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Granzyme M and K release in human experimental endotoxemia

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ABSTRACT

Granzymes are serine proteases involved in killing of tumor cells and virally infected cells. However, granzymes are also upregulated in blood under inflammatory conditions and contribute to cytokine release and processing. Here, we show that granzyme M (GrM) and to a lesser extent GrK are transiently elevated in the circulation following LPS administration in humans. GrM is released upon stimulation of whole blood with LPS or the gram-negative bacteria *Escherichia coli BL21*, *Pseudomonas aeruginosa*, and *Neisseria meningitidis*. GrK is only released upon stimulation with *P. aeruginosa*. Thus, GrM and GrK are differentially released in response to LPS and gram-negative bacteria.

1. Introduction

Severe sepsis is a major health problem, resulting in a significant number of deaths each year (Angus and van der Poll, 2013). The disease is caused by a local microbial infection that spreads to the circulation, resulting in a systemic inflammatory disorder that can cause irreversible organ injury and may ultimately result in death (Angus and van der Poll, 2013). Although mortality rates of sepsis have dropped over the past decades due to improved hospital care of patients, 20–30% of patients with severe sepsis still die, and effective treatment options are lacking (Angus and van der Poll, 2013).

Granzymes are a set of homologous serine proteases expressed in cytotoxic lymphocytes and are involved in the killing of tumor cells and virally infected cells (Bovenschen and Kummer, 2010). In humans, there are five granzymes: granzyme A (GrA), GrB, GrH, GrK, and GrM. Following target cell recognition by cytotoxic lymphocytes, granzymes are directed inside the target cell where they can activate pro-apoptotic pathways by cleavage of various substrates, resulting in apoptosis (Bovenschen and Kummer, 2010; Chowdhury and Lieberman, 2008).

In addition to their cytotoxic potential, granzymes also play a role in inflammation. Levels of soluble granzymes A, B, K, and M are elevated in the circulation under inflammatory conditions, including endotoxemia and sepsis (Wensink et al., 2014; Lauw et al., 2000; Zeerleder et al., 2005; Hollestelle et al., 2011; Rucevic et al., 2007; Buzza and Bird, 2006). GrM and GrA knockout mice survive longer than wild-type (WT) mice, in response to a normally lethal LPS challenge (Anthony et al., 2010; Metkar et al., 2008), and GrM$^{-/-}$ mice express lower levels of cytokines than WT mice upon LPS challenge (Anthony et al., 2010). In addition, we have recently demonstrated that GrK synergistically potentiates cytokine responses from human monocytes induced by LPS or gram-negative bacteria (Wensink et al., 2014). This suggests that granzymes are involved in the (innate) immune response against bacterial infections.

Soluble GrA and GrB are released in serum during experimental human endotoxemia and following stimulation of whole blood with LPS and bacteria (Lauw et al., 2000). However, it remains unknown whether GrK and GrM are also secreted in response to stimulation with LPS or bacteria. Here, we report that injection of LPS into healthy volunteers triggers a temporary increase in soluble GrM and, to a lesser extent, GrK levels. Whole blood stimulation with LPS or three strains of gram-negative bacteria results in GrM release, whereas GrK release is more restricted and is observed only

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upon stimulation with the gram-negative bacterium *Pseudomonas aeruginosa*, but not with *Escherichia coli* BL21, *Neisseria meningitidis*, or LPS.

2. Materials and methods

2.1. Experimental human endotoxemia

Five healthy nonsmoking male volunteers (mean age: 24.4 years, range: 21–30 years) were admitted to the Clinical Research Unit of the Academic Medical Center, Amsterdam. Physical and routine laboratory examination were normal, as well as medical history and electrocardiography. Each volunteer was given a bolus intravenous (i.v.) injection of 4 ng/kg LPS (*E. coli* O113, CC-RE lot 3, National Institutes of Health, USA), administered over 1 min in an antecubital vein. Blood was collected in EDTA tubes immediately before injection, and furthermore at time points 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 12, and 20 h post injection. After centrifugation, plasma was transferred to clean vials and stored at −80 °C until analysis. The clinical response of the volunteers to the LPS injection, as well as their leukocyte and lymphocyte counts, were similar to previously published results (Lauw et al., 2000). The study was approved of by the Medical Ethics Commission of the AMC, Amsterdam, The Netherlands, and written informed consent was obtained from all subjects.

