Granzymes A and B Regulate the Local Inflammatory Response during Klebsiella pneumoniae Pneumonia

M. Isabel García-Laorden a, Ingrid Stroo a, e, Dana C. Blok a, Sandrine Florquin b, Jan Paul Medema a, c, Alex F. de Vos a, Tom van der Poll a, d

a Center for Experimental and Molecular Medicine (CEMM), b Department of Pathology, c Laboratory for Experimental Oncology and Radiobiology (LEXOR), d Division of Infectious Diseases, Academic Medical Center, and e Department of Immunopathology, Sanquin Research at CLB and Landsteiner Laboratory of the Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

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Granzymes · Pneumonia · Klebsiella pneumoniae · Sepsis · Mouse model

Abstract
Klebsiella pneumoniae is a common cause of hospital-acquired pneumonia. Granzymes (gzms), mainly found in cytotoxic lymphocytes, have been implicated as mediators of infection and inflammation. We here sought to investigate the role of gzmA and gzmB in the host response to K. pneumoniae-induced airway infection and sepsis. For this purpose, pneumonia was induced in wild-type (WT) and gzmA-deficient (gzmA-/-), gzmB-/- and gzmAxB-/- mice by intranasal infection with K. pneumoniae. In WT mice, gzmA and gzmB were mainly expressed by natural killer cells. Pneumonia was associated with reduced intracellular gzmA and increased intracellular gzmB levels. Gzm deficiency had little impact on antibacterial defence: gzmA-/- and gzmAxB-/- mice transiently showed modestly higher bacterial loads in the lungs but not in distant organs. GzmB-/- and, to a larger extent, gzmAxB-/- mice displayed transiently increased lung inflammation, reflected in the semi-quantitative histology scores and levels of pro-inflammatory cytokines and chemokines. Most differences between gzm-deficient and WT mice had disappeared during late-stage pneumonia. Gzm deficiency did not impact on distant organ injury or survival. These results suggest that gzmA and gzmB partly regulate local inflammation during early pneumonia but eventually play an insignificant role during pneumosepsis by the common human pathogen K. pneumoniae.

Introduction
Pneumonia is the leading cause of sepsis, one of the most important causes of mortality in intensive care departments. Klebsiella pneumoniae, a Gram-negative bacterium, is one of the main causative pathogens in hospital-acquired pneumonia and is associated with high rates of morbidity and mortality [1, 2]. Emerging bacterial resistance to antibiotics is currently a serious problem for the effective treatment of this micro-organism [3].
Granzymes (gzms) are a family of serine proteases found in the granules of cytotoxic lymphocytes. In humans, 5 different gzms (A, B, H, K and M) exist, while mice express gzms A–G, K, M and N [4]. Granzyme A (gzmA) and gzmB, the most abundant gzms, are expressed constitutively in several cell types including cytotoxic T lymphocytes, natural killer (NK) cells, NK T cells and γδ T cells [5, 6]. Multiple studies have described the role of gzms in eliminating infected, neoplastic or foreign cells; however, the physiological relevance of gzmA cytotoxicity remains controversial [4]. Other evidence suggests that the properties of gzms are diverse and go beyond cytotoxicity. Plasma levels of gzmA and gzmB have been found to be elevated in patients with several parasitic, viral and bacterial diseases [7, 8], severe sepsis [9, 10] and in endotoxemia induced in healthy individuals [11]. The induction of gzmA and gzmB secretion was also shown after stimulation of whole blood with Gram-negative and Gram-positive bacteria [11]. Moreover, diverse extracellular substrates have been identified for gzms [8, 12], and a role for gzmA and gzmB has been reported in mediating cytokine release or maturation [13]. In addition, previous studies have shown that gzmA-deficient (gzmA–/–) and gzmB–/– mice are relatively protected against endotoxin-induced shock [14, 15]. Collectively, these studies suggest a role for gzms in infection and inflammation which goes beyond cytotoxicity.

Current knowledge about the role of gzms in the host response to K. pneumoniae and the pathogenesis of pneumonia and sepsis is highly limited. We sought to investigate this subject by analysing the local and systemic inflammatory responses and damage using a murine model of pneumonia-derived sepsis caused by K. pneumoniae in wild-type (WT) mice and in mice deficient in gzmA, gzmB or both.

