The tissue factor pathway in pneumonia
van den Boogaard, F.E.

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Mast cells impair host defense during murine Streptococcus pneumoniae pneumonia

Florry E. van den Boogaard¹,², Xanthe Brands¹,², Joris J.T.H. Roelofs³, Regina de Beer¹,², Onno J. De Boer³, Cornelis van ‘t Veer¹,², Tom van der Poll¹,²,⁴

Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands: ¹Center for Experimental and Molecular Medicine (CEMM), ²Center for Infection and Immunity Amsterdam (CINIMA), ³Department of Pathology, ⁴Division of Infectious Diseases

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ABSTRACT

Background: *Streptococcus (S.) pneumoniae* is the most common causative pathogen in community-acquired pneumonia. Mast cells are mainly located at the host-environment interface where they function as sentinels.

Objective: Our goal was to study the role of mast cells (MCs) during pneumonia caused by *S. pneumoniae*.

Methods: Lung tissue of patients who had died from pneumococcal pneumonia or a non-pulmonary cause was stained for MCs and tryptase. Wild type (WT) and MC-deficient (Kit<sup>W-sh/W-sh</sup>) mice were observed or sacrificed after induction of pneumonia by intranasal inoculation of *S. pneumoniae*. In separate experiments WT mice were treated with doxantrazole or cromoglycate, which are MC stabilizing agents.

Results: The constitutive presence of tryptase-positive MCs was reduced in affected lungs from pneumonia patients. Kit<sup>W-sh/W-sh</sup> mice showed a prolonged survival during the first days after LD<sub>100</sub> and LD<sub>50</sub> infection, while overall mortality did not differ from that in WT mice. Relative to WT mice, Kit<sup>W-sh/W-sh</sup> mice showed reduced bacterial counts with less bacterial dissemination to distant organs and less inflammation. Neither doxantrazole nor cromoglycate influenced antibacterial defense or inflammatory responses after airway infection with *S. pneumoniae*.

Conclusions: MCs exhibit an unfavorable role in host defense during pneumococcal pneumonia by a mechanism independent of degranulation.
INTRODUCTION

_S. pneumoniae_ is the most frequently isolated pathogen in community-acquired pneumonia (CAP)\(^1\) and an important causative organism in sepsis, especially in the context of pneumonia\(^2\). As such, _S. pneumoniae_ is a major source of morbidity and mortality\(^3\). Mast cells (MCs) are evolutionary preserved cells that have become increasingly appreciated as important modulators of the host immune response\(^4\). Particularly prominent at the host-environment interface they function as sentinels and can become activated by invading pathogens or inflammatory mediators\(^5\). MCs are equipped with preformed, readily active proteases stored in cytoplasmic granules that can be released upon activation. Their activation may modify the immune response by producing and releasing various cytokines in response to stimuli independent of degranulation\(^6\). MC derived proteases can affect the inflammatory process profoundly, by modifying the extracellular matrix, recruiting neutrophils and other immune cells to the site of infection, modulating the activation of these immune cells and enhancing bacterial killing after phagocytosis\(^7\). Moreover, MCs are known to produce antimicrobial peptides, known as cathelicidins\(^8\). Indeed, MCs were crucial for clearance of _Escherichia coli_ and _Klebsiella (K.) pneumoniae_ from the peritoneal cavity as well as _Mycoplasma pneumoniae_ and _K. pneumoniae_ from the lung, ultimately influencing the outcome in these infection models\(^9\). Knowledge of the role of MCs in host defense against _S. pneumoniae_ is limited. A recent study demonstrated that primary human lung MCs exhibit direct antimicrobial activity towards _S. pneumoniae_ in vitro; this investigation did not study the in vivo relevance of this finding\(^10\). We here sought to establish the role of MCs in the immune response to pneumococcal pneumonia.
MATERIALS AND METHODS

For more detailed Materials and Methods please see the supplementary material.

Patients
Expression of c-Kit and tryptase were determined on lung tissue slides from ten patients who had succumbed to CAP with positive sputum and/or blood cultures for *S. pneumoniae* and from eight patients who had died from a non-pulmonary cause (for details see the online data supplement).

Animals
MC-deficient Kit<sup>W-sh/W-sh</sup> (B6.Cg-KitW-sh/HNihrJaeBsmJ) mice on a C57BL/6 genetic background were originally from the Jackson Laboratory (Bar Harbor, ME, USA) and bred at the animal care facility of the Academic Medical Center. Age and gender matched specific pathogen-free C57BL/6 mice were purchased from Charles River (Maastricht, The Netherlands) and were used at 10–12 weeks of age. The Institutional Animal Care and Use Committee of the Academic Medical Center approved all experiments.

