The tissue factor pathway in pneumonia

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CHAPTER 9

Granzyme A impairs host defence during Streptococcus pneumoniae pneumonia

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ABSTRACT

Streptococcus pneumoniae is the most common causative pathogen in community-acquired pneumonia (CAP). Granzyme A (GzmA) is a serine protease produced by many different cell types. We sought to determine the role of GzmA during pneumococcal pneumonia.

GzmA was measured in bronchoalveolar lavage fluid (BALF) harvested from CAP patients from the infected and contralateral uninfected side, and in lung tissue slides from CAP patients and controls. Pneumonia was induced in wild type (WT) and GzmA deficient (GzmA$^{-/-}$) mice by intranasal inoculation of S. pneumoniae. In separate experiments WT and GzmA$^{-/-}$ mice were treated with natural killer (NK) cell depleting antibodies.

In CAP patients, GzmA levels were increased in BALF obtained from the infected lung. Human lungs showed constitutive GzmA expression by both parenchymal and non-parenchymal cells. Upon infection with S. pneumoniae, GzmA$^{-/-}$ mice showed a better survival and lower bacterial counts in BALF and distant body sites compared to WT mice. Although NK cells showed strong GzmA expression, NK cell depletion did not influence bacterial loads in either WT or GzmA$^{-/-}$ mice.

GzmA plays an unfavourable role in host defence during pneumococcal pneumonia by a mechanism that does not depend on NK cells.
INTRODUCTION

Streptococcus (S.) pneumoniae is the most frequently isolated causative pathogen in community-acquired pneumonia (CAP), responsible for up to 60% of bacterial cases\(^1\),\(^2\). CAP is a common illness worldwide, accounting for considerable morbidity and substantial mortality rates ranging from 2 up to 30% as it progresses into sepsis\(^3\). Despite the availability of an extensive arsenal of antibiotics, outcome has not improved over the past decades and therefore adjunctive measures are of vital importance to optimize treatment.

Granzymes are a family of serine proteases that have been characterized in man, rat and mouse. Stored in secretory granules of cytotoxic lymphocytes (CLs) the search for physiological substrates of granzymes has focused on their ability to induce apoptosis. Of the five human granzymes (A, B, H, K, M) the cytotoxic potential of the tryptase granzyme A (GzmA)\(^4\) however has become controversial, as GzmA-induced cytotoxicity was only observed in vitro at super-physiological concentrations in synergy with the pore-forming protein perforin\(^5\)-\(^7\). In addition, GzmA deficient (GzmA\(^{-/-}\)) mice demonstrated normal cytotoxic mechanisms\(^8\). Instead, mounting evidence points towards a role for GzmA in the host inflammatory response. GzmA was shown to cleave pro-IL-1β into its biologically active form\(^9\) and to induce the release of proinflammatory cytokines from monocytes/macrophages, fibroblasts, and epithelial cell lines\(^7\),\(^10\),\(^11\). Furthermore, extracellular matrix proteins are potential substrates for GzmA, which may influence cell migration\(^12\).

GzmA is constitutively expressed by NK, NKT and CD8+ T cells, while other cells require stimulation to induce expression\(^13\). More recently GzmA expression was reported in bronchiolar epithelial cells, alveolar macrophages and type II pneumocytes in human lung tissue with enhanced expression in the latter cell type in chronic obstructive pulmonary disease\(^14\). Plasma GzmA levels were elevated in patients with various parasitic, viral and bacterial infectious diseases\(^12\),\(^15\),\(^16\), severe sepsis caused by different bacterial pathogens\(^17\) and endotoxemia induced in healthy humans\(^16\). Moreover, whole blood stimulation with Gram-negative and Gram-positive bacteria resulted in GzmA release by leukocytes\(^15\). In contrast, GzmA did not seem to play an important role during lymphocytic choriomeningitis virus or Listeria monocytogenes infection\(^6\) and was reported to contribute to lethality during influenza\(^18\) and endotoxic shock\(^7\).

Here we set out to investigate the role of GzmA during pneumococcal pneumonia. For this we studied GzmA expression in lung tissue and measured GzmA levels in BALF samples of patients with CAP. To study the involvement of GzmA in host defence we induced pneumococcal pneumonia in GzmA\(^{-/-}\) and wild-type (WT) mice. In addition, we depleted NK cells to evaluate the contribution of NK cell-derived GzmA to the outcome of this experimental model of CAP.
MATERIALS AND METHODS

For more detailed Materials and Methods please see the online data supplement.

