EXTRACELLULAR GRANZYMES IN INFLAMMATION

EXTRACELLULAIRE GRANZYMEN IN ONTSTEKINGSPROCESSEN

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ANNETTE CORNELIA WENSINK

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PROMOTOREN

Prof. dr. C.E. Hack
Prof. dr. P.J. van Diest

COPROMOTOR

Dr. N. Bovenschen

Zooming in
Zooming out
Nothing I can do without
A lense to see it all up close
To magnify what no one knows

Your Blue Room
Passengers / U2

Colophon

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GRANZYMES REGULATE PROINFLAMMATORY CYTOKINE RESPONSES

Annette C. Wensink[1,2], C. Erik Hack[2] and Niels Bovenschen[1,2]

1 Department of Pathology, University Medical Center Utrecht, Utrecht, 3584 CX, The Netherlands

2 Laboratory of Translational Immunology, University Medical Center Utrecht, Utrecht, 3584 CX, The Netherlands

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Abstract

Granzymes are serine proteases mainly produced by cytotoxic lymphocytes and are traditionally considered to cause apoptosis in tumor cells and virally infected cells. However, the cytotoxicity of several granzymes is currently being debated, and additional, predominantly extracellular, functions of granzymes in inflammation are emerging. Extracellular soluble granzymes are elevated in the circulation of patients with autoimmune diseases and infections. In addition, granzymes are expressed by several types of immune cells other than cytotoxic lymphocytes. Recent research has revealed novel immunomodulatory functions of granzymes. In this review, we provide a comprehensive overview on the role of granzymes in inflammation, highlighting their role in cytokine induction and processing.

Introduction

Our immune defense against tumor cells and virally infected cells is mediated by cytotoxic lymphocytes: Natural Killer (NK) cells, NKT cells, γδ T cell receptor (TCR) cells, and cytotoxic T lymphocytes (CTLs). These cytotoxic cells can induce apoptosis in aberrant cells via the death receptor pathway and the granule-exocytosis pathway (1). The death receptor pathway depends on the engagement of death receptors on the target cell by death receptor ligands on the effector cell, while the granule exocytosis pathway involves the release of a set of cytotoxic proteins. These are stored in granules in cytotoxic lymphocytes and include the pore-forming protein perforin and a family of structurally related serine proteases called granzymes (2, 3). Upon recognition, granzymes are released into the immunological synapse between the cytotoxic lymphocyte and the target cell. The granzymes then enter the target cell with the aid of perforin, and activate various pro-apoptotic pathways by cleavage of intracellular substrates (1, 4).

In humans, five different granzymes exist: granzyme A (GrA), GrB, GrH, GrK, and GrM. This non-sequential nomenclature is explained by the existence of a more extensive granzyme family in rodents. Mice express granzymes A-G, K, M and N, but not H, while rats express granzymes I and J, in addition to granzymes A-C, F, K and M (5). Human granzymes, named after their rodent homologues, are encoded on three distinct chromosomal regions: GrA and GrK on chromosome 5, GrB and GrH on chromosome 14 and GrM on chromosome 19 (6). Granzymes A, B, K and H are expressed in higher quantities in CTLs compared to NK cells, while GrM is more abundant in NK cells (1, 2, 4).

Granzymes are serine proteases that consist of two six-stranded β-barrels that regulate substrate specificity, in the middle of which lies a catalytic triad containing the amino acids serine, histidine, and aspartic acid. Mutation of one of the amino acids in the catalytic triad renders the granzyme catalytically inactive. Although human granzymes are ~40% homologous in amino acid sequence (7), they each cleave their own specific set of substrates, a phenomenon partly caused by differences in the primary substrate specificity or P1 of each granzyme. GrA and GrK display tryptase-like activity, cleaving their substrates after an arginine or a lysine. Similar to caspases, GrB cleaves substrates after an aspartic acid or glutamic acid. GrM cleaves after a leucine or a methionine, and GrH after a tyrosine or a phenylalanine (2, 4). Some granzymes share substrates, but none have exactly the same degradome. Thus, granzymes have substrate specificities that only partially overlap (8). The likeliness that in vitro hydrolysis of a given substrate is physiologically relevant can be estimated by determining the concentration of substrate required for effective hydrolysis

(indicated by the Km), and the time required for the granzyme to hydrolyse one substrate molecule (kcat). When Km is low and kcat is high, the catalysis has a greater probability to be of physiological relevance.

Over the past decades, the dogma has been held that all granzymes induce cell death. However, this dogma has recently been debated, in particular for granzymes A and K (6, 9-11). In addition, increased granzyme levels in serum, plasma, synovial fluid and/or broncheo-alveolar lavage fluid (BALF) have been described in patients suffering from inflammatory diseases, including rheumatoid arthritis (GrA, B), viral infections (GrA, B, K), Plasmodium falciparum infections (GrA, B), experimental endotoxemia or sepsis (GrA, B, K, M), hypersensitivity pneumonitis (GrA, B), and acute airway inflammation (GrK) (12-22). These observations prompted researchers to investigate alternative granzyme functions in inflammation. The first evidence for an extracellular role for granzymes was provided by Sayers et al. (23), who noted that treatment with extracellular rat granzyme causes growth inhibition in tumor cell lines (23). More recently, novel extracellular and perforin-independent functions have been identified (24, 25). It has been demonstrated that granzyme-mediated ECM degradation may contribute to inflammation (26-30). GrB degrades several ECM components, such as vitronectin, laminin, fibronectin, decorin, and the proteoglycans biglycan and betaglycan (31-35). In mouse models, GrB degrades the ECM proteins fibronectin, decorin, fibrillin-1, and vitronectin, and contributes to several disease processes, including delayed wound closure, abdominal aortic aneurysm, and skin aging (36-39). These findings point to a pivotal role of GrB in mediating ECM remodeling in disease. Furthermore, mouse and rat GrA degrade human ECM components (fibronectin and collagen type IV) (40-43). However, it is unclear to what extent these findings have physiological relevance. Also, it is unknown whether human GrA, GrH, GrK, and GrM degrade human ECM substrates.

Apart from their role in ECM degradation, a growing body of evidence now implicates granzymes A, B, K, and M in the production, release, and/or processing of proinflammatory cytokines. This immunomodulatory potential of granzymes will be discussed in the current review.

Granzymes trigger cytokine release and activation

GRANZYME A

Studies in mice show a role for GrA in the inflammatory response to the Gram-negative bacterial cell wall component lipopolysaccharide (LPS). GrA-/- mice better withstand a lethal LPS challenge, in that they survive longer than wild-type (WT) mice (11, 44). Furthermore, human GrA (hGrA) exerts direct effects on several cell types to induce cytokine release in vitro. Extracellular GrA stimulates release of proinflammatory cytokines IL-6 from fibroblast cell lines and IL-8 from fibroblast and epithelial cell lines (45). In addition, extracellular GrA activates primary human monocytes to release IL-1β, TNFα, IL-6, and IL-8 (11, 46). These effects of GrA are dependent on its catalytic activity, implying that at least one downstream signaling protein needs to be cleaved. The effects of GrA are augmented upon intracellular delivery (11), which suggests that GrA substrates necessary for cytokine induction localize in the cell. Until now, however, these substrates have not been identified. hGrA cleaves and activates pro-IL-1β in vitro and was therefore designated an interleukin 1β-converting enzyme (47), but the physiological relevance of this finding has never been shown. Moreover, some have failed to demonstrate such an effect of GrA (11). GrA-induced cytokine release from monocytes is blocked in the presence of caspase-1 inhibitors (11), further indicating that IL-1β maturation upon GrA treatment is not mediated by GrA directly, although the inflammasome is involved (Figure 1). Finally, we have recently found that human recombinant GrA does not induce cytokine release from human monocytes, but synergistically potentiates the LPS-induced cytokine response by these cells (48). This is in line with the observation that mouse macrophages primed with LPS respond to mouse GrA (mGrA) and mGrK (10, 11). We are currently investigating the molecular mechanism underlying this GrA effect. Interestingly, hGrA blocks growth of intracellular mycobacteria in human macrophages (49). Human γδ T cells efficiently inhibit the growth of these bacteria inside macrophages. Recombinant GrA, as well as GrA produced by γδ T cells, induces TNFα production in macrophages, which in turn inhibits the growth of the pathogen (49). The mechanism by which TNFα exerts this effect remains unknown. In this regard, it would be interesting to investigate whether GrA, in addition to potentiating TLR4-mediated cytokine responses to LPS (48), also synergizes with other TLR ligands such as mycobacterial products, to more efficiently activate monocytes and other innate immune cells.

GRANZYME B

Data on GrB knockout mice survival upon LPS challenge are conflicting. Anthony et al. (44) show that deletion of GrB has no effect on survival upon LPS challenge, whereas Metkar et al. (11) show that GrB knockout results in a marked increase in survival that exceeds the effect of GrA deletion. Thus, the effect of GrB on the immune response to LPS remains to be elucidated. The reported differences in survival of GrB-/- mice upon LPS challenge may

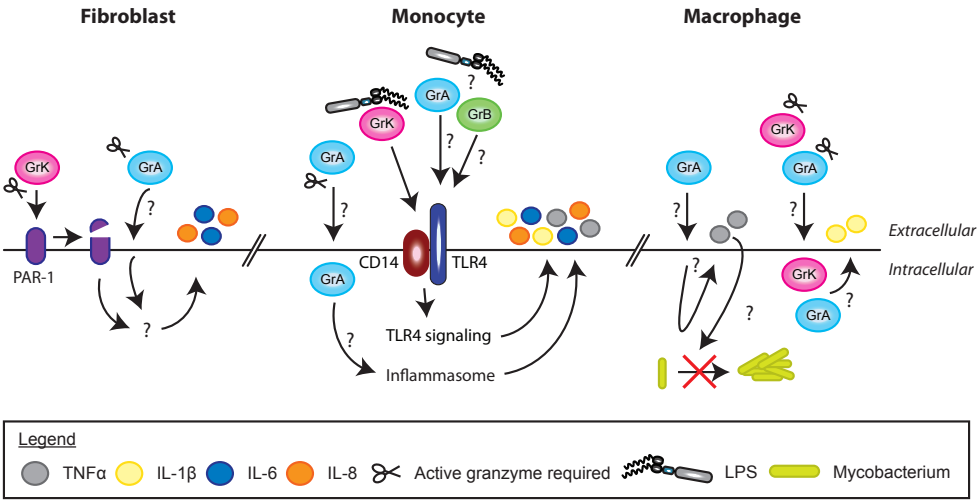

Figure 1.
Overview of effects of granzymes on cytokine secretion by different cell types.

Human or mouse GrA, GrB and GrK induce the release of cytokines from several cell types, including fibroblasts (45, 48, 53), monocytes (11, 46, 48) and macrophages (10, 11, 49). Granzymes cause this release on their own, or potentiate LPS-induced cytokine responses. In some cases, catalytically active granzyme is required for these effects. This cleavage is indicated by scissors. We have reported granzyme functions independent of their catalytic activity (48). Spencer et al. (49) do not report whether GrA activity is required for the observed inhibition of mycobacterial growth.

relate to the age of the mice, the injected amount of LPS per gram of body weight, the source of LPS, or other differences in experimental setup.

Interestingly, Metkar et al. (11) report a worse survival of GrAB double knockout mice compared to GrA or GrB single knockout mice upon LPS challenge (11). Anthony et al. (44), however, found no difference in survival of GrA-/- mice and GrAB-/- mice after LPS injection, consistent with their finding that GrB does not influence survival. GrB-/- and GrAB-/- mice were not directly compared in their experiments (44). The interplay between GrA and GrB in the immune response to LPS is unresolved, and deserves further study. Possibly, different granzymes are preferentially involved in response to different types of LPS.

In vitro evidence that GrB is involved in cytokine release during inflammatory responses is scarce. hGrB itself does not directly induce HeLa and HUVEC cells to produce IL-6 and IL-8 (50). However, we recently demonstrated that hGrB synergistically enhances LPS-induced TNFα release from human monocytes in vitro, but does not induce cytokine release from these cells on its own (48). This function of hGrB is shared with at least hGrK and hGrA (48), a redundancy consistent with a limited effect of mGrB on survival in response to LPS (44). A role for human GrB in the processing of proinflammatory cytokines also has been described. GrB cleaves pro-IL-18 in vitro and ex vivo, at the same position as caspase-1, although with slower kinetics (51, 52). Furthermore, hGrB cleaves the 31 kDa precursor of IL-1α, which enhances the biological activity of the cytokine several fold, an in vitro result that was confirmed in mice (50). Thus, it appears that extracellular GrB is able to cleave and activate several important proinflammatory cytokines. In conclusion, GrB may have more effect on cytokine cleavage and activation than on initial cytokine release.

GRANZYME K

Mice deficient in GrK have not been described yet. Hence, it is currently unknown whether GrK deficiency in mice results in altered responses to LPS challenges. The effects of GrK on different cell types in vitro, however, have been investigated. Like GrA (45), extracellular hGrK releases IL-6 and IL-8 from human lung fibroblasts, dependent on GrK proteolytic activity (48, 53). This effect of GrK involves cleavage of Protease-Activated receptor 1 (PAR-1) (53). Additionally, recombinant mGrK, but not its catalytically inactive pro-form, induces release of IL-1β from peritoneal mouse macrophages (10), an effect also observed for mGrA (11). This effect is seen after priming the cells with LPS, which may indicate an additive or synergistic effect of granzymes and bacterial compounds. Intracellular GrK delivery is required, although extracellular effects are observed at concentrations higher than 600 nM (53). Cytotoxic T

cells isolated from lymphocytic choriomeningitis virus (LCMV)-infected GrA/B-deficient mice, which mainly expressed GrK, also induce IL-1β release from mouse macrophages (10). We have recently shown that extracellular hGrK synergistically potentiates LPS-induced release of the proinflammatory cytokines TNFα, IL-6 and IL-8 in human monocytes in vitro (48). In addition, a combination of GrK and LPS enhances release of TNFα, IL-1β and IL-6 in vivo, compared to LPS alone. This effect is independent of GrK proteolytic activity. GrK liberates individual LPS molecules from micelles and promotes complex formation between LPS and CD14 (48). Taken together, these data indicate that the proinflammatory effects of GrK are diverse and cell-type specific. The pro-inflammatory effects of granzymes A, B, and K on different cell types are summarized in Figure 1.

GRANZYME M

Upon LPS challenge, GrM-/- mice survive longer and produce significantly lower serum levels of the proinflammatory cytokines IL-1α, IL-1β, TNF, and IFNγ, compared to WT mice (44). Perforin knockout partially shows the same phenotype as GrM knockout (44), indicating that granzymes may function intracellularly in the LPS response. However, recombinant mGrM does not cleave pro-IL-1β in vitro, and the molecular role of GrM in promoting responsiveness to an endotoxin challenge remains elusive (44). Consistent with results obtained with single knockout mice, GrM/GrA double knockout mice are even more resistant to LPS than single GrM or GrA knockout mice (44). This additional effect of a double granzyme knockout probably indicates that GrM and GrA function in the immune response to LPS via different mechanisms.

GrM colocalizes with the chemotactic protein Macrophage Inflammatory Protein-1α (MIP-1α) in cytotoxic vesicles of human NK cells (54). GrM knockout leads to impaired MIP-1α secretion from NK cells and macrophages isolated from the liver of mice challenged with LPS or the Gram-positive bacterium *Listeria monocytogenes* (54). Since MIP-1α is important for NK cell recruitment to the liver during infection, the authors propose that GrM regulates this process (54). Similar to the response upon LPS challenge, GrM-/- mice survive longer and exhibit reduced serum levels of cytokines following *L. monocytogenes* infection (54). Interestingly, however, MIP-1α serum levels were not different between WT and GrM-/- serum levels after *L. monocytogenes* infection (54). Apparently, the local effect of GrM knockout on MIP-1α secretion is not reflected systemically. Taken together, GrM appears to be an important regulator of cytokine release in response to infection with Gram-negative as well as Gram-positive bacteria, at least in mice. However, in vitro data explaining the mechanism(s) behind these observations are lacking.

Unresolved questions

WHAT IS THE ORIGIN OF EXTRACELLULAR GRANZYMES?

It is not known how granzymes are released during inflammation. Leakage of granzymes out of the immunological synapse during ongoing cytotoxic lymphocyte responses could explain their extracellular presence. Alternatively, granzymes could be actively secreted during inflammation. GrA and GrB are released upon LPS injection into healthy human volunteers and upon incubation of whole blood cultures with LPS or bacteria (19), suggesting that purposeful degranulation may occur in the absence of cytotoxicity. Alternatively, secretion of granzymes may take place independently from degranulation, since significant amounts of GrA and GrB in cytotoxic lymphocytes are found outside cytolytic granules (55).

The source of extracellular granzymes is also unknown. Cytotoxic lymphocytes may evidently produce granzymes. Anthony et al.(44), observed that NK cell depletion, similar to GrM deletion in WT mice, confers LPS resistance to RAG-1-/- mice. Thus, NK cell-derived mGrM may play an important role in the inflammatory response to LPS (44). However, alternative sources of granzymes also have to be considered. There is ample evidence that both human myeloid dendritic cells (mDCs) and human plasmacytoid DCs (pDCs) express GrB (56-60). Constitutive mRNA and protein expression has been found in pDCs (57, 60), while IL-3, IL-10, and IL-21 upregulate GrB mRNA and protein expression in these cells (57, 58, 60). In mDCs, no constitutive GrB protein expression is reported, but GrB and perforin protein expression can be induced by treatment with TLR7 and TLR8 agonists (59). In contrast, TLR7 and TLR9 agonists inhibit GrB protein expression in pDCs (57-60).

GrB expression by DCs may contribute to their cytotoxic potential, in perforin-dependent as well as -independent ways (61). Human mDCs upregulate perforin protein expression upon stimulation with TLR7 and/or TLR8 ligands, and release perforin together with granzyme B (59). However, no such upregulation is found in pDCs stimulated with TLR7/8 ligands (59) or IL-3/IL-10 (58). GrB-expressing mDCs (59) and pDCs (56, 57, 59) eliminate endothelial cells and tumor cell lines. This cytotoxic activity may provide DCs with extra possibilities to regulate inflammatory processes. Interestingly, human immature DCs eliminate CD8+ T cells via a perforin- and GrA-dependent mechanism (62). Granzyme expression could therefore depend on the developmental stage of the dendritic cell, since mature pDCs do not express GrA (57). Human B cells also express GrB, cells (63-65) and as for DCs, GrB expression is induced by IL-21, albeit in the context of other stimuli, including viral antigens (65). GrB from B cells was shown to inhibit T-cell proliferation via the cleavage of the T cell

receptor ζ chain (63), an extracellular and perforin-independent mechanism also shown for GrB derived from cytotoxic lymphocytes (66). Furthermore, GrB-secreting B cells possess cytotoxic potential in the absence of perforin expression (64), which is thought to contribute to their immunoregulatory potential (66). In contrast, Hagn and coworkers could not detect GrB expression in resting or activated murine B cells (67).

GrB protein expression has also been found in human macrophages (68), basophils (69), mast cells (70) and several other cell types (66). GrB expression in mast cells and basophils was not accompanied by perforin expression (69, 70), indicating perforin-independent and possibly extracellular functions for GrB released by these cells. Further studies are required regarding the cellular origin of granzyme in individual diseases, the regulation of granzyme release and the molecular immunoregulatory mechanisms granzymes may employ.

WHAT ARE THE MOLECULAR MECHANISMS OF GRANZYME-INDUCED CYTOKINE RELEASE?

