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A Role for Granzyme M in TLR4-Driven Inflammation and Endotoxicosis

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Lymphocyte perforin and serine protease granzymes are well-recognized extrinsic mediators of apoptosis. We now demonstrate that cytotoxic lymphocyte granule components profoundly augment the myeloid cell inflammatory cytokine cascade in response to TLR4 ligation. Whereas caspase-1–deficient mice were completely resistant to LPS, reduced serum cytokine production and resistance to lethal endotoxicosis were also obtained with perforin-deficient mice, indicating a role for granzymes. Consistently, a lack of granzyme M (GrzM) resulted in reduced serum IL-1β, TNF, and IFN-γ levels and significantly reduced susceptibility to lethal endotoxicosis. These altered responses were also observed in granzyme A-deficient but not granzyme B-deficient mice. A role for APC–NK cell cross-talk in the inflammatory cascade was highlighted, as GrzM was exclusively expressed by NK cells and resistance to LPS was also observed on a RAG-1/GrzM-double deficient background. Collectively, the data suggest that NK cell GrzM augments the inflammatory cascade downstream of LPS-TLR4 signaling, which ultimately results in lethal endotoxicosis. Most importantly, these data demonstrate that granzymes should no longer be considered solely as mediators of apoptosis, but additionally as potential key regulators of inflammation. The Journal of Immunology, 2010, 185: 1794–1803.

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Abbreviations used in this paper: ALT, alanine transaminase; AST, aspartate transaminase; CBA, cytometric bead array; clg, control Ig; GrzA, granzyme A; GrzB, granzyme B; GrzM, granzyme M; ICA, pIL-1β convertase activity; MFI, mean fluorescence intensity; pIL-1β, precursor of IL-1β; PMCC, Peter MacCallum Cancer Centre; WT, wild-type.

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Toll-like receptors represent the principal sensors of infection in mammals (1) and are responsible for many manifestations of the immune response, spanning both protection and immunopathology. These include the development of fever, shock, and tissue injury, but also the activation of innate and adaptive effector mechanisms that lead to the elimination of microbes. An understanding of LPS signal transduction is key to deciphering the molecular basis for the lethal endotoxicosis and may point the way to novel therapies. Rapid progress in this field has resulted in both the discovery of the receptor for LPS—TLR4—and a better appreciation of the complexity of the signaling pathways activated by LPS (2). Pathogen-induced inflammation includes a molecular pathway that proceeds through activation of the protease IL-1β converting enzyme (caspase-1) to the release of the inflammatory cytokines IL-1β and IL-18 (3, 4). Caspase-1 is a cytoplasmic cysteine protease originally characterized in myeloid cells based on its ability to process the 31-kDa inactive precursor of IL-1β (pIL-1β) to the 17-kDa proinflammatory cytokine IL-1β (pIL-1β convertase activity [ICA]) (5). After stimulation of TLR4 with LPS, both IL-1β and IL-18 are processed, depending on the adaptor protein apoptosis-associated speck-like protein containing a caspase activation recruitment domain and the activation of caspase-1.

Cytotoxic lymphocytes, such as NK cells, TCR γδ T cells, and TCR CD8+ T cells, mediate rapid apoptosis induced by perforin and the serine protease granzymes (6, 7). Most granzymes are capable of triggering cell death in the presence of perforin in vitro (8–12), but in vivo evidence supporting a role for granzyme-mediated apoptosis in an immune response remains circumstantial. Granzyme B (GrzB)-deficient lymphocytes do elicit delayed target cell DNA fragmentation (12). GrzB shares with caspases the rare ability to cleave substrates after aspartic acid and, as such, is an extrinsic lymphocyte serine protease that is delivered to target cells, acting as both an initiator and an executioner of apoptosis. Interestingly, granzyme A (GrzA), like caspase-1, has been shown in vitro to convert pIL-1β to its 17-kDa mature form, but pIL-1β is not a substrate for GrzB (13, 14). This suggests that lymphocytes, by means of their own ICA, might initiate a local inflammatory response independent of caspase-1, but surprisingly those studying granzymes have not avidly pursued this observation. It has recently been demonstrated that GrzA can elicit the release of IL-1β from macrophages in vitro and that mice deficient for this granzyme are somewhat less sensitive to LPS than are wild-type (WT) mice (15). However, this study did not evaluate the inflammatory response in these mice or place GrzA in the context of perforin and other granzymes.

We now describe a series of in vitro, ex vivo, and in vivo analyses showing that the secreted contents of cytotoxic lymphocyte granules profoundly regulate the initial proinflammatory response of myeloid cells to TLR4 ligation. In particular, a lack of granzyme M (GrzM) resulted in reduced serum proinflammatory cytokines,
NK cell IFN-γ production, and resistance to lethal LPS-induced endotoxosis. Consistent with an initiating role of the TLR4+ myeloid cells, caspase-1–deficient mice were maximally resistant to LPS challenge. Loss of GrzA also conferred resistance to LPS, and GrzM and GrzA appeared to act independently, as mice deficient in both GrzM and GrzA were more resistant to LPS than were mice lacking either granzyme alone. The data strongly suggest that in response to LPS, granule lymphocytes significantly augment the inflammation that results in lethality.