Human studies

Subjects
From healthy subjects and from patients with confirmed pneumonia bronchoalveolar lavage fluid (BALF) was harvested. Written informed consent was obtained from all individuals and the study was approved by the institutional medical ethical committee of the St. Antonius Hospital, Nieuwegein, the Netherlands.

Stored lung tissue slides of 10 patients who had succumbed to culture proven S. pneumoniae pneumonia and 9 patients who had died from a non-pulmonary cause were used, according to the ‘Code for Proper Secondary Use of Human Tissue,’ Dutch Federation of Medical Scientific Societies.

Granzyme A ELISA
GzmA protein levels were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) (Pelikine Compact™, Sanquin, Amsterdam, the Netherlands).

Immunohistochemistry
Tissue blocks were taken from human lungs at autopsy and processed for immunohistochemical analysis of GzmA using mouse monoclonal antibodies against GzmA (GA6, M1791; Sanquin, Amsterdam, The Netherlands).

Mouse studies

Experimental study design
The Institutional Animal Care and Use Committee of the Academic Medical Centre approved all experiments. Experiments were conducted with age and gender-matched C57BL/6 WT and Granzyme A (GzmA−/−) mice generously provided by Markus Simon (Max Planck Institute, Freiburg, Germany)⁸. S. pneumoniae serotype 3 (American Type Culture Collection, ATCC 6303, Rockville, MD) was used to induce pneumococcal pneumonia; in separate experiments WT mice were treated with NK1.1 cell (clone PK136) depleting antibody. Processing of samples and quantification of bacterial loads was done as described²⁰,²¹.
Flow Cytometry
Single cell suspensions of lungs were prepared as described elsewhere. Leukocytes were incubated with the following primary antibodies: fluorescein isothiocyanate (FITC)-labeled anti-CD122, peridinin-chlorophyl proteins (PerCP)-Cy5-labeled anti-NK1.1, allophycocyanin (APC)-labeled anti-CD49b and phycoerythrin (PE)-labelled anti-CD3 (all anti-mouse from eBioscience, San Diego, CA). To determine intracellular GzmA expression, cells were fixed, permeabilized and stained with FITC-labelled mouse anti-mouse GzmA mAb (Santa Cruz Biotechnology, Heidelberg, Germany). NK cells were identified based on forward and side scatter (lymphocyte gate) and as CD3-/NK1.1+ cells. The FACS Calibur flow cytometer (BD Biosciences) and FlowJo software (Tree Star, Ashland, OR) were used for analysis.

Assays
Levels of myeloperoxidase (MPO) (Hycult, Uden, the Netherlands), macrophage–inflammatory protein (MIP)–2, keratinocyte-derived cytokine (KC), interleukin (IL)-1β, IL-6, IL-12, tumour necrosis factor alpha (TNF-α), and interferon–gamma (IFN–γ) were determined using commercially available assays (R&D Systems, Abingdon, UK and BD Biosciences, San Jose, CA).

Histopathology and immunohistochemistry
Hematoxylin-and-eosin and neutrophil stainings were performed on lung tissue and scored as described previously.

Statistical analysis
Human data are presented as means ± SEM. Experimental data are expressed as box-and-whisker diagrams. Differences between groups were analysed by (un)paired t-tests, Mann–Whitney U tests or Kruskal-Wallis 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
Granzyme A in BALF and lung tissue of CAP patients
To obtain insight into the local release of GzmA during pneumonia, we measured GzmA in BALF derived from the infected and the uninfected lungs of 6 patients with unilateral CAP and from 8 healthy subjects. CAP patients were 52 ± 6 years of age (mean ± SE, 3 females, 3 males) and all recovered; causative organisms were identified as S. pneumoniae.
in 4, *Mycoplasma pneumoniae* in 1, and *Haemophilus influenzae* in 1 patient. GzmA levels were higher in BALF harvested from the infected lung compared to the uninfected lung from the same individual (Fig. 1A). To gain insight into which cell types express GzmA in human lung tissue during CAP, we stained lung tissue slides from 10 patients who had died from pneumonia caused by *S. pneumoniae* (69 ± 6 years) and from 9 patients who died without known pulmonary disease (62 ± 9 years) with an anti-GzmA antibody. Figure 1B shows representative photomicrographs of these stainings. Positive GzmA staining was found in a variety of pulmonary cells of some, but not all, samples. Positive immunostaining for GzmA was found in pneumocytes, endothelial cells (Figure 1B) and bronchiolar epithelium (not shown). In addition, few alveolar macrophages stained positive for GzmA. Moreover, in CAP patients, GzmA expression was found in intra-alveolar neutrophilic infiltrates (Figure 1B). Besides these GzmA positive neutrophilic infiltrates in CAP patients, there were no differences in GzmA staining between lung tissue slides from patients and control subjects.