Another issue to be clarified is whether granzymes stimulate the release of previously synthesized cytokines stored inside the cell, or whether they exert a stimulatory effect on cytokine mRNA transcription and protein synthesis, or both. Not much is known about the pathways that are activated after extracellular granzyme treatment. GrA can be internalized by monocytes in the absence of perforin, and induces cytokine release (11), but the link between these events is unclear. Upon cleavage of PAR-1 by GrK, ERK1/2 and p38MAPK are activated (53), which then leads to cytokine release from fibroblasts. More research is needed to clarify which signaling pathways are activated by granzymes to trigger cytokine responses. When granzyme catalytic activity is required, identification of intra– or extracellular granzyme substrates is essential. In cases where granzymes potentiate pathways induced by TLR ligands independent of the granzyme catalytic activity (48), the focus should lie on clarifying how granzymes modulate these pathways via interaction with signaling molecules. A common characteristic in LPS-enhancing proteins such as LPS-binding protein (71, 72), granzymes (48), azurocidin (73, 74), High Mobility Group Box-1 protein (75, 76), protamines (77) and apolipoprotein C1 (78, 79) is the presence of cationic patches of arginines and/or lysines, that drive the interaction with LPS. Endocytosis may also play a role, since internalization is required for the stimulating effect of azurocidin on LPS-induced cytokine responses (80). Interestingly, GrA binds to and is internalized by monocytes (11).

IS GRANZYME ACTIVITY INHIBITED IN BLOOD?

Another question is whether extracellular circulating granzymes are active, or whether their activity is reduced due to binding by protease inhibitors. In vivo, the catalytic activity of granzymes and other serine proteases is inhibited by serine protease inhibitors or serpins (81). Inhibition occurs through cleavage of the serpin by granzymes, after which a covalent bond is formed between the serpin and the active site of granzyme (81). Enzyme-serpin complexes are subsequently removed from the circulation by the liver. Removal is rapid and often occurs within minutes after complex formation (82, 83).

Several endogenous serpins for human granzymes have been identified. Furthermore, several general protease inhibitors with the potential to inhibit the catalytic activity of granzymes are present in the human circulation. GrA is inhibited by the serpin Kazal (84), and extracellular GrA is inhibited by anti-thrombin III (ATIII) and α2-macroglobulin (α2M) (85). The granzyme is protected from inhibition when it is complexed to proteoglycans (85). GrB is inhibited by Serpin B9, also known as Proteinase Inhibitor 9 (PI-9) (86). PI-9 is thought to operate intracellularly, but is also present in the circulation where it is complexed to GrB (87). Still, GrB activity is retained in the presence of 80% plasma (88), and therefore it is unclear whether PI-9 inhibits GrB activity in blood. GrB catalytic activity is not reduced by α1-antitrypsin (88). The three major extracellular protease inhibitors present in normal lung fluid (α1-antitrypsin, elafin, and Secretory Leukocyte Protease Inhibitor) do not inhibit GrA or GrB, and GrA is active in BALF (20). GrH is inhibited by Serpin B1 (89), and GrM by Serpin B4 (90). Furthermore, GrM is inhibited by α1-antichymotrypsin (ACT) and α1-proteinase inhibitor (α1PI), although weakly (91). GrK catalytic activity is inhibited by inter-α-trypsin inhibitor (IαI) (92). In sepsis patients, circulating amounts of IαI are lower than in healthy controls (15, 93) Also, the molecular weight of GrK in the serum of sepsis patients is lower than in healthy controls. GrK mainly circulates with a molecular weight of ~26 kDa in sepsis patients (15). This finding implies that GrK is freed from protease inhibitors under inflammatory conditions, although it was not determined whether this GrK is proteolytically active.

In summary, inhibition of catalytic activity of granzymes may be reduced under inflammatory conditions, although this subject requires further study. Apart from intracellular inhibition of GrB by PI-9, the physiological relevance of serpins or other protease inhibitors to inhibit granzyme activity remains unknown. Furthermore, it has to be considered that proteolytic activity may not be essential for all extracellular granzyme functions (48). In this regard, steric hindrance by binding of inhibitors or proteoglycans could be of superior importance.

Conclusions

Increasing evidence now demonstrates that granzymes are involved ECM remodeling and modulation of inflammatory pathways. Granzymes may induce cytokine release from cells, and/or cleave cytokines after cytokine release.

Involvement of GrA, GrB, GrK, and GrM in these processes has been demonstrated in humans and/or mice (11, 44, 48-50, 53, 54). Most of these novel granzyme functions are perforin-independent and extracellular, although effects may be enhanced upon intracellular delivery. Furthermore, granzymes have immunomodulatory functions independent of their catalytic activity (48). Taken together, these data indicate that granzymes have multiple regulatory functions in (innate) pro-inflammatory immune responses via many at least partly overlapping pathways (Figure 1). This functional redundancy underlines the significance of granzymes in immunity and ensures proper granzyme function when one of the granzymes or granzyme-induced mechanisms is inhibited. The notion that multiple granzymes influence inflammatory processes is reminiscent of the cytotoxic potential shared by several granzymes, which is regarded as a safeguard against inhibition of one or more granzymes (4).

Several important questions remain to be solved. First, not all granzymes have been studied in the context of inflammation. It is unknown whether GrH and GrM possess direct cytokine-inducing and/or -processing capacity. Second, in many cases the intracellular mechanisms behind the effects of granzymes on cytokine secretion have not been elucidated. Third, it is not understood how granzyme secretion is regulated under inflammatory conditions and from which cell type(s) granzymes originate. Finally, the regulation of granzyme activity in the circulation also deserves further study. Elucidation of all these different aspects of granzyme biology will help us to better understand the implications of granzyme activity in disease. Once clear molecular mechanisms have been defined for granzymes in inflammatory disease, and their physiological relevance has been demonstrated, granzyme inhibition might in the future contribute to treatment of infectious diseases and auto-immune diseases.

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Aim and scope of the thesis

The aim of this thesis is to identify novel immunomodulatory functions of granzymes.

Chapter

1 In this chapter, the current knowledge about extracellular granzymes in inflammation is reviewed.

2 We provide evidence that GrK synergistically potentiates LPS-induced pro-inflammatory cytokine responses in vitro and in vivo, independent of its catalytic activity. GrK binds to LPS, releases individual LPS molecules from micelles and promotes complex formation between LPS and CD14, thereby modulating LPS-induced TLR4 signaling and lowering the threshold for monocyte activation.

3 Here, we demonstrate that GrA, like GrK, synergistically potentiates LPS-induced cytokine responses from human monocytes independent of its catalytic activity. However, GrA does not bind to LPS and does not effectively liberate individual LPS molecules from micelles. Therefore, mechanisms employed by GrK and GrA to augment LPS-induced cytokine responses appear to be different.

4 This chapter describes the release of GrM and, to a lesser extent, GrK, in a model of human experimental endotoxemia. Furthermore, GrM is released in whole blood cultures upon stimulation with LPS or the Gram-negative bacteria *Pseudomonas aeruginosa*, *Neisseria meningitidis* or *Escherichia coli BL21*. GrK release was found only upon treatment with *Pseudomonas aeruginosa*.

5 In Chapter 5 we describe the upregulation of GrM but not GrK in synovial fluid from rheumatoid arthritis patients. GrM and GrK were not upregulated in serum from rheumatoid arthritis. This indicates that GrM is involved in local disease progression in RA.

6 In this chapter we compare the macromolecular substrate specificities of murine and human GrK (mGrK and hGrK). We show that these granzyme homologues have overlapping but not identical substrate specificities, indicating that caution is required when interpreting data obtained with mGrK. Furthermore, our preliminary experiments indicate that both mGrK and hGrK are not cytotoxic towards tumor cells.

7 Here, we provide evidence that reactivation of Human Cytomegalovirus following allogeneic stem cell transplantation is associated with increased percentages of GrM- and GrB-expressing lymphocytes. Furthermore we demonstrate that GrM levels are elevated in the circulation during HCMV reactivation. This indicates that GrM and GrB may be important in controlling HMCV reactivation after stem cell transplantation.

8 Our findings will be summarized and discussed in Chapter 8.

GRANZYME K SYNERGISTICALLY POTENTIATES LPS-INDUCED CYTOKINE RESPONSES IN HUMAN MONOCYTES

Annette C. Wensink[1,2], Vera Kemp[1], Job Fermie[1], M. Isabel García Laorden[3], Tom van der Poll[3], C. Erik Hack[2] and Niels Bovenschen[1,2]

1 Department of Pathology, University Medical Center Utrecht, Utrecht, 3584 CX, The Netherlands

2 Laboratory of Translational Immunology, University Medical Center Utrecht, Utrecht, 3584 CX, The Netherlands

3 Center for Experimental and Molecular Medicine, Amsterdam Medical Center, University of Amsterdam, Amsterdam, 1100 DD, The Netherlands

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Significance Statement

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 pro-inflammatory 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 systemical bacterial infection.

2

Abstract

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 controling 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-Toll-like receptor 4 signaling during the anti-microbial innate immune response.

Introduction

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.

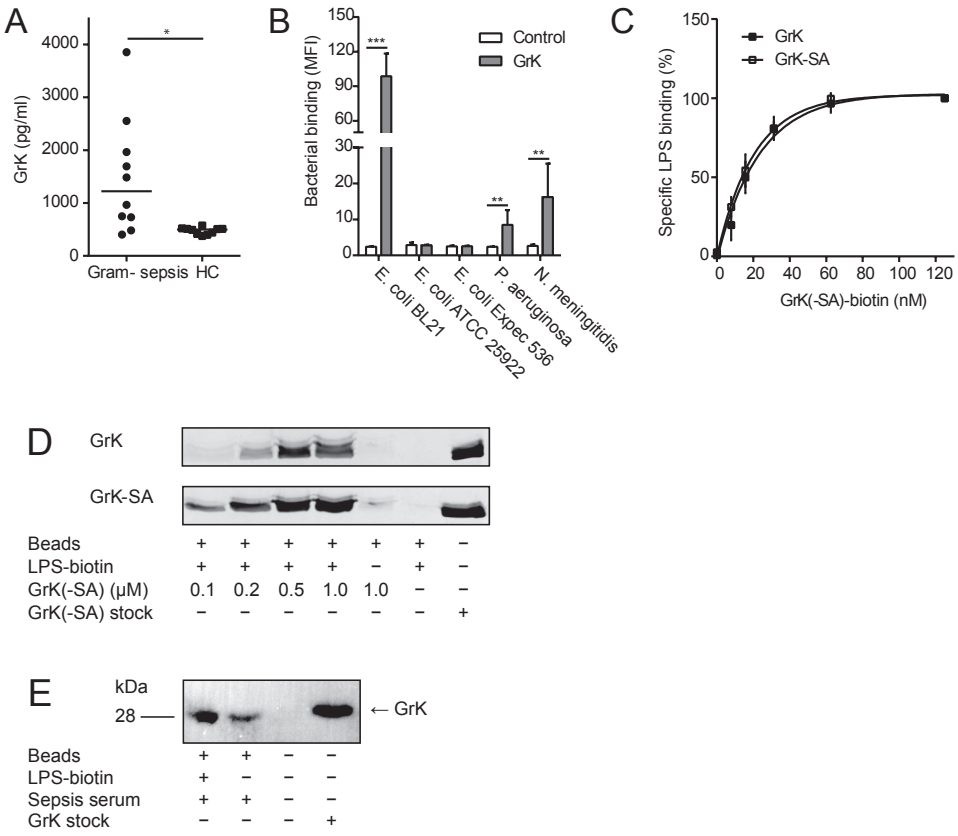
Increasing evidence is emerging that granzymes also exert noncytotoxic extracellular functions during inflammation, including microbial infections. Support for such functions comes from observations that levels of soluble granzymes are elevated in plasma and synovial fluid of rheumatoid arthritis patients (3, 4) and in serum and broncheo-alveolar lavage fluid of patients with bacterial or viral infections (4-8). Furthermore, GrM-/- and GrA-/- mice tolerate a lethal lipopolysaccharide (LPS) challenge better than wildtype (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 pro-inflammatory cytokines by primary monocytes (10-12), and indirectly protects human macrophages from mycobacterial infection by induction of Tumor Necrosis Factor α (TNFα) (13). It also cleaves pro-Interleukin-1β (pro-IL-1β) in vitro (14), while 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 towards 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 though it may contribute to the production of IL-1β, IL-6, and IL-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 pro-inflammatory cytokine release by monocytes both in vitro and in vivo, employing 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 granzyme K are elevated in Gram-negative sepsis Previously, 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 (1224 pg/ml) was significantly elevated compared to the level in serum from healthy donors (n=10, 499.1 pg/ml) (Fig. 1A). These data confirm previous data demonstrating that GrK circulates in Gram-negative sepsis patients.



GRANZYME K BINDS TO GRAM-NEGATIVE BACTERIA

Since 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. GrK bound to *Escherichia coli BL21*, *Pseudomonas aeruginosa*, and *Neisseria meningitidis* with varying intensity, whereas no binding to *E. coli ATCC 25922* and *E. coli Expec 536* was observed under the used experimental conditions (Fig. 1B). 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. 1C). 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 beads, but not uncoated beads, with increasing GrK(-SA) concentrations resulted in a band of increasing intensity migrating at the position of GrK (Fig. 1D). 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. 1E), 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. S1A) and heparin (Fig. S1B), suggesting involvement of electrostatic interactions,

Figure 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 % 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.

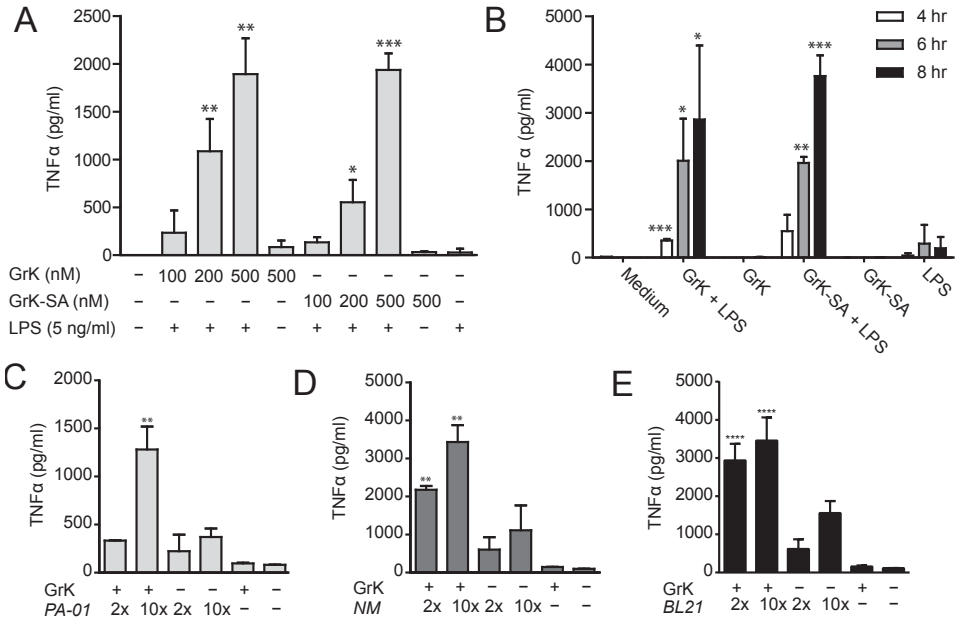


Figure 2.

GrK synergistically potentiates pro-inflammatory TNFα 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 TNFα. 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, as 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 TNFα. 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, as compared with LPS control for the same timepoint). (C-E) Monocytes were incubated with GrK (500 nM) for 6 h with or without *P. aeruginosa* (PA-01) (C), *N. meningitidis HB-1* (NM) (D) or *E. coli BL21 (E),* at 2- or 10-fold excess compared to cell numbers. Supernatants were analyzed for TNFα. 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, as compared with bacteria only).

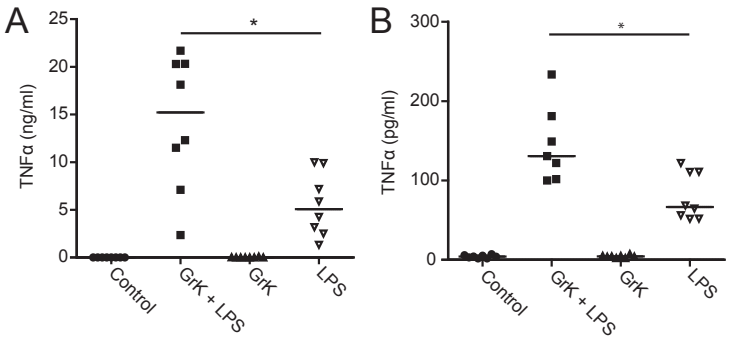


Figure 3.
GrK enhances the LPS-induced TNFα response in vivo.

Wildtype C57BL/6 mice (n=7-8) were injected intraperitoneally (i.p.) with physiological saline, LPS, GrK alone, or GrK with LPS. After 2 (A) or 6 h (B), mice were sacrificed and TNFα levels were measured in plasma. Solid line represents median for each group (*$P < 0.05$, as compared with LPS only, Mann-Whitney U-test).

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, while lipid A was markedly less efficient (Fig. S1C), suggesting that the LPS O-antigen plays a major role in GrK binding. Taken together, these data indicate that GrK binds to the Gram-negative bacterial cell wall component LPS.

GRK SYNERGISTICALLY ENHANCES LPS-INDUCED PRO-INFLAMMATORY CYTOKINE RELEASE FROM MONOCYTES

LPS stimulates TLR4 signaling, leading to pro-inflammatory 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, TNFα production was synergistically enhanced compared to the response to LPS alone (Fig. 2A and B). This synergistic effect of GrK was dose- and time-dependent and was not dependent

on the catalytic activity of GrK, since the GrK-SA mutant showed similar effects (Fig. 2A and B). The effects of GrK(-SA) on LPS-induced TNFα 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 pro-inflammatory cytokines was also enhanced in the presence of GrK. Like for TNFα, GrK and GrK-SA synergistically enhanced LPS-induced IL-6 and IL-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, while no secretion of IL-6 and IL-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 (IFNα) (not detectable) and IFNβ (Fig. S4). Taken together, these results indicate that GrK, independent of its active site, synergistically potentiates the LPS-induced release of pro-inflammatory cytokines from monocytes in the absence of intracellular delivery.

GRK ENHANCES TNFA RELEASE FROM MONOCYTES INDUCED BY GRAM-NEGATIVE BACTERIA

We show that GrK bound to *P. aeruginosa*, *N. meningitidis*, and *E. coli BL21* (Fig. 1B). We therefore investigated the effect of GrK on pro-inflammatory cytokine release from monocytes induced by these Gram-negative bacteria. GrK synergistically potentiated TNFα production caused by all three bacterial species several fold (Fig. 2C-E). Thus, a stimulatory effect of GrK on cytokine production in monocytes is not only observed with LPS, but also with live bacteria.

GRANZYME K SYNERGISTICALLY ENHANCES PRO-INFLAMMATORY LPS-INDUCED TNFα RESPONSE IN MICE

Next, we tested whether the effect of GrK on the pro-inflammatory response to LPS also occurs in vivo. Wild-type C57BL/6 mice were injected intraperitoneally (i.p.) with LPS in the presence or absence of GrK. After 2 h or 6 h, mice were sacrificed and circulating plasma TNFα, IL-6 and IL-1β were measured. Injection of GrK alone did not result in cytokine production. However, TNFα was upregulated upon administration of GrK and LPS together compared to injection of LPS alone, both after 2 h and 6 h (Fig. 3A and B). IL-6 and IL-1β were not elevated upon GrK + LPS injection compared to LPS alone after 2 h (Fig. S5A and C), but were increased after 6 h, although the increase in IL-6 levels was not statistically significant ($P = 0.152$) (Fig. S5B and D). Taken together, these data indicate that GrK synergistically enhances the pro-inflammatory LPS-induced cytokine responses in vivo.