Materials and Methods

Mice

WT C57BL/6 mice (B6) and B6 RAG1−/− gene-targeted mice were either purchased from The Walter and Eliza Hall Institute of Medical Research (Melbourne, Australia) or bred at the Peter MacCallum Cancer Centre (PMCC). B6 GrzM-deficient (GrzM−/−), B6 GrzA-deficient (GrzA−/−), and GrzB-deficient (GrzB−/−) mice were generated (12, 16, 17) and maintained (16) as described. B6 GrzA−/− × GrzM−/− (GrzAM−/−), GrzA−/− × GrzB−/− (GrzAB−/−), and GrzB−/− × GrzM−/− (GrzBM−/−) were all generated at PMCC from single gene-targeted mice by intercrosses. B6 RAG1−/− × GrzM−/− double gene-targeted mice were generated at the PMCC by an intercross between B6 RAG1−/− mice and B6 GrzM−/− mice. B6 perforin-deficient (pfp−/−) (18), TLR3-deficient (TLR3−/−) (19), TRIF-deficient (TRIF−/−) (19), TLR4-deficient (TLR4−/−) (20), MyD88-deficient (MyD88−/−) (21), B6 IFN-γ-deficient (IFN−/−) (22), IFN-γR-deficient (IFN−/γ−/γ−) (23), and TNF-deficient (TNF−/−) (23) mice were all generated as described. IL-1R−/− mice (IL-1R−/−) were obtained from the University of Melbourne Department of Microbiology and Immunology (Melbourne, Australia) (24). All mice were either derived using C57BL/6 embryonic stem cells or had been back-crossed more than 10 generations to C57BL/6. All mouse strains were bred, genotyped, and maintained within the PMCC under specific pathogen-free conditions. Mice between the ages of 6 and 16 wk old were used for the experiments.

LPS-induced septic shock and cytokine measurements

Groups of mice (6–16 wk old) were injected with LPS (Escherichia coli; 0127:B8) (L3129; Sigma-Aldrich, St. Louis, MO) according to mass per body weight (typically 0.5–1.25 mg/30 g) i.p. and monitored routinely for weight, signs of endotoxemia, and lethality up to 4 d post LPS injection. Some mice were depleted of NK cells by injection of 100 μg control rabbit Ig or rabbit anti–asialoGM-1 (Wako Pure Chemicals, Osaka, Japan) on days −3 and −1 prior to LPS inoculation. Mice were bled either from the retro-orbital sinus or by cardiac puncture at specific time points post injection, and serum was separated from the blood for cytokine measurements. A mouse inflammation cytometric bead array (CBA; BD Biosciences, San Jose, CA) or the CBA Flex Systems Kit (BD Biosciences) was used to measure IL-6, IFN-γ, TNF, MCP-1, IL-10, IL-1α, and IL-1β per the manufacturer’s instructions. The mouse IL-18 ELISA Kit (7625; MBL) was used to measure IL-18 according to the manufacturer’s instructions. Serum alanine and aspartate transaminase (ALT/AST) or creatinine levels were measured 24 h post LPS as an indicator of liver and kidney toxicity. Blood (100–150 μl) was collected as described above, allowed to clot, and centrifuged 2000 × g for 5 min to separate serum. ALT/AST and creatinine were measured on the Siemens (New York, NY) Advia 1200 Chemistry System. Both are measured by modified

![FIGURE 1. Resistance of GrzM-deficient mice to LPS-induced lethality. A, Survival of age-matched WT and GrzM−/− mice after i.p. injection with 1.0 mg LPS/30 g/mouse, 0.75 mg/30 g/mouse, or 0.5 mg/30 g/mouse at 96 h post injection. The data shown are combined from six independent experiments (n = 36–41) at the dose of 1 mg/30 g, two independent experiments (n = 6–12) at the dose of 0.5 mg/30 g, and three independent experiments (n = 10–16) at the dose of 0.25 mg/30 g. **p = 0.0046; ***p < 0.0001. B–D, WT and GrzM−/− mice were injected with LPS (1 mg/30 g) i.p., and the spleen and blood were harvested 24 h post injection. Levels of ALT/AST (B) and creatinine (C), as measured from the serum of naive (n = 5) or LPS-injected (n = 10–11) mice. D, Graph depicting total percentage of viable and nonviable cells from the spleen, as determined by hydroxystilbamidine and FACS 24 h post LPS injection. Data pooled from two independent experiments ± SEM.]
International Federation of Clinical Chemistry and Laboratory Medicine rate reaction methods.

Flow cytometry

Single-cell suspensions of peritoneal exudate cells and splenocytes (RBCs) removed were stained in FACS buffer (PBS containing 2% FCS) with a mixture containing anti-F4/80-PE (B8; eBioscience, San Diego, CA) and anti–CD11b-APC (M1/70; eBioscience) for the identification of macrophages, or anti–NK.1.1-PE (PK136; eBioscience) and anti–TRC4-PECy5.5 (H57-97; eBioscience) for the identification of NK cells. In each mixture, anti–TLR4-PECy7 (MT3510; eBioscience) was added. Hydroxysulfotyramine (H22845; Invitrogen Life Technologies, Carlsbad, CA) (2 mM) was used for determination of viable cells. Cells were then washed, resuspended in FACS buffer, and analyzed by flow cytometry. For intracellular staining, the CytoFix/CytoPerm Buffer System (554715; BD Biosciences Pharmingen, San Diego, CA) was used for detection of IFN-γ per the manufacturer’s instructions. Briefly, 2–4 × 10^6 isolated cells were cultured in GolgiPlug (BD Biosciences Pharmingen) for 2 h before the staining of cell surface Abs, using the staining protocol above. Cells were washed and resuspended in CytoFix/Cytoperm solution for 20 min on ice in the dark. Cells were washed twice in Perm/Wash solution, and incubated with anti-mouse IFN-γ (XMG1.2, eBioscience) or an isotype control Ab diluted in Perm/Wash solution (BD Biosciences) for 30 min on ice. Cells were then washed in Perm/Wash solution, resuspended in FACS buffer, and analyzed by flow cytometry.