**Figure 1. Granzyme A levels and expression during community-acquired pneumonia.**

Granzyme A (GzmA) levels in bronchoalveolar lavage fluid (BALF) harvested from the affected and unaffected side of community-acquired pneumonia (CAP) (n = 6) and control patients (n = 8) (A). GzmA immunostaining in tissue slides from control and CAP patients (B). Positive granular GzmA immunostaining was found in pneumocytes (closed arrow), in endothelial cells of pulmonary vessels (open arrow), bronchiolar epithelium and few alveolar macrophages (not shown). In CAP patients positive GzmA staining was observed in intra-alveolar neutrophilic infiltrates (asterisk). Scale bar indicates 200 μm.
Granzyme A reduces survival in murine *S. pneumoniae* pneumonia and has a detrimental effect on bacterial dissemination during *S. pneumoniae* pneumonia

To study the potential influence of GzmA on mortality from pneumococcal pneumonia, GzmA<sup>−/−</sup> and WT mice were observed in a survival study for seven days following intranasal infection with *Streptococcus* (*S.*) *pneumoniae* (*n* = 18 for both groups) observed for seven days (A). **p<0.01 compared with WT mice, log rank test. Number of colony forming units (CFU) in WT (grey boxes) and GzmA<sup>−/−</sup> (open boxes) mice per millilitre bronchoalveolar lavage fluid (BALF), lung homogenates, whole blood and spleen homogenates as indicated 6, 24 or 48 hours after intranasal infection with *S. pneumoniae* (B). Data are expressed as box-and-whisker diagrams (*n* = 8 per group). n.d., none detected. *p<0.05 compared with WT mice.

**Figure 2.** Granzyme A enhances bacterial dissemination and mortality in pneumococcal pneumonia. Survival of wild-type (WT, closed symbols) and granzyme A knock-out (GzmA<sup>−/−</sup>, open symbols) mice infected intranasally with *Streptococcus* (*S.*) *pneumoniae* (*n* = 18 for both groups) observed for seven days (A). **p<0.01 compared with WT mice, log rank test. Number of colony forming units (CFU) in WT (grey boxes) and GzmA<sup>−/−</sup> (open boxes) mice per millilitre bronchoalveolar lavage fluid (BALF), lung homogenates, whole blood and spleen homogenates as indicated 6, 24 or 48 hours after intranasal infection with *S. pneumoniae* (B). Data are expressed as box-and-whisker diagrams (*n* = 8 per group). n.d., none detected. *p<0.05 compared with WT mice.
sal infection with viable *S. pneumoniae*. GzmA$^{-/-}$ mice showed a strong protection from mortality (25%) compared to WT mice (67%, p=0.007) (Fig 2A). We compared bacterial loads in samples from WT and GzmA$^{-/-}$ mice at several time points (6, 24 and 48 hours) after induction of pneumonia to gain insight into the role of GzmA in the host resistance to *S. pneumoniae* (Fig 2B). In the early phase of infection GzmA$^{-/-}$ mice had lower bacterial loads in BALF (6 hours, p<0.05 versus WT mice), whereas in the more advanced stage of pneumonia GzmA$^{-/-}$ mice showed less systemic bacterial dissemination, reflected by lower bacterial counts in blood and spleen (24 hours, both p<0.05 versus WT mice).

