Granzyme K synergistically potentiates LPS-induced cytokine responses in human monocytes

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Granzymes are serine proteases released by cytotoxic lymphocytes to induce apoptosis in virus-infected cells and tumor cells. Evidence is emerging that granzymes also play a role in controlling inflammation. Granzyme serum levels are elevated in patients with autoimmune diseases and infections, including sepsis. However, the function of extracellular granzymes in inflammation largely remains unknown. Here, we show that granzyme K (GrK) binds to Gram-negative bacteria and their cell-wall component lipopolysaccharide (LPS). GrK synergistically enhances LPS-induced cytokine release in vitro from primary human monocytes and in vivo in a mouse model of LPS challenge. Intriguingly, these extracellular effects are independent of GrK catalytic activity. GrK disaggregates LPS from micelles and augments LPS–CD14 complex formation, thereby likely boosting monocyte activation by LPS. We conclude that extracellular GrK is an unexpected direct modulator of LPS–TLR4 signaling during the antimicrobial innate immune response.

Cytotoxic lymphocytes induce apoptosis in virally infected cells or tumor cells via death-receptor ligation or the granule exocytosis pathway. In the latter pathway, cytotoxic lymphocytes release the contents of their intracellular granules into the immunological synapse upon recognition of the target cell. Among the released granule constituents are the pore-forming protein perforin and a set of five serine proteases called granzymes [granzyme A (GrA), GrB, GrH, GrK, and GrM] (1, 2). After entering the target cell, granzymes can induce apoptosis by cleaving specific intracellular substrates.

Increasing evidence is emerging that granzymes also exert noncytotoxic extracellular functions during inflammation, including microbial infections. Support for such functions comes from observations that levels of soluble granzymes are elevated in plasma and synovial fluid of rheumatoid arthritis patients (3, 4) and in serum and broncheoalveolar lavage fluid of patients with bacterial or viral infections (4–8). Furthermore, $GrM^{-/-}$ and $GrA^{-/-}$ mice tolerate a lethal lipopolysaccharide (LPS) challenge better than WT mice (9, 10). Moreover, cytokine responses to LPS are lower in $GrM^{-/-}$ mice than in WT mice (9), implying involvement of granzymes in cytokine production. Indeed, GrA induces the production of several proinflammatory cytokines by primary monocytes (10–12) and indirectly protects human macrophages from mycobacterial infection by induction of tumor necrosis factor α (TNF-α) (13). It also cleaves pro-interleukin-1β (pro-IL-1β) in vitro (14), whereas GrB cleaves and activates pro–IL-1α in vitro and in vivo (15).

Human GrK has been studied only occasionally. This granzyme is expressed by natural killer T (NKT) cells, cytotoxic T cells, and NK cells (5, 16). It exerts cytotoxic activity toward tumor cells (17–21), inhibits influenza virus replication in mice (22, 23), and has an immunoregulatory function in multiple sclerosis (24). GrK may also play an extracellular role during various types of infection. Levels of soluble GrK are increased in the bronchoalveolar lavage fluid during acute airway inflammation (5) and in serum of patients with viral infections or sepsis (6, 8). However, its role in infections is not clear, although it may contribute to the production of IL-1β, -6, and -8 in vitro (25, 26).

In the present study, we demonstrate that human GrK binds to Gram-negative bacteria and to LPS, a major constituent of the Gram-negative bacterial cell wall. We show that extracellular GrK—independent of its catalytic activity—markedly potentiates LPS-induced proinflammatory cytokine release by monocytes both in vitro and in vivo, using a mechanism reminiscent of that of LPS-binding protein. To our knowledge, we are the first to show that a human granzyme binds to LPS and can directly modulate toll-like receptor 4 (TLR4) signaling independent of its catalytic activity. Our study supports a model in which extracellular GrK contributes to the (innate) immune response to bacterial infections.

Results

Circulating Levels of GrK Are Elevated in Gram-Negative Sepsis. It has been reported that circulating levels of soluble GrK are increased in patients with sepsis (6). We measured GrK serum levels in patients with Gram-negative sepsis ($n = 10$). The median GrK level in the sepsis patients (1,224 pg/mL) was significantly elevated compared with the level in serum from healthy donors (499.1 pg/mL; $n = 10$) (Fig. 1*A*). These data confirm previous data demonstrating that GrK circulates in Gram-negative sepsis patients.

Granzyme K Binds to Gram-Negative Bacteria. Because soluble GrK exists in the circulation of patients with Gram-negative sepsis, we hypothesized that GrK may interact with Gram-negative bacteria. Therefore, several Gram-negative bacteria were incubated with biotinylated GrK, and binding was detected by flow cytometry.