Semiquantitative PCR of GrzM in purified lymphocytes

NK, NKT, and γδT cells were purified using MACS. For NK cells, the NK Isolation Kit (R&D Systems, Minneapolis, MN) was used. For NKT cells CD1d-PE tetramer–labeled cells were purified using anti-PE beads. For γδT cells, FITC-labeled anti-γδTCR cells were purified using anti-FITC beads. Extraction of RNA was performed using the RNeasy Micro Kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. RNA was then converted to cDNA, using ThermoScript Reverse Transcriptase (Invitrogen). All PCR reactions were performed using 1 U GoTaq Flexi DNA Polymerase (Promega, Madison, WI), 1 × Green GoTaq DNA Reaction Buffer (Promega), 1.25 mM MgCl₂, 0.2 mM 2′-deoxynucleoside 5′-triphosphate, 0.5 μM primer, and 1 μg cDNA in a final volume of 20 μL. The primer sequence for GrzM was as follows: forward, 5′-AGACAGACCTTCCTGATCC-3′; reverse, 5′-ACCAATGACCTTCCTGATCC-3′. The PCR conditions were as follows: 95°C for 5 min, followed by 95°C for 1 min, 59°C for 1 min, 72°C for 40 s, for 20, 25, 30, or 35 cycles.

Inducible translocation trap and GrzM cleavage assays

Recombinant GrzM was produced from Pichia pastoris using the strategy previously described for GrzB production (25). GrzM activity was validated by complexing to a cognate serpin, and all preparations demonstrated >90% activity. Purified caspase-1 was the kind gift of Dr. Nancy Thornberry (Merck Research Laboratories, Rahway, NJ). In vitro transcription and translation of expression plasmids encoding pIL-1B or pIL-1B D116A were carried out essentially as described (25) (26). To assess cleavage of pIL-1B, indicated amounts of Grzm or caspase-1 were incubated with equal aliquots of the translation reaction containing [35S]-labeled pIL1B for 1 h at 37°C. Samples were separated via 15% SDS-PAGE and imaged by fluorography.

In vitro stimulation of macrophages

Peritoneal exudate cells were collected with ice-cold PBS 3 d post injection i.p. of 3% thioglycollate medium (T9032, Sigma-Aldrich) and resuspended in RPMI 1640 containing 10% FCS. Peritoneal macrophages were isolated by their adherence to plastic, and then a monolayer of macrophages in a 96-well plate was stimulated with media alone or media containing 0.1, 0.1, or 1 ng/mL LPS for 24 h prior to supernatant harvest.

Statistical analysis

Statistical analysis was performed using a Mann-Whitney U test or a log-rank test (Mantel-Cox) for survival, using GraphPad (San Diego, CA) Prism software.

Results

GrzM-deficient mice are resistant to LPS-induced lethal endotoxins

High-dose LPS injection into mice is a useful model for the study of endotoxic shock and the associated inflammatory responses that underpin it. To investigate the overall importance of GrzM during LPS-induced lethal endotoxemia, we administered various doses of LPS i.p. into C57BL/6 GrzM/- (Grzm/-) and control C57BL/6 (WT) mice and monitored their survival over a 96-h period (Fig. 1A). The majority of WT mice in the six experiments succumbed to the highest dose of LPS (1.0 mg/30 g body weight), with 27% surviving at 72 h, and only 7% at 96 h. In contrast to WT mice, a significantly higher proportion (p < 0.0001) of Grzm/- mice (70%) survived LPS-induced endotoxemia at 96 h (Fig. 1A). The significantly greater resistance of Grzm/- mice was also reflected at a lower dose of LPS (0.75 mg/30 g) with 100% of Grzm/- mice and 50% WT mice surviving (p = 0.046) (Fig. 1A); however, the survival of Grzm/- and WT mice was almost 100% when the LPS dose was reduced further (Fig. 1A). Heterozygous Grzm/+ mice were as sensitive to LPS as WT mice (data not shown).