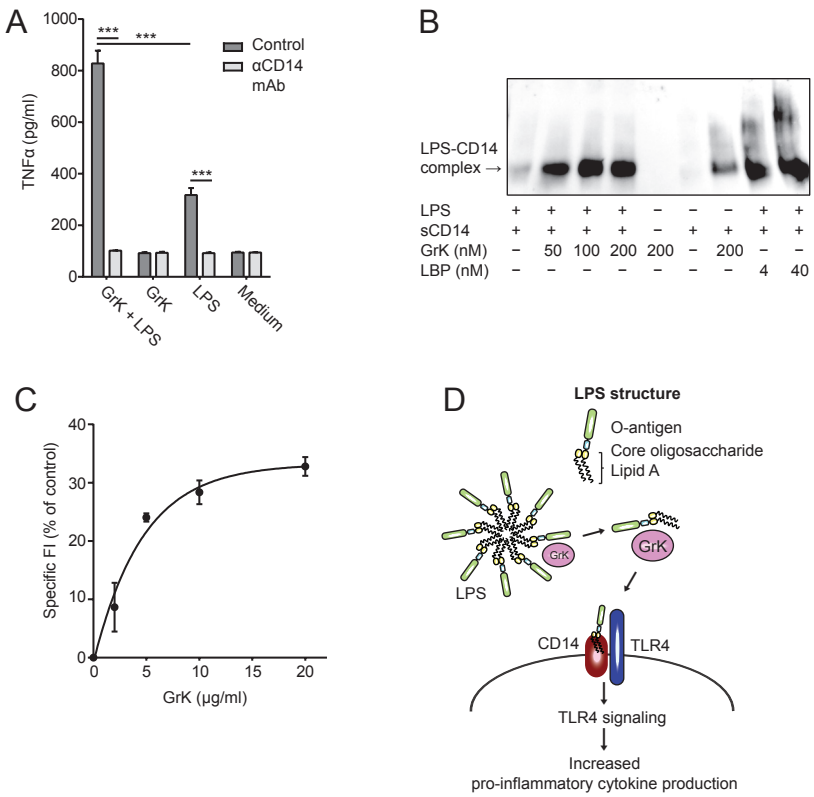


Figure 4.
GrK augments LPS-CD14 complex formation and releases LPS from micelles.

(A) The synergistic effect of GrK and LPS depends on CD14. Human monocytes, pre-treated with or without a neutralising αCD14 monoclonal antibody, were incubated with GrK with or without LPS for 6 h. Supernatants were analyzed for TNFα. 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 pro-inflammatory 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.

THE SYNERGISTIC EFFECT OF GRK AND LPS ON MONOCYTES IS DEPENDENT ON CD14

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, TNFα production was completely inhibited after pre-treatment of human monocytes with a neutralizing αCD14 monoclonal antibody (Fig. 4A). This finding indicates that the synergistic effect of GrK on LPS-induced pro-inflammatory 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. 4B). 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. 4B). 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. 4C). LBP had similar effects, while simultaneous addition of LBP and GrK did not further enhance LPS micelle disaggregation compared to addition of either protein alone (Fig. S6A). Soluble CD14 did not affect GrK- or LBP-mediated LPS micelle disaggregation. Furthermore, LBP competed with GrK for LPS binding in a solid-phase binding assay (Fig. S6B), 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. 4D).

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 pro-inflammatory cytokine response induced by these agents in human monocytes in vitro and in mice (Fig. 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 anti-microbial innate immune response in vivo.

Incubation of monocytes or injection of mice with GrK alone did not induce release of pro-inflammatory 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) of endotoxin, concentrations insufficient to account for the robust synergistic effect of GrK and LPS (Fig. 1-3). Finally, SDS-PAGE analysis revealed that no other proteins were present in our purified granzyme batches (Fig. S7A). These data justify the conclusion that the marked synergistic effect of GrK on the LPS-induced pro-inflammatory 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 pro-inflammatory 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 (600-1000 nM). This study agrees with recent work by Metkar et al. (10) with human GrA, which induces the release of pro-inflammatory 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 IL-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 IL-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, since a catalytically inactive GrK-SA mutant showed similar effects as mature active GrK (Fig. 2). Apparently, granzymes employ 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 to 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 pro-inflammatory cytokine release from monocytes. In this context, it remains unclear why GrK does not bind to all Gram-negative bacteria tested (Fig. 1B). 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 pro-inflammatory cytokine responses, a role that appears similar to the functions of proteins like azurocidin, High Mobility Group protein Box 1, apolipoprotein C1, and LBP, all of which are elevated in plasma during inflammation and play roles in stimulating the LPS-induced pro-inflammatory 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 (Figure S6B).

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 pro-inflammatory 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 pro-inflammatory response in sepsis may be dampened by interfering with the GrK-LPS interaction, for example 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 pro-inflammatory 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 than GrK also interact with LPS and contribute to disease progression 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 pro-inflammatory cytokine release. Disease modulation by targeting granzymes therefore may provide new perspectives for future treatment of acute sepsis.

Material 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 (SIRS) 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 Ser195 has been replaced by Ala) were produced as described previously (48, 49). GrK and GrK-SA were purified by cation exchange chromatography followed by affinity chromatography with Prot A/G beads (Thermo Fisher Scientific, Waltham, USA). Purified granzyme was dialyzed against 20 mM Tris, 150 mM NaCl, pH 7.4. Protein concentrations were measured using a Bradford (BioRad) or Bicinchoninic assay (Thermo Scientific). On SDS-PAGE, purified GrK migrated as a single band of ~25 kD, consistent with monomeric GrK(-SA) (Fig. S7A). GrK, but not GrK-SA, cleaved the synthetic chromogenic substrate Ac-Lys-pNA as well as the known macromolecular substrate SET (21, 48) (Fig. S7B 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 using the Biotin Protein Labeling Kit (Roche) according to the manufacturer's protocol. GrK levels in serum samples from sepsis patients were measured using ELISA (Uscn Life Science Inc., lower detection limit ~3 pg/ml).

BINDING OF GRK TO BACTERIA.

Bacteria were diluted in PBS to OD ~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 2 times in PBS with 1% BSA (wt/vol), and incubated with 1 µg/ml Streptavidin-Phycoerythrin (Southern Biotech) in PBS with 1% BSA (wt/vol) for 30-60 min at 4∞C. Finally, bacteria were washed once with PBS with 1% BSA (wt/vol) 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% (v/v) Tween-20 at 37∞C for 2 h. Bound granzymes were visualized using Streptavidin-polyHRP (Sanquin) followed by 3,3',5,5'-Tetramethylbenzidine (Invitrogen). After adding 1M H2SO4 the OD450 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.

PRO-INFLAMMATORY CYTOKINE RESPONSE IN ISOLATED HUMAN MONOCYTES.

Monocytes (0.5x105/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/well water-soluble tetrazolium-1 reagent was added and OD450 was measured for 90 min. In experiments with bacteria, LPS was replaced with a 2- or 10-fold excess of bacteria compared to the number of monocytes/well. In CD14-neutralising experiments, monocytes were pre-treated for 30 min at 37°C with αCD14 mAb (10 µg/ml) or serumfree medium alone. TNFα, IL-6, and IL-8 levels in culture supernatants were measured using a multiplex assay as described previously (50) on a Luminex FlexMap 3D (BioRad) with xPonent 4.1 software. Data were analyzed using BioPlex Manager 6.1.1 (BioRad). Alternatively, TNFα was measured using ELISA (PeliKine human TNFα ELISA kit, Sanquin).

PRO-INFLAMMATORY CYTOKINE RESPONSE IN MICE.

Female C57BL/6 mice (Charles River, n=7-8 per group), matched in age (10 weeks) and weight, were injected intraperitoneally (i.p.) with LPS (*E. coli O111:B4*, 10 µg/g) with or without human recombinant GrK (500 nM) in physiological saline. After 2 h or 6 h blood was drawn and mice were sacrificed. Blood was centrifuged at 2000 g for 10 min at 4°C and plasma was stored at -80°C. Plasma cytokines were measured using a mouse multiplex assay (BioRad) on a Luminex FlexMap 3D (BioRad) with xPonent 4.2 software. Data were analyzed using BioPlex Manager 6.1.1 (BioRad).

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, The Netherlands.

LPS-CD14 COMPLEX FORMATION.

LPS (1 µg/ml) was incubated with human recombinant CD14 (100 nM) with or without GrK (0-200 nM) or LPS-binding protein (LBP) (4 or 40 nM) in 20 mM Tris, 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 PBS. LPS-BODIPY-FL alone served as a negative control and LPS-BODIPY-FL plus LBP (10 µg/ml) as a positive control. Disaggregation of LPS micelles upon treatment of LPS-BODIPY with 2% SDS (vol/vol) was set at 100%. Fluorescent intensity was measured kinetically for 2 h at 37°C at 520 nm using the FluoStar Omega (BMG Labtech). Data are represented as the percentage of specific Fluorescent Intensity (FI) compared to SDS control.

STATISTICAL ANALYSIS.

Unless indicated otherwise, data are depicted as mean values ± SD and statistical analyses were performed using the independent samples t-test. Two-tailed p-values below 0.05 were considered statistically significant.

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AUTHOR CONTRIBUTIONS

A.C.W., M.I.G.L., T.v.d.P, C.E.H. and N.B. designed research; A.C.W., V.K., J.F. and M.I.G.L performed research; T.v.d.P. contributed new reagents/analytic tools; A.C.W., C.E.H. and N.B. analyzed data; and A.C.W., C.E.H. and N.B. wrote the paper.

The authors declare no conflict of interest.

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SUPPLEMENTARY INFORMATION

Material and methods

REAGENTS.

Cell culture reagents were from Gibco (Life Technologies), human AB serum was from Invitrogen (Life Technologies), and Cell Proliferation Reagent water-soluble tetrazolium-1 (WST-1) was from Roche Applied Science. All compounds for yeast culture were from Becton, Dickinson and Company. Synthetic chromogen substrate (Ac-Lys-pNA) for GrK was from Bachem. Lipopolysaccharide (LPS) (*Escherichia coli 0111:B4*), heparin, Lipid A (diphosphoryl, from Salmonella minnesota, Re595 mutant), delipidated LPS, and LPS coupled to boron-dipyrromethene difluoride (LPS-BODIPY FL) were from Sigma-Aldrich. Biotin-cownjugated LPS (*E. coli 0111:B4*) was from Invivogen. Polyclonal antibody to human nucleosome assembly protein SET was from Alexis Biochemicals (Enzo Life Sciences). Monoclonal antibody to human Cluster of Differentiation 14 (CD14), recombinant soluble CD14 and LPS-binding protein (LBP) were from R&D Systems. All secondary antibodies were from Jackson Immunoresearch. All bacterial strains were kind gifts from the department of Medical Microbiology (UMC Utrecht).

SDS-PAGE, NATIVE PAGE, AND IMMUNOBLOTTING.

Proteins were separated on a 10% or 12% SDS-PAGE gel. For native PAGE, proteins were separated on a 4-20% polyacrylamide gradient gel (BioRad) without SDS or DTT. Total protein staining was performed with Instant Blue (Expedeon). For immunoblotting, proteins were transferred to a PVDF membrane, stained with antibody, and visualized by ECL (GE Healthcare). Imaging was performed on the ChemiDoc Molecular Imager (BioRad).

MONONUCLEAR CELL ISOLATION.

Peripheral blood mononuclear cells (PBMCs) were isolated from human donor blood as described (1). Briefly, fresh blood from healthy volunteers was subjected to Ficoll-Paque Plus (GE Healthcare) density centrifugation. The mononuclear cell fraction was washed three times with RPMI 1640 containing 5% (vol/vol) fetal calf serum (FCS) (Gibco) and 0.2% bicarbonate (wt/vol). PBMCs (5 x 105 cells/well) were incubated for 2 h in RPMI 1640 containing 5% (vol/vol) AB serum, 2 mM L-glutamine, bicarbonate, penicillin and streptomycin (P/S), after which non-adherent cells were removed. Adherent cells were cultured in RPMI 1640 with 2% (vol/vol) AB serum and bicarbonate.

ACTIVATION OF TRIF-RELATED ADAPTOR MOLECULE/TIR-DOMAIN-CONTAINING ADAPTER INDUCING INTERFERON-B PATHWAY IN ISOLATED HUMAN MONOCYTES BY GRK AND LPS.

Monocytes (0.5x105/well) were incubated with GrK (0-500 nM) with or without LPS (5 ng/ml) for 6 h. Supernatants were stored at -20∞C. Tumor Necrosis Factor α (TNFα) levels in culture supernatants were measured using ELISA (PeliKine human TNFα ELISA kit, Sanquin). Interferon α (IFNα) and IFNβ levels were measured using a multiplex assay as described previously (2) on a Luminex FlexMap 3D (BioRad) with xPonent 4.2 software. Data were analyzed using BioPlex Manager 6.1.1 (BioRad).

EFFECT OF GRK ON HUMAN LUNG FIBROBLASTS.

The MRC-5 human lung fibroblast cell line was cultured in DMEM + 10% FCS. One day prior to experiments, cells were seeded at 20,000 cells/well in a 48-well format. Cells were treated for 6 or 24 h with GrK(-SA) in serum-free DMEM. Culture supernatants were stored at -20°C and subsequently analyzed for Interleukin 6 (IL-6) and IL-8 using ELISA (CytoSET, Invitrogen).

TRIGLYCERIDE MEASUREMENT.

The triglyceride content of two different GrK batches was determined using an enzymatic colorimetric assay on the Beckman-Coulter AU5811 (Beckman-Coulter).

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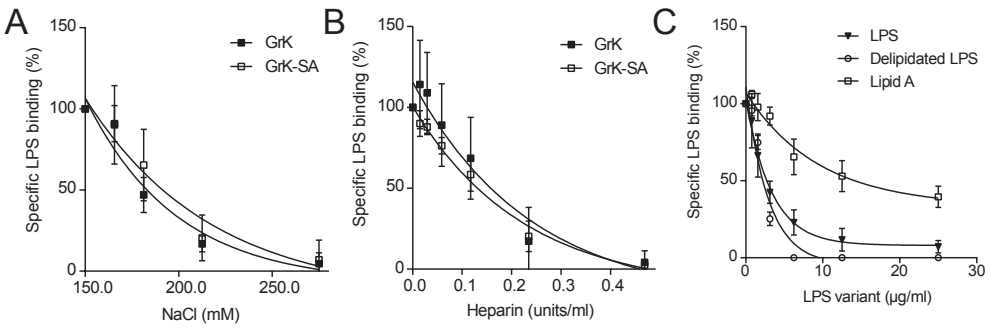

Figure S1.
Binding of GrK to LPS is abrogated by NaCl, heparin and LPS fragments.

(A) GrK-LPS binding is abrogated by NaCl. Immobilized LPS was incubated with biotinylated GrK or GrK-SA with or without NaCl. Data are depicted as specific binding (expressed as % of binding in 150 mM NaCl) and represent mean ± SD of three independent experiments. (B) GrK-LPS binding is abrogated by heparin. Immobilized LPS was incubated with biotinylated GrK or GrK-SA with or without heparin. Data are depicted as specific binding (expressed as % of binding without heparin) and represent mean ± SD of three independent experiments. (C) GrK-LPS binding is abrogated by LPS fragments. Immobilized LPS was incubated with GrK-biotin with or without LPS, Lipid A, or delipidated LPS. Data are corrected for aspecific binding (<5% of total binding) and expressed as % of LPS binding to GrK alone. Data represent mean ± SD of three independent experiments.

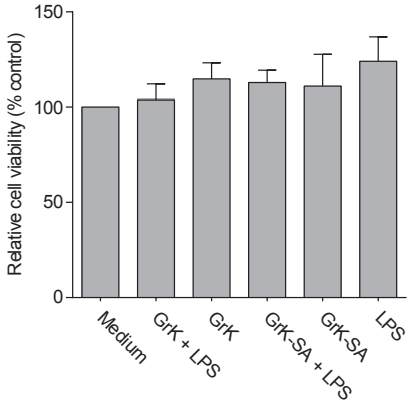

Figure S2.
GrK is not cytotoxic to monocytes.

Extracellular GrK(-SA) is not cytotoxic to monocytes. Human monocytes were incubated for 8 h with the indicated treatments. Relative cell viability was subsequently measured by WST-1 assay. Data (expressed as % of medium control) are depicted as mean ± SD and are representative of at least three independent experiments with normal donors.

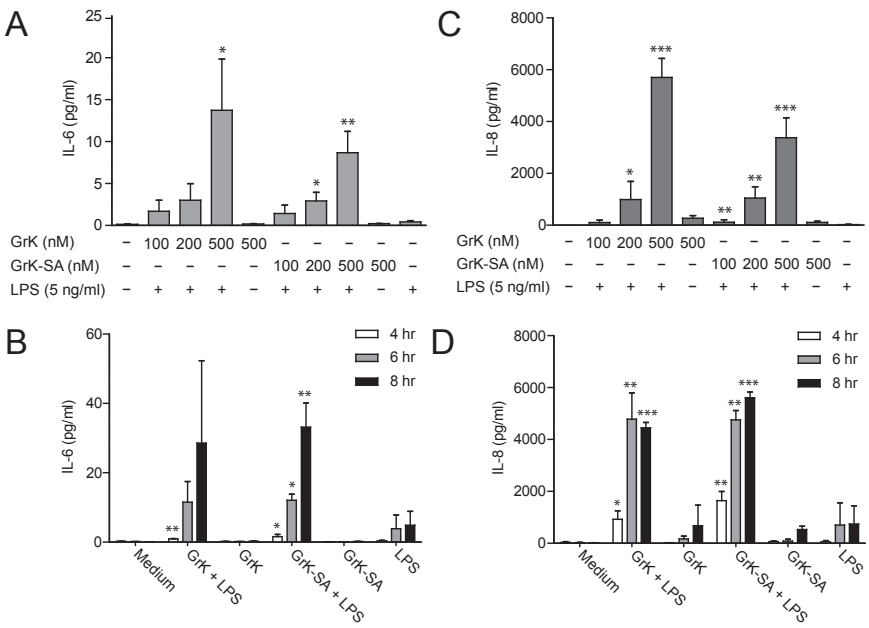

Figure S3.

GrK synergistically potentiates the LPS-induced pro-inflammatory cytokine release from human monocytes.

(A, C) Human monocytes were incubated with the indicated concentrations of isolated GrK or GrK-SA with or without LPS for 6 h. Culture supernatants were analyzed for IL-6 (A), or IL-8 (C). 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, as compared with LPS control). (B, D) Human monocytes were incubated with GrK or GrK-SA (200 nM) with or without LPS for the indicated timeframes. Supernatants were analyzed for IL-6 (B) or IL-8 (D). 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, as compared with LPS control for the same timepoint).

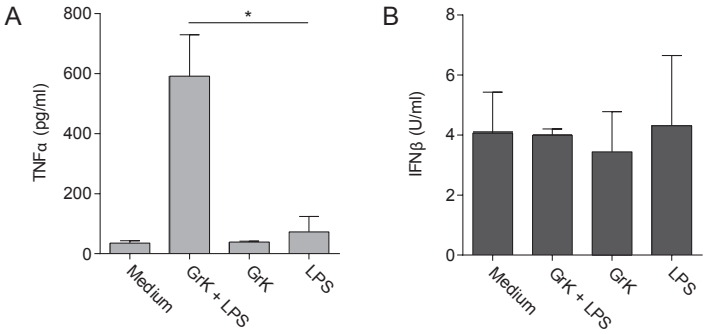

Figure S4.
GrK does not potentiate the MyD88-independent LPS-induced cytokine production in primary human monocytes.

