Platelets: versatile effector cells in pneumonia and sepsis

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Protease Activated Receptor 4 limits bacterial growth and lung pathology during late stage *Streptococcus pneumoniae* induced pneumonia in mice

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Abstract

*Streptococcus (S.) pneumoniae* is a common causative pathogen of pneumonia and sepsis. Pneumonia and sepsis are associated with enhanced activation of coagulation, resulting in the production of several host derived proteases at the primary site of infection and in the circulation. Serine proteases cleave protease activated receptors (PARs), which form a molecular link between coagulation and inflammation. PAR4 is one of 4 subtypes of PARs and is widely expressed by multiple cell types in the respiratory tract implicated in pulmonary inflammation, by immune cells and by platelets. In mice, mouse (m) PAR4 is the only thrombin receptor expressed by platelets. We here sought to determine the contribution of mPAR4 to the host response during pneumococcal pneumonia. Pneumonia was induced by intranasal inoculation with *S. pneumoniae* in mPAR4-deficient (par4−/−) and wild-type mice. Mice were sacrificed after 6, 24 or 48 hours. Blood, lungs, liver and spleen were collected for analyses. *Ex vivo* stimulation assays were performed with *S. pneumoniae* and mPAR4 activating peptides. After 48 hours of infection, higher bacterial loads were found in the lungs and blood of par4−/− mice (P < 0.05), accompanied by higher histopathology scores and increased cytokine levels (P < 0.05) in the lungs. *Ex vivo*, co-stimulation with mPAR4 activating peptide enhanced the whole blood cytokine response to *S. pneumoniae*. In accordance, thrombin inhibition resulted in decreased cytokine release after *S. pneumoniae* stimulation human whole blood. Our findings suggest that mPAR4 contributes to antibacterial defense during murine pneumococcal pneumonia.
Introduction

*S. pneumoniae* is a frequent inhabitant of the upper airways in healthy individuals and a major global cause of human disease \(^1\). The pneumococcus is the most frequent cause of community-acquired pneumonia and a common pathogen in sepsis. Pneumococcal infections can occur at any age, but are most frequent in infants, the elderly and immune compromised patients. Emerging bacterial resistance has further contributed to the fact that the pneumococcus has remained a significant cause of morbidity and mortality worldwide \(^1-3\). Thus, there is an urgent need to expand our knowledge on the pathogenesis of infections caused by this common pathogen.

The inflammatory response elicited by lower respiratory tract infection is associated with the local production of multiple host-derived proteases \(^4\). Serine proteases cleave Protease Activated Receptors (PARs), which are G protein-coupled receptors that are abundantly expressed in the lungs and on immune cells \(^5-7\). Upon cleavage PARs expose a neo-amino terminus, which further serves as a ligand for the same receptor, hereby initiating downstream signaling. Four subtypes of PARs have been identified (PAR1 to PAR4), of which PAR1 and PAR2 have been studied extensively in the context of lung inflammation \(^5\). Recent studies have documented expression of PAR4 in different cell types implicated in pulmonary inflammation, including alveolar epithelial cells \(^8,9\), neutrophils \(^10\), alveolar macrophages \(^11\), platelets \(^12\) and endothelium \(^10,11,14\). Instillation of PAR4 activating peptide (AP) *in vivo* resulted in airway recruitment of neutrophils in mice \(^11\).

Proteases that can activate PAR4 include thrombin, trypsin, tissue factor-factor VIIa-factor Xa, plasmin, cathepsin G, and mannose-binding lectin-associated serine protease-1 \(^15\). Our laboratory has previously documented local activation of coagulation with generation of thrombin during human and murine pneumococcal pneumonia by a mechanism that was dependent on the generation of tissue factor-factor VIIa-factor Xa \(^16,17\). Moreover, ample evidence exists for the local generation of plasmin \(^18\) and cathepsin G \(^19\) during *S. pneumoniae* pneumonia. As such, activation of PAR4 is a likely phenomenon during respiratory tract infection caused by *S. pneumoniae*.