**Granzyme A contributes to the early inflammatory response in the lung**

Invasion of the lower airways by *S. pneumoniae* results in an early inflammatory response that is essential for protective innate immunity$^{2,24}$. In order to obtain information about the early host response in the lungs, we analysed BALF and lung tissue harvested from GzmA$^{-/-}$ and WT mice 6 hours after infection. Considering the importance of neutrophils in host defence, we examined the extent of neutrophil recruitment by determining neutrophil counts in BALF and the number of Ly-6G positive cells in lung tissue. BALF neutrophil numbers were lower in GzmA$^{-/-}$ mice relative to WT mice 6 hours after intranasal inoculation with *S. pneumoniae* (p<0.01, Fig 3A); at this early time point, neutrophils were barely detectable in lung tissue and not different between mouse strains (data not

![Graph showing data](image)

**Figure 3. Granzyme A enhances the early inflammatory response in the lung during pneumococcal pneumonia.** Number of neutrophils (PMN) per millilitre bronchoalveolar lavage fluid (BALF) and levels of keratinocyte-derived cytokine (KC), macrophage–inflammatory protein (MIP)–2, interleukin (IL)-1β, IL-6 and tumour necrosis factor alpha (TNF-α) in lung homogenates of wild-type (WT, grey boxes) and granzyme A knock-out (GzmA$^{-/-}$, open boxes) mice, 6 hours after intranasal infection with *Streptococcus pneumoniae*. Data are expressed as box-and-whisker diagrams (n = 8 per group). ***p<0.001, **p<0.01 and *p<0.05 compared with WT mice.
shown). GzmA has been shown to induce pro-inflammatory mediators in vitro (7;9-11). In accordance with an attenuated early inflammatory response, GzmA−/− mice had lower levels of neutrophil attracting chemokines (KC and MIP-2) and cytokines (TNF-α and IL-1β) in whole lung homogenates harvested 6 hours post infection (Fig 3B). In accordance with previous studies25, H&E staining of lung tissue slides demonstrated only modest signs of pneumonia 6 hours after infection, with no differences between mouse strains (Supplementary Fig 1).

**Granzyme A deficiency has little impact on lung pathology during late stage pneumococcal pneumonia**

During late stage pneumonia perpetuation of lung inflammation can cause collateral damage thereby contributing to an adverse outcome2, 24. We investigated the extent of lung pathology in lungs harvested 48 hours after infection, that is, shortly before the first deaths occurred. Lung pathology tended to be lower in GzmA−/− mice at this late time point, as reflected by lower pathology scores (p<0.1 versus WT mice, Fig 4A; representative slides 4B). Similarly, lungs of GzmA−/− mice contained fewer neutrophils at 48 hours, as determined by the number of Ly-6G positive cells, although the difference with WT mice did not reach statistical significance (p=0.06, Fig 4C; representative slides 4D).

**Figure 4. Granzyme A has little effect on neutrophil influx and lung injury in the late phase of pneumococcal pneumonia.** Total lung histopathology scores (A) with representative microphotographs of haematoxylin and eosin stained lung sections (B) of WT and granzyme A knock-out (GzmA−/−) mice and accumulation of neutrophils in lung tissue expressed as percentage of Ly-6G positive lung tissue surface (C) with representative microphotographs (D) of neutrophil stained lung sections of WT and GzmA−/− mice 48 hours after induction of pneumococcal pneumonia. Data are expressed as box-and-whisker diagrams (n = 8 per group). Scale bar indicates 200 μm. # p<0.1 compared with WT mice.
Hence, these results suggest that GzmA has little influence on lung pathology during late stage pneumococcal pneumonia.

**Figure 5.** Natural Killer cells are the major cellular source of granzyme A.
Granzyme A (GzmA) was stained intracellularly in Natural Killer (NK) cells obtained from lungs and spleens of wild-type (WT) mice before, and 24 hours after infection with *Streptococcus pneumoniae* (SPWT)(A). Lymphocytes from lungs (B) and spleens (C) of WT, infected WT (SPWT) and granzyme A knock-out (GzmA−/−) mice were analysed by flow cytometry and the percentage NK cells of lymphocytes, the percentage of GzmA+ NK cells, and GzmA expression per NK cell in lung expressed as mean fluorescence intensity (MFI) were determined. Data are expressed as flow cytometry plots from representative samples (A) and dot-plots, each dot representing an individual sample (B and C). * p<0.05 as indicated.
Role of NK cells in pneumococcal pneumonia

Since NK cells are a major source of GzmA\textsuperscript{13, 18}, we next determined the contribution of NK cells to GzmA positivity in lungs of mice before and 24 hours after infection with \emph{S. pneumoniae} via the airways (Fig 5). GzmA positivity was almost exclusively found in NK cells in lungs and the hematopoietic compartment (Fig 5A). Whereas the percentage of NK cells did not change after infection in lungs (Fig 5B) or spleens (Fig 5C) of either GzmA\textsuperscript{−/−} or WT mice, the percentage GzmA positive NK cells and the GzmA MFI increased in lungs of infected WT mice; as expected GzmA staining was negative in GzmA\textsuperscript{−/−} mice.