Primary human monocytes were treated with GrK (500 nM) in the presence of absence of LPS (5 ng/ml) for 6 h. TNFα (A) and IFNβ (B) release into the culture supernatant were measured. IFNα could not be detected. Data (mean ± SD, n=3 per treatment) are representative of two independent experiments (*P < 0.05 as compared to LPS control for the same time point).

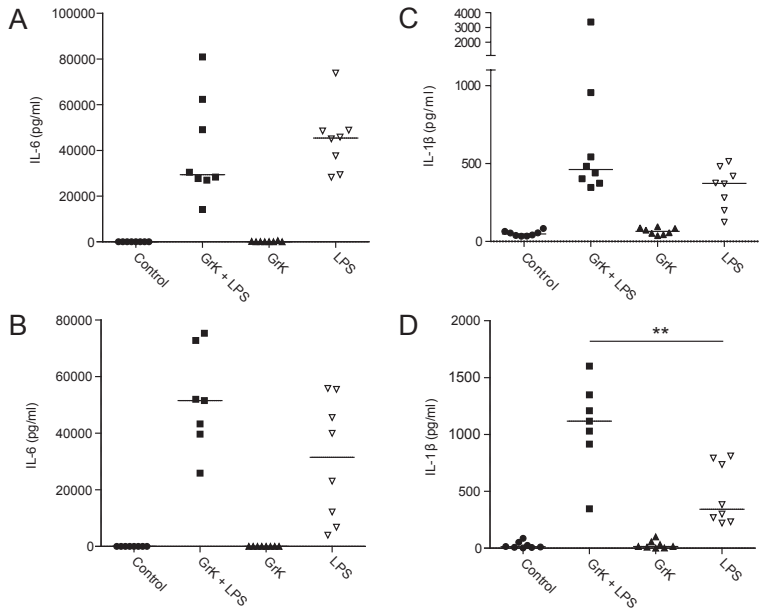

Figure S5.
GrK enhances the LPS-induced cytokine responses in vivo.

Wildtype C57BL/6 mice (n=7-8) were injected intraperitoneally (i.p.) with physiological saline, LPS, GrK alone, or GrK with LPS. After 2 (A, C) or 6 h (B, D), mice were sacrificed and IL-6 and IL-1β levels were measured in plasma (A and B, IL-6; C and D, IL-1β). Solid line represents median for each group (**P < 0.01, as compared with LPS only, Mann-Whitney U-test).

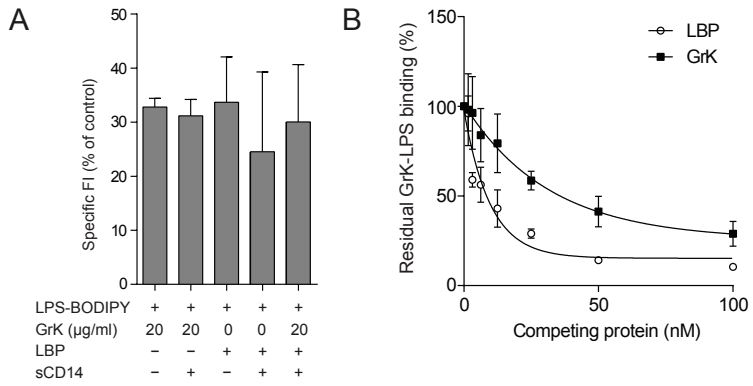

Figure S6.
GrK and LPB do not synergize to promote LPS micelle disaggregation

(A) LBP and GrK do not collaborate to induce LPS micelle disaggregation. LPS-BODIPY was incubated with GrK or LBP or the two proteins together. The increase in mean FI was measured and data are depicted as described in Fig 4C. Data represent mean ± SD (n=2-6). (B) GrK-LPS binding is abrogated by LBP. Immobilized LPS was incubated with 10 nM biotinylated GrK with or without unlabeled GrK or LBP. Data are depicted as specific binding (expressed as % of maximal binding) and are representative of two independent experiments.

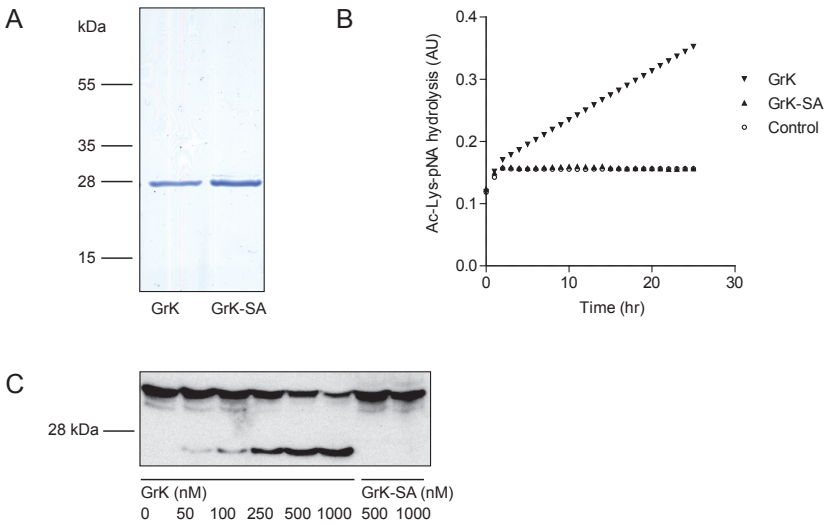

Figure S7.
GrK is pure and active.

(A) SDS-PAGE analysis of purified GrK and GrK-SA batches. Figure shows a representative total protein staining of a GrK (left) and GrK-SA (right) batch. (B) GrK, but not GrK-SA, hydrolyzes the chromogenic substrate Ac-Lys-pNA. GrK(-SA) (2 μM) was incubated with Ac-Lys-pNA (2 mM) and the OD405 was measured kinetically for 25 h. AU, arbitrary units. (C) GrK, but not GrK-SA, hydrolyzes the macromolecular substrate SET. GrK(-SA) was incubated with Jurkat cell lysate for 2 h; samples were blotted for SET.

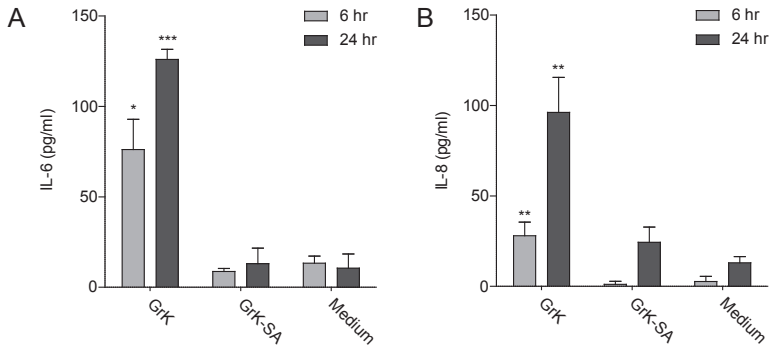

Figure S8.
GrK induces IL-6 and IL-8 release from human lung fibroblasts, dependent on its catalytic activity.

MRC-5 human lung fibroblast cells were treated with GrK(-SA) (500 nM) for 6 h. IL-6 (A) and IL-8 (B) release into the culture supernatant were measured using ELISA. Data (mean ± SD, n=3 per treatment) are representative of three independent experiments (*P < 0.05; **P < 0.01; ***P < 0.001, as compared with GrK-SA control for the same time point).

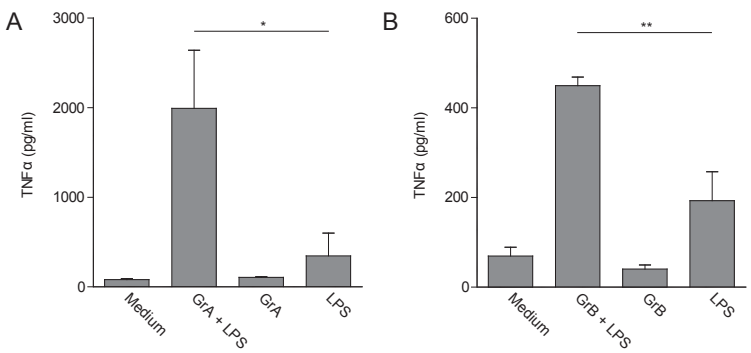

Figure S9.
GrA and GrB synergistically potentiate the LPS-induced TNFα response in primary human monocytes.

Primary human monocytes were treated for 6 h with 500 nM GrA (A) or GrB (B) in the presence or absence of 5 ng/ml LPS. TNFα levels in the culture supernatants were analyzed using ELISA. Data (mean ± SD, n=3 per treatment) are representative of three independent experiments (*P < 0.05; **P < 0.01, compared to LPS control).

GRANZYMES A AND K DIFFERENTIALLY POTENTIATE LPS-INDUCED CYTOKINE RESPONSES FROM MONOCYTES

Annette C. Wensink[1,2], Job Fermie[1], C. Erik Hack[2] and Niels Bovenschen[1,2]

1 Department of Pathology, University Medical Center Utrecht, Utrecht, 3584 CX, The Netherlands

2 Laboratory of Translational Immunology, University Medical Center Utrecht, Utrecht, 3584 CX, The Netherlands

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Abstract

Granzymes are serine proteases that upon release from cytotoxic cells induce apoptosis in tumor cells and virally infected cells. In addition, a role of granzymes in inflammation is emerging. Recently, we have demonstrated that granzyme K (GrK) potentiates lipopolysaccharide (LPS)-induced cytokine responses from monocytes. GrK interacts with LPS, disaggregates LPS micelles, and stimulates LPS-CD14 binding and Toll-like receptor 4 signaling. Here, we show that human granzyme A (GrA) also potentiates cytokine responses in human monocytes initiated by LPS or Gram-negative bacteria. Like for GrK, this effect is independent of GrA catalytic activity, and GrA binds to certain Gram-negative bacterial strains. Unlike GrK, GrA does not bind to LPS, and has little influence on LPS micelle disaggregation. We conclude that GrA and GrK differentially modulate LPS-Toll-like receptor 4 signaling in monocytes, suggesting functional redundancy amongst granzymes in the anti-bacterial innate immune response.

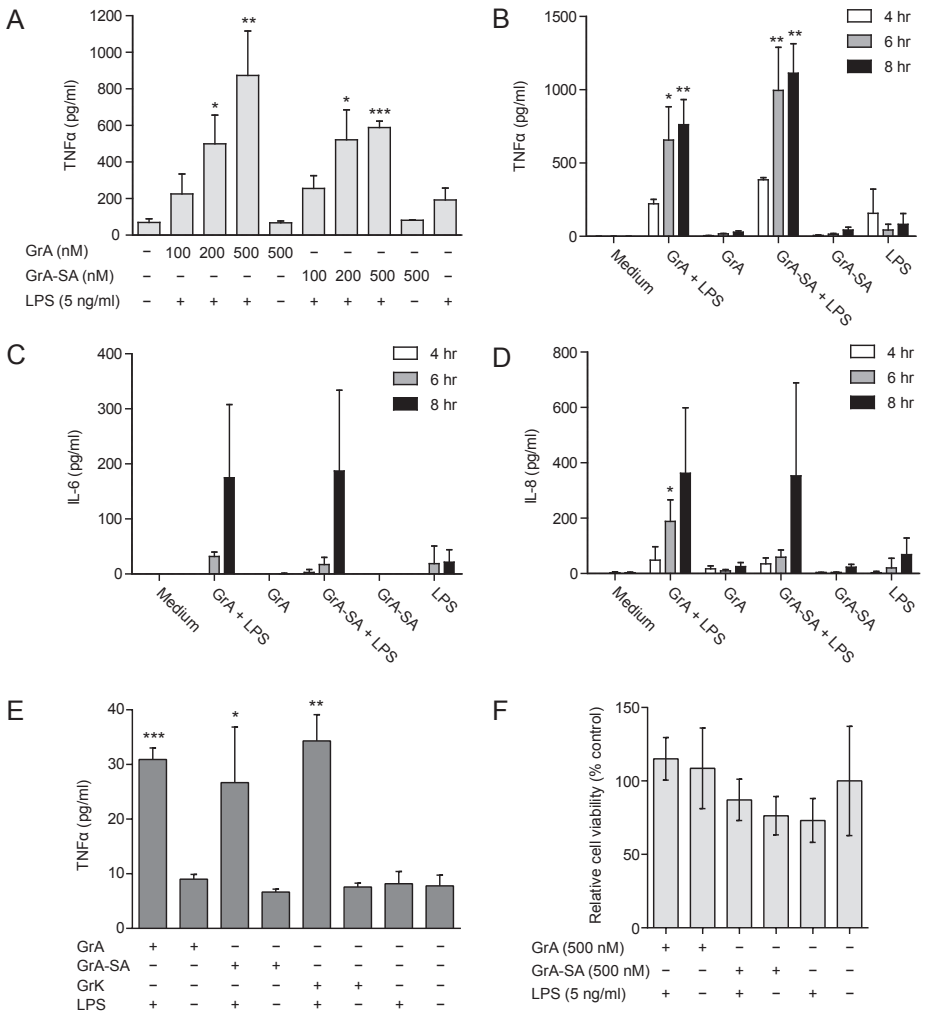
Introduction

Granzymes are a family of structurally related serine proteases best known for their ability to induce apoptosis in tumor cells or virus-infected cells (1). There are five human granzymes: granzyme A (GrA), GrB, GrH, GrK, and GrM. Granzymes are stored in granules in cytotoxic lymphocytes (including cytotoxic T lymphocytes, Natural Killer (NK) cells, NKT cells, and γδ T cells) and are released into the immunological synapse upon recognition of a target cell by a cytotoxic lymphocyte. They subsequently enter the target cell with the aid of the pore-forming protein perforin, and cleave various intracellular substrates that prompt the target cell to undergo apoptosis (2, 3). However, the cytotoxic potential of some granzymes has been debated (4-6), and evidence suggests that granzymes fulfill additional extracellular functions. Increased levels of soluble granzymes are found in plasma and synovial fluid of rheumatoid arthritis patients (7, 8) and in serum and broncheo-alveolar lavage fluid of patients suffering from bacterial or viral infections (8-12). Although the functional consequences of this granzyme release remain incompletely understood, granzymes have been implicated in cytokine release or processing (5, 6, 13-17). This suggests that granzymes are involved in the inflammatory response to infections.

Compared to healthy controls, levels of soluble GrA are elevated in serum from sepsis patients (7, 8, 10, 18), and lipopolysaccharide (LPS) injection into healthy volunteers results in GrA release (11, 19). Increased intracellular GrA levels in cytotoxic lymphocytes correlate with disease severity in sepsis patients (20), and GrA-/- mice are more resistant to LPS challenges than WT mice (6). GrA releases the inflammatory cytokines IL-6, IL-8, IL-1β, and TNFα from human monocytes (6, 21), IL-6 and IL-8 from fibroblasts (22), and IL-8 from A549 epithelial cells (23). Furthermore, GrA induces human macrophages to produce TNFα, which indirectly protects them from mycobacterial infection (24). Collectively, these data suggest that GrA plays a role in disease progression of infections and sepsis.

Recently, we have demonstrated that extracellular GrK potentiates LPS-induced release of inflammatory cytokines from monocytes, and that this effect is independent of the catalytic activity of GrK (13). We showed that GrK binds to LPS and releases single LPS molecules from LPS micelles, thereby lowering the threshold for monocyte activation (13). In the present study, we addressed the question whether GrA mediates similar effects. We show that GrA alone does not induce cytokine release from human primary monocytes. Like GrK, GrA potentiates cytokine responses induced by LPS, independent of its catalytic activity. In

contrast to GrK, GrA does not bind LPS to and does not efficiently remove LPS molecules from micelles. Apparently, granzymes employ different mechanisms to augment LPS-induced cytokine responses from monocytes. This points to functional redundancy amongst granzymes in the anti-bacterial innate immune response.



Results

GRA ENHANCES LPS-INDUCED CYTOKINE RESPONSES FROM MONOCYTES

We have recently demonstrated that GrK potentiates LPS-induced cytokine responses from primary human monocytes, independent of its catalytic activity (13). To investigate whether GrA has a similar effect, GrA or its catalytically inactive mutant (GrA-SA) was incubated with monocytes in presence or absence of a suboptimal stimulatory dose of LPS. Treatment of monocytes with extracellular GrA alone for 0-8 hr did not result in cytokine release (Figure 1). However, incubation of monocytes with GrA in combination with a suboptimal stimulatory dose of LPS enhanced TNFα release compared to the response to LPS alone in a dose- and time-dependent manner (Figure 1A and B). This effect was independent of GrA catalytic activity, since treatment of monocytes with catalytically inactive GrA-SA showed similar effects as wild-type GrA (Figure 1A and B). IL-6 and IL-8 release were also enhanced in response to combined treatment with GrA and LPS, compared to LPS control (Figure 1C and D). Again, cytokine secretion was independent of GrA catalytic activity, and treatment of monocytes with granzyme alone did not result in substantial IL-6 or IL-8 release (Figure 1C and D). Moreover, GrA had comparable potency to enhance LPS-induced TNFα release as GrK (Figure 1E). Treatment of monocytes with GrA alone did not induce cell death (Figure 1F). We conclude that GrA, like GrK, enhances LPS-induced cytokine responses in human monocytes.

Figure 1.
GrA enhances the LPS-induced pro-inflammatory cytokine release from monocytes.

A) Human monocytes were incubated with increasing concentrations of GrA(-SA) with or without LPS (5 ng/ml) for 6 h. TNFα levels in the culture supernatants were determined. Data are expressed as mean ± SD and are representative of at least three independent experiments with normal donors (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, compared to LPS control). B, C, D) Human monocytes were treated with GrA(-SA) (400 nM) with or without LPS (2.5 ng/ml) for 4, 6 or 8 h. Cytokines TNFα (B), IL-6 (C), and IL-8 (D) were detected in the culture supernatants. Data are expressed as mean ± SD and are repres entative of at least three independent experiments with normal donors (*$p < 0.05$; **$p < 0.01$, as compared with LPS control for the same time point). E) The magnitude of the GrA synergistic effect is similar to that of GrK. Human monocytes were incubated with GrA(-SA) or GrK (400 nM) with or without LPS (2.5 ng/ml) for 6 h, after which TNFα levels in the culture supernatants were measured. Data are expressed as mean ± SD and are representative of six independent experiments with normal donors (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, compared to LPS control). F) GrA is not cytotoxic to human monocytes. Human monocytes were incubated with GrA(-SA) (500 nM) for 0-6 hr. Relative cell viability was subsequently determined in a WST-1 assay. Data (n=3 per treatment) are depicted as mean ± SD (% of medium control) and are representative of at least two independent experiments with normal donors.

THE EFFECT OF GRA ON LPS-INDUCED CYTOKINE PRODUCTION DEPENDS ON CD14

LPS activates monocytes via binding to CD14 and subsequent transferal to TLR4 (25), and mice lacking CD14 are highly insensitive to LPS (26). If potentiation of LPS-induced cytokine release by GrA is entirely mediated via CD14, neutralization of CD14 would abolish this effect. To examine this, monocytes were pre-incubated with or without a neutralizing CD14 antibody or isotype control and then treated with GrA with or without LPS. Pre-incubation with αCD14 antibody completely abolished the synergistic effect of GrA and LPS (Figure 2), while the isotype control had no effect. Thus potentiation of LPS-induced cytokine responses by GrA is dependent on CD14-TLR4 signaling.