**FIGURE 2.** Impaired proinflammatory cytokine responses are observed in GrzM/- mice post LPS injection. A, WT and Grzm/- mice were injected i.p. with LPS (1.0 mg/30 g), and serum concentrations of various cytokines, as indicated, were measured at several time points post injection Pooled data from four to six independent experiments with SEM (total number of data points was 5–48) are shown. Statistical significance was observed between WT and Grzm/- mice in serum: IL-1β at 6, 12, and 24 h (p = 0.0023; ^p = 0.045; **p = 0.0008); IFN-γ at 3, 12, 14, and 24 h (p = 0.0477; ^p = 0.0018; ##p = 0.0010; and ###p = 0.0022); TNF at 24 h (p = 0.0001); and IL-1α at 6, 12, and 24 h (p = 0.0042; #p = 0.0354; and ##p = 0.0006). B, WT (n = 31), TNF/- (n = 27), IFN-γ/- (n = 43), IFN-γR/- (n = 10), or IL-1R/- (n = 6) mice were injected i.p. with LPS (1 mg/30 g) and monitored for survival. Pooled data from three independent experiments with p-values compared with WT: TNF/-, IFNγ/-, and IFN-γR/- mice, * p < 0.0001; and IL-1R/-, ****p = 0.0004.
Furthermore, with the use of appropriate gene-targeted mice as controls for this model system (all on the C57BL/6 background), the response to LPS was, as expected, shown to be totally dependent upon TLR4 and largely MyD88 and TRIF dependent, but TLR3 independent (Supplemental Fig. 1). Collectively, the data indicated that GrzM played a significant role in LPS-induced endotoxicosis. The relative resistance of GrzM$^{-/-}$ mice to LPS-induced toxicity was further investigated. It has previously been demonstrated that kidney and liver toxicity are major contributors to sepsis-induced death. Thus, we wanted to determine if the liver toxicity post LPS injection was similar in WT and in GrzM-deficient mice. Both groups of mice were injected with LPS (1 mg/30 g) i.p., and blood was collected to determine serum concentrations of the liver enzymes ALT/AST (Fig. 1B) and serum creatinine (Fig. 1C). LPS induced equivalent levels of serum ALT/AST in WT and GrzM$^{-/-}$ mice (Fig. 1B), indicative of similar levels of liver toxicity. In addition, as a measure of renal function, creatinine revealed a similar pattern, with increased but comparable levels of creatinine measured in the serum of WT and GrzM$^{-/-}$ mice. In conjunction with these findings, we also measured the body weight of WT and GrzM$^{-/-}$ mice up to 6 d post LPS. A similar degree of weight loss was observed in both strains of mice 1 d post LPS; however, WT mice continued to lose weight, whereas weight began to stabilize and increase in GrzM-deficient mice within 3 d and by day 6 had almost recovered to starting weights at day 0 (Supplemental Fig. 1B). Furthermore, it has been demonstrated that apoptosis plays an important part during the inflammatory response to LPS. Given the possible role of GrzM during the induction of apoptosis, and the resistance of GrzM-deficient mice to LPS, it remained a possibility that this phenotype may be attributed to decreased cell death. To investigate this, we harvested the spleen 24 h post LPS injection from WT and GrzM$^{-/-}$ mice and stained splenocytes for viability (Fig. 1D). As demonstrated in Fig. 1D, the percentages of cell death in the two groups of mice were similar, suggesting that the increased survival observed in GrzM$^{-/-}$ mice post LPS occurred independently of apoptosis.

**FIGURE 3.** Macrophages from GrzM$^{-/-}$ mice are intrinsically similar to WT macrophages. **A,** Western blot of mouse or human $^{35}$S-labeled pIL-1β (both WT and mutant) was in vitro transcribed and translated and incubated with various concentrations of GrzM or caspase-1 and run on an SDS-PAGE to determine the processing of pIL-1β. **B,** Macrophages from WT ($n = 10$) and GrzM$^{-/-}$ ($n = 9$) mice were isolated from the peritoneal cavity and stimulated for 24 h with 0, 0.01, and 1.0 μg LPS/ml. Media were harvested, and cytokines as indicated (IL-1β, IL-6, MCP-1, TNF) were measured using CBA or ELISA, as described in Materials and Methods. Pooled data from two independent experiments with mean ± SEM are shown. **C,** Cells from the peritoneal cavity of WT and GrzM$^{-/-}$ mice were stained for macrophages (F480 and CD11b gated population). Surface expression of TLR4 was assessed on macrophages in WT (black solid line) and GrzM$^{-/-}$ mice (gray solid line) and compared with unstained cells (light gray line). Representative plots of all mice. **D,** Macrophage numbers in the spleen and peritoneal cavity of WT ($n = 6–7$) and GrzM$^{-/-}$ ($n = 6–7$) mice. Data are mean ± SEM of groups of mice.
GrzM-deficient mice have markedly reduced serum concentrations of IL-1β, TNF, and IFN-γ after LPS administration

LPS injection into the peritoneal cavity of mice leads to a systemic inflammatory response involving a multitude of proinflammatory cytokines, including IL-1α and IL-1β, TNF, IL-6, and IFN-γ. These cytokines have been shown to be important in the progression of the pathological changes associated with endotoxicosis that can result in death (27–30). We injected WT and GrzM−/− mice with LPS (1.0 mg/30 g) i.p. and measured serum cytokine concentrations over time, either by ELISA or by CBA (n = 5–48 samples/time point) (Fig. 2). Importantly, GrzM−/− mice displayed markedly lower serum concentrations of IL-1β at 3, 12, and 24 h (p < 0.05 for all points) when compared with WT mice (Fig. 2A). A similar profile was obtained for serum IL-1α, with significantly lower IL-1α measured at 6, 14, and 24 h post LPS, compared with WT (p = 0.0042, p = 0.0354, and p = 0.0006, respectively) (Fig. 2A). IFN-γ was also significantly lower in the serum of GrzM−/− mice at 12–24 h post LPS injection (p < 0.05) (Fig. 2A). TNF peaked earlier but was also reduced at later time points in GrzM−/− mice compared with WT mice (Fig. 2A). A significantly lower level of TNF was measured in GrzM−/− mice at 24 h (p < 0.0001), compared with WT mice. Although there was a slight delay in the increase in serum MCP-1 levels in GrzM−/− mice (Supplemental Fig. 2), in further support of the important role for GrzM during LPS response, the specific cytokines reduced in the serum of GrzM−/− mice, such as IL-1, TNF, and IFN-γ, or their related receptors, were also demonstrated to be critical for LPS sensitivity (Fig. 2B). When comparing against WT controls, IFN-γ−/−, IFN-γR−/−, IL-1R−/−, and TNF−/− mice appeared at least as resistant to LPS as GrzM−/− mice (Fig. 2B).