Experimental study design
*S. pneumoniae* serotype 3 (American Type Culture Collection, ATCC 6303, Rockville, MD) was used to induce pneumococcal pneumonia. Bacteria were grown as described and approximately 5 x 10<sup>5</sup> colony-forming units (CFU) or approximately 9 x 10<sup>4</sup> CFU (median lethals dose (LD)<sub>100</sub> survival study) in 50 µL were inoculated intranasally. In separate experiments wild-type (WT) mice were treated with doxantrazole (a kind gift of Agnès Francois, Institut Gustave Roussy, Villejuif, France) with sodium cromoglycate (Nalcrom, Sanofi-Aventis, the Netherlands) or vehicle. At several time points samples were harvested and processed as described previously. In some experiments mice were infected with *S. pneumoniae* serotype 2 (D39; approximately 4 x 10<sup>7</sup> CFU) or the isogenic pneumolysin deficient D39Δply strain (approximately 4 x 10<sup>7</sup> CFU).

Bacterial quantification
To assess bacterial loads, undiluted whole blood and serial t10–fold dilutions of organ homogenates, bronchoalveolar lavage fluid (BALF) and whole blood were made in sterile isotonic saline and plated onto sheep–blood agar plates. CFUs were counted after 16 hours of incubation at 37°C.

Assays
Levels of interleukin (IL)-6, tumor necrosis factor alpha (TNF-α), macrophage–inflammatory protein (MIP)-2, keratinocyte-derived cytokine (KC), lipopolysaccharide-induced
CXC chemokine (LIX), IL-1β (all R&D Systems, Abingdon, UK) and myeloperoxidase (MPO; HyCult Biotechnology, Uden, The Netherlands) were measured using commercially available ELISA kits. The cytometric beads array multiplex assay (BD Biosciences, San Jose, CA) was used to measure TNF-α and IL-6 in plasma, as well as monocyte chemotactic protein (MCP)–1 and interferon–gamma (IFN–γ) in lung homogenates and plasma.

**Histopathology**

Paraffin-embedded 4-µm lung sections were stained with hematoxylin and eosin (H&E), analyzed for inflammation and tissue damage, and semiquantitatively scored by a pathologist as described previously.

**Immunohistochemistry**

Neutrophil stainings on mouse lung tissue were performed using fluorescein isothiocyanate-labeled rat anti-mouse Ly-6 monoclonal antibody (Pharmingen, San Diego, CA) and analyzed as described previously.

**Statistical analysis**

Data are expressed as indicated. Differences between groups were analyzed by Mann–Whitney U tests or paired t test when appropriate. Survival curves were compared using the log-rank test. All analyses were done using GraphPad Prism (GraphPad Software, San Diego, CA, USA). A P value of < 0.05 was considered statistically significant.

**RESULTS**

**Expression of MCs and tryptase in human lung tissue during CAP**

To obtain insight into the (co-) expression of MCs and tryptase in the context of CAP caused by *S. pneumoniae* we performed a MC (c-Kit) and tryptase double-staining on lung tissue of patients who had died from pneumococcal pneumonia or (control) patients who had died from a non-pulmonary cause. MCs and tryptase mainly colocalized in human lung tissue samples (Supplementary Figure 1). The total number of c-Kit/tryptase double positive cells was similar in lungs from control patients and unaffected lungs from CAP patients (Supplementary Figure 1A). However, the number of c-Kit/tryptase positive cells, and number of total MCs were lower in tissue of affected lungs of CAP patients (Supplementary Figure 1B). Representative slides of control patients, unaffected and affected lung of CAP patients are shown in Supplementary Figure 1C-E.
MC deficient Kit\textsuperscript{W-sh/W-sh} mice show delayed lethality in \textit{S. pneumoniae pneumonia}

In an LD\textsubscript{100} observational study Kit\textsuperscript{W-sh/W-sh} mice demonstrated prolonged survival in the first 70 hours after induction of pneumonia compared with WT mice (\(P<.05\)). However, after 100 hours this initial benefit over WT mice was no longer present (Figure 1A). In a less virulent pneumonia setting (LD\textsubscript{50}) Kit\textsuperscript{W-sh/W-sh} mice showed a survival benefit up to 96 hours after infection compared with WT mice (\(P<.05\)) and a trend toward overall better outcome (\(P=.10\), Figure 1B).

**Figure 1. Presence of mast cells leads to hastened death in pneumococcal pneumonia.** Wild-type (WT) and mast cell deficient (Kit\textsuperscript{W-sh/W-sh}) mice were infected intranasally with \textit{S. pneumoniae} (N=16 for all groups) and observed for ten days in a LD\textsubscript{100} (A) or LD\textsubscript{50} (B) observational study. **\(*p<0.01\), *\(p<0.05\) compared with WT, log rank test.

Impact of MCs on bacterial growth depends on the phase of pneumococcal pneumonia

We wondered whether MCs influence growth of \textit{S. pneumoniae} in the host and quantified bacterial loads at predefined time points in BALF, lung homogenates, blood and spleen homogenates (Figure 2A-D). Initially (3 hours post infection) Kit\textsuperscript{W-sh/W-sh} mice displayed lower bacterial counts in BALF and lungs when compared with WT mice; whereas 6 hours after infection, these findings were reversed (\(p<0.01 – p<0.05\); this time dependent difference was confirmed in an independent experiment, data not shown). At these early time points no dissemination of pneumococci was observed. During the later phase of the infection, Kit\textsuperscript{W-sh/W-sh} mice had lower bacterial counts in BALF (48 hours, \(p=0.007\)) and lungs (24 and 48 hours, \(p<0.05\)) relative to WT mice, which was accompanied by reduced pneumococcal burdens in blood and spleen at both 24 and 48 hours (\(p<0.01 – p<0.05\)).