Knowledge on the role of PAR4 in lung inflammation *in vivo* is highly limited. We here sought to determine the contribution of mouse (m)PAR4 to the host response during pneumococcal pneumonia. For this we infected mPAR4 deficient (par4\(^{-/-}\)) mice \(^20\) with viable *S. pneumoniae* via the airways and compared several outcome parameters with those in wild-type (WT) mice.
Materials and Methods

Animals

Specific pathogen-free C57Bl/6 mice were purchased from Harlan Sprague-Dawley (Horst, the Netherlands). Par4\(^{-/-}\) mice were purchased as embryos from Mutant Mouse Regional Resource Centers (MMRRC) and backcrossed 8 times into the C57Bl/6 background in the Animal Research Institute Amsterdam (ARIA) of the Academic Medical Center. Experimental groups were age- and sex matched, and housed in the ARIA facility under standard care. All experiments were conducted with mice between 10 and 12 weeks of age. The Institutional Animal Care and Use Committee of the Academic Medical Center approved all experiments.

Experimental study design

Pneumonia was induced by intranasal inoculation with S. pneumoniae D39 (serotype 2, 1 x 10\(^6\) colony forming units (CFU) in 50 mL isotonic saline) using previously described methods \(^{16-18}\). Mice were euthanized 6, 24 or 48 hours after induction of pneumonia (N = 8 mice per group at each time point in each experiment) or observed for 12 days (N = 18 per group). Where applicable, t = 0 hours values were obtained in uninfected (“untouched”) mice. Blood was obtained from the inferior vena cava and diluted 4:1 with citrate; 200 mL was kept at room temperature, from which platelets counts were determined with the Sysmex XE 5000 (Sysmex corporation, Kobe, Japan). Lung, spleen and liver were harvested and homogenised in five volumes of sterile isotonic saline. The left lung was fixed in 10% buffered formalin and embedded in paraffin.

For bacterial quantification blood and organ homogenates were serially diluted by 10-fold in sterile isotonic saline and plated onto sheep-blood agar plates. Following 16 hours of incubation at 37°C CFU were counted. CFU results shown for 24 and 48 hours are pooled data obtained from two independent experiments (thus representing 16 mice per group at these time points). For further measurements, homogenates were diluted 1:1 with lysis buffer (300 mM NaCl, 30 mM Tris, 2 mM MgCl\(_2\), 2 mM CaCl\(_2\), 1% (v/v) Triton X-100, pH 7.4) with protease inhibitor mix (Complete protease inhibitor cocktail tablets, Roche, Basel, Switserland) and incubated for 30 minutes on ice, followed by centrifugation at 680 g for 10 minutes. Supernatants were stored at -20°C until analysis.

Histopathology

Four-micrometer sections of the left lung lobe were stained with hematoxylin and eosin (H&E). Slides were coded and scored by a pathologist blinded for group identity for the following parameters: interstitial inflammation, endothelialitis, bronchitis, oedema, pleuritis and presence of thrombi. All parameters were rated separately from 0 (condition absent) to 4 (most severe condition). The total histopathological score was expressed as the sum of the scores of the individual parameters, with a maximum of 24. Granulocyte stainings
were made using the following procedure: Endogenous peroxidase activity was quenched by a solution of 0.3% H$_2$O$_2$ in Methanol. Slides were then digested by a solution of pepsin 0.025% (Sigma-Aldrich, St. Louis, MO, USA) in 0.1 M HCl. After being rinsed, the sections were incubated in Ultra V Block (Thermo Scientific, Fremont, CA, USA) and then exposed to FITC-labeled anti-mouse Gr-1 monoclonal antibody (BD PharMingen, San Diego, CA, USA). After washes, slides were incubated with a rabbit anti-FITC antibody (Nuclilab, Ede, the Netherlands) followed by further incubation with Brightvision poly-horseradish peroxidase anti Rabbit IgG (Immunologic, Duiven, the Netherlands), rinsed again and developed using Bright DAB (Immunologic, Duiven, the Netherlands). The sections were counterstained with methyl green (Sigma Aldrich, St. Louis, MO, USA), hydrated and mounted in Pertex (Histolab, Gothenburg, Sweden).