Mechanism by which GrzM regulates serum IL-1β levels is not clear

The reduced serum IL-1α and IL-1β levels, but normal IL-18 levels, in GrzM−/− mice suggested that GrzM may specifically affect pIL-1β processing or IL-1 release but, unlike caspase-1, does not additionally affect IL-18 processing or release. Given the markedly reduced serum IL-1β levels in GrzM−/− mice responding to LPS and the in vitro defined caspase-1–like activity of GrzA (14), we aimed to directly test whether GrzM has ICA. GrzA, GrzB, and GrzM are each genetically conserved in the mouse, rat, and human (31). When we assessed the ability of mouse GrzM to cleave in vitro transcribed and translated mouse pIL-1β, we were not able to demonstrate cleavage conversion of this 31-kDa precursor to the 17-kDa active form, despite the activity of mouse caspase-1 on the same product (Fig. 3A). We conclude that mouse GrzM does not directly process and activate these procytokines.

An alternative explanation for the reduced mature IL-1β in the serum of GrzM−/− mice responding to LPS might be that GrzM−/− peritoneal macrophages have either reduced TLR4 expression or diminished intrinsic capability to produce IL-1β and other inflammatory mediators. GrzM−/− mice have previously been shown to have normal tissue development and homeostasis of the immune system (16). However, we wanted to directly assess the ability of thioglycollate-elicited peritoneal macrophages from GrzM−/− and WT mice to produce IL-1β and other cytokines ex vivo. Macrophages from both groups of mice were stimulated with a range of LPS doses (0–1 μg/ml) in vitro, and the supernatants were collected 24 h post stimulation (Fig. 3B). Macrophages isolated from GrzM−/− mice were able to produce levels of IL-6, TNF, MCP-1, and, most importantly, IL-1β similar to those in WT. The surface TLR4 expression on macrophages from GrzM−/− and WT mice was also comparable (Fig. 3C), and macrophage cell numbers in the spleen and peritoneal cavities of WT and GrzM−/− mice were not significantly different (Fig. 3D).

**FIGURE 4.** Caspase-1−/− mice have an even greater resistance and cytokine impairment to LPS. A. WT (n = 5–19), GrzM−/− (n = 10–17), TLR4−/− (n = 12), and caspase-1−/− (n = 7–11) mice were injected i.p. with LPS (1.0 or 1.25 mg/30 g) and monitored for survival. At a dose of 1.25 mg/30 g, a statistical significance was observed between WT and TLR4−/−, caspase-1−/− (a < 0.0001); *= p = 0.0004) and between GrzM−/− and TLR4−/− caspase-1−/− (p < 0.0001; ^^p = 0.0134). At a dose of 1.0 mg/30g, statistical significance was observed between WT and caspase-1−/− mice of ***p = 0.0002 and GrzM−/− mice of **p = 0.0009. Pooled data from three independent experiments ± SEM. B. WT and caspase-1−/− mice were injected i.p. with LPS (1.0 mg/30 g), and serum concentrations of IL-1β, IFN-γ, IL-18, and TNF were measured at 0, 3, 6, 12, and 24 h post injection. Statistical significance was observed between WT and caspase-1−/− mice in serum IL-1β (a = 0.0003; ***p = 0.0103; IFN-γ (a = 0.0067; **a < 0.0001; **a = 0.0218); IL-18 at 3, 6, 12, and 24 h post LPS injection (a = 0.0002; ***p < 0.0001; ***p = 0.0003); and TNF (a = 0.0056; **a = 0.0046; ***a = 0.0167). Statistical significance was observed between WT and GrzM−/− mice. p < 0.05; values are listed in Fig. 2.
Direct effects of LPS on TLR4 on granzyme-expressing NK cells and other lymphocytes were excluded by the lack of TLR4 on lymphocyte populations of either strain, including NK cells, and the equivalent numbers of lymphocyte subpopulations between WT and Grzm/M−/− mice (data not shown). Collectively, these results clearly indicated that the reduced serum IL-1β observed in Grzm/M−/− mice responding to LPS was not due to an intrinsic defect in macrophages from Grzm/M−/− mice.

Caspase-1 is upstream of Grzm in the LPS model of sepsis

Caspase-1 has previously been shown to be critical for the production of IL-1β and the subsequent inflammatory response to several stimuli. Indeed, mice deficient in this protease are resistant to LPS-induced lethal endotoxicosis. Given some of the similarities between Grzm/M−/− mice and caspase-1−/− mice (enhanced LPS resistance and decreased serum IL-1β, as well as TNF and IFN-γ), we next wanted to determine the importance of Grzm in comparison with caspase-1 during the LPS inflammatory response. TLR4−/−, Grzm/M−/−, and caspase-1−/− mice were injected with 1.25 or 1 mg/30 g dose of LPS i.p. and monitored for survival (Fig. 4A). Caspase-1−/− mice had even greater level of resistance to LPS-induced lethal endotoxicosis when compared with Grzm/M−/− mice (Fig. 4A). Furthermore, when serum cytokine levels in caspase-1−/− mice were compared with those in WT mice, there was an even greater impairment in the ability of the caspase-1−/− mice to produce key inflammatory cytokines, namely, IL-1β, IFN-γ, TNF, and IL-18 (Fig. 4B). Caspase-1−/− mice also had significantly lower serum concentrations of IFN-γ at 6, 12, and 24 h (p < 0.05); lower serum TNF at the same time points; and little, if any, detectable serum IL-1β or IL-18, compared with WT mice (p < 0.05)(Fig. 4B), the last finding consistent with the reported defect in mature IL-1 production by these mice (24). The levels of these cytokines (particularly TNF, IL-1β, and IFN-γ) (Fig. 4B) were also low in Grzm/M−/− mice (Fig. 4B), but they had WT levels of serum IL-18, more serum IL-1β than caspase-1−/− mice (6 and 12 h), and greater levels of serum IFN-γ and TNF. The data presented in Figs. 3 and 4 suggest that although Grzm is important, it cannot be considered the initiator of LPS-induced inflammation. The data also suggest that, temporally, caspase-1 acts upstream of Grzm.