Materials and Methods

Animals
C57BL/6 WT mice were purchased from Charles River Laboratories Inc. (Maastricht, The Netherlands). gzmA–/–, gzmB–/– and gzmAxB–/– mice on a C57BL/6 background were kindly provided by Dr. M.M. Simon (Max Planck Institute, Freiburg, Germany) [16]. Experimental groups were age- and gender-matched, and housed in the Animal Research Institute, Amsterdam, under standard care. All experiments were conducted on mice that were 10–12 weeks of age. The Animal Care and Use Committee of the Academic Medical Center approved all experiments.

Experimental Study Design
Pneumonia was induced by intranasal inoculation with 4 × 10^3 colony-forming units (CFU) of K. pneumoniae serotype 2 (ATCC 43816; American Type Culture Collection, Manassas, Va., USA) in 50 μl of saline solution as previously described [17, 18]. Mice were followed for a maximum of 14 days (n = 20 mice per group) or, in separate experiments, euthanized at 6, 16 or 48 h after infection (n = 8 mice per group). Blood was drawn into heparinized tubes, and the lung, spleen and liver were harvested and processed as described [17, 18]. To analyse induction of gzmA and gzmB, groups of WT mice were euthanized before and after infection, and blood and lung were harvested and processed for flow cytometry (n = 5).

Histopathology
Lung sections (4 μm thick) were stained with haematoxylin and eosin and analysed by a pathologist who was blinded for groups. To score lung inflammation and damage, a semi-quantitative scoring system of the following parameters was used [18]: interstitial inflammation, endothelialitis, bronchitis, oedema, pleuritis and the presence of thrombi. Each parameter was rated separately on a scale of 0 (condition absent) to 4 (very severe). The total lung inflammation score was expressed as the sum of the scores of the individual parameters, with a maximum of 24. In addition, pulmonary infiltrate was scored as a percentage of total lung surface occupied by confluent infiltrates. Liver injury was scored according to the following parameters: interstitial inflammation (also on a scale of 0–4), number of thrombi and the percentage of hepatocellular necrosis.

Assays
Interleukin (IL)-6, IL-10, tumour necrosis factor (TNF)-α, interferon (IFN)-γ, IL-1β, cytokine-induced neutrophil chemoattractant (KC, CXCL1), macrophage inflammatory protein 2 (MIP-2, CXCL2) and myeloperoxidase (MPO) were measured in the supernatants of lung homogenates by ELISA (R&D Systems, Abingdon, UK) in accordance with the manufacturer’s instructions. Plasma levels of IL-6, IL-10, TNF-α, IFN-γ, IL-12-p70 and monocyte chemotactic protein 1 (MCP-1) were measured by cytometric bead array multiplex assay (BD Biosciences, San Jose, Calif., USA) according to the manufacturer’s recommendations. Lactate dehydrogenase (LDH), aspartate aminotransferase (ASAT) and alanine transaminase (ALAT) levels were measured in plasma with kits from Sigma (St. Louis, Mo., USA) by a Hitachi analyser (Boehringer Mannheim, Mannheim, Germany).