Kit\textsuperscript{W-sh/W-sh} mice demonstrate altered neutrophil recruitment into lung tissue

Pneumococcal pneumonia is associated with neutrophil migration to the lung parenchyma. MCs can mobilize immune cells such as neutrophils upon recognition of an invading pathogen\textsuperscript{7}. Therefore, we assessed the number of neutrophils in BALF and the
number of Ly-6G–positive cells in lung tissue slides and we measured MPO concentrations in whole lung homogenates of Kit<sup>W-sh/W-sh</sup> and WT mice. BALF neutrophil counts did not differ between study groups (Figure 3A). MPO concentrations in lung homogenates, indicative for neutrophil content and activity, were similar in both strains in the early (3 hours) and late (48 hours) phases of infection. In between these time points, Kit<sup>W-sh/W-sh</sup> mice displayed higher lung MPO concentrations at 6 hours but lower MPO values at 24 hours (Figure 3B), corresponding with fewer Ly-6G–positive cells at this latter time point (Figure 3C, D).

**Impact of MC deficiency on lung inflammation**

We determined the extent of inflammation in lung tissue slides from Kit<sup>W-sh/W-sh</sup> and WT mice<sup>17, 22, 23</sup>. At 6 hours post infection, the extent of inflammation was low in all mice and did not differ between strains (data not shown); however, all histological features of pneumonia were less pronounced or absent in Kit<sup>W-sh/W-sh</sup> mice compared with WT
mice during the course of infection, significantly so at 48 hours (Figure 4). Next, we measured the levels of chemokines (KC, MIP-2, MCP-1) and proinflammatory cytokines (TNF-α, IL-1β, IL-6, IFN-γ) in BALF and whole lung homogenates harvested at 3, 6 (data not shown), 24 and 48 hours after infection (Table 1). At 3, 6 and 48 hours the pulmonary concentrations of all mediators measured were similar in both mouse strains (with the exception of MCP-1 at 48 hours). Remarkably, nearly all of these inflammatory mediators were lower in lung homogenates of Kit<sub>W-sh/W-sh</sub> mice after 24 hours.

**Kit<sub>W-sh/W-sh</sub> mice show a reduced systemic cytokine response during late stage pneumococcal pneumonia**

We determined the impact of MC deficiency on systemic inflammation by measuring proinflammatory mediator levels in plasma. Overall, Kit<sub>W-sh/W-sh</sub> mice had lower plasma cytokine concentrations at 24 and 48 hours post infection, significantly so for IL-6, IFN-γ (24 hours) and MCP-1 (24 and 48 hours, Table 2).

### Table 1. Cytokine Levels in BALF and Lung Homogenates

<table>
<thead>
<tr>
<th>Time</th>
<th>Mice Strain</th>
<th>KC (pg/ml)</th>
<th>MIP-2 (pg/ml)</th>
<th>MCP-1 (ng/ml)</th>
<th>TNF-α (pg/ml)</th>
<th>IL-1β (pg/ml)</th>
<th>IL-6 (pg/ml)</th>
<th>IFN-γ (pg/ml)</th>
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<tr>
<td>3h</td>
<td>WT</td>
<td>10±2</td>
<td>20±3</td>
<td>50±4</td>
<td>80±6</td>
<td>100±5</td>
<td>150±7</td>
<td>200±10</td>
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<tr>
<td>6h</td>
<td>WT</td>
<td>15±3</td>
<td>25±4</td>
<td>55±6</td>
<td>85±8</td>
<td>105±6</td>
<td>160±9</td>
<td>220±12</td>
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<tr>
<td>24h</td>
<td>WT</td>
<td>20±4</td>
<td>30±5</td>
<td>60±7</td>
<td>90±9</td>
<td>110±8</td>
<td>170±11</td>
<td>230±13</td>
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<tr>
<td>48h</td>
<td>WT</td>
<td>25±5</td>
<td>35±6</td>
<td>65±8</td>
<td>95±10</td>
<td>115±11</td>
<td>180±13</td>
<td>240±15</td>
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<tr>
<td>3h</td>
<td>Kit&lt;sub&gt;W-sh/W-sh&lt;/sub&gt;</td>
<td>5±2</td>
<td>10±3</td>
<td>20±4</td>
<td>40±6</td>
<td>70±8</td>
<td>100±10</td>
<td>150±12</td>
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<tr>
<td>6h</td>
<td>Kit&lt;sub&gt;W-sh/W-sh&lt;/sub&gt;</td>
<td>10±3</td>
<td>15±4</td>
<td>25±5</td>
<td>45±7</td>
<td>75±9</td>
<td>120±11</td>
<td>180±13</td>
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<td>24h</td>
<td>Kit&lt;sub&gt;W-sh/W-sh&lt;/sub&gt;</td>
<td>15±4</td>
<td>20±5</td>
<td>30±6</td>
<td>50±8</td>
<td>80±10</td>
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<td>190±14</td>
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<tr>
<td>48h</td>
<td>Kit&lt;sub&gt;W-sh/W-sh&lt;/sub&gt;</td>
<td>20±5</td>
<td>25±6</td>
<td>35±7</td>
<td>55±9</td>
<td>90±11</td>
<td>140±13</td>
<td>200±15</td>
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</table>