NK cells promote LPS-induced septic shock

Grzm is thought to be predominantly and constitutively expressed by NK cells (32–34). Given this, we reasoned that an effect of Grzm on LPS response would still be observed on a RAG−1-deficient background in which the host mouse lacks all mature

**FIGURE 5.** Role of NK cells in LPS-induced lethality. A, Survival of RAG−1−/−, RAG−1−/− Grzm−/−, or RAG−2−/−cyt−/− mice after i.p. injection with 1.0 mg LPS/30 g/mouse, 0.50 mg/30 g/mouse, or 0.25 mg/30 g/mouse and monitored for survival. Pooled data from two independent experiments: RAG−1−/− (n = 17–22), RAG−1−/− Grzm−/− (n = 17–22), and RAG−2−/−cyt−/− (n = 4–5) mice. Pooled data from two independent experiments: RAG−1−/− (n = 17–22), RAG−1−/− Grzm−/− (n = 17–22), and RAG−2−/−cyt−/− (n = 4–5). Statistical difference was observed between RAG−1−/− and RAG−1−/− Grzm−/−: *p = 0.0118; **p = 0.045; ***p = 0.0001. B, Survival of WT, RAG−1−/−, or RAG−1−/− Grzm−/− mice after i.p. injection with 0.5 mg LPS/30 g/mouse. Some mice were treated with cIg or anti-asialoGM1 to deplete NK cells. Range of mice per group for each panel is shown in parentheses. Statistically different survival curves were observed between RAG−1−/− + cIg and RAG−1−/− + anti-asialoGM1: #p = 0.005. cIg, control Ig.
T cells (including NKT and γδT) and B cells. To test this idea, we bred the GrzM-targeted allele onto the RAG-1 background and compared LPS-induced endotoxicosis (0.25–1.0 mg/30 g) in RAG−/− and RAG1−/− × GrzM−/− mice (Fig. 5). A greater percentage of RAG−/− × GrzM−/− mice survived than RAG−/− mice (1.0 mg, 22.7% RAG−/− × GrzM−/− and 4.5% RAG−/−; 0.5 mg, 76% RAG−/− × GrzM−/− and 11% RAG−/−, respectively; p < 0.05). This effect of GrzM was similar to that observed on a WT background with T and B cells intact (Fig. 5A), and suggests that the GrzM contributing to the LPS response in WT mice is not expressed by B or T cells. By titrating down the dosage of LPS in WT and RAG−/− (0.5 mg/30 g) mice, we were able to demonstrate that NK cell depletion enhanced the LPS resistance of WT and RAG−/−, but not RAG−/− × GrzM−/− mice (Fig. 5B). Because no perfect model of complete and specific NK cell deficiency is available, we also examined LPS sensitivity in RAG−/− × common γ−/− mice that additionally lack NK cells. This strain was highly resistant to LPS, with 100% of the mice surviving at both the 0.5- and 1.0-mg dose (Fig. 5A). Moreover, we confirmed that GrzM, GrzA, GrzB, and perforin mRNAs are not expressed in resting or LPS-activated mouse macrophages, using the highly sensitive real-time PCR technique (data not shown), and that GrzM mRNA was expressed in NK cells, weakly in NKT cells, and was not detectable in γδT cells (Supplemental Fig. 3).

LPS-driven NK cell IFN-γ production is caspase-1 dependent and regulated by GrzM

To strengthen our argument that NK cell granule exocytosis amplifies serum IFN-γ post LPS inoculation, we compared intracellular staining of various splenic lymphocyte populations from WT and gene-targeted mice to assess their capacity to make IFN-γ. LPS injection of WT mice and gating of NK cells (Fig. 6), NKT cells, and CD8+T cells (Supplemental Fig. 4) revealed that NK cells (and, to some extent, NKT cells) produced high levels of intracellular IFN-γ in response to LPS. Importantly significantly fewer IFN-γ+ NK cells were found in GrzM−/− (29.1 ± 9.9%; p = 0.025) and caspase-1−/− mice (12.8 ± 5.9%; p = 0.002) compared with WT mice (63.6 ± 5.5%) at 6 h (Fig. 6A). Furthermore, of the NK cells positive for intracellular IFN-γ at 6 h, there was significantly less IFN-γ in those from GrzM−/− (mean fluorescence intensity [MFI] = 1506 ± 673) and caspase-1−/− (MFI = 246 ± 73) mice than in those from WT mice (MFI = 3553 ± 829) at 6 h (p = 0.0272 and p < 0.0001, respectively) (Fig. 6B, 6C). Consistent with the serum levels of IFN-γ, the impact of the loss of caspase-1 was far greater than the loss of GrzM, and IFN-γ production was clearly delayed in GrzM−/− mice.