Flow Cytometry
Blood erythrocytes were lysed with ice-cold isotonic NH₄Cl solution (155 mM NH₄Cl, 10 mM KHCO₃ and 0.1 mM EDTA, pH 7.4); the remaining cells were washed and counted using a haemocytometer. Lungs were cut and digested for 30 min at 37°C in HBSS (Invitrogen, Leek, The Netherlands) with 0.1 mg/ml DNase I (Roche, Almere, The Netherlands) and 1 mg/ml collagenase D (Roche) and passed through a 70-μm cell strainer, after which red blood cells were lysed; the remaining cells were counted. Immunostaining for cell surface molecules was performed for 25 min at 4°C in the dark, using directly labelled antibodies against CD3 (PerCP-Cy5.5; BD Pharmingen, San Jose, Calif., USA), CD4 (AF700; BD Pharmingen), CD8 (APC-Cy7; BioLegend, San Diego, Calif., USA), γδTCR (APC; Bioscience, San Diego, Calif., USA) and NK1.1 (PE-Cy7; Bioscience). For the intracellular staining,
cells were fixed for 20 min in Cytofix/Cytoperm (BD Bioscience, San Jose, Calif., USA) at 4°C in the dark and resuspended in perm/wash buffer containing the antibodies against gzmA (PE; Santa Cruz Biotechnology, Dallas, Tex., USA) and gzmB (PE-CF594; BD Horizon, San Jose, Calif., USA). These 2 antibodies were tested in samples from the 3 knockout strains, to exclude cross-reaction with other gzms. Only minor unspecific gzmA staining (mostly in CD3– and NK1.1– cells) was observed in 
\[gzmA^{--}\] and \[gzmAxB^{--}\] mice (data not shown). All antibodies were used in concentrations recommended by the manufacturer. The cells were analysed by flow cytometry with a FACSCanto device (BD Bioscience). FlowJo software (Tree Star Inc., Ashland, Oreg., USA) was used to analyse the data. Live cells were gated in the forward-scatter versus side-scatter dot plot. Cells were selected as CD3+CD4+ (CD4+ T), CD3+CD8+ (CD8+ T), CD3+γδTCR+ (γδ T), CD3+NK1.1+ (NK1.1+ T) and CD3–NK1.1+ (NK), and the expression of gzmA and gzmB was analysed in these populations. The results are expressed as percentage of cells of the specific population expressing the corresponding gzm and as the median fluorescence intensity (MFI). Alternatively, cells were selected as positive for each gzm and the percentages of the above-mentioned lymphocyte populations were analysed within the gzm-positive live cells.

**Statistical Analysis**

Data in figures are expressed as box and whiskers, showing the smallest observation, lower quartile, median, upper quartile and largest observation (n = 5 at each time point).

**Fig. 1.** Lymphocyte source of intracellular gzmA and gzmB in the lungs and blood of WT mice before and after infection with *K. pneumoniae* via the airways. Mice were infected with \(4 \times 10^3\) CFU *K. pneumoniae* intranasally and sacrificed before and at 6, 16 and 48 h after infection. Percentage of the total gzmA (a, c) or gzmB (b, d) expressed by different lymphocyte subsets from the lungs (a, b) and blood (c, d) are shown. Box-and-whisker diagrams depict the smallest observation, lower quartile, median, upper quartile and largest observation (n = 5 at each time point).
largest observation, or as individual data points. Comparisons were performed using the Kruskal-Wallis test for multiple groups and the Mann-Whitney U test for 2 independent groups. Survival was compared by Kaplan-Meier analysis followed by a log-rank test. Analyses were done using GraphPad Prism v4.0 and the SPSS Statistical Package v15.0. Statistical significance was taken as p < 0.05.

**Results**

**Expression of Granzymes A and B during K. pneumoniae Pneumonia**

To evaluate a possible role for gzmA and gzmB in pneumonia, we first sought to ascertain their expression before and during *Klebsiella* pneumonia. We infected WT mice intranasally, and analysed the intracellular expression of gzmA and gzmB in lymphocytes in whole lungs and blood obtained before and 6, 16 and 48 h after infection using flow cytometry. Only a low proportion of the live cells from the lungs and blood expressed gzmA and gzmB (data not shown). We focused on lymphocyte subsets previously implicated in gzm expression, i.e. NK, NK1.1+, T, γδ T, CD8+ T and CD4+ T cells [8] (the gating strategy for each subpopulation is shown in online suppl. fig. 1; see www.karger.com/doi/10.1159/000443401 for all online suppl. material). As shown in figure 1, amongst these lymphocyte subsets, NK cells were by far the most abundant for both gzmA and gzmB expression.
prominent cellular source of gzmA and gzmB in both the lungs and the blood, both before and after infection with *Klebsiella*. The percentage of the NK cells from the lungs and blood expressing gzmA initially did not change after infection with *Klebsiella*; however, there was a strong decrease after 48 h (fig. 2a). The MFI of these cells (i.e. the amount of gzmA expressed by gzmA+ NK cells) showed a clear decreasing trend over time (fig. 2b). On the contrary, the expression of gzmB by NK cells was very low in uninfected mice and increased strongly after infection (fig. 2c, d). Only a small percentage of γδ T cells and an even smaller amount of CD8+ T and CD4+ T cells expressed gzmA or gzmB in both lungs and blood (online suppl. table 1).