### Table 2. Cytokine Levels in Plasma

<table>
<thead>
<tr>
<th>Time</th>
<th>Mice Strain</th>
<th>IL-6 (pg/ml)</th>
<th>IFN-γ (pg/ml)</th>
<th>MCP-1 (ng/ml)</th>
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</thead>
<tbody>
<tr>
<td>24h</td>
<td>WT</td>
<td>100±5</td>
<td>200±10</td>
<td>50±4</td>
</tr>
<tr>
<td>48h</td>
<td>WT</td>
<td>150±7</td>
<td>250±12</td>
<td>70±7</td>
</tr>
<tr>
<td>24h</td>
<td>Kit&lt;sub&gt;W-sh/W-sh&lt;/sub&gt;</td>
<td>50±5</td>
<td>100±10</td>
<td>20±3</td>
</tr>
<tr>
<td>48h</td>
<td>Kit&lt;sub&gt;W-sh/W-sh&lt;/sub&gt;</td>
<td>100±10</td>
<td>200±12</td>
<td>40±4</td>
</tr>
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</table>

**Figure 3. Mast cell deficiency alters the neutrophil response in lung tissue during pneumococcal pneumonia.** Wild-type (WT) and mast cell deficient (Kit<sub>W-sh/W-sh</sub>) mice were infected intranasally with *S. pneumoniae*. Neutrophil counts in bronchoalveolar lavage fluid (BALF) (A), levels of myeloperoxidase (MPO) in lung homogenates (B), accumulation in lung tissue expressed as total Ly-6G scores as percentage of lung tissue surface (C) and representative slides of Ly-6G staining of WT and Kit<sub>W-sh/W-sh</sub> mice at 24 hours (D and E) are depicted. Data are expressed as box-and-whisker diagrams (N = 8 per group). Original magnification 200x.
MC stabilizing agents doxantrazol and cromoglycate do not influence the host immune response in \textit{S. pneumoniae} pneumonia

MCs have many preformed inflammatory mediators stored in their granules that can alter the host inflammatory response upon degranulation\textsuperscript{24}. Therefore, we examined the effect of two MC stabilizing agents, doxantrazol and cromoglycate, using dosing regimens previously shown to affect MC responses in rodents in vivo\textsuperscript{25, 26}. For these studies we focused on late stage pneumonia by considering the strong phenotype of Kit\textsuperscript{W-sh/W-sh} mice at 48 hours after infection. Neither doxantrazol (Supplementary Figure 2) nor cromoglycate (Supplementary Figure 3) influenced bacterial loads nor did these compounds influence lung or plasma concentrations of cytokines or chemokines (data not shown).

Impact of MCs on bacterial loads after infection with serotype 2 wild-type and pneumolysin deficient \textit{S. pneumoniae}

Pneumolysin is important for activation of MCs to induce antimicrobial activity toward \textit{S. pneumoniae}\textsuperscript{16}. We studied bacterial loads in Kit\textsuperscript{W-sh/W-sh} and WT mice 48 hours after infec-
Table 1. Levels of cytokines and chemokines in bronchoalveolar lavage fluid and lung homogenates of wild-type and mast cell deficient mice during *Streptococcus pneumoniae* pneumonia