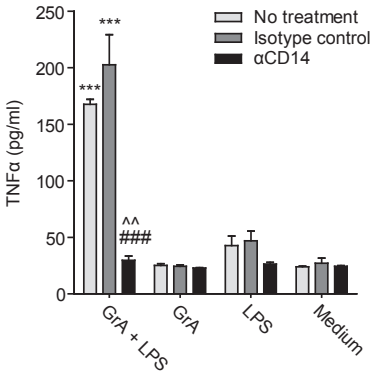


Figure 2.
The synergistic effect of GrA on the LPS-induced TNFα release is dependent on CD14.

Human monocytes were treated with GrA (500 nM) with or without LPS (5 ng/ml) for 6 h in serum-free medium. Cells were pretreated with a neutralizing αCD14 antibody, an isotype control or serum-free medium alone (*** p <0.001, compared to LPS control; ## p <0.001, compared to GrA + LPS without antibody pre-treatment; ^^ p <0.01, compared to GrA + LPS with isotype pre-treatment). Data (n=3 per treatment) are depicted as mean ± SD and are representative of three independent experiments with normal donors.a

GRA ENHANCES TNFA RELEASE INDUCED BY GRAM-NEGATIVE BACTERIA

LPS is an important constituent of the Gram-negative bacterial cell wall. We therefore wondered whether treatment of monocytes with GrA and live Gram-negative bacteria also potentiates cytokine release, compared to treatment with bacteria alone. Monocytes were treated with GrA alone, Gram-negative bacteria (*E. coli BL21*, *Pseudomonas aeruginosa* or *Neisseria meningitidis*) alone, or GrA combined with bacteria. As expected, treatment with only GrA failed to induce cytokine release (Figure 2). Treatment with bacteria resulted in a clear cytokine release, which was enhanced several fold when GrA was added (Figure 2A-C). This demonstrates that GrA also augments cytokine release generated by live Gram-negative bacteria.

GRA BINDS TO GRAM-NEGATIVE BACTERIA

GrK binds to several Gram-negative bacteria (13). To determine whether GrA also binds to Gram-negative bacteria, several bacteria were incubated with or without biotinylated GrA and binding was measured by flow cytometry. GrA bound to *E. coli BL21*, *Pseudomonas aeruginosa* (PA-01) and *Neisseria meningitidis* (NM) (Figure 3A), the same Gram-negative bacteria that are bound by GrK (13). Like GrK (13), GrA did not bind to *E. coli ATCC 25922* or *E. coli Expec 536* (Figure 3A). For some bacterial strains (PA-01 and NM), two distinct populations of bacteria were observed, one that bound GrA and one that did not. This possibly reflects heterogeneity amongst these bacteria. These data indicate that GrA binds to some Gram-negative bacteria.

GRA DOES NOT BIND TO LPS

We have shown that GrK binds to LPS, and that this binding contributes to the synergistic effect of GrK on the LPS-induced cytokine release from monocytes (13). Considering that GrA binds to Gram-negative bacteria and also enhances LPS-induced cytokine responses in monocytes, we investigated whether this granzyme also binds to LPS. First, biotinylated GrA(-SA) was incubated on immobilized LPS in a solid phase binding assay and specific binding was determined, using GrK as a positive control. In marked contrast with GrK, GrA

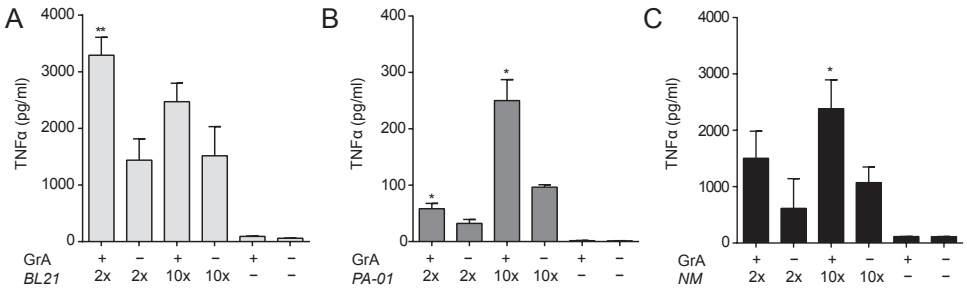


Figure 3.
GrA enhances TNFα production by human monocytes induced by Gram-negative bacteria.

Monocytes were incubated with GrA (500 nM) for 6 h with or without *E. coli BL21* (A), *P. aeruginosa* (PA-01) (B), or *N. meningitidis HB-1* (NM) (C) at 2- or 10-fold excess compared to cell numbers. Supernatants were analyzed for TNFα. Data are depicted as mean ± SD (n=3 per donor) and are representative of at least three independent experiments with normal donors (**p < 0.01; **p < 0.01, compared with bacteria only).

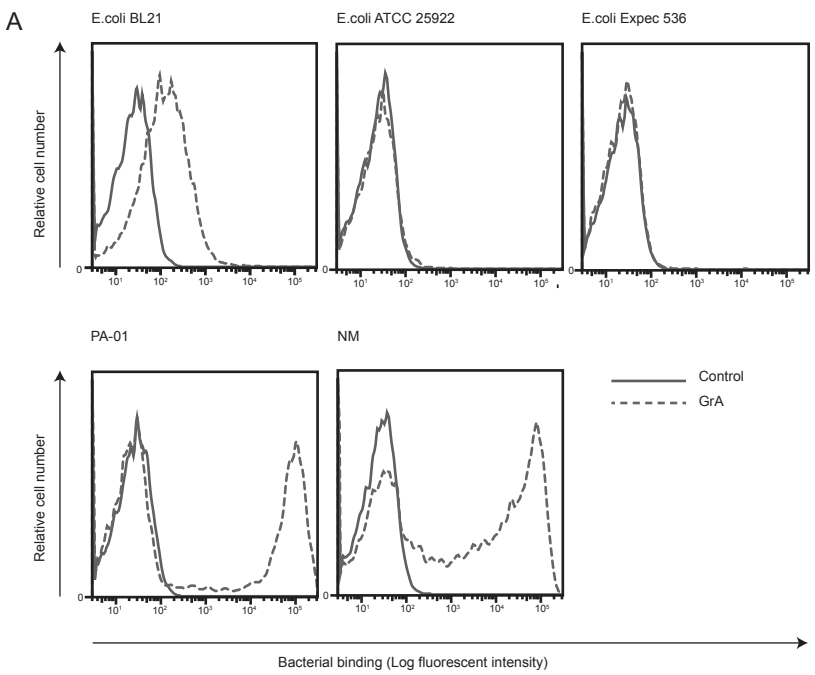
and GrA-SA did not bind to LPS (Figure 3B). Second, a pull-down assay was performed in which biotinylated LPS was coupled to streptavidin-coated beads that were incubated with GrA, GrA-SA, or GrK as a positive control. Bound protein was analyzed by SDS-PAGE. In contrast to GrK, which efficiently bound LPS, binding of GrA or GrA-SA to LPS-coated beads was barely observed (Figure 3C). Finally, surface plasmon resonance experiments were performed, using immobilized GrA and GrK to which LPS was applied. Whereas LPS bound to GrK in a dose-dependent manner (Figure 3D), binding of LPS to immobilized GrA was hardly detectable even at the highest LPS concentration used (Figure 3E). In summary, these data show that GrA does not bind to LPS.

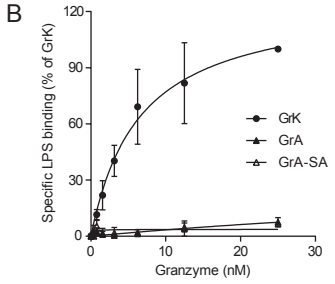
GRA INEFFICIENTLY REMOVES INDIVIDUAL LPS MOLECULES FROM MICELLES

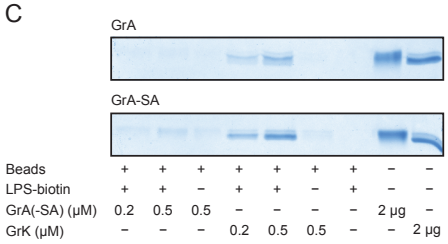
LPS is an amphipathic molecule, forming micelles in aqueous solutions, and we have previously demonstrated that GrK liberates individual LPS molecules from these micelles (13). To study the behavior of GrA in this respect, we incubated GrA with LPS-BODIPY FL. The fluorescence of this compound is quenched when LPS is in micelles and increases upon removal from the micelle. When GrK (positive control) was added to LPS-BODIPY FL, a clear increase in FI was observed (Figure 4A). An increase in FI was also observed when GrA was added, but ~4-fold less efficient as compared with GrK. The FI of LPS-BODIPY alone remained constant during measurements (Figure 4A). Experiments with increasing concentrations of GrA and GrK showed similar results (Figure 4B). We conclude that GrA can disaggregate LPS micelles to some extent, but markedly less efficient than GrK.

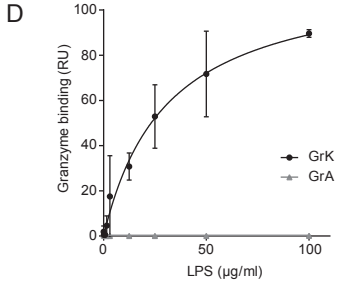
Figure 4.
GrA binds several Gram-negative bacteria but not LPS

A) GrA binds to Gram-negative bacteria. GrA–biotin binding to *E. coli BL21*, *E. coli ATCC 25922*, *E. coli Expec 536*, *P. aeruginosa* (PA-01), or *N. meningitidis HB-1* (NM) was detected by flow cytometry. Data are representative of at least three independent experiments. B) GrA does not bind to LPS in a solid-phase binding assay. LPS was immobilized and incubated with biotinylated GrK, GrA or GrA-SA. Data are depicted as specific binding (depicted as % of maximum GrK binding) and represent mean ± SD of three independent experiments. C) GrA does not bind to LPS in a pull-down assay. LPS-biotin was coupled to streptavidin-sepharose beads. After washing, beads were incubated with GrK, GrA or GrA-SA. Bound protein was eluted from the beads and visualized by SDS-PAGE followed by total protein staining. Results are representative for three independent experiments. D) GrA does not bind to LPS in a surface plasmon resonance assay. Immobilized GrA and GrK were incubated with LPS for 10 min. (association), followed by a buffer flow (dissociation). The association of LPS to GrA and GrK at t = 10 min is shown. Results represent mean ± SD of three independent experiments. RU, response units. E) Direct comparison of LPS (100 µg/ml) binding to immobilized GrK and GrA. Results are representative of three independent experiments. RU, response units.


A
E.coli BL21
E.coli ATCC 25922
E.coli Expec 536
PA-01
NM
Relative cell number
Control
GrA
Bacterial binding (Log fluorescent intensity)


B
Specific LPS binding (% of GrK)
GrK
GrA
GrA-SA
Granzyme (nM)


C
GrA
GrA-SA
Beads
LPS-biotin
GrA(-SA) (μM)
GrK (μM)
2 μg


D
Granzyme binding (RU)
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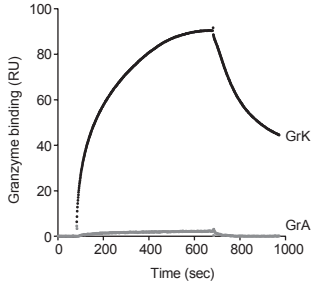
E
Granzyme binding (RU)
GrK
GrA
Time (sec)

Discussion

Growing evidence points to a role for granzymes in infection and inflammation (1, 27). Levels of soluble granzymes are increased in the circulation during inflammation and contribute to cytokine release and processing (6, 13, 15, 16, 28, 29). GrA may be important in the disease progression of sepsis (11, 20), but its extracellular functions are incompletely understood. Here, we demonstrate that GrA augments LPS-induced cytokine responses in human monocytes (Figure 1 and 3), in a CD14-dependent fashion (Figure 2). Unlike GrK, however, GrA does not bind to LPS (Figure 4), and does not efficiently remove LPS from micelles (Figure 5). Therefore, GrA augments LPS-induced cytokine responses from human monocytes via mechanisms at least partially different from those employed by GrK (13).

Treatment of monocytes with GrA alone did not induce cytokine production (Figure 1 and 3). These results are in accordance with our previous data obtained with GrK (13), but stand in contrast to results published by Metkar et al. (6). These authors demonstrate that low doses of GrA (~50-200 nM) induce production of the cytokines TNFα, IL-1β, IL-6, and IL-8 in human primary monocytes, which effect depends on GrA catalytic activity and is enhanced upon intracellular GrA delivery (6). We here show that cytokine production by GrA requires co-stimulation with low doses of LPS (Figure 1) or Gram-negative bacteria (Figure 3), and is not dependent on GrA catalytic activity (Figure 1). We have no clear explanation for the discrepancy between our results and those of Metkar et al. (6) other than that monocyte differentiation status, monocyte cell numbers, or the recombinant granzyme source may have been different in the experimental conditions. Mouse GrA (6) and mouse GrK (5) induce IL-1β release in mouse macrophages that have been sensitized with LPS prior to the experiment. Furthermore, GrA-/- and GrM-/- mice survive longer than WT mice when challenged with LPS (6, 17), and GrM-/- mice produce less cytokine upon LPS injection, compared to WT mice (17). These results indicate that granzymes enhance the innate immune response to LPS, at least via potentiating LPS-induced cytokine responses (13).

Spencer et al. (24) report that GrA released by γδ T cells induces production of TNFα in human macrophages infected with mycobacteria. This TNFα production in turn inhibits growth of the intracellular mycobacteria (24). The authors show that TNFα produced by the macrophages, and not by the γδ T cells, is responsible for this effect (24). It has been reported that mycobacteria activate infected macrophages to produce TNFα via TLR2 (30). Furthermore, infected monocytes and macrophages frequently undergo apoptosis (31, 32). Thus, it is feasible that GrA enhances TNFα production induced by mycobacterial products,

released from infected or apoptotic macrophages. This opens the possibility that granzymes also enhance immune responses to TLR ligands other than LPS.

Previously, we have demonstrated that GrK binds to LPS and to several Gram-negative bacteria (13). We hypothesized that GrK binding to Gram-negative bacteria is mediated via their LPS moieties and that differences in LPS structure between different bacterial strains could explain differences in GrK binding intensity. In the present study, we found that GrA binds the same Gram-negative bacteria as GrK (Figure 4, (13)), whereas GrA does not bind to LPS (*E. coli* B111:O4) (Figure 4). Whether granzyme binding to Gram-negative bacteria depends on the LPS subtype or is driven by other bacterial molecules remains an open question that deserves further study.

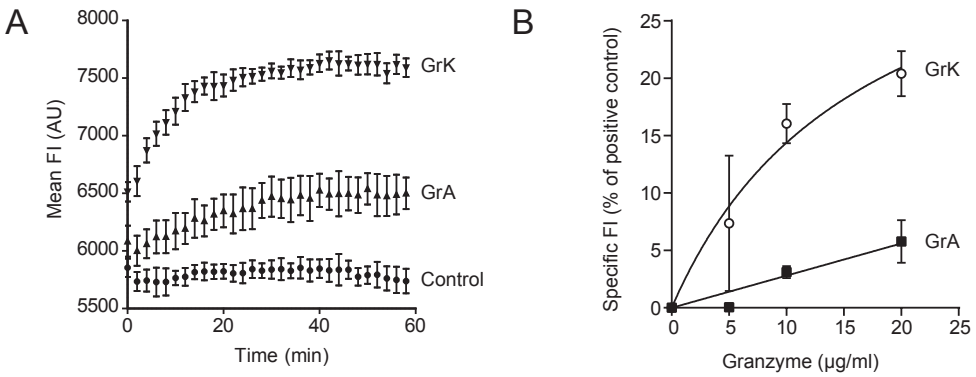

Figure 5.
GrA does not efficiently remove LPS from micelles.

GrA is less effective in removing LPS from its micelles than GrK. A) LPS-BODIPY, of which the fluorescent intensity (FI) increases upon removal from LPS micelles, was incubated for 90 minutes at 37°C, after which granzymes (20 µg/ml) or extra PBS (LPS-BODIPY control) were added. The FI was then measured for an additional 60 minutes. Results are depicted as mean ± SD (n=3) and are representative of two independent experiments. Explanation of legends in figure: GrA = LPS-BODIPY with GrA; GrK = LPS-BODIPY with GrK, Control + LPS-BODIPY alone. B) LPS–BODIPY was incubated with GrA or 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).

Since GrA does not bind to LPS, and does not efficiently liberate LPS molecules from micelles, other mechanisms likely contribute to the synergistic effect of GrA on LPS-induced cytokine production. Azurocidin (an inactive serine protease structurally related to granzymes) also enhances the LPS-induced cytokine release from human monocytes (33) and internalization is a prerequisite for this effect (34, 35). Furthermore, GrA binds to and is internalized by monocytes (6). This opens the possibility that GrA influences LPS signaling intracellularly in a perforin-independent manner. Intracellular delivery of GrA and GrK by perforin or perforin analogs shows beneficial effects on granzyme-induced cytokine responses via unknown mechanisms (5, 6). Further research is required to identify the molecular mechanism by which GrA enhances cytokine production.

In conclusion, GrA and GrK (13) likely use differential mechanisms to augment TLR4 signaling during bacterial infections. Apparently, granzymes augment inflammation in manners sufficiently different from each other to provide back-up mechanisms. This ensures a proper innate immune response when one or more granzymes are blocked. The possibility of functional redundancy further underlines the potential importance of granzymes in augmenting the anti-bacterial innate immune response.

Experimental procedures

3

REAGENTS

Cell culture reagents were from Gibco (Life Technologies), unless stated otherwise. Cell proliferation reagent (WST-1 reagent) was from Roche Applied Science. Human AB serum was from Invitrogen (Life Technologies). All yeast culture compounds were from Becton, Dickinson and Company. Synthetic chromogen substrats Z-Phe-Arg-pNA for GrA and Ac-Lys-pNA were from Bachem. LPS (*Escherichia coli 0111:B4*) and LPS-BODIPY FL were from Sigma-Aldrich. Biotin-conjugated LPS (*E. coli 0111:B4*) was from Invivogen. Polyclonal antibody to human nucleosome assembly protein SET was from Alexis Biochemicals (Enzo Life Sciences). Monoclonal antibody to human CD14 was from R&D Systems. IgG1 isotype control used in CD14-neutralising experiments was anti-human serpin B13 antibody (clone 4A9D) (36). Secondary antibodies were obtained from Jackson Immunoresearch. All bacterial strains were kind gifts from the department of Medical Microbiology (UMC Utrecht).

SDS-PAGE

Proteins were separated on a 10% or 12% SDS-PAGE gel, and total protein staining was performed with Instant Blue (Expedeon).