**Granzyme A and Granzyme B Deficiency Has Little Impact on Bacterial Growth and Dissemination during *K. pneumoniae Pneumonia***

To investigate whether gzmA and gzmB influence local bacterial outgrowth and dissemination to distant organs, we determined bacterial loads in lung homogenates as well as in the blood, spleen and liver of WT, gzmA−/−, gzmB−/− and gzmAxB−/− mice at predefined time points after infection (fig. 3). Gzm deficiency had little if any impact on antibacterial defence; at 16 h after infection, gzmA−/− and gzmAxB−/− mice had modestly but statistically significantly higher bacterial burdens in their lungs but not in the blood or distant organs. Bacterial loads were similar in all mouse strains at 6 and 48 h.

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**Fig. 3.** Bacterial loads in WT, gzmA−/−, gzmB−/− and gzmAxB−/− mice during *K. pneumoniae* pneumonia. Mice were infected with 4 × 10⁵ CFU *K. pneumoniae* intranasally. To determine bacterial loads in the lungs (a), blood (b), spleen (c) and liver (d), mice were sacrificed at 6, 16 and 48 h after infection. Box-and-whisker diagrams depict the smallest observation, lower quartile, median, upper quartile and largest observation (n = 7–8 per group at each time point). Dashed lines represent the lower detection limit of CFU. *p < 0.05 (Mann-Whitney U test).
**Fig. 4.** Histopathology of the lungs of WT, gzmA−/−, gzmB−/− and gzmAxB−/− mice during *K. pneumoniae* pneumonia. Mice were infected with 4 × 10^5 CFU *K. pneumoniae* intranasally and sacrificed at 6, 16 and 48 h after infection. a Semi-quantitative histology total score of lung slides is represented. Staining on representative lung slides of WT (b), gzmA−/− (c), gzmB−/− (d) and gzmAxB−/− (e) mice 16 h after *K. pneumoniae* infection. HE. ×10. Scale bars: 200 μm. Box-and-whisker diagrams depict the smallest observation, lower quartile, median, upper quartile and largest observation (n = 7–8 per group at each time point). * p < 0.05, ** p < 0.01, *** p < 0.001 (Mann-Whitney U test).
5,000 IL-6 (pg/ml)

6 h 16 h 48 h

WT

gzmA−/−
gzmB−/−
gzmAxB−/−

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**Effect of Granzyme A and Granzyme B Deficiency in Lung Inflammation during K. pneumoniae Pneumonia**

This model of respiratory tract infection is associated with a gradually developing inflammatory response at the primary site of infection that eventually results in severe lung inflammation with the characteristic histological features of pneumonia [17, 18]. To obtain insight into the role played by gzmA and gzmB, we semi-quantitatively analysed lung histology slides prepared from gzmA−/−, gzmB−/− and gzmAxB−/− mice at 6, 16 and 48 h after infection (fig. 4).

At the earliest time point (6 h) relatively little pathology was observed in all groups, with gzmA−/− mice showing a slightly higher total lung pathology score than WT mice (fig. 4a). Remarkably, while at 16 h after infection the extent of lung pathology had modestly increased in WT mice, the gzmB−/− mice and, to a larger extent, gzmAxB−/− mice, presented with strongly increased lung pathology. At the later time point (48 h), shortly before the first mice died (see below), all mouse strains demonstrated profound lung pathology with no relevant differences between groups.