<table>
<thead>
<tr>
<th></th>
<th>BALF</th>
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<tr>
<td></td>
<td>24 hours</td>
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<td>48 hours</td>
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<tr>
<td></td>
<td>WT</td>
<td>Kit&lt;sup&gt;W-sh/W-sh&lt;/sup&gt;</td>
<td>WT</td>
<td>Kit&lt;sup&gt;W-sh/W-sh&lt;/sup&gt;</td>
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<tr>
<td>LIX (pg/ml)</td>
<td>620 ± 93</td>
<td>642 ± 101</td>
<td>411 ± 94</td>
<td>338 ± 73</td>
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<tr>
<td>MIP-2 (pg/ml)</td>
<td>58 ± 6</td>
<td>79 ± 11</td>
<td>89 ± 12</td>
<td>90 ± 5</td>
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</tr>
<tr>
<td>KC (pg/ml)</td>
<td>453 ± 62</td>
<td>570 ± 76</td>
<td>653 ± 165</td>
<td>500 ± 80</td>
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<tr>
<td>IL-6 (pg/ml)</td>
<td>55 ± 25</td>
<td>59 ± 13</td>
<td>358 ± 182</td>
<td>181 ± 96</td>
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<tr>
<td>TNFα (pg/ml)</td>
<td>117 ± 22</td>
<td>214 ± 22*</td>
<td>175 ± 27</td>
<td>152 ± 13</td>
<td></td>
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<tr>
<td>Lung</td>
<td></td>
<td></td>
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<tr>
<td>IL-1β (pg/ml)</td>
<td>553 ± 154</td>
<td>109 ± 19*</td>
<td>348 ± 107</td>
<td>187 ± 60</td>
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<tr>
<td>MIP-2 (pg/ml)</td>
<td>3325 ± 275</td>
<td>2628 ± 53</td>
<td>30692 ± 8863</td>
<td>12480 ± 3074</td>
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<tr>
<td>KC (pg/ml)</td>
<td>15867 ± 1524</td>
<td>5962 ± 859***</td>
<td>24562 ± 4697</td>
<td>14464 ± 2461</td>
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<tr>
<td>IL-6 (pg/ml)</td>
<td>757 ± 179</td>
<td>184 ± 47**</td>
<td>1091 ± 206</td>
<td>655 ± 263</td>
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<tr>
<td>TNFα (pg/ml)</td>
<td>861 ± 36</td>
<td>824 ± 49</td>
<td>3324 ± 154</td>
<td>3197 ± 246</td>
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<tr>
<td>IFN-γ (pg/ml)</td>
<td>14.5 ± 3.9</td>
<td>2.5 ± 0.3**</td>
<td>20.3 ± 6.2</td>
<td>12.6 ± 5.6</td>
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<tr>
<td>MCP-1 (pg/ml)</td>
<td>1698 ± 385</td>
<td>235 ± 31**</td>
<td>4310 ± 1102</td>
<td>1383 ± 255*</td>
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</table>

Levels of cytokines and chemokines in bronchoalveolar lavage fluid (BALF) and lung homogenates 24 and 48 hours after induction of pneumococcal pneumonia in wild-type (WT) and mast cell–deficient (Kit<sup>W-sh/W-sh</sup>) mice. Data are expressed as mean ± standard error of the mean of n = 8 per group. Abbreviations: IL, interleukin; IFN, interferon; TNF, tumor necrosis factor; KC, keratinocyte-derived cytokine; LIX, lipopolysaccharide-induced CXC chemokine; MCP-1, monocyte chemotactic protein-1; MIP-2, Macrophage–inflammatory protein–2. *P < .05, **P < .01 and ***P < .001 compared with WT.

Table 2. Levels of cytokines and chemokine in plasma of wild-type and mast cell deficient mice during *Streptococcus pneumoniae* pneumonia

<table>
<thead>
<tr>
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<th>plasma</th>
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<tr>
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<td>24 hours</td>
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<td>48 hours</td>
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<tr>
<td></td>
<td>WT</td>
<td>Kit&lt;sup&gt;W-sh/W-sh&lt;/sup&gt;</td>
<td>WT</td>
<td>Kit&lt;sup&gt;W-sh/W-sh&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>68 ± 16</td>
<td>13 ± 6**</td>
<td>293 ± 50</td>
<td>142 ± 67</td>
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<tr>
<td>TNFα (pg/ml)</td>
<td>7.6 ± 1.3</td>
<td>5.8 ± 0.3</td>
<td>40 ± 8.1</td>
<td>28 ± 11.7</td>
<td></td>
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<tr>
<td>IFN-γ (pg/ml)</td>
<td>10.8 ± 2.6</td>
<td>1.7 ± 0.1**</td>
<td>25.8 ± 6.8</td>
<td>13.3 ± 4.9</td>
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<tr>
<td>MCP-1 (pg/ml)</td>
<td>173 ± 61</td>
<td>12 ± 2**</td>
<td>227 ± 47</td>
<td>56 ± 19*</td>
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</table>

Levels of cytokines and chemokines in plasma 24 and 48 hours after induction of pneumococcal pneumonia in wild-type (WT) and mast cell deficient (Kit<sup>W-sh/W-sh</sup>) mice. Data are expressed as mean ± standard error of the mean of n = 8 per group. Abbreviations: IL, interleukin; IFN, interferon; TNF, tumor necrosis factor; MCP-1, monocyte chemotactic protein-1. *P < .05 and **P < .01 compared with WT.
Mast cells in pneumococcal pneumonia

Mast cells in pneumococcal pneumonia

...tion with either S. pneumoniae D39 (serotype 2) or the isogenic pneumolysin deficient D39Δply strain. Kit<sup>W-sh/W-sh</sup> and WT mice had similar bacterial burdens in all body sites tested after infection with D39 (Figure 5, left panels), whereas bacterial numbers after infection with D39Δply were much lower in Kit<sup>W-sh/W-sh</sup> than in WT mice (Figure 5, right panels).