Significance

Granzymes are serine proteases released by cytotoxic lymphocytes and induce cell death in virus-infected cells and tumor cells. However, granzymes also exist extracellularly in the blood circulation of patients with autoimmune diseases and infections and may contribute to inflammation. Here, we show that human granzyme K (GrK) binds to Gram-negative bacteria and to lipopolysaccharide (LPS), a Gram-negative bacterial cell wall component. Our data indicate that GrK lowers the threshold for monocyte activation by LPS, in that GrK synergistically increases LPS-induced release of proinflammatory cytokines in vitro and in vivo. In conclusion, GrK modulates the innate immune response against LPS and Gram-negative bacteria and may contribute to the pathogenesis of diseases associated with a local or systemic bacterial infection.

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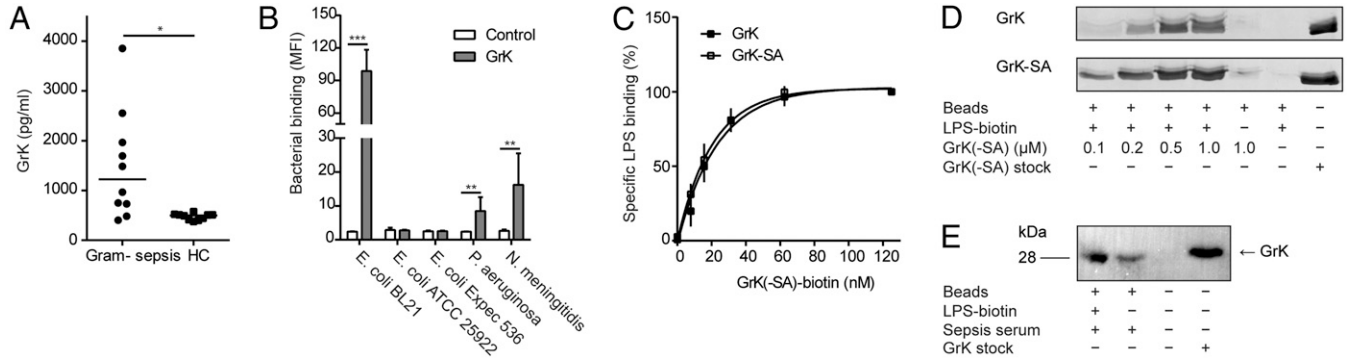

Fig. 1. GrK binds to Gram-negative bacteria and LPS. (*A*) Soluble GrK circulates in patients with Gram-negative sepsis. Serum from Gram-negative sepsis patients (n = 10) and healthy controls (n = 10) was analyzed for soluble GrK. *P < 0.05. (*B*) GrK binds to Gram-negative bacteria. GrK–biotin binding to bacteria was detected by flow cytometry. Data represent mean ± SD of three independent experiments. **P < 0.01; ***P < 0.001. (*C*) GrK binds to LPS in a solid-phase binding assay. Immobilized LPS was incubated with biotinylated GrK or GrK-SA. Data are depicted as specific binding (expressed as percent of maximal binding) and represent mean ± SD of three independent experiments. (*D*) GrK binds to LPS in a pull-down assay. LPS-coated beads were incubated with GrK or GrK-SA. Bound protein was visualized by SDS/PAGE. (*E*) Native GrK binds to LPS. Serum from a sepsis patient was incubated on LPS-coated beads. Bound protein was visualized by GrK immunoblotting.

GrK bound to *Escherichia coli BL21*, *Pseudomonas aeruginosa*, and *Neisseria meningitides* with varying intensity, whereas no binding to *E. coli ATCC 25922* or *E. coli Expec 536* was observed under the used experimental conditions (Fig. 1*B*). These data indicate that GrK binds to Gram-negative bacteria.