We next set out to deplete WT and GzmA\textsuperscript{−/−} mice from NK cells using a NK1.1 depleting antibody. This treatment resulted in a 97% reduction of the NK1.1 (CD122\textsuperscript{+} / CD3\textsuperscript{−}) lymphocyte population in lungs of WT mice (versus 94% in GzmA\textsuperscript{−/−} mice) and 92%

Figure 6. Depletion of Natural Killer cells does not impact on bacterial loads in wild-type or granzyme A knock-out mice during pneumococcal pneumonia. Wild-type (WT) and granzyme A knock-out (GzmA\textsuperscript{−/−}) mice were infected intranasally with \emph{Streptococcus pneumoniae} and treated with Natural Killer 1.1 cell depleting antibody (NK depl) or placebo (co); samples were obtained 24 hours post-infection. Number of colony forming units (CFU) in WT control mice (WT co, grey boxes), NK depleted WT mice (WT NK depl, grey striped boxes), GzmA\textsuperscript{−/−} mice (GzmA\textsuperscript{−/−} co, open boxes), and NK depleted GzmA\textsuperscript{−/−} mice (GzmA\textsuperscript{−/−} NK depl, open striped boxes) per millilitre lung homogenates (A), whole blood (B) and spleen homogenates (C). Data are expressed as box-and-whisker diagrams (n = 8 per group). ***p<0.001, **p<0.01 and *p<0.05 as indicated.
reduction in blood (versus 91% in GzmA−/− mice) (Table 1). In line with the initial 24-hour time point experiment (Fig 2), GzmA−/− mice had lower bacterial loads in lungs (p=0.06), spleen (p=0.006) and blood (p<0.001) compared to WT mice (Fig 6). However, NK cell depletion did not influence bacterial loads in either WT or GzmA−/− mice (Fig 6). In this experiment, GzmA−/− mice (relative to WT mice) showed lower concentrations of cytokine and chemokine levels in whole lung homogenates, significantly so for KC (Table 2). NK cell depletion did not impact on lung cytokine or chemokine levels in either WT or GzmA−/− mice.

Table 1. NK cell depletion rates in lung and blood of wild-type and granzyme A knock-out mice.

<table>
<thead>
<tr>
<th></th>
<th>WT control</th>
<th>WT NK depl</th>
<th>GzmA−/− control</th>
<th>GzmA−/− NK depl</th>
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</thead>
<tbody>
<tr>
<td>Lung CD122+/CD3−</td>
<td>10.0 ± 0.5</td>
<td>0.3 ± 0.1***</td>
<td>8.2 ± 1.0</td>
<td>0.4 ± 0.1***</td>
</tr>
<tr>
<td>% depletion</td>
<td>97%</td>
<td>94%</td>
<td></td>
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<tr>
<td>Blood CD122+/CD3−</td>
<td>3.4 ± 0.4</td>
<td>0.3 ± 0.0 (92%)***</td>
<td>4.2 ± 0.5</td>
<td>0.4 ± 0.1 (91%)***</td>
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<tr>
<td>% depletion</td>
<td>92%</td>
<td>91%</td>
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Percentage of NK cells, defined as CD122+/CD3− lymphocytes, in wild-type (WT) and granzyme A knock-out (GzmA−/−) control and Natural Killer cell depleted (NK depl) WT and GzmA−/− mice and percentage of NK cell depletion at time of infection. *** indicates P < 0.001 versus WT; ### indicates P<0.001 versus GzmA−/−

Table 2. Levels of cytokines and chemokines in lung homogenates of wild-type and granzyme A knock-out mice with/without NK cells during Streptococcus pneumoniae pneumonia.