Macrophage stainings were performed as previously described 21 subsequently using rat-anti-F4/80 antibody (MCA497GA; AbD Serotec, Oxford, UK), (FAB2)-anti-rat IgG (#6130-01; ITK Diagnostics, Uithoorn, the Netherlands) and Powervision PolyHRP-anti-rabbit IgG (DPVM-55HRP; Immunologic, Duiven, the Netherlands). Slides were stained using 3,3′ diaminobenzidine dihydrochloride (BS04-999; Immunologic, Duiven, the Netherlands) and counterstained with hematoxylin (Sigma-Aldrich, St. Louis, MO, USA). Ly-6G and F4/80 expression in the lung tissue were quantified by digital image analysis 22. In short, lung sections were scanned using the Olympus Slide system (Olympus dotSlide, Tokyo, Japan) and TIF images, spanning the full tissue section were generated. In these images Ly-6G positivity and total surface area were measured using Image J (U.S. National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij); the amount of Ly-6G positivity was expressed as a percentage of the total surface area.

**Assays**

Mouse interleukin (IL)-6, tumor necrosis factor alpha (TNF-α), keratinocyte-derived cytokine (KC) and IL-1β were measured using commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA). In plasma, mouse IL-6, TNF-α, interferon gamma (IFN-g), IL-10, monocyte chemotactic protein (MCP-1) and IL-12p70 were determined using a commercially available cytometric beads array multiplex assay (BD Biosciences, San Jose, CA, USA). Myeloperoxidase (MPO; Hycult, Uden, the Netherlands), Platelet Factor 4 (PF4; R&D Systems, Minneapolis, MN, USA) and thrombin-antithrombin complexes (TATc; Kordia, Leiden, the Netherlands) were measured by ELISA according to manufacturers’ instructions. In human samples, TNFα and IL-8 were measured by commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA).

**Mouse whole blood stimulation assay**

Heparinized mouse whole blood was 1:1 diluted with IMDM medium (Lonza, Basel, Switzerland) and stimulated with *S. pneumoniae* D39 (10⁶ CFU/mL), 130 mM mPAR4 activating peptide (PAR4 AP AYPGKF-NH2, American peptide company, Vista, CA, USA), or *S. pneumoniae* D39
and mPAR4 AP and incubated at 37°C. After 6 hours, samples were serially diluted by 10-fold in sterile isotonic saline and plated onto sheep-blood agar plates for bacterial quantification. Remaining sample was spun 5 minutes at 1250 rpm and supernatant was stored at -20°C until analysis. Results are pooled data of two independent experiments.

**Multiple platelet function analyser**

Thrombocyte responsiveness to thrombin or ADP was determined in mouse whole blood using the Multiplate 5.0 Analyzer (Dynabite Medical, Munich, Germany). Blood was withdrawn from the inferior vena cava, 4:1 diluted with citrate and stimulated immediately with 8 nM thrombin (Enzyme Research Laboratories (ERL), Southband, IN, USA), 10 nM ADP (BIO/DATA Corporation, HorsHam, PA, USA) or 0.9% NaCl in the presence of 0.5 mg/ml fibrin polymerase inhibitor (high potency, American Diagnostica, Stamford, CT, USA). Platelet response was measured during 10 minutes following stimulation.

**Human whole blood stimulation assay**

For human whole blood clotting (by recalcification) experiments, fresh blood was collected in citrate in 6 ml vacutainer (BD Vacutainer®, Franklin Lakes, NJ, USA) tubes. Beforehand 12 ml sterile PS tubes (Greiner Bio One, Monroe, NC, USA) preloaded with 150 ml IMDM medium supplemented with HEPES, 12 mM CaCl$_2$ and 0.1% human albumin and lepirudin (20 mg/g, Refludan, Celgene, NJ, USA) or vehicle control were placed in a 37°C water bath. At the time of blood draw 30 ml IMDM medium or 30 ml IMDM medium containing 1 x 10$^6$ CFU’s bacteria was added to the tubes and immediately thereafter 150 ml citrated blood. Tubes were mixed by vortexing briefly at low speed and incubated for 2 hours at 37°C. After incubation 1.2 ml ice-cold sterile 0.9% NaCl was added to each tube and tubes were vortexed vigorously. Blood clots were homogenised with a tissue homogenizer (Biospec Products, Bartlesville, OK, USA). Immediately thereafter samples were centrifuged at 3000 RPM at 4°C for 10 minutes and the supernatant was stored at -20°C until analysis. Results are representative of two independent experiments using blood of different donors.