Granzyme K Binds to LPS. Considering that GrK binds some Gram-negative bacteria, we investigated whether GrK interacts with LPS (*E. coli* 0111:B4) and whether this binding involves the GrK catalytic center. Immobilized LPS was incubated with GrK and GrK-SA (a catalytically inactive GrK mutant) in a solid-phase binding assay. Both GrK and GrK-SA bound to LPS in a concentration-dependent manner (Fig. 1*C*). To further substantiate the binding of GrK(-SA) to LPS, a pull-down assay was performed. Biotinylated LPS was coupled to streptavidin-coated Sepharose beads, after which the beads were incubated with GrK(-SA). Bound proteins were analyzed by SDS/PAGE. Incubation of LPS-coated, but not uncoated, beads with increasing GrK(-SA) concentrations resulted in a band of increasing intensity migrating at the position of GrK (Fig. 1*D*). This finding indicates that GrK specifically binds to LPS and that this binding is independent of its catalytic center. To determine whether native GrK also binds to LPS, serum from a selected Gram-negative sepsis patient was incubated on LPS-coated beads. Indeed, native GrK bound to LPS (Fig. 1*E*), indicating that binding can occur in vivo. To further investigate the nature of the GrK–LPS interaction, we assessed the effects of NaCl and heparin. Binding of recombinant GrK (-SA) to LPS was dose-dependently inhibited by NaCl (Fig. S1*A*) and heparin (Fig. S1*B*), suggesting involvement of electrostatic interactions, possibly within the putative heparin-binding site of GrK (27). LPS consists of a lipid A moiety, which inserts into the bacterial cell wall, a core oligosaccharide, and an O-antigen that protrudes from the bacterial cell membrane (28). To determine which part(s) of LPS bind(s) GrK, we tested lipid A and delipidated LPS (LPS without lipid A fatty acid tails) for their ability to compete with recombinant GrK binding to LPS. Full-length LPS and delipidated LPS competed with GrK binding to immobilized LPS to a similar extent, whereas lipid A was markedly less efficient (Fig. S1*C*), suggesting that the LPS O-antigen plays a major role in GrK binding. Together, these data indicate that GrK binds to the Gram-negative bacterial cell-wall component LPS.

GrK Synergistically Enhances LPS-Induced Proinflammatory Cytokine Release from Monocytes. LPS stimulates TLR4 signaling, leading to proinflammatory cytokine release (29). Therefore, we studied the effect of GrK on the LPS-induced cytokine release by human primary monocytes. Treatment of monocytes with extracellular GrK or GrK-SA for up to 8 h did not influence cell viability (Fig. S2). Incubation of human monocytes with GrK or GrK-SA alone for 6 h did not result in cytokine production (Fig. 2). However, when monocytes were incubated with GrK combined with a suboptimal stimulatory dose of LPS, TNF-α production was synergistically enhanced compared with the response to LPS alone (Fig. 2 *A* and *B*). This synergistic effect of GrK was dose- and time-dependent and was not dependent on the catalytic activity of GrK, because the GrK-SA mutant showed similar effects (Fig. 2 *A* and *B*). The effects of GrK(-SA) on LPS-induced TNF-α secretion were observed in at least 20 independent experiments performed with 20 separate donors and with at least seven independently isolated GrK batches. Next, we determined whether secretion of other proinflammatory cytokines was also enhanced in the presence of GrK. As for TNF-α, GrK and GrK-SA synergistically enhanced LPS-induced IL-6 and -8 release from monocytes in a dose- and time-dependent manner (Fig. S3). Treatment of human monocytes with LPS alone induced low levels of cytokine production, whereas no secretion of IL-6 and -8 was found upon incubation with granzyme alone (Fig. S3). Finally, under these conditions, we did not detect potentiating effects of LPS and GrK on the release of TRIF-related adaptor molecule/TIR-domain-containing adapter inducing interferon-β–dependent type I interferons, including interferon α (IFN-α) (not detectable) and IFN-β (Fig. S4). Together, these results indicate that GrK, independent of its active site, synergistically potentiates the LPS-induced release of proinflammatory cytokines from monocytes in the absence of intracellular delivery.

GrK Enhances TNF-α Release from Monocytes Induced by Gram-Negative Bacteria. We show that GrK bound to *P. aeruginosa*, *N. meningitides,* and *E. coli BL21* (Fig. 1*B*). We therefore investigated the effect of GrK on proinflammatory cytokine release from monocytes induced by these Gram-negative bacteria. GrK synergistically potentiated TNF-α production caused by all three bacterial species severalfold (Fig. 2 *C*–*E*). Thus, a stimulatory effect of GrK on cytokine production in monocytes is observed not only with LPS, but also with live bacteria.

GrK Synergistically Enhances Proinflammatory LPS-Induced TNF-α Response in Mice. Next, we tested whether the effect of GrK on the proinflammatory response to LPS also occurs in vivo. WT C57BL/6 mice were injected intraperitoneally (i.p.) with LPS in