<table>
<thead>
<tr>
<th></th>
<th>WT control</th>
<th>WT NK depl</th>
<th>GzmA−/− control</th>
<th>GzmA−/− NK depl</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIP-2 (pg/ml)</td>
<td>3871 ± 711</td>
<td>7393 ± 2049</td>
<td>2429 ± 437</td>
<td>2001 ± 261</td>
</tr>
<tr>
<td>KC (pg/ml)</td>
<td>10387 ± 1569</td>
<td>16884 ± 3155</td>
<td>3200 ± 1350#</td>
<td>3263 ± 1169##</td>
</tr>
<tr>
<td>IL-1β (pg/ml)</td>
<td>525 ± 134</td>
<td>922 ± 225</td>
<td>324 ± 163</td>
<td>134 ± 42##</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>4410 ± 1035</td>
<td>7788 ± 1900</td>
<td>1985 ± 1044</td>
<td>1203 ± 734##</td>
</tr>
<tr>
<td>TNFα (pg/ml)</td>
<td>443 ± 74</td>
<td>775 ± 143</td>
<td>284 ± 92</td>
<td>158 ± 25</td>
</tr>
<tr>
<td>IFN-γ (pg/ml)</td>
<td>18.7 ± 5.6</td>
<td>6.1 ± 1.3</td>
<td>5.9 ± 2.9</td>
<td>1.9 ± 0.7</td>
</tr>
</tbody>
</table>

Levels of cytokines and chemokines in lung homogenates of wild-type (WT) and granzyme A knock-out (GzmA−/−) mice and Natural Killer cell depleted (NK depl) WT and GzmA−/− mice 24 hours after induction of pneumococcal pneumonia. Data are expressed as mean ± SEM of n = 8 per group. IL, interleukin; IFN, interferon; TNF, tumour necrosis factor; KC, keratinocyte-derived cytokine; MIP-2, Macrophage–inflammatory protein–2. * indicates p < 0.05 vs WT control; ## indicates P < 0.01 versus WT NK depleted.
DISCUSSION

Research on the function of granzymes has mainly focused on their ability to cause target cell death. However, the role of GzmA as inducer of target cell death is controversial and recent studies have pointed to a role of GzmA in the regulation of innate immunity during infectious and inflammatory conditions. Here we report GzmA expression in BALF and lungs of CAP patients and show that GzmA deficiency during experimental CAP caused by *S. pneumoniae* is associated with reduced bacterial growth and improved survival.

GzmA is constitutively expressed by NK, NKT and γδT cells, by approximately half of circulating CD8+ cells and few CD4+ cells. Additionally, GzmA expression can be induced in mast cells, macrophages, T regulatory cells and human B cells; however, the possible function of GzmA within non-lymphoid cells thus far has not been established. We aimed to look at GzmA expression in human lung tissue in the setting of CAP caused by *S. pneumoniae*. In line with a previous report, we demonstrated GzmA expression by bronchial epithelial cells, pneumocytes, tissue macrophages and a small number of alveolar macrophages. Notably, endothelial cells also demonstrated GzmA expression. Although enhanced GzmA expression by type II pneumocytes in patients with severe COPD points towards a role for GzmA in chronic lung inflammation, we did not observe any difference in GzmA expression between CAP patients and patients who died from a non-pulmonary cause apart from GzmA positive neutrophilic infiltrates in the former group. We did detect elevated GzmA levels in BALF obtained from the infected lung of patients with unilateral CAP. Together these data indicate that GzmA is constitutively expressed by multiple cell types in human lungs, and that infection results in local release of GzmA.

The generation of GzmA deficient mice has provided the opportunity to gain more insight in to the functional role of GzmA in infectious diseases. Previous studies have indicated that the involvement of GzmA in the host response to infection varies dependent on the causative organism. GzmA−/− mice were more susceptible to ectromelia infection and the parasite *Trypanosoma cruzi* than WT mice. However, GzmA did not seem to play an important role during infection with either lymphocytic choriomeningitis virus or *Listeria monocytogenes* and GzmA−/− mice were protected from death during influenza infection. GzmA−/− mice were resistant to endotoxic shock, illustrating the proinflammatory properties of this protease. In the present study we infected GzmA−/− and WT mice with viable *S. pneumoniae* and found reduced bacterial numbers in BALF in the early phase and in the systemic compartment at 24 hours post infection with protection from mortality in an observational study. Of note, the observed difference in systemic bacterial counts 24 hours post-infection was no longer present at 48 hours, which may reflect maximal systemic bacterial loads in both study groups.
data suggest that the presence of GzmA facilitates pneumococcal multiplication and dissemination, leading to increased mortality.