**Statistical analysis**

Data are expressed as box and whisker plots showing the smallest observation, lower quartile, median, upper quartile and largest observation. Comparisons between groups were tested using the Mann-Whitney U test. For survival studies Kaplan-Meier analyses followed by log rank test were performed. All analyses were done using GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA, USA). P-values < 0.05 were considered statistically significant.
Results

Par4/− mice demonstrate enhanced bacterial growth in the lungs and blood during late stage pneumococcal pneumonia

To study the role of mPAR4 in S. pneumoniae induced pneumonia, we quantified bacterial loads at the primary site of infection (lungs) and distant body sites (blood, spleen and liver) in WT and par4/− mice at predefined end points after intranasal inoculation with S. pneumoniae (Figure 1). Bacterial loads were similar in both mouse strains at 6 and 24 hours after intranasal infection, in all body compartments. However, after 48 hours of infection a higher bacterial load was observed in the lungs and blood of the par4/− mice (P < 0.05 versus WT mice). Similar trends were found in spleen and liver, albeit not statistically significant.

Par4/− mice show enhanced lung inflammation

This model of pneumococcal pneumonia is associated with interstitial inflammation, endothelialitis, edema, inflammatory infiltrates, pleuritis, and rarely thrombus formation 23. To determine the impact of mPAR4 on the induction of these inflammatory alterations, we semi-quantitatively scored lung histology slides obtained from par4/− and WT mice 6, 24 or 48 hours after infection with S. pneumoniae (Figure 2A). Whereas the extent of pulmonary

Figure 1: Enhanced bacterial growth in the lungs of par4/− mice in the late phase of pneumococcal pneumonia. WT (dark grey) and par4/− mice (white) were infected with S. pneumoniae via the intranasal route and euthanized at the indicated time points thereafter. Bacterial counts were determined in lungs (A), blood (B), spleen (C) and liver (D). Data are expressed as box- and whisker plots depicting the smallest observation, lower quartile, median, upper quartile and largest observation. 24 and 48 hour data are pooled results of two independent experiments. N = 8 mice per strain in each experiment. * P < 0.05.
inflammation was similar in both mouse strains at early time points after infection, at 48 hours par4⁻/⁻ mice displayed significantly more inflammation in their lungs (P < 0.05 versus WT mice), which was caused by enhanced interstitial inflammation, endothelialitis and oedema. Figure 2B shows representative lung tissue slides prepared from par4⁻/⁻ and WT mice at the indicated time points after infection.

Considering that neutrophils play a key role in host defense against respiratory tract infection by S. pneumoniae (1;24), we next examined the impact of mPAR4 on neutrophil recruitment by quantification of Ly-6G+ cells in lung tissue slides and by measuring MPO concentrations in whole lung homogenates. No significant differences were however found in the percentage of Ly-6G+ cells (Figure 3A and B) or MPO levels (Figure 3C). To obtain more insight in the cell type driving host defense in par4⁻/⁻ and WT mice, we additionally stained macrophage F4/80 on lung tissue slides. No differences were however found in the percentage of F4/80 positive cells at any of the time points (quantification of F4/80 positive cells in Figure 3D, slides not shown).

Cytokines and chemokines play an eminent role in the regulation of inflammation during pneumonia 1,24. Therefore, we measured cytokines (TNF-α, IL-1β, IL-6) and a chemokine (KC) in whole lung homogenates as an additional readout for pulmonary inflammation (Table 1). There appeared to be a tendency towards delayed KC release in par4⁻/⁻ mice after 6 hours. While the lung levels of the other mediators were similar in both mouse strains at 6 and 24 hours.
hours after infection, at 48 hours they were higher in par4−/− mice, significantly so for TNF-α and IL-1β.

Impact of mPAR4 deficiency on the systemic cytokine response

To obtain insight in the role of mPAR4 in systemic inflammation, we measured the plasma concentrations of pro-inflammatory cytokines (TNF-α, IL-6, IL-12, IFN-g), an anti-inflammatory cytokine (IL-10) and a chemokine (MCP-1) (Table 2). The levels of these mediators remained relatively low in WT mice throughout the course of the infection. While at 6 and 24 hours par4−/− mice showed a systemic cytokine response that was similar to that in WT mice, at 48 hours all mediator levels were higher in par4−/− mice with the exception of IL-12; however, plasma
Table 1