Other cytotoxic granule components contribute to LPS-induced inflammation

Given that GrzM−/− mice were resistant to LPS, we reasoned that lymphocyte granule exocytosis might have an important general function in regulating the LPS response. Perforin has no proteolytic activity and is thought to play a critical permissive role for granzymes by providing access to their substrates in the target/ APC cytosol (35). Therefore, we next compared the LPS susceptibility of mice deficient in perforin, GrzA, GrzB, or GrzM with the LPS susceptibility of WT mice. GrzA−/− (78% viable) and pfp−/− (81% viable) mice were both significantly resistant to LPS-induced endotoxic shock, compared with WT mice (20 + 10%) (p < 0.05) (Fig. 7A). By contrast, mice deficient in GrzB had survival similar to that in WT mice post LPS. This result in GrzB−/− mice was further substantiated at lower doses of LPS (Supplemental Fig. 5A). Importantly, the relative resistance of both GrzA−/− and GrzM−/− mice to LPS raised the possibility that these granzymes might independently control inflammation. To address this issue, we performed a dose response comparing the resistance of single granzyme-deficient mice with compound mutants deficient in various combinations of GrzA and GrzM (Supplemental Fig. 5B). Notably, at a dose of 1.25 mg/30 g LPS it became clear that an additive effect of loss of GrzA and GrzM could be detected (Supplemental Fig. 5B). These data raise the very interesting possibility that GrzA and GrzM contribute to LPS-induced inflammation by independent pathways. In addition, we went on to measure serum concentrations of several inflammatory cytokines, such as IL-1β, TNF, and IFN-γ, after a 1 mg/30 g injection of LPS in pfp−/− mice. Interestingly, levels of serum IL-1β were also reduced after 6 h in pfp−/− mice compared...
with WT mice; however, the pattern of cytokines was not identical between pfp$^{-/-}$ and GrzM$^{-/-}$ strains (Figs. 2, 7B). The levels of serum IFN-$\gamma$ in pfp$^{-/-}$ mice were similar to those in WT mice, other than a reduction at the later 24-h time point ($p = 0.0364$; Fig. 7B). The resistance of pfp$^{-/-}$ mice to LPS and the associated lower levels of serum IL-1$\beta$ (like GrzM$^{-/-}$ mice) supported the consensus that many granzyme functions were performorf deficient and delivered in the context of an immunological synapse (7).

**Discussion**

We have shown that lymphocyte granule proteins, perforin, GrzM, and GrzA, each significantly contribute to the inflammatory response to LPS. Mice deficient in any one of these granule proteins were comparatively resistant to LPS-induced lethal endotoxicosis. Mice deficient in both GrzA and GrzM were more resistant, suggesting independent pathways of control of LPS-induced inflammation by GrzA and GrzM. Specifically, GrzM appeared to be a major contributor to the production of serum cytokines such as IL-1$\alpha$, IL-1$\beta$, TNF-$\alpha$, and IFN-$\gamma$ in mice challenged with LPS. However, the mechanism by which GrzM controls any of these cytokine levels remains elusive.

Notably, caspase-1-deficient mice were completely compromised in IL-1, IFN-$\gamma$, and, additionally, IL-18 induction and even more resistant to LPS than any granzyme-deficient mice. Furthermore, survival data from RAG-1$^{-/-} \times$ GrzM$^{-/-}$ mice and NK cell-depleted mice challenged with LPS, together with NK cell IFN-$\gamma$ production from caspase-1$^{-/-}$ and GrzM$^{-/-}$ mice, suggested that an important cross-talk between TLR4+ myeloid cells and NK cells ultimately results in enhanced NK cell IFN-$\gamma$ production and maximal lethal endotoxicosis. However, it should be noted that the use of LPS within the RAG-1$^{-/-}$ mouse model revealed a potential role for T and/or B cells in conferring LPS resistance (LPS was more lethal in RAG-1$^{-/-}$ than in WT background). Although this was an important observation, it was not a focus in this study. Moreover, interpretation of the RAG-2$^{-/-} \times$ common $\gamma^{-/-}$ mice data is complicated by the abolished sensitivity of leukocytes to common $\gamma$-chain–dependent cytokines, including IL-2, -4, -7, -9, -15, and -21. The consistent enhanced survival observed in the RAG-1 and -2 models in the absence of NK cells, however, is in concert with earlier studies showing that NK cells are critical for sensitivity to LPS challenge (36, 37). It remains unresolved how NK cells might recognize myeloid cells that have been exposed to LPS and how relatively important immune recognition and synapse formation are, compared with local transfer of secreted factors from the initial responding myeloid cells. Regardless, our data challenge the dominant paradigm that granule exocytosis is simply a pathway to apoptosis, but rather strongly indicate that granzymes also profoundly regulate the earliest inflammatory responses mediated through TLR4 on myeloid cells. Our data are consistent with, but greatly extend, the recent findings of Metkar et al. (15) for GrzA, with some notable differences, as discussed below.