2.2. Whole blood stimulation with LPS or gram-negative bacteria

Whole blood from healthy volunteers (other than described in the previous paragraph) was collected in sodium heparin Vacutainers (Becton Dickinson). Blood was diluted in serum-free RPMI 1640 to a final dilution of 1:5 (blood:RPMI). LPS (*E. coli* 0111:B4) (0–50 ng/ml) was added and samples were incubated for 0–24 h under standard incubator conditions (37 °C, 5% CO_2, relative humidity (RH) 95%). After incubation, blood was collected and centrifuged at 4 °C for 5 min at 500 g. Plasma supernatants were transferred to clean eppendorf tubes and stored at −20 °C until analysis. Alternatively, whole blood was stimulated with live gram-negative bacteria added in a 2-fold excess compared to PBMC numbers (∼1 × 10E6 PBMCs/ml in whole undiluted blood). *Escherischia coli BL21* (E.coli BL21), *P. aeruginosa 01* (PA-01), or *Neisseria meningitides* HB-1 (NM) were used. All bacterial strains were a kind gift from the Medical Microbiology Department (UMC, Utrecht). Bacteria were grown overnight on TSASB plates at 37 °C, except for *N. meningitidis*, which was grown on GB-Choco plates (37 °C, 5% CO_2, RH 95%). Bacteria were diluted in PBS to OD ∼0.5 at 660 nm (representing approximately 5 × 10E8 bacteria/ml), and added in the correct concentration to whole blood pre-diluted as described above. After incubation, plasma samples were obtained as described above.

2.3. Granzyme and cytokine measurements in plasma and serum samples

GrM and GrK levels in samples were determined using ELISA (Uscn Life Science Inc., lower detection limit for both assays ∼3 pg/ml). The specificity of the ELISAs was confirmed using Western Blot for GrM and GrK (data not shown). TNFα and IL6 were measured in EDTA anticoagulated plasma by Luminex multiplex assay using BioPlex 200 (BioRad, Hercules, CA).

2.4. Granzyme M FACS staining

PBMCs were isolated from healthy volunteers using Ficoll density gradient. Cells were cultured in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells (5 × 10E5) were treated with or without 20 ng/ml LPS for 18 h. Cells were incubated with one of two cell-surface marker combinations prior to determine GrM protein levels in different cell subsets: (1) CD3-Pacific Blue (Biolegend), CD8-PerCP, CD56-PE-Cy7, CD14-V500 (BD), CD16-APC (eBioscience); (2) CD3-Pacific Blue, CD4-PE-Cy7 (Biolegend), CD8-PerCP (BD). Cells were subsequently permeabilized for intracellular GrM staining. After fixation and permeabilization, cells were incubated with Alexa Fluor 488-conjugated anti-GrM (clone 4B2G4) as we described previously (Bovenschen et al., 2014; de Koning et al., 2010). Samples were measured at FACS Canto II and analyzed using FACSdiva software. The monocytes were identified based on characteristic properties of the cells in the forward (FSC) and side scatter (SSC) and then divided in CD14+ and CD16+ monocytes. Within the lymphocyte gate based on FSC/SSC, the T cells were gated on CD3+ and then divided in CD4+ or CD8+. NK cells were gated as CD3-CD16 + CD56+. NKT cells are gated as CD3 + CD56+. Within these populations, the percentage of GrM+ cells was defined.