PRODUCTION, PURIFICATION AND CHARACTERIZATION OF GRANZYMES

Human granzymes A and K, and the GrA catalytically inactive mutant (GrA-SA), in which the active site residue Ser195 has been replaced by Ala, were produced and characterized as described before (37, 38). Briefly, cDNA encoding granzyme A or K was cloned into the yeast expression vector pPIC9 (Invitrogen). The catalytically inactive GrA-SA mutant was generated using QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's protocol. Plasmids were transformed into the GS115 strain of *Pichia pastoris* (Invitrogen) and granzymes were expressed in conditioned media for 72 hr. Granzymes were purified by cation exchange chromatography followed by affinity chromatography with Prot A/G beads (Thermo Fisher Scientific). Purified granzymes were dialyzed against 20 mM Tris, 150 mM NaCl, pH 7.4. Alternatively, granzymes were dialyzed against 1x PBS for use in surface plasmon resonance experiments or for biotinylation (see below). Protein concentrations were measured using a Bradford (BioRad) or Nanodrop (Thermo Scientific) assay and granzymes were stored at -80°C until use. All active granzymes cleaved their respective synthetic chromogenic substrates (Ac-Lys-pNA for GrK and Z-Phe-Arg-pNA for GrA), while inactive GrA-SA did not cleave Z-Phe-Arg-pNA (data not shown). In addition, GrA and GrK both cleaved their known macromolecular substrate SET (37, 39) (data not

shown). This indicates that GrA and GrK are catalytically active whereas GrA-SA is not. Granzyme batches were not contaminated with endotoxin [<0.5 EU/mL (~0.05 ng/ml), final concentration] as determined by LAL assay (Thermo Scientific). Granzymes were biotinylated using the Biotin Protein Labeling Kit (Roche) according to the manufacturer's protocol.

SOLID-PHASE BINDING ASSAYS WITH GRANZYMES AND LPS

LPS (10 µg/ml in PBS) was incubated overnight at 4°C on 96-well plates (Greiner Bio-One GmbH), and incubated with various concentrations of biotinylated GrA(-SA) in PBS with 0.1% (v/v) Tween-20 at 37°C for 2 hr. GrK was used as a positive control. Bound granzymes were visualized by incubation with Streptavidin-polyHRP (Sanquin), followed by TMB (Invitrogen). The reaction was stopped by adding 1M H2SO4 and OD450 was measured.

LPS-GRK PULL-DOWN ASSAY

LPS-biotin (50 µg/ml) was coupled to Streptavidin-coated beads (Amersham BioSciences). After extensive washing the beads were incubated with recombinant GrA(-SA) or GrK for 1 hr at RT or overnight at 4°C by head over head rotation. Bound protein was eluted from the beads with 2x concentrated Laemmli buffer, and analyzed by SDS-PAGE followed by Instant Blue total protein staining.

SURFACE PLASMON RESONANCE ANALYSIS

Real-time binding experiments were performed on the Biacore T100 (GE Healthcare). Granzymes were immobilized on CM5 sensor-chip surface via amine-coupling at 3001 (GrA) and 2866 (GrK) response units, using manufacturer's instructions. One control flow channel was routinely activated and blocked in the absence of protein. Association of LPS (0-100 µg/ml) was assessed in triplicate in PBS for 10 min, at a flow rate of 5 µl/min at 37°C. Dissociation was allowed for 5 min in the same buffer flow. Sensor chips were regenerated using several pulses of 50 mM Tris (pH 7.4), 1 M NaCl at a flow rate of 20 µl/min. Data were corrected for aspecific binding of LPS to the control channel, which was <10% of specific binding.

BINDING OF GRA TO BACTERIA

Bacteria were diluted in PBS to OD ~0.5 (660 nm), spun down, resuspended in PBS supplemented with 0.1% (w/v) BSA and mixed with biotinylated granzyme (0-20 µg/ml). The mixtures were incubated for 1 hr at 37°C. Bacteria were washed 2 times with PBS supplemented with 1% BSA, and incubated with 1 µg/ml Streptavidin-PE (Southern Biotech) in PBS supplemented with 1% BSA for 60 min at 4°C. Bacteria were washed once with PBS with 1% BSA and analyzed by flow cytometry.

MONONUCLEAR CELL ISOLATION

Peripheral blood mononuclear cells (PBMCs) were obtained from human donor blood as described previously (Sower et al., 1996a). In short, Ficoll-Paque (GE Healthcare) densitiy centrifugation was used to separate fresh blood from healthy volunteers into layers. The mononuclear cell fraction was collected and washed three times with RPMI 1640 containing 5% (v/v) fetal calf serum (FCS) (Gibco) and 0.2% bicarbonate (w/v). Cells (0.5 x 106 cells/well) were incubated for 2 hr in 48-well culture plates in RPMI 1640 containing 5% (v/v) AB serum, 2 mM L-glutamine, bicarbonate, penicillin and streptomycin (P/S), after which non-adherent cells were removed. Adherent cells were subsequently cultured in RPMI 1640 supplemented with 2% AB serum and bicarbonate for up to one week before the experiment.

Alternatively, monocytes were purified from the PBMC layer using Magnetic-activated cell sorting (MACS). The PBMC layer was washed once with RPMI 1640 containing 5% FCS and bicarbonate and once with ice-cold PBS containing 0.5% FCS and 2 mM EDTA. Monocytes were subsequently isolated using a CD14 MACS kit (Miltenyi Biotec). The positive fraction was washed once with RPMI 1640 containing 5% AB serum, 2 mM L-glutamine, 0.2% bicarbonate and P/S and once with serum-free RPMI containing 0.2% bicarbonate.

PRO-INFLAMMATORY CYTOKINE RESPONSE IN ISOLATED HUMAN MONOCYTES

Monocytes (0.5x105/well) were incubated with granzyme (0-500 nM) with or without LPS (0-5 ng/ml) in serum-free medium for 0-8 hours depending on the experiment. After incubation, supernatants were collected and stored at -20°C. Cells were subjected to a WST-1 assay to determine relative cell viability. To each well, 250 µl WST-1 reagent was added and the increase in OD450 was measured for 90 min. Wells without cells were used as a negative control. Experiments with bacteria, GrA(-SA), and monocytes were done in the same way, except that LPS was replaced with bacteria, added in a 2-10 fold excess compared to the number of monocytes per well. The effect of αCD14 mAb was tested in a similar experiment except that monocytes were pretreated for 30 min at 37°C with αCD14 mAb (10 µg/ml), isotype control (anti-human serpin B13 antibody, 10 µg/ml), or serumfree medium alone. TNFα, IL-1β, IL-6, and IL-8 levels in culture supernatants were measured using a multiplex assay as described previously (de Jager et al., 2005) on a Luminex FlexMap 3D (BioRad) with xPonent 4.2 software. Data were analyzed using BioPlex Manager 6.1.1 (BioRad). Alternatively, TNFα was measured using ELISA (PeliKine human TNFα ELISA kit, Sanquin).

EFFECT OF GRA ON LPS MICELLE FORMATION

LPS micelle formation was studied using LPS-BODIPY-FL as described (Heinzelmann and Bosshart, 2005). The fluorescence of LPS-BODIPY-FL increases upon disaggregation

of LPS micelles. Increasing concentrations of granzyme (0-20 µg/ml) were added to LPS-BODIPY-FL (7.5 µg/ml) in 250 µl PBS. LPS-BODIPY-FL alone was used as a negative control and LPS-BODIPY-FL plus GrK served as a positive control. Disaggregation of LPS micelles upon treatment of LPS-BODIPY with 2% SDS was set at 100%. Fluorescent intensity of all samples was measured kinetically for 2 hr at 37°C at 520 nm using the FluoStar Omega apparatus (BMG Labtech).

STATISTICAL ANALYSIS

Unless indicated otherwise, data are depicted as mean values ± SD and statistical analyses were performed using the independent samples t-test. Two-tailed p-values below 0.05 were considered statistically significant.

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AUTHOR CONTRIBUTIONS

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3

GRANZYME M AND K RELEASE IN HUMAN EXPERIMENTAL ENDOTOXEMIA

Annette C. Wensink[1,2], Maryse A. Wiewel[3], Tom van der Poll[3], C. Erik Hack[2] and Niels Bovenschen[1,2]

1 Department of Pathology, University Medical Center Utrecht, Utrecht, 3584 CX, The Netherlands

2 Laboratory of Translational Immunology, University Medical Center Utrecht, Utrecht, 3584 CX, The Netherlands

3 Center for Experimental and Molecular Medicine, Amsterdam Medical Center, University of Amsterdam, Amsterdam, 1100 DD, The Netherlands

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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 *Pseudomonas aeruginosa*. Thus, GrM and GrK are differentially released in response to LPS and Gram-negative bacteria.

Introduction

Severe sepsis is a major health problem, resulting in a significant number of deaths each year (1). 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 (1). 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 (1).

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 (2). 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 (2, 3).

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 (4-9). GrM and GrA knockout mice survive longer than wild-type (WT) mice, in response to a normally lethal LPS challenge (10, 11), and GrM-/- mice express lower levels of cytokines than WT mice upon LPS challenge (10). In addition, we have recently demonstrated that GrK synergistically potentiates cytokine responses from human monocytes induced by LPS or Gram-negative bacteria (4). 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 (5). 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 upon stimulation with the Gram-negative bacterium *Pseudomonas aeruginosa*, but not with *Escherichia coli BL21*, *Neisseria meningitidis*, or LPS.

Results

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 (Figure 1A, B). A major peak in GrM levels was observed after ~2 h, with a minor peak following after ~5-8 h (Figure 1A). A less clear but recognizable rise in GrK levels was observed in several donors (Figure 1B). When GrK and GrM levels in individual volunteers are compared, peaks in GrK and GrM levels largely coincided (Figures 1C-G). These data indicate that GrM and to a lesser extent GrK are released in human experimental endotoxemia.

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 (Figure 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 (Figure 2B). In one donor, minor amounts of GrK were released (~30 pg/ml after 24h incubation with 50 ng/ml LPS). No granzyme was detected without incubation or after incubation of untreated blood (Figure 2A, B). These data indicate that GrM, but not GrK, is released in response to LPS stimulation.

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 *Escherichia coli* (*E. coli*) *BL21*, *Pseudomonas aeruginosa* (PA-01), or *Neisseria meningitidis* (NM), added in a two-fold excess compared to peripheral blood mononuclear cell (PBMC) numbers. All three bacterial species caused a marked GrM release at 6h and 24h following incubation (Figure 2C). Interestingly, GrK was only released upon treatment with PA-01, and only after 24h of incubation (Figure 2D). Soluble GrK or M were not released in blood incubated without bacteria (Figure 2C, D). These data demonstrate that GrM and GrK are differentially released in whole blood upon incubation with Gram-negative bacteria.

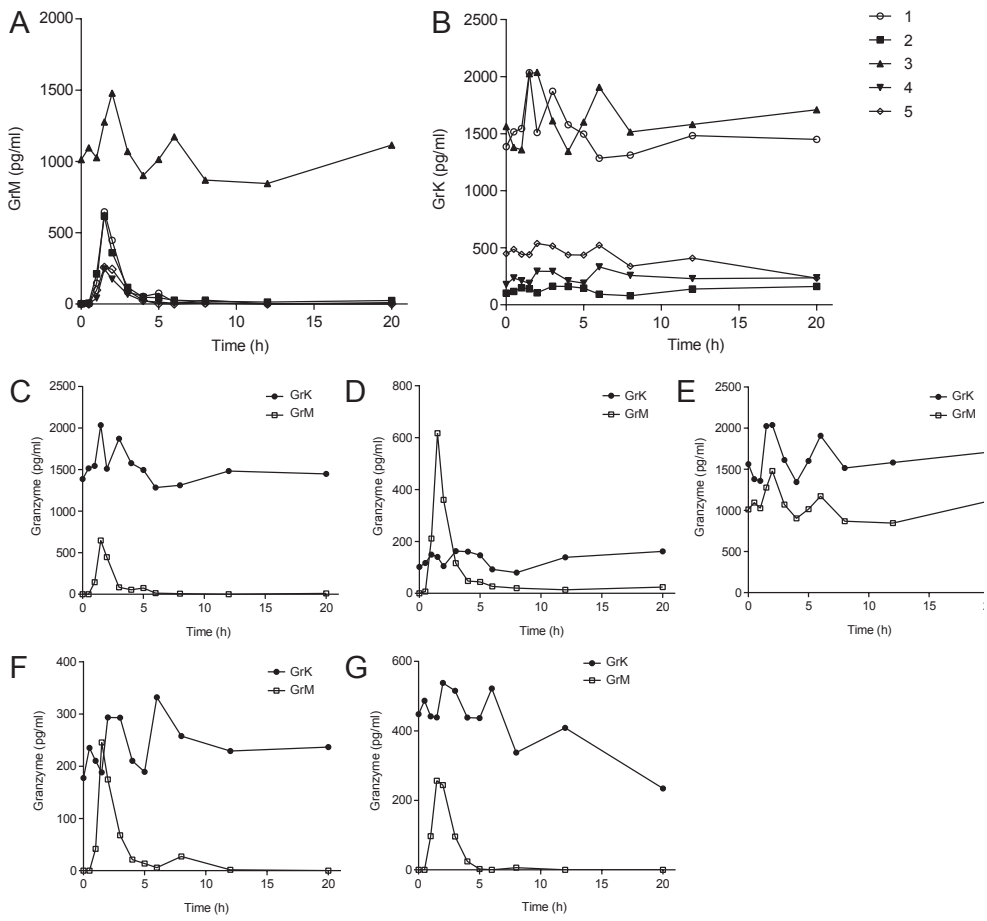

Figure 1.
GrM and GrK are released in human experimental endotoxemia.

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 levels in each volunteer is depicted in figures C)-G) (volunteer 1-5, respectively).

Discussion

It is well established that granzymes A, B, K, and M are elevated in the blood circulation during endotoxemia and sepsis (4-9). Evidence is emerging that these extracellular granzymes play a role during the process of inflammation, for instance via induction of pro-inflammatory cytokine responses (4, 11). Like for GrA and GrB (5), 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 (Figures 1 and 2). In healthy volunteers, a transient rise in GrM levels was observed (Figures 1 and 2), accompanied by a modest temporary rise in GrK levels (Figure 1). These results agree with reports demonstrating that GrM and GrK are elevated in the circulation during sepsis (4, 7, 8). In whole blood cultures, GrM was released upon stimulation with LPS or Gram-negative bacteria, and remained high for at least 24 h (Figure 2). GrK was released upon treatment with the Gram-negative bacterium *Pseudomonas aeruginosa*, but not in response to *Escherichia coli BL21*, *Neisseria meningitidis*, or LPS (*Escherichia coli 0111:B4*) (Figure 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 (Figure 1), consistent with GrM baseline levels detected in blood plasma in vitro (Figure 2). In contrast, soluble GrK is clearly detectable in serum of healthy volunteers at the start of the measurements (Figure 1). This finding is in agreement with results published by us and others (4, 8, 12), but differs from the low GrK levels we found in plasma in the absence of stimulation in vitro (Figure 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 (2). NK cells respond to LPS, and express intracellular TLR4, although surface expression of TLR4 is probably weak (13, 14). However, LPS treatment decreases NK cell degranulation (13), and injection of LPS into healthy volunteers is associated with a marked decrease in NK cell numbers (5). 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 (2). These cytotoxic lymphocytes may also contribute to granzyme release upon LPS or bacterial stimulation. GrB expression has been found in a number of cell types other than cytotoxic lymphocytes, including dendritic cells, mast cells, basophils, and B cells (15), and

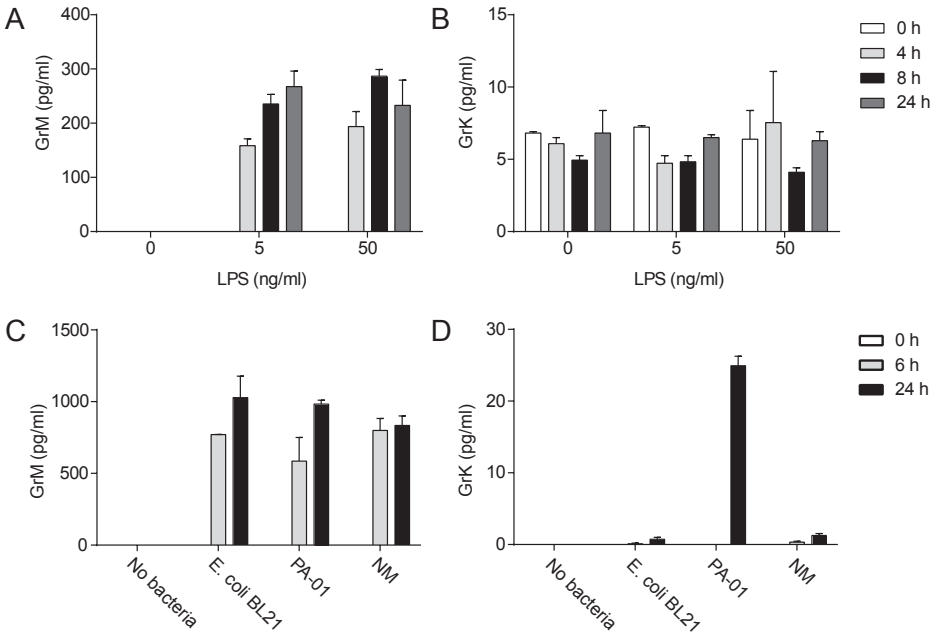

Figure 2.
GrM and GrK release in whole blood stimulated with LPS or Gram-negative bacteria.

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, D) 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 (C) and GrK (D) levels in the culture supernatant were determined. Data are representative of three (PA-01) or two (*E. coli BL21* and NM) independent experiments.

it is possible that these cell types also express GrM and/or GrK. Finally, non-hematopoietic cells may contribute to granzyme release in vivo, which may explain the absence of GrK release under some of the tested in vitro conditions (Figure 2).

How LPS and Gram-negative bacteria cause granzyme release remains to be elucidated. Lauw et al. (5) 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 (5). This suggests that LPS-induced cytokines trigger cellular granzyme release. Since granzymes also induce cytokine release (4, 11), 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 (1). 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.

Methods

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 hr 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 (5). 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.

WHOLE BLOOD STIMULATION WITH LPS OR GRAM-NEGATIVE BACTERIA

Whole blood from healthy volunteers 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 (*Escherichia coli 0111:B4*) (0-50 ng/ml) was added and samples were incubated for 0-24 h under standard incubator conditions (37°C, 5% CO2, 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 (~1x10E6 PBMCs/ml in whole undiluted blood). *Escherischia coli BL21* (*E. coli BL21*), *Pseudomonas aeruginosa 01* (PA-01), or *Neisseria meningitidis 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 *Neisseria meningitidis*, which was grown on GB-Choco plates (37°C, 5% CO2, RH 95%). Bacteria were diluted in PBS to OD ~0.5 at 660 nm (representing approximately 5x10E8 bacteria/ml), and added in the correct concentration to whole blood prediluted as described above . After incubation, plasma samples were obtained as described above.

GRM AND GRK LEVELS 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).