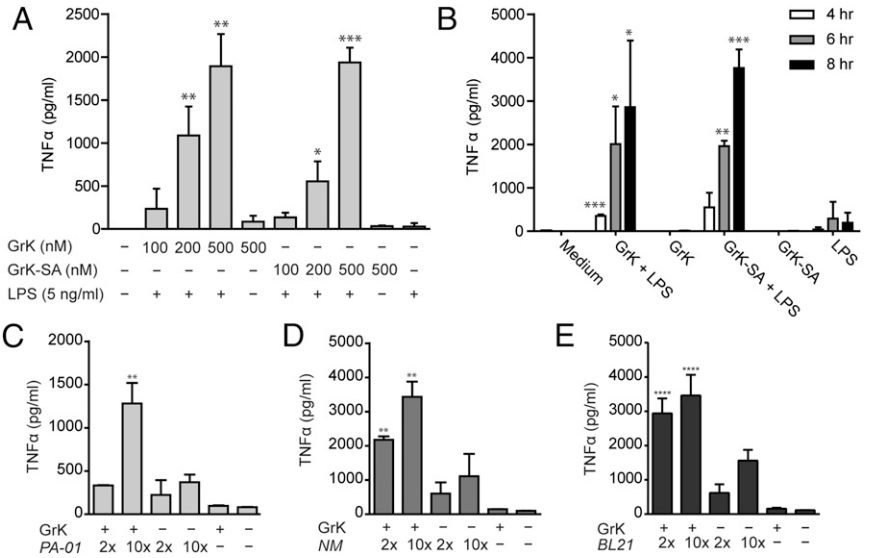

Fig. 2. GrK synergistically potentiates proinflammatory TNF-α release from human monocytes induced by LPS and Gram-negative bacteria. (*A*) Human monocytes were incubated with indicated concentrations of isolated GrK or GrK-SA with or without LPS for 6 h. Culture supernatants were analyzed for TNF-α. Data are depicted as mean ± SD ($n = 3$ per donor) and are representative of at least three independent experiments with normal donors. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (compared with LPS control). (*B*) Human monocytes were incubated with GrK or GrK-SA (200 nM) with or without LPS for indicated timeframes. Supernatants were analyzed for TNF-α. Data are depicted as mean ± SD ($n = 3$ per donor) and are representative of at least three independent experiments with normal donors. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (compared with LPS control for the same time point). (*C*–*E*) Monocytes were incubated with GrK (500 nM) for 6 h with or without *P. aeruginosa* (PA-01) (*C*), *N. meningitides HB-1* (NM) (*D*), or *E. coli BL21* (*E*), at 2- or 10-fold excess compared with cell numbers. Supernatants were analyzed for TNF-α. Data are depicted as mean ± SD ($n = 3$–6 per donor) and are representative of at least three independent experiments with normal donors. **$P < 0.01$; ****$P < 0.0001$ (compared with bacteria only).

the presence or absence of GrK. After 2 or 6 h, mice were killed, and circulating plasma TNF-α, IL-6, and -1β were measured. Injection of GrK alone did not result in cytokine production. However, TNF-α was up-regulated upon administration of GrK and LPS together, compared with injection of LPS alone, both after 2 and 6 h (Fig. 3 *A* and *B*). IL-6 and -1β were not elevated upon GrK + LPS injection compared with LPS alone after 2 h (Fig. S5 *A* and *C*), but were increased after 6 h, although the increase in IL-6 levels was not statistically significant ($P = 0.152$) (Fig. S5 *B* and *D*). Together, these data indicate that GrK synergistically enhances the proinflammatory LPS-induced cytokine responses in vivo.

The Synergistic Effect of GrK and LPS on Monocytes Is Dependent on Cluster of Differentiation 14. LPS activates monocytes via binding to cluster of differentiation 14 (CD14) and subsequent TLR4 signaling. If potentiation of LPS signaling by GrK is fully dependent on the CD14–TLR4 signaling pathway, neutralization of the LPS–CD14 interaction would be expected to block the synergistic effect of GrK. Indeed, TNF-α production was completely inhibited after pretreatment of human monocytes with a neutralizing αCD14 monoclonal antibody (Fig. 4*A*). This finding indicates that the synergistic effect of GrK on LPS-induced proinflammatory cytokine release is mediated via CD14 and subsequent TLR4 signaling.

GrK Augments LPS–CD14 Complex Formation and Releases LPS from Its Micelle Conformation. Effective activation of monocytes depends on LPS transfer to CD14 via the serum protein LPS-binding protein (LBP), which promotes complex formation between LPS and CD14 and thereby lowers the threshold for monocyte activation by LPS (30, 31). We wondered whether GrK promotes LPS–CD14 complex formation in a manner similar to that of LBP. GrK was incubated with LPS and recombinant CD14 for 2 h, while LBP served as a positive control. LPS–CD14 complex formation was visualized by native PAGE and immunoblotting for CD14 (30, 32). Intriguingly, like LBP, GrK stimulated complex formation between LPS and CD14 in a dose-dependent manner (Fig. 4*B*). Incubation of GrK and CD14 without LPS resulted in a faint band, likely representing a minor amount of complex formation between the two proteins (Fig. 4*B*). Triglyceride contamination of GrK was below detection level (<0.1 mmol/L), ruling out the possibility that lipids account for this observed band. LPS is an amphipathic molecule that forms micelles in an aqueous environment. LBP pulls LPS out of its micelle conformation and delivers single LPS molecules to CD14 (30). To determine whether GrK disaggregates LPS from micelles, we used LPS coupled to boron-dipyrromethene difluoride (LPS-BODIPY FL), of which the fluorescence depends on the LPS aggregation state (33). Addition of GrK dose-dependently increased LPS–BODIPY FL fluorescence, indicating that GrK disturbs LPS micelle conformation (Fig. 4*C*). LBP had similar effects, whereas simultaneous addition of LBP and GrK did not further enhance LPS micelle disaggregation compared with addition of either protein alone (Fig. S6*A*). Soluble CD14 did not affect GrK- or