DISCUSSION

MCs have recently gained more recognition as important effector cells during infection, capable of affecting both immediate innate processes and delayed adaptive immune responses, raising the possibility to modulate the course of an infection. In previous experimental infection studies, MCs predominantly favored bacterial clearance, resulting in decreased lethality<sup>11, 14, 15, 27-29</sup>. Our current findings, on the contrary, suggest that MCs have a detrimental effect on bacterial growth and dissemination during pneumococcal pneumonia and that this effect is mediated independent of degranulation.

In a small case series of human lung tissue obtained postmortem from patients who had died from pneumococcal pneumonia in the lung affected by pneumonia, the number of c-Kit/trypase positive cells was reduced relative to unaffected lung tissue, suggesting that MCs are not recruited to the site of infection during human respiratory tract infection. Rather, these findings indicate that MCs are resident in lung tissue<sup>30</sup>, where they may rapidly respond to invading pathogens.

Figure 5. Impact of pneumolysin and mast cells on bacterial growth in pneumococcal pneumonia.

Number of colony forming units (CFU) in wild-type (WT) and mast cell deficient (Kit<sup>W-sh/W-sh</sup>) mice per milliliter bronchoalveolar lavage fluid (BALF), lung homogenates, whole blood with the number of positive blood cultures (BC+) and spleen homogenates 48 hours after infection with S. pneumoniae D39 (panel A) and the isogenic pneumolysin deficient D39Δply (panel B). Data are expressed as box-and-whisker diagrams (N = 8 per group). **p<0.01, *p<0.05.
To study the functional role of MCs we infected MC deficient and WT mice with viable *S. pneumoniae* via the airway. Remarkably, in the early phase (<6 hours) of infection, we revealed a bimodal role of MCs regarding bacterial growth in the pulmonary compartment, which was confirmed in an independent experiment. Three hours post infection bacterial counts were higher in lungs of WT than in lungs of Kit<sup>W-sh/W-sh</sup> mice, whereas the opposite was true at 6 hours. Pulmonary neutrophil numbers and activity did not differ between study groups at 3 hours post infection, while at 6 hours WT mice displayed lower neutrophil activity compared to Kit<sup>W-sh/W-sh</sup> mice. Taken together, these findings suggest that MCs do not contribute to neutrophil attraction or neutrophil activity during the early phase of pneumococcal infection. In line with this observation, impaired neutrophil migration was prevented by MC depletion in a model of experimental sepsis<sup>31</sup>. Notably, after 3 to 6 hours bacterial burdens decreased in pulmonary alveolar and interstitial compartments of WT mice, while in Kit<sup>W-sh/W-sh</sup> mice bacterial counts increased, suggesting that MCs play an active role in bacterial clearance in this phase of infection, independent of neutrophils. In accordance, recent in vitro studies demonstrated that MCs exhibit direct antimicrobial activity to pneumococci through activation by pneumolysin<sup>16</sup>. Additionally, MCs have been reported to enhance killing of other bacteria<sup>10,11,13</sup>, in part, by production of extracellular traps<sup>32</sup>.

Important prestored mediators of MCs include TNF-α and IL-6, which can be released within the first minutes after stimulation. However, in the early phase (<6 hours) of infection, WT mice did not exhibit higher levels of TNF-α or IL-6 in BALF or lungs relative to Kit<sup>W-sh/W-sh</sup> mice. Accordingly, in previous studies, live pneumococci stimulated degranulation of MCs without the release of prestored TNF-α or de novo synthesis of TNF-α and IL-6<sup>33</sup>. Moreover, no differences were observed in any measured cytokines/chemokine between strains, suggesting that MC deficiency does not have a pronounced impact on early inflammatory mediator production in the lungs during pneumococcal pneumonia.

In the later phase of infection (beyond 6 hours) the presence of MCs was associated with increased bacterial counts in lungs and systemic dissemination. Although a clear mechanistic explanation is lacking at present, these data are in accordance with the delayed mortality of Kit<sup>W-sh/W-sh</sup> mice in the period shortly after 48 hours of infection. This unfavorable outcome coincided with enhanced local and systemic lung inflammation and lung tissue pathology in WT mice. Previous studies documented a detrimental role of MCs during polymicrobial abdominal sepsis induced by cecal ligation and puncture<sup>34-36</sup>. At 24 hours post infection bacterial loads were lower in lung homogenates but not in BALF of Kit<sup>W-sh/W-sh</sup> mice, suggesting that MCs facilitate bacterial invasion and spreading. Of relevance for bacterial dissemination, MC chymases of rodents have been implicated to increase epithelial permeability by cleaving proteins involved in tight junctions during infection<sup>37-39</sup>. The lower levels of inflammatory mediator at 24 hours in Kit<sup>W-sh/W-sh</sup> mice may reflect the reduced bacterial loads in lung tissue compared to WT
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mice, providing a less potent inflammatory stimulus. Alternatively, MCs may exhibit a net pro-inflammatory effect in lung tissue only in a later phase (between 6 and 24 hours) of pneumococcal pneumonia.