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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INCREASED INTRA-ARTICULAR GRANZYME M LEVELS CORRELATE WITH PROINFLAMMATORY CYTOKINE LEVELS IN RHEUMATOID ARTHRITIS

Annette C. Wensink[1,2], Joel A. van Roon[2,3], Timothy R.D.J. Radstake[2,3], Mark. H. Wenink[2,3], C. Erik Hack[2] and Niels Bovenschen[1,2]

1 Department of Pathology, University Medical Center Utrecht, Utrecht, 3584 CX, The Netherlands

2 Laboratory of Translational Immunology, University Medical Center Utrecht, Utrecht, 3584 CX, The Netherlands

3 Department of Rheumatology, University Medical Center Utrecht, Utrecht, 3584 CX, The Netherlands

Submitted

To the editor,

Granzymes are serine proteases produced by cytotoxic lymphocytes (cytotoxic T cells, γδ T cells, Natural Killer (NK) cells, and NKT cells). Humans have five granzymes: granzyme A (GrA), GrB, GrH, GrK, and GrM, which can cause apoptosis in tumor cells and virally infected cells (1). Soluble granzyme levels are elevated in the blood of patients with infections or auto-immune diseases (2). These extracellular granzymes fulfill proinflammatory functions, including extracellular matrix degradation and proinflammatory cytokine release (3). Levels of GrA and GrB are elevated in rheumatoid arthritis (RA) synovial fluid and plasma (2). GrB is expressed in RA synovial tissue, but not in control osteoarthritis (OA) tissue (4), and single-nucleotide polymorphisms in the GrB gene influence the rate of joint destruction in RA (5). However, nothing is known about other granzymes in RA. Here, we measured GrK and GrM levels in unmatched serum and synovial fluid from patients with RA or Psoriatic arthritis (PsA). Clinical and demographic patient data are summarized in Table 1. Serum GrK and GrM levels in RA and PsA were similar to those in healthy controls (figure 1A and B). Furthermore, GrK levels in RA or PsA synovial fluid were not elevated compared to levels in control OA patients (figure 1C). In contrast, GrM levels were increased in the synovial fluid of RA ($p<0.01$), but not significantly in PsA patients ($p=0.25$), compared to OA patients (figure 1D). This suggests that GrM is released specifically in the synovial tissue or fluid in RA, but apparently not at levels to produce increased quantities in the circulation. No correlation between GrM levels in SF and circulating C-Reactive Protein (CRP) levels or Erythrocyte Sedimentation Rate (ESR) was found. However, GrM levels correlated significantly with the cytokines X-CL1 ($p<0.05$, $r=0.4908$), TNFα ($p<0.01$, $r=0.5706$), and IL-25 ($p<0.05$, $r=0.4604$) and notably with the IFN/CD8-related cytokine IL-29 ($p<0.01$, $r=0.5750$), in RA synovial fluid (figure 1E-H).

Our data point to differential production of GrK and GrM in RA, and suggest a role for GrM in local inflammatory reactions occurring in the RA joint. GrM is expressed by NK cells, NKT cells, γδ T cells, and CD8+ T cells(1), known to be enriched in the synovial membrane and fluid in RA (6-8). In line with GrA, that induces release of the proinflammatory cytokines TNFα, IL-1β, IL-6, and IL-8 from monocytes (9), GrM knockout mice have impaired proinflammatory cytokine responses to LPS (10). Hence, these reports and the present data indicate that GrM may contribute to disease progression by inducing a multitude of proinflammatory cytokines. Consistent with this, GrM correlates with IL-29, TNFα, X-CL1 and IL-25 levels (Figure 1), suggesting that GrM may induce a positive feedback loop of cytokine secretion that aggravates inflammation.

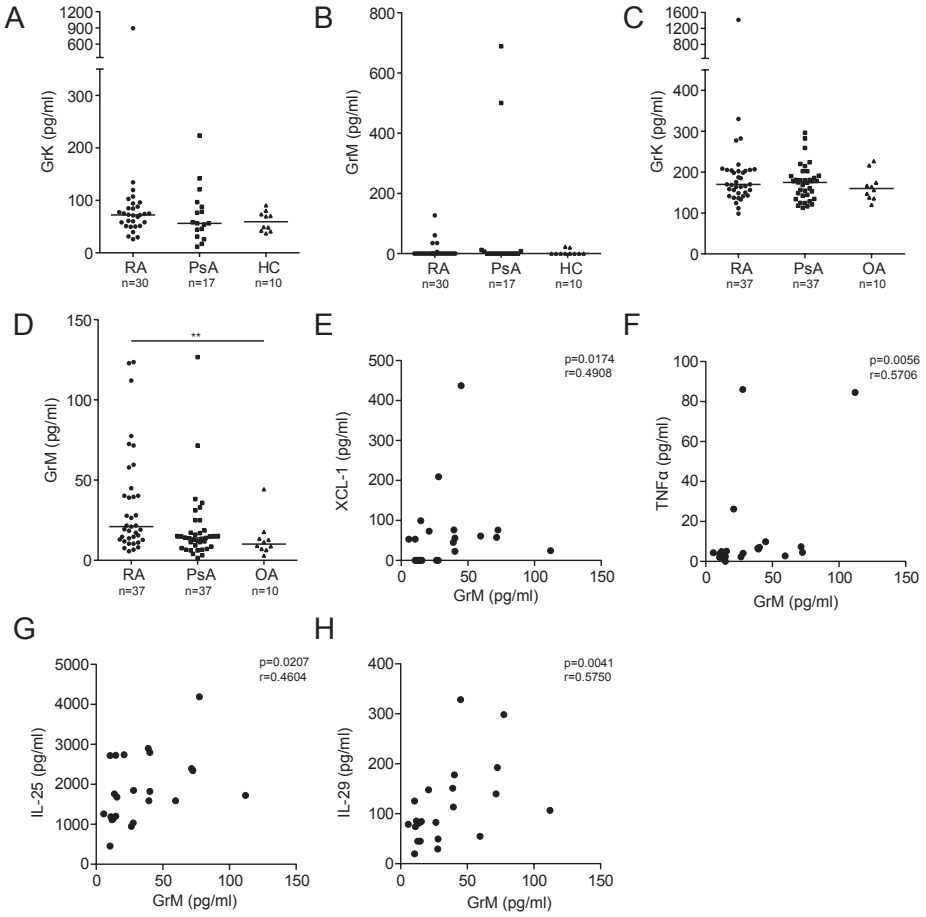

Figure 1.
GrM levels in synovial fluid of RA patients are elevated and correlate with proinflammatory cytokine expression.

(A,B) Serum levels of GrK (A) and GrM (B) in RA (n=30) and PsA (n=17) patients, and healthy controls (n=10). (C,D) Synovial fluid levels of GrK (C) and GrM (D) in RA (n=37), PsA (n=37), and OA (n=10) patients. Soluble granzymes were measured using ELISA (Uscn Life Science Inc., lower detection limit ~3 pg/ml). All samples except sera from healthy controls were pretreated with Heteroblock (Omega Biologicals), 150 µg/ml in undiluted samples, for 1 h at room temperature. Synovial fluids and sera were not matched. Median granzyme levels are depicted and statistical analysis was performed using the Mann-Whitney U-test. $P < 0.05$ was considered statistically significant. $^{**}p < 0.01$ compared to OA control. E) GrM levels correlate with IL-29 expression in RA synovial fluid. Cytokines were measured using a Luminex assay as described previously (12) on a Luminex FlexMap 3D (BioRad) with xPonent 4.2 software. Data were analyzed using BioPlex Manager 6.1.1 (BioRad).

Since inflamed joints are infiltrated with macrophages extensively (11), GrA-induced release of proinflammatory cytokines from these cells (9) may contribute to joint inflammation. Cleavage of extracellular matrix components by GrA and GrB is also thought to contribute to tissue destruction in RA (2). Local release of GrM in RA may have similar effects on cytokine release, cytokine processing, or ECM degradation and contribute to immunopathology in RA. Further studies are warranted to confirm and extend these interesting findings.

Table 1. Clinical and demographic patient characteristics.

Data	RA		PsA		OA
	Serum (n=30)	SF (n=37)	Serum (n=17)	SF (n=37)	SF (n=10)
Age (average + range)	61.1 (35-84)	54 (19-83)	50.8 (29-70)	48 (31-73)	63 (53-78)
Gender (m/v)	8/22	N.R.	7/10	N.R.	N.R.
ESR (mm/1st hour)*	14.25 ± 8.4	18.3 ± 17.2	N.R.	44.3 ± 33.3	11.25 ± 8.7
CRP (mg/L)**	3.5 ± 1	12.1 ± 12.8	29.5 ± 40.3	42.2 ± 34.1	6.3 ± 1.2
Immunosuppressive drugs (no/yes/N.R.)	N.R.	33/37/1	16/17	25/37/1	8/1/1

N.R. = Not Registered.

For serum measurements serum from healthy volunteers was used as a control.

* Erythrocyte Sedimentation Rate (ESR) was available in the following patients: RA serum 28/30; PsA serum 0/17; RA synovial fluid 35/37; PsA synovial fluid 37/37 and OA synovial fluid 4/10 patients.

**C-Reactive Protein (CRP) levels were available in the following patients: RA serum 4/30; PsA serum 4/17; RA synovial fluid 16/37; PsA synovial fluid 14/37 and OA synovial fluid 3/10 patients.

Footnotes

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CONTRIBUTORS

AW performed experiments and data analysis and wrote the paper; JvR, TR and MW provided patient material; JvR, CEH and NB wrote the paper.

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COMPETING INTERESTS

None.

PATIENT CONSENT

Samples were collected at the UMC Utrecht. Informed consent was obtained from all patients or one of their family members.

ETHICS APPROVAL

The use of patient samples was approved of by the Ethics committee of the University Medical Center, Utrecht, The Netherlands

PROVENANCE AND PEER REVIEW

Not commissioned; externally peer reviewed.

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HUMAN AND MOUSE GRANZYME K ARE NONCYTOTOXIC AND HAVE PARTIALLY OVERLAPPING SUBSTRATE SPECIFICITIES

Annette C. Wensink[1,2], Roderick R. Oude Vrielink[1,2], Stefanie A.H. de Poot[1], Jan Meeldijk[2], Dieter E. Jenne[3], C. Erik Hack[2] and Niels Bovenschen[1,2]

1 Department of Pathology, University Medical Center Utrecht, Utrecht, 3584 CX, The Netherlands

2 Laboratory of Translational Immunology, University Medical Center Utrecht, Utrecht, 3584 CX, The Netherlands

3 Comprehensive Pulmonary Center, Helmholtz Center Munich, D-85764 Neuherberg, Germany

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Abstract

Granzymes are serine proteases involved in the clearance of tumor cells and virally infected cells. Humans have five granzymes, whereas mice express ten. Remarkably, human GrK (hGrK) efficiently eliminates tumor cells, while mouse GrK (mGrK) is not cytotoxic at all. Therefore, although both proteases display considerable amino acid sequence homology, it remains unknown whether hGrK and mGrK share macromolecular substrates and biological functions. In the present study, we directly compared the pro-apoptotic potential and macromolecular substrate specificities of hGrK and mGrK. We show that both mGrK and hGrK are not cytotoxic towards several human and murine tumor cell lines. Both proteases cleave the well-established hGrK substrates SET and hnRNPK. Furthermore, using a fluorescent 2 dimensional difference gel electrophoresis proteomic approach, we demonstrate that hGrK and mGrK display highly restricted, partially overlapping (39%) macromolecular substrate specificities. Thus, caution is required when extrapolating data from mouse models to human GrK biology.

Introduction

The immune defense against tumor cells and virally infected cells is predominantly mediated by cytotoxic lymphocytes [CD8+ T cells, Natural Killer (NK) cells, NKT cells, and γδ T cells], which efficiently eliminate aberrant cells via induction of apoptosis (1). Induction of apoptosis is achieved via two routes: the death receptor pathway and the granule-exocytosis pathway. The death receptor pathway depends on engagement of death receptors on the target cell by death-inducing ligands on the effector cell to activate pro-apoptotic pathways within the target cell (2). The granule exocytosis pathway involves the release of cytotoxic proteins from vesicles in the effector cell into the immunological synapse that is formed between the target and effector cell upon recognition (3, 4). These cytotoxic proteins include a set of homologous serine proteases called granzymes and the pore-forming protein perforin. Granzymes enter the target cell with the aid of perforin, after which they cleave various intracellular substrates, thereby activating pathways that prompt the target cell to undergo apoptosis (3, 4). Humans have five granzymes: granzyme A (GrA), GrB, GrH, GrM, and GrK. Cytotoxicity has been demonstrated for all five granzymes (1), although the pro-apoptotic potential of GrA and GrK has been debated lately (5-7). Mice do not express GrH, but additionally express granzymes C-G and N (8). The cytotoxic potential of murine granzymes is not well studied (8).

Human GrK (hGrK)-mediated cytotoxicity towards tumor cells is caspase-independent (9-11) and occurs via multiple pathways. One involves cleavage of valosin-containing protein, leading to accumulation of ubiquitinated proteins, ER stress, and cell death (12). Another pathway comprises cleavage of the redox/DNA repair enzyme ApeI which causes oxidative stress in target cells and induces apoptosis (9). Furthermore, hGrK treatment causes single-strand DNA breaks followed by cell death via hGrK-mediated degradation of the nucleosome assembly protein SET and subsequent release of DNase NM23H1 (10). hGrK-mediated cleavage of p53 leads to apoptosis in tumor cells (13), and hGrK is also reported to cleave Bid and cause mitochondria-dependent cell death (11). hGrK also protects against influenza virus via cleavage and inactivation of several host cell nuclear import proteins that assist viral entry into the nucleus of infected host cells (14). In addition, hGrK induces IL-6 and IL-8 release from human lung fibroblasts (15), and we have recently demonstrated that GrK synergistically potentiates LPS-induced cytokine responses from human monocytes (16).

Mouse GrK (mGrK) protects against infections with influenza virus and lymphocytic choriomeningitis virus (LCMV) in vivo (6, 17). Remarkably, mGrK-expressing CD8-enriched

lymphocytes from GrAB-/- mice are not cytotoxic towards tumor cells, but instead induce IL-1β release from mouse macrophages (6). Intracellular delivery of recombinant mGrK in tumor cells also does not trigger cell death (6). This implies that mGrK is not cytotoxic towards tumor cells, but contributes to the elimination of infections.

Out of all murine granzymes, mGrK displays the highest amino acid sequence homology with hGrK. Nevertheless, it remains unknown whether both proteases share macromolecular substrates and biological functions. In the present study, we directly compared the pro-apoptotic potential and macromolecular substrate specificities of hGrK and mGrK. We show that hGrK and mGrK are not cytotoxic and that both proteases display highly restricted, partially overlapping macromolecular substrate specificities.

Material and methods

REAGENTS

All cell culture reagents were from GIBCO (Invitrogen). Polyclonal antibody to the human nucleosome assembly protein SET was from Alexis Biochemicals (Enzo Life Sciences). Polyclonal antibody to human hnRNP K was from Abcam. These antibodies cross-react with the mouse homologues of their targets. All secondary antibodies were from Jackson Immunoresearch. SLO (streptolysin O) was from Aalto Bio Reagents. Reagents for two-dimensional Difference Gel Electrophoresis (2D DIGE) were from GE Healtcare and BioRad.

CELL CULTURE AND LYSATE PREPARATION

The human HeLa and murine C26 and C2C12 tumor cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal Calf Serum (FCS) (vol/vol), 100 units/ml penicillin and 100 µg/ml streptomycin (wt/vol) (Invitrogen). The human tumor cell line Jurkat was maintained in Roswell Park Memorial Institute 1640 (RPMI-1640) medium supplemented with 10% FCS (vol/vol), pen/strep and 2 g/L sodium bicarbonate (wt/vol). To obtain cell lysate for 2D-DIGE or immunoblotting, HeLa or Jurkat cells (~6x10E6) were washed and lysed in 150 mM NaCl and 20 mM Tris, pH7.4, by repeated freeze-thawing (3 times). Lysates were centrifuged for 10 min at 20,000 g at 4 °C. Protein concentration was determined using the Bradford method, after which cell-free protein extracts were stored at –80 °C.

PRODUCTION, PURIFICATION AND CHARACTERIZATION OF RECOMBINANT GRANZYMES

Human and mouse GrK were purified from *Escherichia coli* (*E. coli*) inclusion bodies as described (18, 19). Human GrK, GrK-SA (in which the active site residue Ser195 has been replaced by Ala) and GrB from *Pichia pastoris* (*P. pastoris*) were produced and purified as described previously (16, 20). SDS-PAGE analysis was performed using 12% gels followed by Instant Blue total protein staining (Expedeon). Granzyme activity was determined using the synthetic chromogenic substrate Ac-Lys-pNA (Bachem). Granzyme (500 nM) was mixed with substrate (2 mM) and incubated for 15 h at 21°C. Hydrolysis of the substrate was measured kinetically at OD405 using an Anthos Zenyth 340 rt microtiter plate reader (Anthos). Assays were performed in buffer containing 100 mM Tris, 200 mM NaCl, and 0.01% Tween, pH 7.4.

MACROMOLECULAR SUBSTRATE CLEAVAGE ASSAYS

HeLa or Jurkat cell lysate (10 µg) was mixed with increasing concentrations of mGrK or hGrK(-SA) (0 – 1000 nM) for 4 h at 37°C. Protein samples were separated using SDS-PAGE,

after which proteins were transferred to a PVDF membrane, stained with antibody, and visualized by ECL or ECL Prime (GE Healthcare). Imaging was performed on the ChemiDoc Molecular Imager (BioRad).

FLUORESCENT 2-DIMENSIONAL DIFFERENCE GEL ELECTROPHORESIS (2D DIGE)

HeLa cell lysate (100 µg per condition) was incubated with 250 nM mGrK or hGrK for 4 h at 37°C. As a control (mock), untreated lysate was used. Subsequently, proteins were precipitated, washed, solubilized, labelled [using either the red fluorescent dye indodicarbocyanine (Cy5) or the green fluorescent dye indocarbocyanine (Cy3)], rehydrated, and isoelectrically focused as we have described previously (20). The strips were reduced and overlaid on a 12% SDS-PAGE gel as described (20). After running the second dimension, images were acquired on a Typhoon 9410 scanner (GE Healthcare). Each condition was performed five times, during which dye swaps were included to correct for possible preferential labelling of proteins. The internal standard method was used to perform inter-gel analyses as described (21). Relative quantification and statistics of matched spots in the gels was performed using Decyder DIA and BVA software (GE Healthcare). $P<0.05$ (Student's t test) was considered statistically significant.

CELL VIABILITY ASSAYS

Cells were grown to confluence in 96-well tissue-culture plates. Cells were washed twice in serum-free DMEM, after which they were incubated at 37 °C with a sublytic dose of SLO (Jurkat, 0.1 µg/ml SLO; HeLa 0.3 µg/ml SLO; C26 and C2C12, 0.5 µg/ml SLO) and mGrK or hGrK (0-700 nM), or hGrB (0-500 nM) in serum free buffer containing 20 mM Tris and 150 mM NaCl for 30 min. Adherent cells were washed twice with supplemented DMEM. Non-adherent cells were not washed but instead provided with an excess of supplemented RPMI. Thereafter, cells were incubated in the appropriate medium for another 20 h at 37 °C. Cell viability was assessed using the WST-1 method (Roche Applied Sciences) per the manufacturer's instructions. Alternatively, cell viability was determined using flow cytometry. Cells were stained with Annexin V-fluos (AnnV, Invitrogen) and propidium iodide (PI) (Invitrogen) for 15 min in a buffer containing 140 mM NaCl, 4 mM KCl, 0.75 mM MgCl2, 1.5 mM CaCl2 and 10 mM Hepes (pH 7.4). Flow cytometry was performed on a FACSCalibur™ instrument (BD Biosciences) and results were analyzed using CellQuest Pro software (BD Biosciences). Cells that were negative for both Annexin V and PI were considered viable. The percentage of viable cells after treatment with SLO only was set at 100% and the percentages of viable cells in other conditions were calculated accordingly.

Results

ISOLATED RECOMBINANT MGRK AND HGRK ARE PURE AND ACTIVE

Mouse and human GrK display a considerable amino acid sequence homology of 76%, and the serine, histidine and aspartic acid residues that constitute the catalytic triad are conserved (Figure 1A). To characterize both proteases, isolated recombinant mGrK and hGrK (from *E. coli*) were analyzed by SDS-PAGE followed by total protein staining (Figure 1B). Both granzymes migrated as a single band of ~25 kDa, consistent with monomeric GrK. Virtually no other proteins were visible on the gel, indicating that mGrK and hGrK were devoid of protein contamination. Next, the catalytic activity of both granzymes was determined by measuring hydrolysis of the small chromogenic substrate Ac-Lys-pNA (Figure 1C). Mouse and human GrK hydrolyzed this synthetic substrate with similar efficiency. No hydrolysis of the substrate was observed in absence of granzyme. These data indicate that recombinant mGrK and hGrK are pure and active.