A pro-inflammatory role for GzmA was first suggested when GzmA was shown to cleave pro-IL-1β into its biologically active form⁹. Subsequent studies have shown that GzmA induces the release of proinflammatory cytokines such as IL-1β, IL-6, IL-8 and TNF-α from monocytes/macrophages, fibroblasts, and epithelial cell lines⁷, ¹⁰, ¹¹. In addition, GzmA⁻/⁻ mice showed a reduced inflammatory response during parasitic infection²⁷. In line with these studies, GzmA⁻/⁻ mice demonstrated less neutrophil influx in BALF 6 hours post-infection, together with lower levels of IL-1β, TNFα, KC and MIP-2 in lung homogenates. In the late stage of pneumonia (48 hours post infection), apart from elevated levels of KC, no major differences were found in levels of chemokines/cytokines between WT and GzmA⁻/⁻ mice in the pulmonary compartment, nor in the systemic compartment (data not shown), implying a limited role for GzmA in this phase. Several extracellular matrix proteins, such as fibronectin, laminins, basement membrane collagen type IV and the membrane bound protease-activated receptor (PAR)-2 are substrates of GzmA¹⁸, and their degradation may contribute to tissue damage. Nonetheless, in the present study only a tendency towards reduced lung histopathology was found in the absence of GzmA.

NK cells are considered the main source of GzmA¹³, ¹⁸ and in naïve mice NK cells were mainly GzmA positive. During pneumonia the percentage of NK cells did not increase locally or systemically, however, the percentage of GzmA positive NK cells, GzmA mRNA expression in lung tissue (data not shown) and GzmA expression per NK cell increased. Remarkably, depletion of NK cells did not alter bacterial loads in either GzmA⁻/⁻ or WT mice. A previous study reported a detrimental role for NK cells in pneumonia caused by serotype 2 S. pneumoniae; of note, however, these investigations were performed with a less virulent bacterial strain (D39), administered at much higher doses as used here (10⁶ versus 5x10⁴ CFU) to a different mouse strain (BALBc)³¹. In contrast, NK cells were important for protective immunity during Klebsiella pneumonia³². The current results suggest that in our model of severe pneumonia caused by a highly virulent pneumococcal strain NK cells may either require GzmA to exert a protective role, or that NK cells do not play an important role in host defence and that another cellular source of GzmA may account for the adverse outcome of WT mice relative to GzmA⁻/⁻ mice. In this regard CD8⁺ T cells are an unlikely GzmA source, considering that antibody induced CD8⁺ T-cell depletion increased, rather than reduced bacterial loads³³ (and our own unpublished results).

This study is the first to investigate the role of GzmA in host defence against CAP. We here link observational data in humans showing pulmonary GzmA expression with functional studies in experimental pneumococcal pneumonia using GzmA⁻/⁻ mice. Our results point to a detrimental role for GzmA in respiratory tract infection caused by S. pneumoniae that is independent of NK cells.
REFERENCES


SUPPLEMENTARY MATERIAL

MATERIALS AND METHODS

Human studies

Immunohistochemistry
Tissue blocks were taken from human lungs at autopsy, fixed without inflation in 10% phosphate-buffered formalin, and embedded in paraffin wax. Sections (4 μm) were cut and processed for immunohistochemical analysis of GzmA. For this, deparaffinized sections were boiled in 10 mM citrate buffer for antigen retrieval and treated with 5% bovine serum albumin in tris-buffered saline (TBS) to reduce background staining. Mouse monoclonal antibodies against GzmA (GA6, M1791; Sanquin, Amsterdam, The Netherlands) were used followed by biotin-conjugated rabbit anti-mouse IgG antibody (E-0413; Dako Cytomation, Glostrup, Denmark). After applying alkaline-phosphatase–labeled avidin-biotin complex (ABC-AP, K-0376; Dako Cytomation), enzymatic reactivity was visualized using Vector Blue (Vector Laboratories, Burlingame, CA) and sections were counterstained with Nuclear Fast Red (Vector Laboratories) and mounted. Spleen tissue was used as a positive control. Negative controls for nonspecific binding by omitting the primary detecting antibodies or applying normal mouse IgG instead of the primary antibodies revealed no signal.