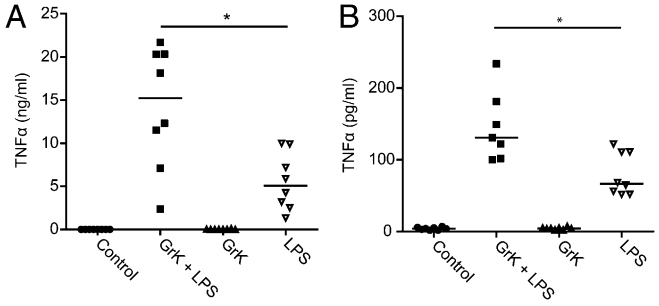

Fig. 3. GrK enhances the LPS-induced TNF-α response in vivo. WT C57BL/6 mice ($n = 7$ or 8) were injected i.p. with physiological saline, LPS, GrK alone, or GrK with LPS. After 2 (*A*) or 6 (*B*) h, mice were killed, and TNF-α levels were measured in plasma. Solid line represents the median for each group. *$P <$ 0.05 (compared with LPS only; Mann–Whitney *u* test).

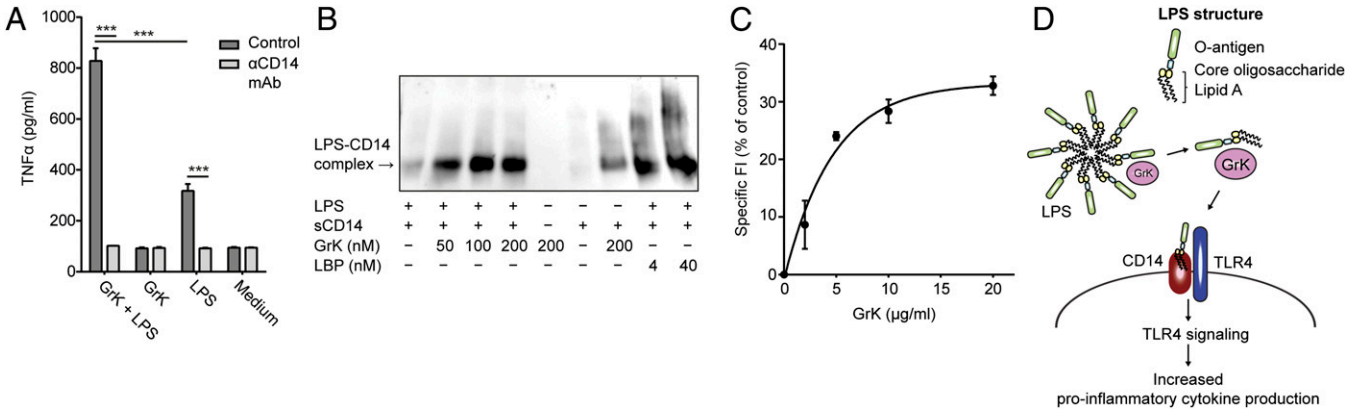

Fig. 4. GrK augments LPS–CD14 complex formation and releases LPS from micelles. (*A*) The synergistic effect of GrK and LPS depends on CD14. Human monocytes, pretreated with or without a neutralizing αCD14 monoclonal antibody, were incubated with GrK with or without LPS for 6 h. Supernatants were analyzed for TNF-α. Data are depicted as mean ± SD ($n = 3$ per donor) and are representative of at least three independent experiments. ***$P < 0.001$. (*B*) GrK promotes complex formation between LPS and CD14. LPS was incubated with human sCD14 with or without GrK or LBP. Samples were separated by native PAGE. CD14–LPS complexes were visualized by immunoblotting for CD14. Results are representative of three independent experiments. (*C*) GrK mobilizes LPS from micelles. LPS–BODIPY, of which the fluorescent intensity (FI) increases upon removal from LPS micelles, was incubated with GrK and the mean FI was measured. Data are corrected for the FI of LPS–BODIPY alone and depicted as percentage of the FI of LPS–BODIPY treated with 2% SDS. Data represent mean ± SD ($n = 6$). (*D*) Schematic model of the potentiation of LPS-induced proinflammatory cytokine release from monocytes by GrK. GrK liberates individual LPS molecules from LPS micelles and likely delivers these to CD14, thereby lowering the threshold for monocyte activation.