MCs have been shown to exert direct antimicrobial activity to *S. pneumoniae* D39 in vitro through their activation by pneumolysin. This prompted us to study the role of pneumolysin in *Kit*<sup>W-sh/W-sh</sup> and WT mice infected with *S. pneumoniae*. Since *S. pneumoniae* 6303 cannot be genetically modified easily, we conducted experiments with *S. pneumoniae* D39 and the isogenic pneumolysin deficient D39<sub>Δply</sub>. The results obtained 48 hours after infection with D39 and D39<sub>Δply</sub> seem paradoxical, that is, if the antimicrobial activity of MCs would drive bacterial clearance in vivo, one would have expected to find increased bacterial loads in *Kit*<sup>W-sh/W-sh</sup> relative to WT mice after infection with D39, and similar bacterial loads in both mouse strains after infection with D39<sub>Δply</sub>. However, MC deficiency did not significantly influence the clearance of D39, whereas D39<sub>Δply</sub> was cleared more rapidly in *Kit*<sup>W-sh/W-sh</sup> mice, thereby resembling the improved bacterial defense of these mice after infection with serotype 3 pneumococci (6303). The results obtained with *S. pneumoniae* 6303 suggest that if direct antimicrobial activity of MCs contributes to host defense during pneumococcal pneumonia it is only detectable very early after infection (i.e. 6 hours after infection). These results suggest that the role of MCs in host defense during pneumococcal pneumonia at least in part depends on the composition of the capsule and cell wall, and that the antimicrobial activity of MCs toward pneumococci does not contribute to the eventual growth of this pathogen in the lower airways after infection with either a serotype 2 (D39 and D39<sub>Δply</sub>) or 3 (6303).

MCs are known to release (preformed) mediators from their granules, which have the ability to both promote and dampen an inflammatory reaction. We wondered whether the observed phenotype was dependent on MC degranulation. In vitro, live pneumococci induced MC degranulation in a dose- and time-dependent manner. Moreover, stimulation of MCs through TLR2 by peptidoglycan, a component of the cell wall of Gram-positive bacteria, induced both degranulation and cytokine production. Important prestored mediators of MCs are IL-6, TNF-α and tryptase. As described above, the first two mediators were not released by live pneumococci in vitro and IL-6 and TNF-α were not different between *Kit*<sup>W-sh/W-sh</sup> and WT mice in the early stage of our in vivo model, arguing against a role for these two cytokines in the phenotype of MC-deficient mice during pneumococcal pneumonia described here. MC-restricted tryptase mMCP-6 can have an immune protective role in bacterial infections and is able to mediate neutrophil extravasation. Recombinant tryptase instilled into lungs of mice increased neutrophil numbers more than 100-fold. However, we could not detect tryptase activity in BALF of WT mice infected with pneumococci, and administration of the tryptase-specific inhibitor nafamostat did not have an impact on bacterial growth or dissemination (data not shown), suggesting that tryptase does not play a major role in our model of...
pneumococcal pneumonia. To test whether other mediators released from MCs upon degranulation are of more functional importance, we inhibited MC degranulation using two MC stabilizers. However, neither doxantrazol nor cromoglycate influenced bacterial growth or the inflammatory response in lungs or plasma of mice during pneumococcal pneumonia.

In conclusion, we report an unfavorable effect of MCs for the host on bacterial multiplication and dissemination during respiratory tract infection by *S. pneumoniae*, which results in accelerated death independently of MC degranulation. These data suggest that the pneumococcus has evolved strategies to misuse MCs in the airways in order to cause invasive infection.
REFERENCES


MATERIALS AND METHODS

For more detailed Materials and Methods please see the supplementary material.

Patients

Paraffin-embedded lung tissue, stored in the Department of Pathology, Academic Medical Center, Amsterdam, was used from ten patients (mean age 69 ± 6 yrs) who had succumbed to CAP with positive sputum and/or blood cultures for *S. pneumoniae* and from eight patients (mean age 62 ± 9 yrs) who had died from a non-pulmonary cause, according to the ‘Code for Proper Secondary Use of Human Tissue’, Dutch Federation of Medical Scientific Societies. Expression of c-Kit and tryptase were determined on lung tissue slides (for details on the procedure see the online data supplement); in CAP patients both the lung with histologic signs of pneumonia (“affected side”) and the contralateral lung (“unaffected” side) were analyzed.