<table>
<thead>
<tr>
<th></th>
<th>Wildtype</th>
<th>par4&lt;sup&gt;-/-&lt;/sup&gt;</th>
<th>p-value</th>
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<td><strong>Lung homogenate, t = 6h (pg/mL)</strong></td>
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<td></td>
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<tr>
<td>TNF-α</td>
<td>445 (280 - 496)</td>
<td>415 (254 - 552)</td>
<td>0.92</td>
</tr>
<tr>
<td>IL-1β</td>
<td>339 (309 - 417)</td>
<td>496 (168 - 595)</td>
<td>0.57</td>
</tr>
<tr>
<td>IL-6</td>
<td>546 (303 - 690)</td>
<td>428.3 (199 - 555)</td>
<td>0.28</td>
</tr>
<tr>
<td>KC</td>
<td>4778 (1970 - 7914)</td>
<td>2604 (796 - 4509)</td>
<td>0.13</td>
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<td><strong>Lung homogenate, t = 24h (pg/mL)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>303 (198 - 1034)</td>
<td>249 (197 - 605)</td>
<td>0.57</td>
</tr>
<tr>
<td>IL-1β</td>
<td>297 (200 - 1152)</td>
<td>521 (216 - 844)</td>
<td>0.96</td>
</tr>
<tr>
<td>IL-6</td>
<td>599 (304 - 2102)</td>
<td>622 (316 - 1685)</td>
<td>0.65</td>
</tr>
<tr>
<td>KC</td>
<td>1633 (664 - 6884)</td>
<td>3140 (732 - 4724)</td>
<td>0.92</td>
</tr>
<tr>
<td><strong>Lung homogenate, t = 48h (pg/mL)</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>31 (26 - 201)</td>
<td>385 (132 - 827)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>IL-1β</td>
<td>52 ( 30 - 147)</td>
<td>493 (113 - 905)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>IL-6</td>
<td>71 (58 - 709)</td>
<td>1609 (311 - 3089)</td>
<td>0.07</td>
</tr>
<tr>
<td>KC</td>
<td>653 (385 - 4514)</td>
<td>4902 (1173 - 7805)</td>
<td>0.16</td>
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</table>

Table 1: Lung cytokine and chemokine concentrations. Data are medians and interquartile ranges (N = 8 per strain at each time point). * P < 0.05.

Table 2

<table>
<thead>
<tr>
<th></th>
<th>Wildtype</th>
<th>par4&lt;sup&gt;-/-&lt;/sup&gt;</th>
<th>p-value</th>
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<tr>
<td><strong>Plasma, t = 6h (pg/mL)</strong></td>
<td></td>
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<tr>
<td>TNF-α</td>
<td>BD</td>
<td>BD</td>
<td>-</td>
</tr>
<tr>
<td>IL-6</td>
<td>10 (8 - 24)</td>
<td>8 (BD - 11)</td>
<td>0.23</td>
</tr>
<tr>
<td>IL-10</td>
<td>BD</td>
<td>BD</td>
<td>-</td>
</tr>
<tr>
<td>IL-12</td>
<td>BD</td>
<td>BD</td>
<td>-</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>1 (1 - 2)</td>
<td>1 (1 - 1)</td>
<td>0.67</td>
</tr>
<tr>
<td>MCP-1</td>
<td>15 (11 - 23)</td>
<td>17 (12 - 19)</td>
<td>0.96</td>
</tr>
<tr>
<td><strong>Plasma, t = 24h (pg/mL)</strong></td>
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<td></td>
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<tr>
<td>TNF-α</td>
<td>10 (BD - 36)</td>
<td>8 (6 - 50)</td>
<td>0.75</td>
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<tr>
<td>IL-6</td>
<td>28 (BD - 519)</td>
<td>85 (BD - 270)</td>
<td>1.00</td>
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<tr>
<td>IL-10</td>
<td>BD</td>
<td>BD</td>
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</tr>
<tr>
<td>IL-12</td>
<td>BD</td>
<td>BD</td>
<td>-</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>11 (2 - 68)</td>
<td>21 (4 - 93)</td>
<td>0.64</td>
</tr>
<tr>
<td>MCP-1</td>
<td>87 (28 - 812)</td>
<td>177 (30 - 1202)</td>
<td>0.88</td>
</tr>
<tr>
<td><strong>Plasma, t = 48h (pg/mL)</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>14 (5 - 58)</td>
<td>86 (36 - 138)</td>
<td>0.08</td>
</tr>
<tr>
<td>IL-6</td>
<td>25 (BD - 250)</td>
<td>508 (126 - 1775)</td>
<td>0.06</td>
</tr>
<tr>
<td>IL-10</td>
<td>BD</td>
<td>11 (BD - 29)</td>
<td>0.33</td>
</tr>
<tr>
<td>IL-12</td>
<td>40 (BD - 90)</td>
<td>40 (BD - 105)</td>
<td>0.94</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>43 (2 - 2546)</td>
<td>219 (54 - 1073)</td>
<td>0.08</td>
</tr>
<tr>
<td>MCP-1</td>
<td>447 (16 - 4448)</td>
<td>1089 (606 - 3378)</td>
<td>0.37</td>
</tr>
</tbody>
</table>