LBP-mediated LPS micelle disaggregation. Furthermore, LBP competed with GrK for LPS binding in a solid-phase binding assay (Fig. S6*B*), indicating that GrK and LBP do not bind LPS simultaneously. These data indicate that GrK, independent from LBP, promotes complex formation between LPS and CD14, most likely by mobilizing individual LPS molecules from micelles and transferring these to CD14 (Fig. 4*D*).

Discussion

Granzymes are serine proteases known for their capacity to induce apoptosis in tumor cells and virally infected cells. However, evidence supporting a role for granzymes in inflammation is emerging (34, 35). Circulating soluble granzymes A, B, K, and M are increased in inflammatory conditions, including sepsis (3–8, 36). Their extracellular functions in infection, however, remain unclear. Here, we demonstrate that human GrK binds to Gram-negative bacteria and to LPS and synergistically enhances the proinflammatory cytokine response induced by these agents in human monocytes in vitro and in mice (Figs. 1–3). GrK promotes LPS–CD14 complex formation, likely by disturbing the LPS micelle conformation and facilitating transfer of individual LPS molecules to CD14 (Fig. 4). Intriguingly, to our knowledge, we have demonstrated for the first time a granzyme function not related to its catalytic activity. Thus, extracellular GrK is an unexpected direct modulator of LPS–TLR4 signaling during the antimicrobial innate immune response in vivo.

Incubation of monocytes or injection of mice with GrK alone did not induce release of proinflammatory cytokines, ruling out the possibility that our granzyme preparations were contaminated with endotoxins. Indeed, our granzyme preparations contained <0.5 endotoxin units (EU)/mL (~0.05 ng/mL) endotoxin, concentrations insufficient to account for the robust synergistic effect of GrK and LPS (Figs. 1–3). Finally, SDS/PAGE analysis revealed that no other proteins were present in our purified granzyme batches (Fig. S7*A*). These data justify the conclusion that the marked synergistic effect of GrK on the LPS-induced proinflammatory cytokine release from monocytes and in mice is dependent on the granzyme and not on contaminations in the preparations used.

In contrast to our findings, two recent studies show that GrK alone directly induces proinflammatory cytokine release in vitro. Joeckel et al. have demonstrated that active mouse granzyme K, but not its inactive zymogen, triggers secretion of IL-1β from murine macrophages (26). This effect is largely dependent upon intracellular delivery, although extracellular effects are observed at high GrK concentrations (600–1,000 nM). This study agrees with recent work by Metkar et al. (10) with human GrA, which induces the release of proinflammatory cytokines from human monocytes. This effect of human GrA is enhanced when the protease is delivered intracellularly and is fully dependent on the catalytic activity of the granzyme. In another study, Cooper et al. have shown that extracellular human GrK alone induces IL-6 and -8 release from human lung fibroblasts (25). Like for GrA (10), in the studies by both Joeckel et al. (26) and Cooper et al. (25), GrK catalytic activity is an absolute prerequisite to trigger cytokine responses, indicating that GrK cleaves one or more substrates to exert these effects. Indeed, we were able to confirm that GrK induced IL-6 and -8 release in human lung fibroblasts in a manner dependent on GrK catalytic activity (Fig. S8). In marked contrast, we found that GrK potentiated the LPS-induced cytokine release from monocytes independent of its catalytic activity, because a catalytically inactive GrK-SA mutant showed similar effects as mature active GrK (Fig. 2). Apparently, granzymes use different mechanisms to induce cytokines in different cell types.

We demonstrate that human GrK and GrK-SA bound to Gram-negative bacteria and LPS (Fig. 1). This interaction was blocked by addition of NaCl, heparin, and, predominantly, by an LPS fragment (delipidated LPS) that harbors the core oligosaccharide and the O-antigen. This finding suggests that the putative heparin-binding site (27) on GrK plays a role in binding LPS O-antigens that protrude out of LPS micelles formed in aqueous solutions. Consistent with this hypothesis, GrK released LPS from its micelle conformation and promoted complex formation between LPS and CD14 (Fig. 4). We hypothesize that GrK-mediated release of LPS from its micelle structure facilitates the transfer of individual LPS molecules to CD14. This process lowers the threshold for monocyte activation by LPS, resulting in enhanced TLR4 signaling and increased proinflammatory cytokine release from monocytes. In this context, it remains unclear why GrK does not bind to all Gram-negative bacteria tested (Fig. 1*B*). This result might be related to the type and structure of LPS that varies among different Gram-negative bacterial strains. Alternatively, other bacterial-wall components

may affect GrK–LPS binding. Further study is required to distinguish between these possibilities.