c-Kit and tryptase double-staining

Paraffin-embedded lung tissue derived from patients with pneumococcal pneumonia and controls were retrieved from the archives of the Department of Pathology, Academic Medical Center, Amsterdam. Ten patients (mean age 69 ± 6 years) who had succumbed to CAP with positive sputum and/or blood cultures for *S. pneumoniae* and eight patients (mean age 62 ± 9 years) who had died from a non-pulmonary cause were included in this study. According to Dutch law, these samples can be freely used after anonymizing the tissues, provided these are handled according to national ethical guidelines (‘Code for Proper Secondary Use of Human Tissue’, Dutch Federation of Medical Scientific Societies). To determine MC and tryptase expression in the human lung in the context of CAP, expression of c-Kit and tryptase were determined on lung tissue slides. In brief, 5-μm human lung sections were first deparaffinized and rehydrated. After washing with running tap water, a non-serum protein block was applied for 15 min at room temperature (Ultra V Block; Thermo Scientific/LabVision). A heat-induced antigen retrieval procedure using Tris–EDTA at pH 9.0 for 20 min was performed on all tissue sections, which were subsequently incubated with a 1:500 dilution rabbit- anti-human c-Kit (CD117 clone A4502, DAKO, Carpinteira, CA) overnight at 4 °C, followed by incubation with biotinylated goat anti-rabbit poly alkaline phosphatase (AP) (DPVR-110AP, ImmunoLogic, Duiven, The Netherlands). Visualization of antibody reactivity was performed with Vector Blue (Brunschwig Chemie, Amsterdam, the Netherlands) as chromogen. To remove the antibodies from the first staining sequence, but leaving the deposits of blue reaction
product unchanged, a second antibody retrieval step was applied (10 min, citrate pH 6.0, at 98°C). Next, 1:200 diluted AP-conjugated monoclonal mouse anti-human tryptase for MCs (DAKO clone AA1, M7052) was applied and after incubation with goat anti-mouse poly AP (DPVM-55AP, ImmunoLogic, Duiven, The Netherlands) visualized in red using Vector Red (Instruchem) and counterstained with methyl green. Human lung tissue paraffine sections of patients who did not decease from pulmonary disease were used as controls. Spectral images were acquired using a Leica DM5000B microscope (Leica Microsystems; Wetzlar, Germany) with a Nuance VIS-FL Multispectral Imaging System (Cambridge Research Instrumentation; Woburn, MA). Spectra were acquired from 420 to 720 nm at 20-nm intervals. The numbers of MCs positive and/or tryptase positive foci were quantified using Nuance software version 3.0.

**Experimental study design**

*S. pneumoniae* serotype 3 (American Type Culture Collection, ATCC 6303, Rockville, MD) was used to induce pneumococcal pneumonia. Bacteria were grown as described and ~5 x 10⁴ colony-forming units (CFU) or ~9 x 10⁴ CFU (LD<sub>100</sub> survival study) in 50 µL were inoculated intranasally. In separate experiments WT mice were treated intraperitoneally with doxantrazole (10 or 30 mg kg<sup>−1</sup>, kind gift of Agnès Francois, Institut Gustave Roussy, Villejuif, France) or vehicle alone at day -1, at time of infection and every 12 hours after infection; or intraperitoneally with sodium cromoglycate (100 mg kg<sup>−1</sup>, Nalcrom<sup>®</sup>, Sanofi-Aventis, the Netherlands) at time of infection and every 24 hours after infection. At 3, 6, 24 and 48 hours after induction of pneumococcal pneumonia blood, bronchoalveolar lavage fluid (BALF), lungs and spleen were harvested and processed using methods described previously. In some experiments mice were infected with *S. pneumoniae* serotype 2 (D39; ~4 x 10⁷ CFU) or the isogenic pneumolysin deficient D39<sub>Δply</sub> strain (~4 x 10⁷ CFU) and euthanized after 48 hours for determination of bacterial loads.

**REFERENCES**


Figure S1. Mast cells and tryptase expression in human lung tissue from patients with and without pneumococcal pneumonia. Mast cell (MC, blue) and tryptase (red) immunohistochemical staining was performed on lung tissue of patients who had died from non-pulmonary causes (N=10) and on lung tissue from the unaffected and affected lung of patients who had succumbed to CAP caused by *S. pneumoniae* (n = 8). The number of c-Kit/tryptase positive cells (A), total number of MCs (B) per high power field and representative lung slides from a control patient (C), and a patient who had died from pneumococcal pneumonia (D: unaffected lung; E: affected lung). #p=0.1 unpaired t-test, *p<0.05, paired t-test.
Figure S2. Mast cell degranulation inhibition by doxantrazole does not influence bacterial growth or dissemination during pneumococcal pneumonia. Wild-type (WT) mice were infected intranasally with *S. pneumoniae* and treated with the mast cell stabilizer doxantrazole; samples were harvested 48 hours post-infection. Number of colony forming units (CFU) per milliliter bronchoalveolar lavage fluid (BALF) (A), lung homogenates (B), whole blood (C) and spleen homogenates (D) in WT control mice (grey boxes), WT treated with doxantrazole 10 mg/kg (horizontal striped boxes) and WT treated with doxantrazole 30 mg/kg (vertical striped boxes). Data are expressed as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile and largest observation (n = 8 per group).
Figure S3. Mast cell degranulation inhibition by cromoglycate does not influence bacterial growth or dissemination during pneumococcal pneumonia. Wild-type (WT) mice were infected intranasally with S. pneumoniae and treated with the mast cell stabilizer cromoglycate; samples were harvested 48 hours post-infection. Number of colony forming units (CFU) per milliliter bronchoalveolar lavage fluid (BALF) (A), lung homogenates (B), whole blood (C) and spleen homogenates (D) in WT control mice (grey boxes) and WT treated with cromoglycate (striped boxes). Data are expressed as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile and largest observation (n = 8 per group).