Table 2: Plasma cytokine concentrations. Data are medians with interquartile ranges (N = 8 per strain at each time point). BD = below detection limit.
cytokines differences did not reach statistical significance ($P = 0.08$ for TNF-$\alpha$, $P = 0.06$ for IL-6, $P = 0.08$ for IFN-g).

**Whole blood mPAR4 activation enhances cytokine response to *S. pneumoniae* in vitro.**

To determine whether mPAR4 activation influences cytokine response in mice, we incubated heparinised whole blood with *S. pneumoniae* in the presence or absence of mPAR4 AP and determined cytokine release and CFU outgrowth after 6 hours of incubation. Whole blood stimulation with *S. pneumoniae* alone resulted in the release of TNF-$\alpha$ but not of KC. Levels of both TNF-$\alpha$ and KC significantly increased when co-incubated with mPAR4 AP (Figure 4A,B). This however did not result in enhanced anti-bactericidal capacity of the blood, as CFU outgrowth under both conditions was similar (Figure 4C).

**Impact of mPAR4 deficiency on endothelial cell activation**

PAR4 is expressed by endothelial cells and the vascular endothelium plays a major role in sepsis pathogenesis. To test whether mPAR4 activation is important for endothelial cell activation during pneumonia, we measured (soluble) E-selectin in plasma and whole lung homogenates. Whereas plasma soluble E-selectin concentrations were similar in *par4* and

![Figure 4: mPAR4 AP enhances cytokine response in mouse whole blood to *S. pneumoniae* in vitro.](image-url)

Mouse whole blood was stimulated with mPAR4 AP (light grey), $10^6$ CFU’s *S. pneumoniae* (dark grey), *S. pneumoniae* and mPAR4 AP (black) or medium control for 6 hours. TNF-$\alpha$ (A) and KC (B) concentrations were measured in the supernatant. Bacterial counts were determined directly after incubation (C). Results are pooled data of two independent experiments. N = 4 mice, * $P < 0.05$. 

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WT mice at all time points (Figure 5A), total E-selectin levels in lung tissue were higher in par4−/− mice at 48 hours post infection (P < 0.05 versus WT mice) (Figure 5B).

**Impact of mPAR4 deficiency on platelet responses**

Thrombin is a potent activator of PAR4 that is produced during pneumococcal pneumonia. Importantly, since mouse platelets do not express mPAR1, mPAR4 exclusively mediates thrombin-induced platelet activation in mice. To confirm that par4−/− platelets are unresponsive to thrombin, citrated whole blood from par4−/− and WT mice was stimulated with thrombin, ADP or NaCl control *in vitro*. Thrombin induced aggregation in WT, but not in par4−/− blood (Figure 6A); as expected par4−/− thrombocytes responded normally to ADP (data not shown). After *in vivo* infection with *S. pneumoniae*, platelet counts did not differ between par4−/− and WT mice (Figure 6B). In addition, the plasma levels of PF4, an index of platelet activation, increased similarly in both groups after induction of pneumonia, although par4−/− mice tended to have lower plasma PF4 concentrations at 48 hours after infection (Figure 6C; P = 0.08 versus WT mice). The generation of thrombin was confirmed in the present experiments by the detection of elevated plasma TATc levels (Figure 6D). Remarkably, at 24 hours post infection par4−/− mice displayed lower plasma TATc levels (P < 0.05 versus WT mice), whereas they showed a trend toward higher plasma TATc concentrations at 48 hours (P = 0.09). Similar differences were found with regard to TATc concentrations in whole lung homogenates, with par4−/− mice having lower levels at 24 hours (P < 0.05 versus WT mice) and higher values at 48 hours (P = 0.05 versus WT mice) (Figure 6E).