GrK potentiates LPS-induced proinflammatory cytokine responses, a role that appears similar to the functions of proteins like azurocidin, high-mobility group protein B1, apolipoprotein C1, and LBP, all of which are elevated in plasma during inflammation and play roles in stimulating the LPS-induced proinflammatory response in vivo (32, 37–41). Interestingly, GrK is structurally related to azurocidin [also known as heparin-binding protein (HBP) or CAP-37], which also is a family member of the serine proteases but has lost its ability to cleave substrates (42). Although the exact mechanism by which azurocidin potentiates the LPS-induced cytokine response remains unknown, azurocidin—like GrK—exerts this effect independently of serine protease catalytic activity. It is therefore tempting to speculate that azurocidin employs the same mechanism to enhance LPS-induced cytokine release as described here for GrK. Moreover, our findings in conjunction with the studies just discussed reflect the possible redundancy of proteins that potentiate the cytokine-stimulating activity of LPS during inflammation. Support for such a compensatory backup comes from our finding that GrK and LBP do not bind LPS simultaneously (Fig. S6*B*).

Severe sepsis is a major health problem, affecting at least 1.8 million people worldwide each year (43, 44). The syndrome is caused by uncontrolled local microbial infections that spread to the bloodstream and cause the production of excessive amounts of proinflammatory cytokines, acute phase proteins, and reactive oxygen species. This immune response results in a disproportionate systemic inflammation that may cause irreversible tissue damage and, eventually, death (45). The initial excessive proinflammatory response in sepsis may be dampened by interfering with the GrK–LPS interaction, for example, by using monoclonal antibodies. The physiological importance of the synergistic effect of GrK and LPS is demonstrated by our in vivo data, showing that GrK contributes to the initial systemic response to LPS (Fig. 3). Apart from GrK, at least GrA, GrM, and GrB levels are also upregulated during (acute) sepsis (7, 36, 46). Interestingly, GrM$^{-/-}$ mice have reduced serum levels of several proinflammatory cytokines upon LPS challenge (9). Furthermore, both GrM$^{-/-}$ and GrA$^{-/-}$ mice have better survival rates than WT mice when injected with a lethal dose of LPS (9, 10), and this effect is even stronger in GrM/GrA double-knockout mice (9). These findings open the possibility that other granzymes besides GrK also interact with LPS and contribute to disease progression by using a mechanism that resembles that of GrK. In accordance with this hypothesis, we found that purified GrA and GrB also potentiated the LPS-induced cytokine response of monocytes (Fig. S9). Thus, granzymes may have evolved to be mutually redundant in their capacity to trigger TLR4-dependent proinflammatory cytokine release. Disease modulation by targeting granzymes therefore may provide new perspectives for future treatment of acute sepsis.

Materials and Methods

Human Serum Samples. Serum samples from patients with sepsis, which were obtained previously (47), were used. All patients or one of their family members had given informed consent. Patients fulfilled the criteria for systemic inflammatory response syndrome and had evidence for Gram-negative infection. All samples were anonymized. The use of the samples was approved of by the ethics committee of the Academic Medical Center, Amsterdam. Serum samples from healthy donors were obtained from the University Medical Center Utrecht. All donors had given informed consent, and all samples were anonymized.

Production, Purification, and Characterization of GrK. Human GrK and its catalytically inactive mutant, GrK-SA (in which the active site residue Ser^{195} has been replaced by Ala) were produced as described (48, 49). GrK and GrK-SA were purified by cation-exchange chromatography followed by affinity chromatography with Prot A/G beads (Thermo Fisher Scientific. Purified granzyme was dialyzed against 20 mM Tris and 150 mM NaCl (pH 7.4). Protein concentrations were measured by using a Bradford (Bio-Rad) or bicinchoninic acid (Thermo Scientific) assay. On SDS/PAGE, purified GrK migrated as a single band of ∼25 kDa, consistent with monomeric GrK(-SA) (Fig. S7*A*). GrK, but not GrK-SA, cleaved the synthetic chromogenic substrate Ac-Lys-pNA as well as the known macromolecular substrate SET (21, 48) (Fig. S7 *B* and *C*), indicating that GrK, but not GrK-SA, is catalytically active. GrK(-SA) batches were not contaminated with endotoxin [<0.5 EU/mL (∼0.05 ng/mL), final concentration] as determined by Limulus amebocyte lysate assay (Thermo Scientific). GrK(-SA) was biotinylated by using the Biotin Protein Labeling Kit (Roche) according to the manufacturer's protocol. GrK levels in serum samples from sepsis patients were measured by using ELISA (Uscn Life Science; lower detection limit ∼3 pg/mL).