To obtain further insight into the local inflammatory response and the impact of gzmA and gzmB deficiency, we measured the levels of pro-inflammatory (IL-6, TNF-α, IFN-γ and IL-1β) and anti-inflammatory (IL-10) cytokines and chemokines (KC and MIP-2) in lung homogenates (fig. 5). Consistent with the increased lung pathology at 16 h after infection, gzm-deficient animals (most notably, the gzmAxB−/− mice) displayed elevated lung levels of pro-inflammatory cytokines and chemokines relative to WT mice at this time point. Increased lung inflammation was also reflected in elevated levels of MPO in the lungs (reflecting the neutrophil content) in gzmAxB−/− mice at 16 h after infection, although these differed significantly only versus gzmB−/− mice. Differences between the mouse strains had largely subsided at 48 h after infection, although IL-1β and MPO remained higher in the gzmAxB−/− mice. Of interest, soon after the induction of pneumonia (i.e. at 6 h), when lung inflammatory mediator levels were still low, gzmA-deficient mice showed clearly lower levels of IL-1β and KC than the other groups, and gzmAxB−/− animals demon-

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**Granzymes and K. pneumoniae Pneumonia**

**Fig. 6.** Plasma levels of ASAT, ALAT and LDH in WT, gzmA−/−, gzmB−/− and gzmAxB−/− mice during K. pneumoniae pneumonia. Mice were infected with 4 × 10^3 CFU K. pneumoniae intranasally. Figure shows plasma levels ASAT, ALAT and LDH 48 h after infection. Box-and-whisker diagrams depict the smallest observation, lower quartile, median, upper quartile and largest observation (n = 7–8 per group). No significant differences were observed between groups by Mann-Whitney U test.

**Fig. 7.** Survival in WT, gzmA−/−, gzmB−/− and gzmAxB−/− mice during K. pneumoniae pneumonia. Mice were infected with 4 × 10^3 CFU K. pneumoniae intranasally and were monitored for 14 days for mortality. Each symbol represents an individual mouse (n = 20 per group). No significant differences were observed between groups by log-rank test.
Granzyme A and Granzyme B Deficiency Does Not Influence Distant Organ Injury during *K. pneumoniae* Pneumonia

This model of severe pneumonia is associated with development of distant organ injury during late-stage infection [17, 18]. To determine the role played by gzm, we measured the plasma concentrations of ASAT and ALAT (both parameters of hepatocellular injury) and LDH (indicative for cellular injury in general). While these injury markers remained unaltered up to 16 h after infection in all groups (data not shown), their plasma levels were increased 48 h after infection; values did not differ between mouse strains, however (fig. 6). In accordance, the extent of liver pathology, determined by the semi-quantitative scoring system described in the Methods section, did not differ between groups (online suppl. fig. 2).

Granzyme A and Granzyme B Deficiency Does Not Influence Mortality during *K. pneumoniae* Pneumonia

The *Klebsiella* strain used here is highly virulent, causing high mortality rates at low infectious doses [17, 18]. Consistent with the transient effect on lung pathology and the unaltered distant organ injury, the gzm-deficient mice had similar mortality rates to the WT mice (fig. 7).

Discussion

The respiratory tract is the most common primary site of infection in sepsis, and *K. pneumoniae* is a clinically important Gram-negative pathogen in hospital-acquired pneumonia. Previous studies reported elevated plasma levels of gzmA and gzmB in patients with severe sepsis [9, 10]. Since Gram-negative bacteria, as well as lipopolysaccharide (LPS), are potent inducers of gzmA and gzmB release in vitro [11], we sought to investigate the role of these gzms in the host response to *Klebsiella* in vivo. To this end, we used a mouse model of *K. pneumoniae* pneumonia in WT mice and mice deficient in gzmA and/or gzmB. Although gzmB deficiency, in particular, transiently enhanced lung inflammation during *Klebsiella* pneumonia, gzm-deficient mice displayed a largely unaltered pattern of bacterial dissemination, distant organ damage and mortality when compared to WT mice. These data suggest that gzmA and gzmB play a modest role in the pathogenesis of Gram-negative pneumonia-derived sepsis.

To our knowledge, we present, for the first time, the pattern of intracellular expression of gzmA and gzmB by different lymphocyte populations before and after the induction of Gram-negative infection. NK cells represented the predominant cell type expressing gzms. While gzmA levels decreased in NK cells during *Klebsiella* sepsis, gzmB expression increased in these cells, suggesting a differential regulation of these mediators. Using human whole blood, our group previously demonstrated release of gzmA and gzmB upon exposure to bacterial agonists [11]. We could not confirm gzm release in vivo during *Klebsiella* infection due to the lack of mouse-specific immune assays with sufficient sensitivity. Hence, it remains to be established whether the decreased intracellular gzmA expression is the result of gzmA release.