3. Results

3.1. GrM and GrK are released in human experimental endotoxemia

Human endotoxemia was modeled by injecting five healthy volunteers with LPS intravenously. Serum was collected at different time points post LPS injection, and granzymes were measured. Both GrK and GrM serum levels showed a temporary rise upon LPS injection (Fig. 1a and b). A major peak in GrM levels was observed after ∼2 h, with a minor peak following after ∼5–8 h (Fig. 1a). A less clear but recognizable rise in GrK levels was observed in several donors (Fig. 1b). When GrK and GrM levels in individual volunteers are compared, peaks in GrK and GrM levels largely coincided (Fig. 1c–g). As expected, TNFα (Fig. 1h) and IL6 (Fig. 1i) levels in serum transiently increase following LPS injection, with peak levels that coincide with the peak levels of serum granzymes. However, there is no correlation between the absolute responses of granzymes and proinflammatory cytokines to LPS (at least in these 5 patients), suggesting that the different granzyme responses among individuals cannot be explained by different responses to LPS. These data indicate that GrM and to a lesser extent GrK are released in human experimental endotoxemia.

3.2. GrM, but not GrK, is released in LPS-treated whole blood cultures

We next addressed whether LPS can trigger GrK and GrM release in whole blood. Incubation of whole blood with LPS induced a clear time-dependent increase in soluble GrM levels (Fig. 2a), observed in four out of five donors. In contrast, no GrK release was observed under the same circumstances in four out of five donors tested (Fig. 2b). In one donor, minor amounts of GrK were released (∼30 pg/ml after 24 h incubation with 50 ng/ml LPS). No granzyme was detected without incubation or after incubation of untreated blood (Fig. 2a and b). Subsequently, we measured intracellular GrM expression in several PBMC subsets of four independent donors upon LPS treatment. The amount of GrM-positive cells did not change in NK cell, NKT cell, CD8+ T cell, CD4+ T cell, and CD14+ monocyte subsets (Fig. 2c). Surprisingly, however, CD16+ monocytes did express GrM (unstimulated), and this GrM expression increased following LPS treatment (in three out of four donors). Taken together, these data indicate that GrM, but not GrK, is released in response to LPS stimulation.

