](http://www.jimmunol.org/)

**Figure 9.** PAFR−/− mice demonstrate increased hepatocellular injury. Aspartate aminotransferase (AST; A) and alanine aminotransferase (ALT; B) levels in plasma from Wt and PAFR−/− mice were measured 6 h after intranasal inoculation with 10⁶ CFUs of *P. aeruginosa*. Data are means ± SEM of 8–11 mice per genotype. *\( p < 0.05 \) vs Wt mice. Dotted lines represent the mean values obtained from normal (uninfected) mice.
cytokine levels were similar in both mouse strains (data not shown). PAFR deficiency did not influence mortality (Fig. 10G).

Discussion

P. aeruginosa pneumonia with systemic dissemination of bacteria and septic shock is associated with high morbidity and mortality (3). Host defense in severe pneumonia is an established domain of the innate immune system since the rapid response to invading pathogens is essential for the host to survive. PAFR has been shown to be involved in host defense during bacterial infections via several ways. First of all, PAF and its receptor play an important role in the ability of a host to deal with infections by facilitating phagocytosis of engulfed microorganisms (8-10). In addition, PAFR activation may underline the tissue injury/damage associated with the infection and/or inflammation (11–13). In the present study, we sought to obtain insight into the contribution of PAFR during severe P. aeruginosa pneumonia. Our key finding was that PAFR contributes to an effective early antibacterial host response during P. aeruginosa infection. Indeed, PAFR deficiency caused an enhanced outgrowth of P. aeruginosa at the primary site of infection along with increased spreading of bacteria to other body compartments and more severe lung damage. This reduced resistance against P. aeruginosa in PAFR<sup>−/−</sup> mice could at least in part be explained by a reduced phagocytosis capacity of PAFR<sup>−/−</sup> neutrophils.

Pneumonia caused by P. aeruginosa is characterized by the recruitment of phagocytic cells, especially neutrophils, to the site of infection (40–42). In our study, neutrophil influx into the lung interstitium was increased in the PAFR<sup>−/−</sup> mice 6 h after P. aeruginosa administration. A possible explanation for the increased pulmonary neutrophil influx in PAFR<sup>−/−</sup> mice is that it was the consequence of the increased proinflammatory stimulus provided by the higher bacterial load. In addition, the elevated local concentrations of the CXC chemokine MIP-2, which is known to contribute to neutrophil attraction to sites of the bacterial infection (36, 37), may have contributed. Remarkably, MIP-2 levels in BALF of PAFR<sup>−/−</sup> mice were not increased, whereas lung and BALF concentrations of KC, another CXC chemokine contributing to neutrophil recruitment (36, 37), were lower in the PAFR<sup>−/−</sup> mice. The finding of reduced KC levels in the presence of a higher bacterial burden can at least in part be explained by a diminished capacity of PAFR<sup>−/−</sup> alveolar macrophages to release this chemokine upon stimulation with Pseudomonas LPS. Earlier, it was described that the administration of a PAFR antagonist inhibited the increase of KC lung levels in mice injected with a venom (43), which is in line with our study. The observation that the neutrophil influx and vascular permeability in the venom model were also decreased in the mice in which PAFR was blocked, while these (and other) parameters were increased in our Pseudomonas model, could be due to higher bacterial load in our model. However, it is interesting that KC levels in the PAFR<sup>−/−</sup> mice were consistently lower, while MIP-2 and cytokine levels were higher, suggesting a more specific relation between PAFR and KC release/degradation. Additional experiments are needed to investigate whether PAFR is important for KC release and signaling and whether this is pathogen (or stimulus) specific. Together, the higher bacterial load (either directly or more indirectly via MIP-2 in lung homogenate) could have contributed to the increased neutrophil influx in the lung interstitium in PAFR<sup>−/−</sup> mice, but additional chemokine-independent effects on cell recruitment seem more likely. Despite the increase in the neutrophil influx, the clearance of P. aeruginosa was diminished in the PAFR<sup>−/−</sup> mice. One could speculate that this might be due to a decreased activation of the neutrophils of the PAFR<sup>−/−</sup> mice. Previously, Au et al. (44) found that pretreatment with a PAFR antagonist blocked the increase in the expression of the activation-dependent epitope on CD11/CD18 in human neutrophils stimulated with zymosan.

Moreno et al. (45) showed that blockade of PAFR signaling was associated with an enhancement of neutrophil migration to the infectious site in a model of polymicrobial sepsis, which is in line with our findings. Because in their study PAFR blockade was associated with decreased bacterial outgrowth, the increased neutrophil migration to the peritoneal cavity during PAFR blockade was
unlikely to be the consequence of a higher bacterial load. Moreover, Moreno et al. (45) showed in an ex vivo experiment that PAFR blockade increased the rolling and adhesion of neutrophils on endothelial cells harvested 4 h after induction of sepsis. Additional experiments are needed to investigate whether PAFR blockade enhances neutrophil rolling and adhesion during *P. aeruginosa* pneumonia.