Mouse studies

Animals
Granzyme A (GzmA<sup>−/−</sup>) mice on a C57Bl/6 background were originally obtained from Dr. M. M. Simon<sup>1</sup> and bred at the animal care facility of the Academic Medical Centre. C57Bl/6 mice were purchased from Charles River (Maastricht, The Netherlands) and all experiments were conducted with 10 to 12-week old age-and gender-matched mice. 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) were grown as described<sup>2,3</sup> and ~5 x 10<sup>4</sup> colony-forming units (CFU) in 50 μL were inoculated intranasally to induce pneumococcal pneumonia. Mice were observed in a survival study or sacrificed at 6, 24 or 48 hours after induction of pneumococcal pneumonia. NK cell depletion was performed by intraperitoneal (i.p) administration of 500 μg NK1.1 cell
depleting antibody (clone PK136) 2 and 1 days prior to induction of pneumonia. Lung, spleen, blood and BALF cells were isolated using methods described previously\(^2,3\).

Total cell numbers in BALF were determined by an automated cell counter (Coulter Counter, Coulter Electronics, Hialeah, FL). Differential cell counts were performed on cytospin preparations stained with a modified Giemsa stain (Diff-Quick; Dade Behring AG, Düdingen, Switzerland).

**Bacterial quantification**

To assess bacterial loads undiluted whole blood and serial ten-fold dilutions of whole blood, organ homogenates and BALF were made in sterile isotonic saline and plated onto sheep–blood agar plates. Following 16 hours of incubation at 37°C colony forming units (CFU) were counted.

**Flow Cytometry**

Lung cell suspensions were obtained by crushing lungs through a 40-μm cell strainer (Becton Dickinson, Franklin Lakes, NJ) as described previously\(^4\). Erythrocytes were lysed by ammonium chloride and the cells were washed twice and resuspended in FACS staining buffer (PBS with 0.5% BSA, 0.01% Na-azide and 0.02% potassium-EDTA). Leukocytes were incubated for 30 min at 4°C with the following primary antibodies: fluorescein isothiocyanate (FITC)-labeled anti-CD122, peridinin-chlorophyl proteins (PerCP)-Cy5-labeled anti-NK1.1, allophycocyanin (APC)-labeled anti-CD49b and phycoerythrin (PE)-labeled anti-CD3 (all anti-mouse from eBioscience, San Diego, CA). To determine intracellular GzmA expression, cells were fixed and permeabilized with fixation/permeabilization solution (BD Biosciences, Franklin Lakes, NJ) and stained with FITC-labeled mouse anti-mouse GzmA mAb (Santa Cruz Biotechnology, Heidelberg, Germany) in the presence of anti-CD16/CD32 block (2.4G2, Biocers BV, Utrecht, The Netherlands) to prevent nonspecific antibody binding. GzmA expression in NK1.1 cells was determined by flow cytometry; NK cells were identified based on forward and side scatter (lymphocyte gate) and as CD3-/NK1.1+ cells. The FACSCalibur flow cytometer (BD Biosciences) and FlowJo software (Tree Star, Ashland, OR) were used for analysis.

**Histopathology and immunohistochemistry**

Four-micrometer sections of the left lung lobe were stained with hematoxylin and eosin (H&E). All slides were coded and scored by a pathologist who was blinded for group identity for the following parameters: interstitial inflammation, endothelialitis, bronchitis, edema and pleuritis. Confluent (diffuse) inflammatory infiltrate was quantified separately and expressed as percentage of the lung surface. The remaining parameters were rated separately on a scale from 0 (condition absent) to 4 (severe condition). Neu-
trophil stainings were performed using an anti-mouse Ly-6G monoclonal antibody (BD Pharmingen, San Diego, CA) and analyzed as described previously.

Figure S1. Granzyme A has little effect on lung injury in the early phase of pneumococcal pneumonia. Total lung histopathology scores (A) with representative microphotographs of haematoxylin and eosin stained lung sections (B) of wild-type (WT) and and granzyme A knock-out (GzmA−/−) mice 6 hours after induction of pneumococcal pneumonia. Data are expressed as box-and-whisker diagrams (n = 8 per group). Scale bar indicates 200 μm.
REFERENCES