We then analysed the effect of gzmA and gzmB deficiency on the host response during *Klebsiella* pneumonia-derived sepsis. This model resembles the clinical scenario of a gradually evolving infection in the airways followed by bacterial dissemination to distant body sites and sepsis, making it suitable for the study of both the early protective innate immune response at the primary site of infection and the late detrimental consequences of exaggerated inflammation responsible for collateral local and systemic tissue injury. Overall, bacterial loads did not differ between gzm-deficient and WT mice at any site, with the exception of modestly higher bacterial burdens in the lungs of *gzmA*–/– and *gzmAxB*–/– mice at 16 h after infection. Thus, gzms played little, if any, role in the control of local infection and subsequent dissemination to distant organs. In agreement, *gzmA*–/– mice did not present differences in *Listeria monocytogenes* growth [19] or in bacteraemia and dissemination after *Brucella microti* infection [20] when compared to WT mice.

Early (6 h) after infection, when the inflammatory response in the lung was still limited, gzm-deficient mice had lower pulmonary levels of IL-1β, MIP-2 and KC. This finding is in line with previous reports revealing the role of gzms in the production, release and/or processing of pro-inflammatory cytokines [8, 13, 14, 21]. The modestly reduced pro-inflammatory mediator levels in the gzm-deficient mice may, at least in part, explain the lower MPO concentrations, indicative of total neutrophil con-

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tent, in these animals at 6 h after infection. In addition, considering that a brisk inflammatory reaction in the lungs is important for antibacterial defence in this model [17, 22, 23], the impaired early response could have contributed to the higher bacterial loads in the lungs of gzmA−/− and gzmAxB−/− mice at 16 h after infection. Notably, at this intermediate time point (16 h), when the lung infection had advanced to distant sites but was not yet accompanied by distant organ damage, gzmB and gzmAxB deficiency were associated with enhanced pulmonary inflammation. In addition, gzmAxB−/− mice, in particular, showed higher levels of pro-inflammatory cytokines and chemokines in whole-lung homogenates. The mechanism by which gzms contribute to the regulation of lung inflammation during Klebsiella pneumonia remains to be established. Earlier studies also reported enhanced inflammatory responses in gzm-deficient mice during viral infection [24, 25] and bleomycin-induced pulmonary fibrosis [26]. Eventually, gzm deficiency did not impact on the outcome of Klebsiella sepsis, as reflected by the similar bacterial counts in all organs and comparable distant organ damage at a later time point (48 h) after infection as well as unaltered mortality in gzm-deficient and WT mice. Together, these results suggest that the role of gzmA and gzmB in the host response during Klebsiella sepsis is modest and transient. In this respect, it is worth mentioning, however, that we cannot exclude possible compensatory mechanisms by other gzms, such as gzmM and gzmK, taking part in the results obtained in the gzmAxB−/− mice [15, 27, 28]. Previous studies reported the reduced mortality of gzmB−/− mice during polymicrobial peritonitis [29] and of gzmA−/− mice during B. mii-
croti sepsis [20], suggesting differential roles of gzms depending on the primary site of infection and/or the causative pathogen. Of note and relevant for the responsiveness to Gram-negative bacteria, earlier reports on the role of gzms in LPS-induced shock are partially contradictory. One study showed an increased resistance to LPS-induced mortality of gzmA−/− and gzmB−/− mice but not of gzmAxB−/− mice [14], while a subsequent study found gzmA−/− but not gzmB−/− mice to be resistant to LPS-induced lethality [15].

In conclusion, we studied the function of gzmA and gzmB in a clinically relevant model of Gram-negative pneumonia-derived sepsis. While gzm deficiency was associated with an enhanced local inflammatory response during early-stage pneumonia, our results argue against an important role for gzms in the pathogenesis of severe pneumonia and sepsis caused by a common human pathogen.

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Disclosure Statement

The authors declare that they have no conflicts of interest.

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