In our *P. aeruginosa* pneumonia model, PAFR−/− mice had higher levels of cytokines in their lung homogenates, BALF, and plasma compared with WT mice 6 h after infection. These differences could in part be due to the increased bacterial load in the PAFR−/− mice. However, an (additional) intrinsic effect is very well possible, because we found that in vitro stimulation with LPS from *P. aeruginosa* led to more TNF-α in supernatants of alveolar macrophages from PAFR−/− mice compared with WT mice. In addition, Soares et al. (10) showed that the concentrations of TNF-α in BALF from PAFR−/− mice were diminished after *K. pneumoniae* infection, suggesting a more (pathogen) specific effect from (LPS from) *P. aeruginosa* on PAFR−/− cells. Therefore, the increased cytokine levels in the PAFR−/− mice in our *P. aeruginosa* model could partially be explained by a higher bacterial load and by an intrinsic effect of (LPS from) *P. aeruginosa* on cells deficient in PAFR.

Earlier it has been demonstrated that pulmonary coagulopathy is an important feature of pulmonary infection (38, 39), with changes in fibrin generation being restricted to the site of infection in patients with community-acquired pneumonia caused by *S. pneumoniae* (46) or ventilator-associated pneumonia caused by several pathogens, among others *P. aeruginosa* (47, 48). Pulmonary coagulopathy now gains more and more interest as a new target in therapeutic studies of acute lung injury of pneumonia (49–51). PAF can mediate procoagulant activity on the surface of endothelial cells possibly partly via PAFR (51). Moreover, Yokota et al. (52) showed with an in vivo study that administration of a PAF antagonist inhibits LPS-induced disseminated intravascular coagulation in rats. To determine whether our *P. aeruginosa* model is associated with activation of the coagulation system locally and systemically, we measured TAT-c and D-dimer levels in the bronchoalveolar space and plasma and performed fibrinogen lung stainings. TAT-c as well as D-dimer levels were increased in BALF and plasma 6 h after *P. aeruginosa* inoculation compared with healthy mice. In line, there was more fibrin deposition in the lungs of the mice with *P. aeruginosa* pneumonia. PAFR deficiency enhanced all of these procoagulant phenomena, indicating that PAFR is not essential for coagulation activation during *Pseudomonas* pneumonia. In this context, it is important to emphasize that PAFR signaling plays an eminent role in lung injury caused by sterile inflammatory stimuli (11–13). In our pneumonia model, the higher bacterial loads might have overlaid this effect of PAFR deficiency, resulting in net increased lung inflammation and damage in the lungs of PAFR−/− mice compared with that of the WT mice. Our study indicates that the increased bacterial loads in the PAFR−/− mice can at least in part be explained by a reduced capacity of PAFR−/− neutrophils to phagocytose *Pseudomonas*. In line, results from other in vitro investigations suggest a role of PAF and its receptor during phagocytosis of different pathogens (8, 9).

Although PAFR deficiency was associated with higher bacterial loads early (6 h) after inoculation, later during the course of the infection (24 h) both mouse strains appeared capable of partially clearing the infection as demonstrated by similar bacterial loads in lungs at this time point. Nonetheless, PAFR−/− mice still demonstrated evidence of increased lung inflammation. PAFR deficiency did not influence mortality. In this respect, it should be noted that the mortality curves after infection of previously healthy mice with this *Pseudomonas* strain are very steep; whereas low doses do not cause lethality, doses that do cause lethality almost invariably do so. Hence, we consider this model less suitable to determine the impact on mortality and rather make use of it to study early host defense mechanisms (21, 24).

Several earlier studies investigated the role of PAFR in bacterial pneumonia. Our laboratory reported on the impact of PAFR deficiency in respiratory tract infection caused by pathogens that express phosphorylcholine (a ligand for PAFR) in their cell wall (17–19). These studies revealed that *S. pneumoniae* uses PAFR to cause invasive infection (17, 18), whereas this receptor does not play a role of importance during *H. influenzae* pneumonia (19). In addition, Soares et al. (10) demonstrated that PAFR deficiency impairs host defense against *Klebsiella pneumoniae* pneumonia, resulting in higher bacterial loads during the course of the infection. The current study is the first to establish that PAFR is important for antibacterial defense against *Pseudomonas* pneumonia. Moreover, our results extensively document the fact that PAFR is not essential for the induction of excessive lung inflammation and injury. Indeed, the increased bacterial loads in PAFR−/− mice were associated with exaggerated inflammation, coagulation, and injury despite the absence of PAFR-mediated signaling. These data further illustrate the existence of a delicate balance between inflammation and anti-inflammation, in which a certain degree of inflammation is required to combat invading pathogens and exaggerated inflammation can result in severe tissue injury.

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**Disclosures**

The authors have no financial conflict of interest.

**References**