**mPAR4 deficiency does not significantly influence survival during pneumococcal pneumonia**

To determine whether the increased bacterial loads and enhanced lung inflammation in par4−/− mice impact on mortality during *S. pneumoniae* induced pneumonia, we carried out a
survival study in which we followed $par4^{-/-}$ and WT mice for 12 days after infection (Figure 7). Although $par4^{-/-}$ mice tended to die earlier and to a greater extent (4/18 $par4^{-/-}$ versus 7/18 WT mice survived), the difference between groups was not significant ($P = 0.14$).
Thrombin enhances human whole blood cytokine response to *S. pneumoniae*

Thrombin is the most potent activator of human and mouse PAR1 and PAR4. To test whether PAR activation by thrombin enhances cytokine response to *S. pneumoniae* in human whole blood, we stimulated human whole blood with 10^6 CFU *S. pneumoniae* in the presence or absence of thrombin inhibitor lepirudin. Endogenous thrombin release was ensured by recalcification of the citrated blood. In line with the detected cytokine upregulation by mPAR4 AP in stimulated mouse blood, thrombin inhibition significantly decreased TNF-α and IL-8 response to *S. pneumoniae* in human whole blood (Figure 8).

**Discussion**

Pneumococcal pneumonia represents a major health burden worldwide. The PAR family forms a molecular link between coagulation and inflammation, host responses that during severe infection contribute to antibacterial defense on the one hand and collateral tissue damage on the other hand. Considering the wide-spread distribution of PAR4 on multiple cell types in the respiratory tract and previous documentation of the induction of multiple PAR4.
activating proteases during pneumonia, we here sought to determine the role of mPAR4 in the host response to pneumococcal pneumonia. Our main findings are that mPAR4 signaling contributes to host defense in *S. pneumoniae* pneumonia, as reflected by higher bacterial loads 48 hours after infection in *par4*/*−* mice, accompanied by enhanced lung inflammation, as indicated by histopathology and cytokine concentrations. *In vitro* experiments show that mPAR4 activation enhances whole blood cytokine release to *S. pneumoniae*. mPAR4 deficiency did not significantly influence survival. In human whole blood, inhibition of the main PAR1 and 4 activating protease thrombin resulted in decreased cytokine release after *S. pneumoniae* stimulation.

Our study does not provide insight into the cell types that drive the role of mPAR4 in pneumococcal pneumonia. PAR4 is expressed on different cell types important for an adequate host response in lower respiratory tract infection, including alveolar epithelial cells, neutrophils, endothelium, platelets, and alveolar macrophages. *In vitro* studies have shown that epithelial cells release various pro-inflammatory cytokines upon PAR4 stimulation, which in theory may facilitate neutrophil recruitment and antibacterial defense during infection of the airways. In accordance, instillation of PAR4 AP into the airways resulted in a modest influx of neutrophils in the lungs of mice. In this work we show *in vitro* that mPAR4 activation, in co-incubation with *S. pneumoniae*, significantly increased whole blood cytokine release. *In vivo*, this was supported by a tendency towards delayed KC release in the lungs of *par4*/*−* mice after 6 hours. This did not result in a difference in neutrophil or macrophage influx, as MPO levels and the number of Ly-6G and F4/80 positive cells in lung tissue were similar in *par4*/*−* and WT mice. During late pneumonia, lung and plasma cytokine concentrations followed bacterial loads, and cytokine levels increased in the more severely ill *par4*/*−* mice.

Human and mouse endothelial cells respond to PAR4 stimulation *in vitro* with increased levels of calcium and phosphorylated ERK and actin filament formation. mPAR4 did not play a role in endothelial cell activation in our pneumonia model, because E-selectin levels were similar in *par4*/*−* and WT mice early after infection and even higher in the former strain at 48 hours. Likely, induction of E-selectin is primarily driven by the bacterial burden, matching histopathology scores.