Binding of GrK to Bacteria. Bacteria were diluted in PBS to OD at ∼0.5 (660 nm), centrifuged, resuspended in PBS with 0.1% (wt/vol) BSA, mixed with biotinylated granzyme (0–20 μg/mL), and incubated for 1 h at 37 °C. Bacteria were washed two times in PBS with 1% BSA and incubated with 1 μg/mL Streptavidin–phycoerythrin (Southern Biotech) in PBS with 1% BSA for 30–60 min at 4 °C. Finally, bacteria were washed once with PBS with 1% BSA and analyzed by flow cytometry.

Solid-Phase Binding Assays with GrK and LPS. LPS (10 μg/mL in PBS) was coated onto 96-well plates (Greiner Bio-One GmbH) overnight at 4 °C and incubated with biotinylated GrK or GrK-SA in PBS with 0.1% (vol/vol) Tween-20 at 37 °C for 2 h. Bound granzymes were visualized by using Streptavidin–poly-HRP (Sanquin) followed by 3,3′,5,5′-tetramethylbenzidine (Invitrogen). After adding 1 M H_2SO_4 the OD_{450} was measured.

LPS–GrK Pull-Down Assay. LPS–biotin (50 μg/mL) was coupled to Streptavidin-coated beads (Amersham BioSciences). Beads were incubated with recombinant GrK, GrK-SA, or serum from a selected Gram-negative sepsis patient for 1 h at room temperature or overnight at 4 °C, rotating. Bound protein was eluted with 2x concentrated Laemmli buffer, and analyzed by SDS/PAGE followed by Instant Blue total protein staining.

Proinflammatory Cytokine Response in Isolated Human Monocytes. Monocytes (0.5×10^5 per well) were incubated with granzyme (0–500 nM) with or without LPS (5 ng/mL) in serum-free RPMI 1640 for 0–8 h. Supernatants were stored at −20 °C. To determine relative cell viability, 250 μL per well of water-soluble tetrazolium-1 reagent was added, and OD_{450} was measured for 90 min. In experiments with bacteria, LPS was replaced with a 2- or 10-fold excess of bacteria compared with the number of monocytes per well. In CD14-neutralizing experiments, monocytes were pretreated for 30 min at 37 °C with α-CD14 mAb (10 μg/mL) or serum-free medium alone. TNF-α, IL-6, and -8 levels in culture supernatants were measured by using a multiplex assay as described (50) on a Luminex FlexMap 3D (Bio-Rad) with xPonent software (Version 4.1). Data were analyzed by using BioPlex Manager (Version 6.1.1; Bio-Rad). Alternatively, TNF-α was measured by using ELISA (PeliKine human TNF-α ELISA kit; Sanquin).

Proinflammatory Cytokine Response in Mice. Female C57BL/6 mice (Charles River; $n = 7$ or 8 per group), matched in age (10 wk) and weight, were injected i.p. with LPS (*E. coli* O111:B4; 10 μg/g) with or without human recombinant GrK (500 nM) in physiological saline. After 2 or 6 h, blood was drawn, and mice were killed. Blood was centrifuged at $2,000 \times g$ for 10 min at 4 °C, and plasma was stored at −80 °C. Plasma cytokines were measured by using a mouse multiplex assay (Bio-Rad) on a Luminex FlexMap 3D (Bio-Rad) with xPonent software (Version 4.2). Data were analyzed by using BioPlex Manager (Version 6.1.1; Bio-Rad).

All animal experiments were carried out in accordance with the guidelines of the Dutch Experiments on Animal Act and were approved by the Institutional Animal Care Use Committee of the Academic Medical Center, Amsterdam.

LPS–CD14 Complex Formation. LPS (1 μg/mL) was incubated with human recombinant CD14 (100 nM) with or without GrK (0–200 nM) or LBP (4 or 40 nM) in 20 mM Tris and 150 mM NaCl for 2 h at 37 °C. LPS–CD14 complex formation was analyzed by native PAGE followed by immunoblotting for human CD14 as described (30).

Effect of GrK on LPS Micelle Formation. LPS–BODIPY-FL (7.5 μg/mL) was incubated with GrK (0–20 μg/mL) with or without human recombinant CD14 (7.5 μg/mL) in 250 μL of PBS. LPS–BODIPY-FL alone served as a negative control, and LPS–BODIPY-FL plus LBP (10 μg/mL) was a positive control. Disaggregation

of LPS micelles upon treatment of LPS–BODIPY with 2% (vol/vol) SDS was set at 100%. Fluorescent intensity (FI) was measured kinetically for 2 h at 37 °C at 520 nm by using the FluoStar Omega (BMG Labtech). Data are represented as the percentage of specific FI compared with SDS control.

Statistical Analysis. Unless indicated otherwise, data are depicted as mean values ± SD, and statistical analyses were performed by using the independent samples *t* test. Two-tailed *P* values <0.05 were considered statistically significant.

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