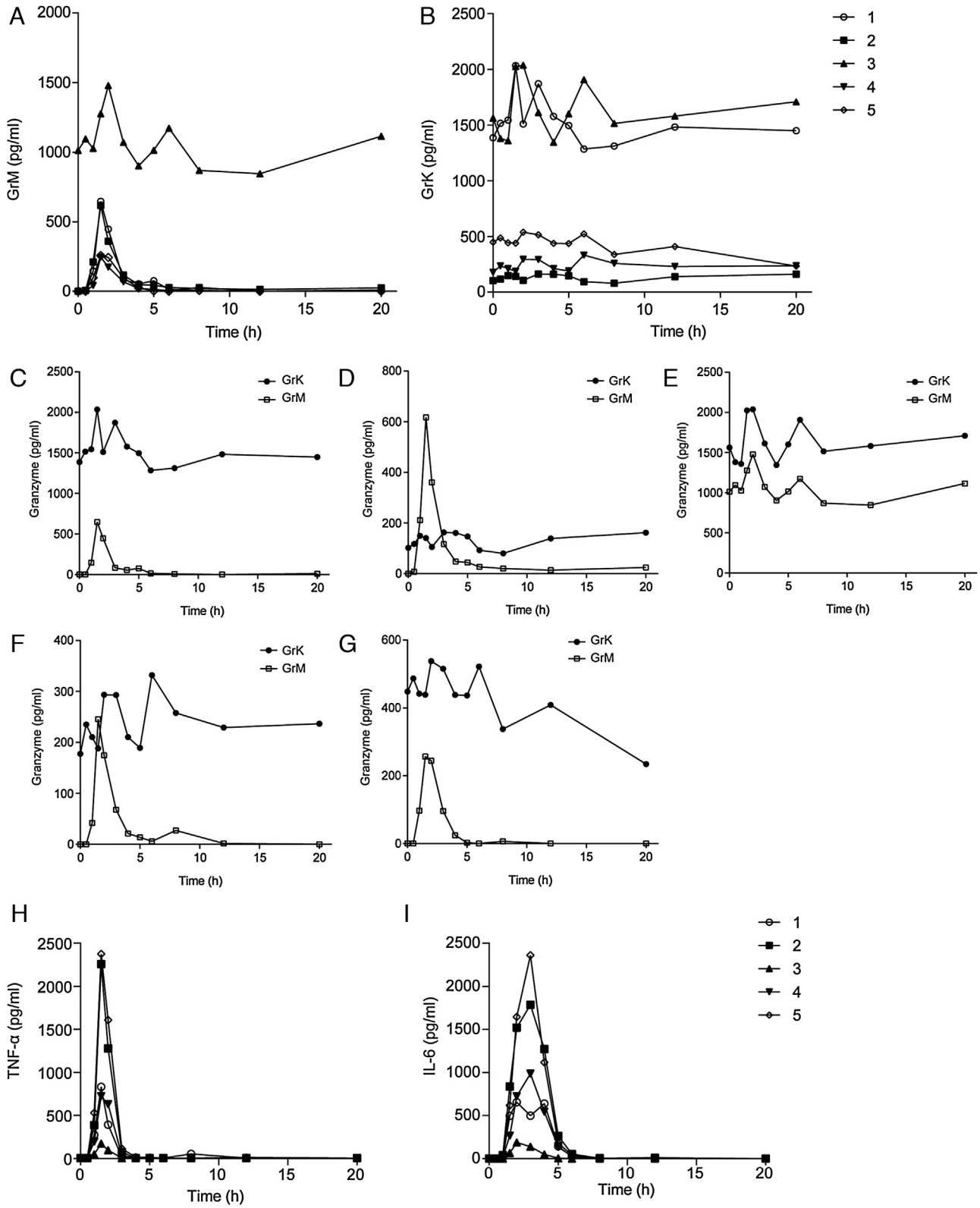

Fig. 1. GrM and GrK are released in human experimental endotoxemia.
Five male subjects were injected intravenously with LPS at $t = 0$ and blood was drawn at different time points after injection. Sera were analyzed for GrM (a) and GrK (b) levels using ELISA. The course of circulating GrK and GrM (c-g), and TNFα (h) and IL6 (i) levels in each volunteer is depicted in figures (volunteer 1–5, respectively).

3.3. GrM and GrK are differentially released in whole blood treated with gram-negative bacteria

LPS is an important cell wall constituent of gram-negative bacteria. Therefore, GrK and GrM release in whole blood upon incubation with gram-negative bacteria was investigated. Whole blood was stimulated with *E. coli* BL21, *P. aeruginosa* (PA-01), or *N. meningitidis* (NM), added in a 2-fold excess compared to peripheral blood mononuclear cell (PBMC) numbers. All three bacterial species