In mice, mPAR signaling in platelets is exclusively mediated via mPAR4 under costimulation of mPAR3 since mouse platelets do not express mPAR1. In humans, platelet PAR signaling is mainly hPAR1 dependent, which makes extrapolation of the role of PAR4 signaling in platelets from mice to humans difficult. However, because of the lack of mPAR1 on mouse platelets, *par4*/*−* mice provide a unique opportunity to study the impact of thrombin-induced platelet activation during infection. PF4 is stored in platelet a-granules and secreted upon platelet activation. In our study, plasma PF4 concentrations equally rose in WT and *par4*/*−*
mice initially, while after 48 hours of infection, a trend towards lower PF4 levels – indicative of decreased platelet activation - was found in par4−/− mice. Although the difference with WT mice did not reach statistical significance, this finding is remarkable considering the concurrently elevated TATc levels – indicative of enhanced thrombin generation - in par4−/− mice. Platelets have been implicated as major players in the innate immune response through the release of regulatory proteins such as cytokines and chemokines, platelet-leukocyte interactions, induction of neutrophil extracellular trap release, and the secretion of antimicrobial peptides. PF4 itself has been identified as such an antimicrobial peptide, and is in addition known to bind to S. pneumoniae, inducing antibody formation and enhancing phagocytosis. We therefore suggest that thrombin-induced platelet activation via mPAR4 contributes to limitation of bacterial outgrowth during late stage pneumococcal pneumonia.

During infection, coagulation activation is triggered via tissue factor, which together with Factor VIIa mediates thrombin generation via cleavage of prothrombin by Factor Xa. Thrombin is a potent activator of PAR4 and an important platelet agonist. Moreover, thrombin activation of platelets results in coagulation factor assembly on the platelet surface, facilitating the coagulation cascade and creating an additional thrombin burst. Bacterial multiplication and dissemination in pneumococcal pneumonia lead to enhanced thrombin generation in the lungs and plasma, as reflected by elevated TATc levels (present study and 16,17). Of interest, in par4−/− mice the rise in plasma and lung TATc concentrations was less profound during the first 24 hours after infection, suggesting that the thrombin induced coagulation burst via platelets may contribute to the extent of coagulation activation during the early phase of pneumococcal pneumonia. Later in infection TATc levels increased in par4−/− mice and even exceeded the levels measured in WT mice, most likely driven by the higher bacterial burden and indicating that mPAR4 does not play a significant role herein anymore.

Our study is the first to study the role of mPAR4 in infection. Previous investigations reported on the role of mPAR4 in lung and systemic inflammation. Instillation of a PAR4 AP into the lungs resulted in a modest influx of neutrophils; this treatment did not affect airway pressure, lung water or lung vascular permeability to protein in spontaneously breathing or ventilated mice. In addition, mPAR4 deficiency did not protect in bleomycin-induced pulmonary fibrosis. In a mouse model of systemic inflammation and disseminated intravascular coagulation, PAR4 inhibition by the administration of a pepducin protected against multiorgan failure in a neutrophil dependent manner; this latter study did not report confirmatory data using par4−/− mice.

Considering the interspecies variety in PAR1 and 4 expression between mice and humans, extrapolation of the mouse whole blood stimulation assay to the human situation should be done with care. Comparisons however could be made using thrombin, since this serine protease is the most potent activator of both human and mouse PAR1 and 4. In line with our
mouse whole blood experiments, where mPAR4 activation resulted in enhanced cytokine response to \textit{S. pneumoniae}, thrombin inhibition by lepirudin reduced cytokine levels after \textit{S. pneumoniae} stimulation in human whole blood. These data suggest that in humans stimulation of hPAR1 and hPAR4 enhances cytokine release elicited by \textit{S. pneumoniae}.

In conclusion, we show that mPAR4 contributes to host defense during pneumococcal pneumonia, as reflected by increased pulmonary bacterial growth and higher histopathology scores in \textit{par4}/- mice during late stage infection. Of interest, our laboratory recently reported that \textit{par1}/- mice displayed an improved host defense response during pneumonia caused by \textit{S. pneumoniae}, although in that study another serotype was used \cite{38}. Further studies are warranted to elucidate the interplay between different PARs in the innate immune response during severe infection.

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References

Protease activated receptor 4 limits bacterial growth and lung pathology during late stage Streptococcus pneumoniae induced pneumonia in mice.