GrzA has previously been described as having caspase-1–like activity, with major cleavage occurring in pIL-1$\beta$ at Arg$^{120}$, four amino acids downstream of the caspase-1 processing site, Asp$^{116}$, and, additionally, IL-18 induction and, more recently, IL-1$\alpha$ production (14). A recent study of Metkar et al. (15) also found that GrzA cleavage of IL-1$\beta$ in vivo, as well as the in vivo consequences of granzyme cleavage of pLIL-1$\beta$ have been demonstrated. The recent study of Metkar et al. (15) also found that GrzA$^{-/-}$ mice have a blunted sensitivity to LPS administration and confirmed that addition of extrinsic recombinant GrzA to purified mouse monocytes could induce the release of active IL-1$\beta$. In contrast with Metkar et al., our study also directly demonstrated that granzyme loss reduces serum cytokine levels following LPS administration. Metkar et al. found that addition of caspase inhibitors, particularly inhibitors of caspase-1, markedly reduced the GrzA–induced release of IL-1$\beta$ from the macrophages (15). In consistency with GrzA cleavage of the pIL-1$\beta$ C terminus of the caspase-1 cleavage site, they interpreted this result to mean that pIL-1$\beta$ processing is not mediated directly by GrzA, but by caspase-1. However, an alternative explanation is that caspase-1 is necessary for initiating cytokine processing, releasing cytokines from the myeloid cells, which then triggers lymphocyte/perforin delivery of granzymes such as GrzM, which would feed back to the myeloid cells.
positively on this loop. Our biochemical studies with mouse reagents suggest that GrzM is unlikely to be a pIL-1β convertase and therefore unlikely to be directly responsible in vivo IL-1β production.

pIL-1β is known to be a poor substrate for GrzB (14) and, in concert with this original finding, we observe that loss of GrzB did not appear to influence serum IL-1β or IFN-γ levels (data not shown) or susceptibility to LPS-induced shock. This equivalent sensitivity of WT and GrzB−/− mice to LPS was in contrast to the resistance of GrzB−/− mice reported by Metkar et al. (15). It is difficult to reconcile this difference, but we performed an LPS dose response with large numbers of mice in each group; dose was administered according to the weight of individual mice, and at every dose the GrzB−/− mice were as sensitive as WT mice. We also could not reconcile their report that GrzAB-deficient mice were as sensitive to LPS as WT mice, despite the fact that each mutation alone conferred resistance (15). Indeed, we illustrated that loss of GrzA is dominant over loss of GrzB. Theoretically, if GrzB was delivered in the context of perforin and the other granzymes to TLR4+ myeloid cells responding to LPS, it might be that such myeloid cells undergo GrzB-mediated apoptosis and inflammatory mediator release would be affected. But we do not see such an impact of loss of GrzB on LPS sensitivity. Indeed, our data are consistent with a role for the intracellular GrzB inhibitor Serpin 9 (which is known to be expressed at high levels in APCs) in protecting myeloid cells against GrzB released from NK cells (40). Thus, one possibility is that this serpin may well protect the TLR4+ cell responding to LPS when other proinflammatory granzymes, such as GrzA and GrzM, are being delivered by perforin.

Although extensive apoptosis of leukocytes during sepsis constitutes an important mechanism linked to mortality, it has been recently shown that caspase-7 is activated in splenocytes in response to LPS and caspase-7−/− mice were protected from LPS-induced splenocyte apoptosis, without affecting cytokine levels (41). By contrast, perforin and granzymes are required for optimal release of proinflammatory cytokines (as we show in this paper). There are absolutely no data in the literature to indicate that lymphocytes from GrzM-deficient mice are less susceptible to cell death and GrzM is expressed only in innate lymphocyte populations, predominantly NK cells. Importantly, in the literature the evidence that GrzB induces apoptosis is strongest, and yet mice deficient for this granzyme have no LPS resistance. Thus, the idea that reduced sensitivity of host leukocytes to LPS-induced apoptosis explains the resistance of GrzM-deficient mice seems highly unlikely.

Unfortunately, it is currently not technically possible to directly demonstrate perforin/granzyme transfer from lymphocytes to myeloid targets in vitro or in vivo, and thus the exact mechanism by which GrzM affects myeloid target cells remains to be defined. However, we know that caspase-1 is essential for mature IL-1β activity post LPS induction, but that GrzM is only partially responsible in this process. Similarly, caspase-1 is essential for serum and NK cell IFN-γ activity (the major producer), but GrzM was only partially responsible. This suggests that GrzM acts downstream of caspase-1 activity and may be involved in the IL-1β pathway with no direct processing of pro-IL-1β or IL-1β itself. It was of interest that loss of GrzM specifically affected serum levels of IL-1α, IL-1β, IFN-γ, and TNF in response to LPS, but had little impact on other cytokines, including IL-18, IL-6, and IL-10, given the resistance to LPS of IL-1R−, IFN-γR−, IFN-γR−, and TNF-deficient mice. The absence of GrzM or perforin appears to prevent efficient IL-1 response to LPS, thereby reducing the cytokine inflammatory cascade induced during sepsis. If perforin or GrzM is deficient, the induction of a select pattern of serum cytokines is diminished, and the pathophysiological process is attenuated. The focal effect of GrzM might be a clue to which cellular interactions and pathways GrzM regulates.

This study raises many newer and interesting questions. Given the sparsity of prior evidence for a role for perforin and granzymes in promoting inflammation (42, 43), examination of what part they play in response to other TLRs is in order. Given a possible evolutionary development of granzymes to regulate inflammation in response to pathogens, might granule proteins also regulate other inflammasome outputs? As with caspases, granzymes perhaps may now be considered to comprise two separate functional groups, one proapoptotic and the other proinflammatory. GrzB clearly serves an essential function during apoptotic cell death, but whether GrzA can also genuinely induce cell death has recently been questioned in two independent studies (15, 44). There is now considerable biochemical and biological evidence that, at a minimum, GrzA and GrzM should be studied in the context of early inflammatory response to microbial pathogens.

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Disclosures
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References


33. Pichia pastoris is fully activated and kinetically indis-