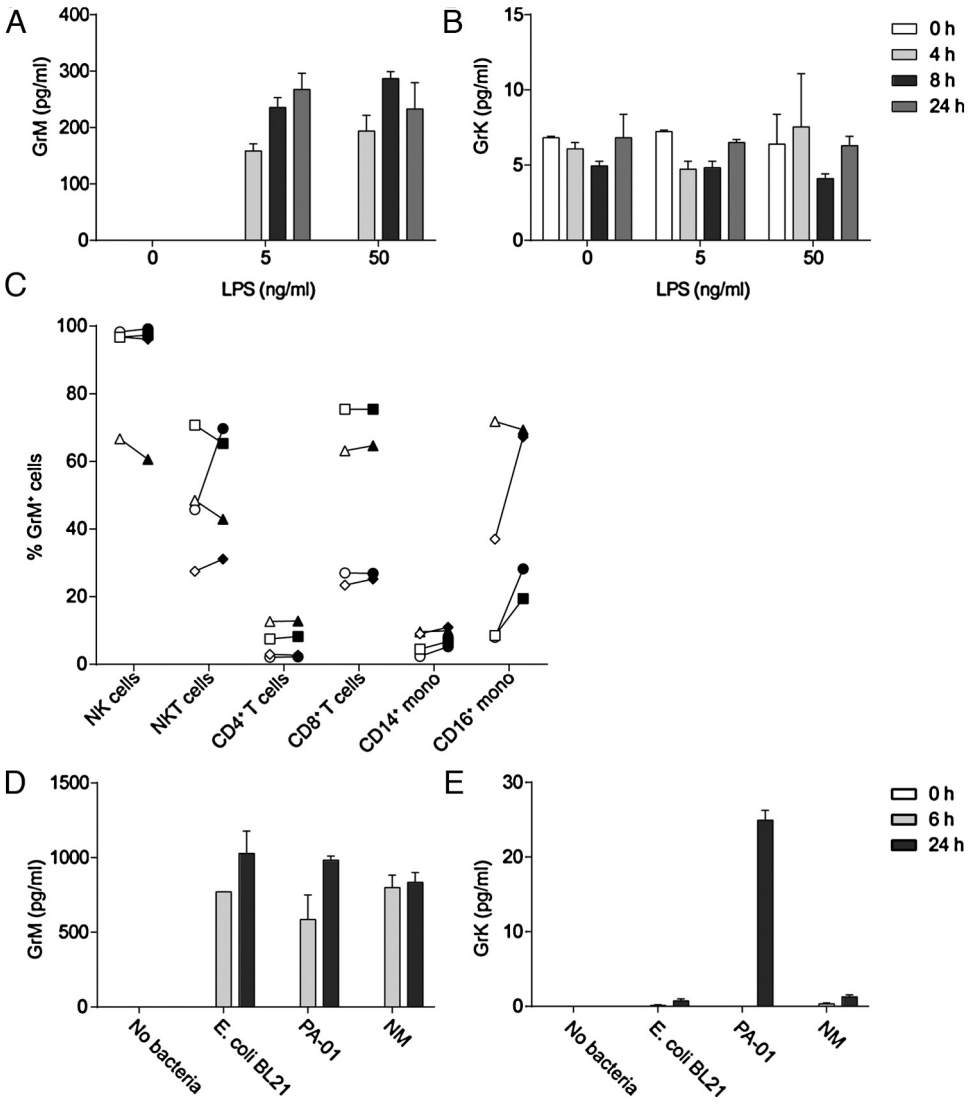

Fig. 2. GrM and GrK release in whole blood stimulated with LPS or gram-negative bacteria.
(a, b) GrM, but not GrK, is released in whole blood upon LPS stimulation. Whole blood, diluted 1:5 (blood:medium), was incubated with LPS (0–50 ng per ml). At different time points GrM (a) and GrK (b) levels in culture supernatant were determined using ELISA. Data are representative of five independent experiments. (c) Isolated PBMCs (four independent donors) were treated with LPS (20 ng/ml) (closed symbols) or without LPS (open symbols) for 18 h. Indicated cell subsets were FACS stained for intracellular GrM. (d, e) GrM and GrK are released in whole blood incubated with gram-negative bacteria. Whole blood, final dilution 1:5 (blood:medium), was treated with *Escherichia coli* BL21 (*E. coli* BL21), *Pseudomonas aeruginosa* 01 (PA-01), or *Neisseria meningitidis* HB-1 (NM) added in two-fold excess compared to PBMC numbers. At different time points GrM (d) and GrK (e) levels in the culture supernatant were determined. Data are representative of three (PA-01) or two (*E. coli* BL21 and NM) independent experiments.

caused a marked GrM release at 6 h and 24 h following incubation (Fig. 2d). Interestingly, GrK was only released upon treatment with PA-01, and only after 24 h of incubation (Fig. 2e). Soluble GrK or M were not released in blood incubated without bacteria (Fig. 2d and e). These data demonstrate that GrM and GrK are differentially released in whole blood upon incubation with gram-negative bacteria.

4. Discussion

It is well established that granzymes A, B, K, and M are elevated in the blood circulation during endotoxemia and sepsis (Wensink et al., 2014; Lauw et al., 2000; Zeerleder et al., 2005; Hollestelle et al., 2011; Rucevic et al., 2007; Buzza and Bird, 2006). Evidence is emerging that these extracellular granzymes play a role during the process of inflammation, for instance via induction of pro-inflammatory cytokine responses (Wensink et al., 2014; Metkar et al., 2008). Like for GrA and GrB (Lauw et al., 2000), we report here that GrM and GrK are secreted during human experimental endotoxemia and in whole blood cultures stimulated with LPS or gram-negative bacteria (Figs. 1 and 2). In healthy volunteers, a transient rise in GrM levels was observed (Figs. 1 and 2), accompanied by a modest temporary rise in GrK levels (Fig. 1). These results agree with reports demonstrating that GrM and GrK are elevated in the circulation during sepsis (Wensink et al., 2014; Hollestelle et al., 2011; Rucevic et al., 2007). In whole blood cultures, GrM was released upon stimulation with LPS or gram-negative bacteria, and remained high for at least 24 h (Fig. 2). GrK was released upon treatment with the gram-negative bacterium *P. aeruginosa*, but not in response to *E. coli* BL21, *N. meningitidis*, or LPS (*E. coli* 0111:B4) (Fig. 2). Thus, in vitro granzyme release induced by LPS or gram-negative bacteria depends on the bacterial type and seems more restricted for GrK than for GrM, at least for the bacteria tested in this study.

Except for one donor, GrM levels in healthy volunteers were undetectable before LPS injection (Fig. 1), consistent with GrM

baseline levels detected in blood plasma in vitro (Fig. 2). In contrast, soluble GrK is clearly detectable in serum of healthy volunteers at the start of the measurements (Fig. 1). This finding is in agreement with results published by us and others (Wensink et al., 2014; Rucevic et al., 2007; Bade et al., 2005), but differs from the low GrK levels we found in plasma in the absence of stimulation in vitro (Fig. 2). Thus, basal circulating GrK and to a lesser extent GrM levels vary amongst individuals.

It remains unknown from which cell type(s) GrM and GrK are released upon stimulation with LPS or bacteria. Candidates include NK cells, as they store large amounts of GrM and GrK (Bovenschen and Kummer, 2010). NK cells respond to LPS, and express intracellular TLR4, although surface expression of TLR4 is probably weak (Kanevskiy et al., 2013; Souza-Fonseca-Guimaraes et al., 2012). However, LPS treatment decreases NK cell degranulation (Kanevskiy et al., 2013), and injection of LPS into healthy volunteers is associated with a marked decrease in NK cell numbers (Lauw et al., 2000). Furthermore, we show that the percentage of GrM-positive NK cells in PBMCs does not change following LPS treatment (Fig. 2). This argues against a role for NK cells in granzyme secretion. Other cell types that express GrM and GrK include NKT cells, γΔ T cells, and CD8+ T cells (Bovenschen and Kummer, 2010). These cytotoxic lymphocytes may also contribute to granzyme release upon LPS or bacterial stimulation, although the percentage of GrM-positive NKT cells and CD8+ T cells in PBMCs do not change upon LPS treatment (Fig. 2). GrB expression has been found in a number of cell types other than cytotoxic lymphocytes, including dendritic cells, mast cells, basophils, and B cells (Hagn and Jahrsdorfer, 2012), and it is possible that these cell types also express GrM and/or GrK. Interestingly, our data show – for the first time – that GrM also is expressed by monocytes and that its expression increases in CD16+ monocytes upon LPS stimulation (Fig. 2). Whether or not the latter cells also contribute to the release of GrM requires further study. Finally, we cannot fully exclude the possibility that other (non-hematopoietic) cells may contribute to granzyme release in vivo, which may explain the absence of GrK release in vitro while it is detected in vivo (Fig. 2).

How LPS and gram-negative bacteria cause granzyme release remains to be elucidated. Lauw et al. (2000) showed that release of GrA and GrB in response to the gram-negative bacterium *Burkholderia pseudomallei* in whole blood cultures is inhibited by adding neutralizing monoclonal antibodies to TNFα or IL-12 (Lauw et al., 2000). This suggests that LPS-induced cytokines trigger cellular granzyme release. Since granzymes also induce cytokine release (Wensink et al., 2014; Metkar et al., 2008), a positive feedback loop may enhance release of both granzymes and cytokines. Further research is required to address this possibility.

Excessive cytokine production in response to LPS or gram-negative bacteria can contribute to the development of severe sepsis (Angus and van der Poll, 2013). Currently, there are no therapies available to modulate the immune response during sepsis, and the only treatment option is to administer antibiotics. One of the major challenges in treating sepsis is to increase the patients' chances of survival by dampening the immune response to the pathogen. Inhibition of granzymes expression or blocking proinflammatory granzyme functions may contribute to this goal.

Conflicts of interest

The authors declare no conflicts of interest.

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