BOTH MGRK AND HGRK CLEAVE HUMAN SET AND HUMAN HNRNP K

We and others have previously established that human GrK cleaves the macromolecular substrates heterogeneous nuclear ribonucleoprotein K (hnRNP K) (22) and the nucleosome assembly protein SET (10, 20). Whether these substrates are also cleaved by mouse GrK is unknown. To directly compare the efficiency with which mGrK and hGrK cleave hnRNP K and SET, human tumor cell lysate was incubated with both granzymes, and samples were analyzed by immunoblotting. Both mGrK and hGrK cleaved hnRNP K (Figure 2A and B) and SET (Figure 2C and D) with similar efficiency. Cleavage of human hnRNP K by mGrK and hGrK resulted in cleavage fragments of similar size, indicating that both proteases cleave human hnRNP K at the same position or closely adjacent positions (Figure 2A and B). The largest cleavage fragment was ~37 kDa in size, while a smaller cleavage fragment of ~34 kDa appeared at higher granzyme concentrations. Upon incubation with high concentrations of mGrK (1000 nM), an even smaller cleavage fragment appeared around 32 kDa, while the largest cleavage fragment was completely degraded. These events were not clearly discernible for hGrK, suggesting that mGrK has (an) additional cleavage site(s). No cleavage was observed without granzyme. Since human and murine hnRNP K are 100% identical in amino acid sequence, it is probable that murine hnRNP K is also cleaved by both mGrK and hGrK, likely at the same positions as human hnRNP K. Cleavage of human SET by mGrK resulted in two cleavage fragments of around 22 kDa, while only one cleavage fragment was observed for hGrK (Figure 2C and D). It is not clear to which mGrK cleavage fragment this hGrK cleavage fragment corresponds. No cleavage was observed with GrK-SA or in

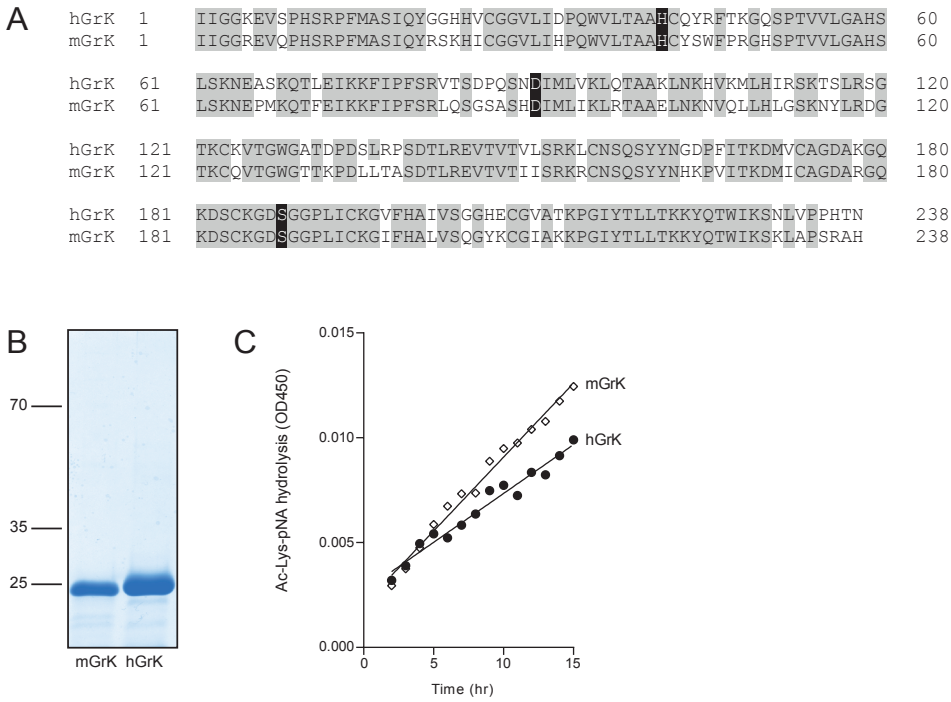

Figure 1.
Recombinant mGrK and hGrK are pure and active.

A) Sequence alignment of mature mGrK (top) and hGrK (bottom). Residues shared between mGrK and hGrK are boxed in grey. Residues constituting the catalytic triad are depicted in white and boxed in black. B) SDS-PAGE analysis of mGrK (10 µg) and hGrK (20 µg) followed by total protein staining. C) mGrK and hGrK (both 500 nM) were incubated with the synthetic chromogenic substrate Ac-Lys-pNA and pNA hydrolysis was measured kinetically for 15 h. Data are depicted as specific absorption (corrected for absorption with buffer only). Slopes were 0.0047 for hGrK (R2 0.97) and 0.00070 for mGrK (R2 0.99).

absence of granzyme. Since human and mouse SET also share extensive amino acid sequence homology (98%), it is likely that murine SET is degraded by both mGrK and hGrK with similar dynamics as human SET. Taken together, these data indicate that human SET and human hnRNP K are substrates for both hGrK and mGrK.

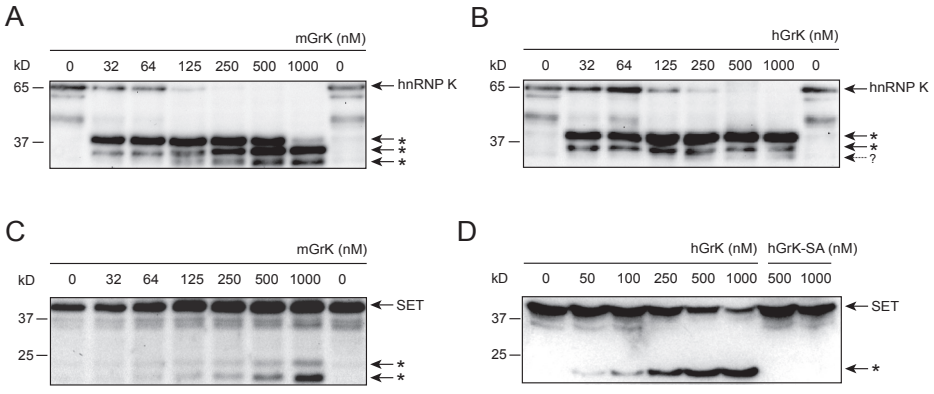

Figure 2.
Both mGrK and hGrK cleave human SET and human hnRNP K.

HeLa (panel A-C) or Jurkat (panel D) cell lysate was incubated with the indicated concentrations of mGrK or hGrK(-SA) for 4 h (panels A-C granzymes from *E. coli*, panel D granzymes from *P. pastoris*). Samples were immunoblotted for hnRNP K (mGrK, panel A; hGrK, panel B) or SET (mGrK, panel C; hGrK, panel D). Representative blots for two independent experiments are shown. Cleavage products are indicated with an *.

HGRK AND MGRK SHOW RESTRICTED AND PARTIALLY OVERLAPPING MACROMOLECULAR SUBSTRATE SPECIFICITIES

To compare the macromolecular substrate specificities of mGrK and hGrK, a two-dimensional Difference Gel Electrophoresis (2D DIGE) proteomic approach was used. This approach quantitatively inspects the proteome for granzyme macromolecular substrates and concomitant cleavage products. HeLa cell lysate was incubated with mGrK, hGrK, or buffer (mock treatment) and was subsequently labeled using red or green fluorescent labels. Two representative 2D gels of cell lysate treated with mGrK (Figure 3A) or hGrK (Figure 3B) are shown. In these gels, cell lysate treated with granzyme was labeled red and mock treated lysate was labeled green. Protein spots that decrease in intensity after granzyme treatment appear green and indicate possible full-length granzyme substrates. Protein spots that emerge after granzyme treatment are red and indicate fragments of cleaved proteins. Proteins unaffected by the treatment will be present in equal abundance in both

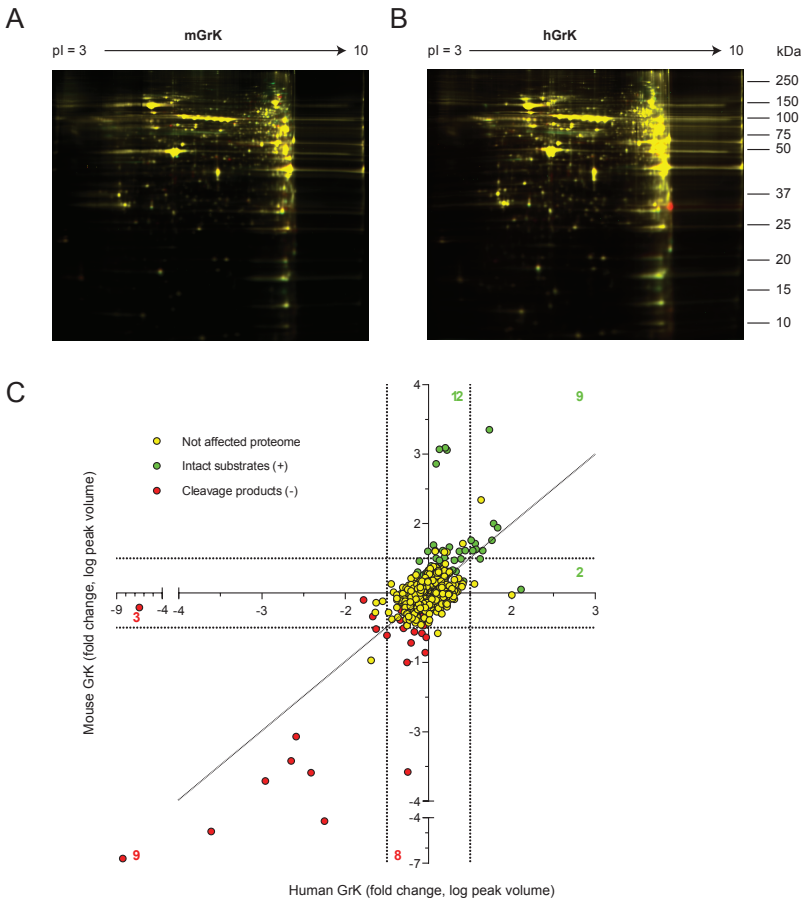

Figure 3.
hGrK and mGrK show restricted and partially overlapping macromolecular substrate specificities.

HeLa cell lysate (100 ug) was treated with 250 nM mGrK or hGrK, or buffer (negative control) for 4 h. Samples were stained with Cy5 (red; granzyme treated lysate) or Cy3 (green; mock treated lysate) and subjected to 2D DIGE analysis. A-B) Two representative 2D gels after mGrK (A) or hGrK (B) treatment are shown. Proteins present in equal abundance in untreated versus granzyme-treated samples appear as yellow spots in the gel. Proteins digested by either mGrK or hGrK will be present to a lesser extent in granzyme-treated sample and will thus appear as green spots in the gel, while cleavage fragments are absent from the mock-treated sample and will thus appear red in the gel. C) Changes in log peak volume were calculated in gels prepared from mGrK/mock and hGrK/mock treated HeLa cell lysate. Subsequently, gels were matched and hGrK/mock log peak volume ratios (x axis) were plotted against mGrK/mock log peak volume ratios (y axis). Protein spots are labeled yellow (proteome not affected by granzyme treatment), green (intact substrates that disappear upon granzyme treatment) or red (cleavage products that appear upon granzyme treatment). Protein spot intensity was regarded as changed when log peak volume ratios were larger than 1.5 (dotted lines) and p>0.05.

samples and will be visible as yellow spots in the overlay. Nearly 2200 individual proteins were identified in the HeLa cell lysate, of which 23 decreased in abundance after mGrK and hGrK treatment (Figure 3C). After mGrK treatment, 21 spots (~0.95% of total proteome) decreased in intensity while 11 spots (~0.5% of total proteome) decreased in intensity after hGrK treatment (change in log peak volume > 1.5-fold, $p<0.05$) (Figure 3C). This relatively low number of cleaved proteins indicate that mGrK and hGrK both display a highly restricted macromolecular substrate specificity. When mGrK- and hGrK-mediated cleavage of proteins in HeLa lysate was compared, 12 unique substrates for mGrK and 2 unique substrates for hGrK were identified (Figure 3C). Interestingly, 9 substrates (~39% of the total number of substrates) were shared between the two granzymes. Furthermore, 9 cleavage fragments were shared by mGrK and hGrK, strongly suggesting that the accompanying substrates are cleaved at the same position by both granzymes. Several cleavage fragments appear with comparable intensity after mGrK or hGrK treatment (Figure 3C), indicating that mGrK and hGrK hydrolyze these substrates with similar efficiency. Taken together, these data indicate that hGrK and mGrM display highly restricted partially overlapping macromolecular substrate specificities.

BOTH MGRK AND HGRK ARE NOT CYTOTOXIC IN VITRO

To determine the (relative) cytotoxicity of mGrK and hGrK towards human and murine tumor cells, living HeLa (human cervix carcinoma), Jurkat (human T cell lymphoma), C2C12 (mouse myoblast), and C26 (mouse colon carcinoma) cells were treated with purified mGrK and hGrK (from *E. coli*) in the presence of the perforin-analog Streptolysin O (SLO) for intracellular delivery. Human GrB and GrK produced in *Pichia pastoris* were also included in this analysis. After overnight incubation, cell viability was determined by staining the cells for the apoptotic markers Annexin V (AnnV) and Propidium Iodide (PI), followed by flow cytometry. Both mGrK and hGrK failed to induce apoptosis in the human tumor cell lines HeLa and Jurkat (Figure 4A and B) and the mouse tumor cell lines C2C12 and C26 (Figure 4C and D). Results obtained for hGrK were not influenced by the source of the granzyme (*E. coli* or yeast *P. pastoris*) (Figure 4A-D). Under the same circumstances, GrB readily induced cell death. This result was confirmed using the WST-1 assay, an alternative approach to study cell viability. Cells were treated with granzyme and SLO, followed by overnight incubation. Thereafter, WST-1 reagent was added and cell viability was measured. Again, mGrK and hGrK did not induce apoptosis in any of the tested cell lines (Figure 4E-H, while hGrB (100-500 nM) caused extensive apoptosis. These data indicate that hGrK and mGrK are not cytotoxic towards human and mouse tumor cell lines under conditions that human GrB is.

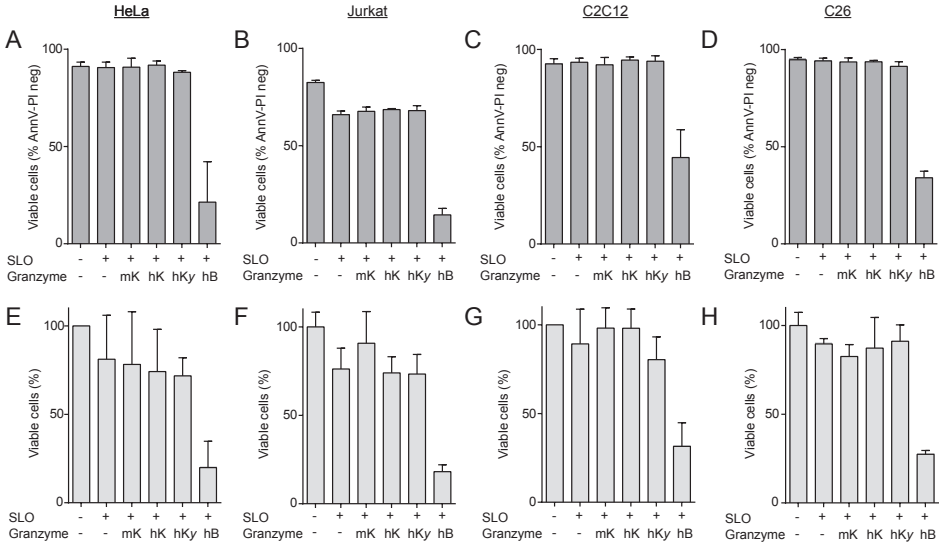

Figure 4.
Both mGrK and hGrK are not cytotoxic towards human and mouse tumor cell lines.

HeLa, Jurkat, C2C12, and C26 cells were treated with mGrK (mK, 700 nM) or hGrK (hK, 700 nM) from *E. coli*, or hGrK (hKy, 700 nM) from *P. pastoris*, in the presence of a sublytic dose of the perforin-analog Streptolysin O (SLO). hGrB (hB, 500 nM on C26 cells and 100 nM on other cells) with SLO was included as positive control. Cells were treated with granzyme for 30 minutes and then incubated another 20 h, after which cell viability was assessed. A-D) Cells were stained with Annexin V (AnnV) and propidium iodide (PI) and analyzed using flow cytometry. AnnV-PI- cells were regarded as living. Results are depicted as mean ± SD (n=4 from two independent experiments; C26 n=3 from one experiment). E-H) Cells were incubated with WST-1 reagent, the OD450 was measured for 60 min, and the slope was calculated as a measure for living cells. Data are depicted as mean ± SD (n=3 from three separate experiments; C26 n=3 from one experiment) and as percentage of cell viability measured in untreated cells.

Discussion

Granzymes are cytotoxic proteases involved in the clearance of tumor cells and virally infected cells (1, 4). In humans, five granzymes exist, while in mice there are 10 (8). Mice are often used as a model when studying granzyme functions. However, it is not known whether murine and human GrK cleave the same set of substrates, or have otherwise similar physiological functions. Here, we show – for the first time – that the degradomes of hGrK and mGrK partially overlap (for 39%), indicating that several substrates are conserved in mouse and human (Figure 3). However, also differences in substrates were also observed, suggesting that the macromolecular substrate specificities of mGrK and hGrK have diverged during evolution, which has also been demonstrated for human granzymes A, B, and M (23-28). Since the physiological functions of hGrK and mGrK may therefore also differ, caution is warranted when extrapolating results obtained in mice to the human situation.

Although it has been demonstrated that all granzymes are cytotoxic (1), the cytotoxic potential of especially GrA and GrK has recently been disputed (5-7). Fan and coworkers have extensively demonstrated that hGrK is pro-apoptotic (9-13), whereas Joeckel et al. (6) show that mGrK is not cytotoxic. Our data indicate that both mGrK and hGrK are not cytotoxic towards the human and mouse tumor cell lines that we tested, using conditions under which GrB induces apoptosis in these lines (Figure 4). No cell death was observed in HeLa, Jurkat, C2C12, or C26 cells after treatment with either mGrK or hGrK. Although GrB induced apoptosis under the experimental conditions used, it remains important to demonstrate intracellular delivery of GrK. Furthermore, knockout or overexpression of GrK in cytotoxic cells, potentially in combination with other granzymes, and the effects thereof on tumor cell killing in vitro and in vivo is required to unequivocally demonstrate whether GrK is pro-apoptotic.

We show that mGrK and hGrK both cleave hnRNP K in tumor cell lysate (Figure 2). HnRNP K is a pan-granzyme substrate that is cleaved by all five human granzymes under physiological conditions, and is essential for tumor cell survival (22). Under certain conditions, loss of hnRNP K in tumor cells leads to spontaneous caspase-dependent and caspase-independent cell death (22). Furthermore, cleavage of SET disrupts the nucleosome assembly function of this protein, causing nuclear relocation of the DNAse NM23H1, which leads to generation of DNA nicks and apoptosis (10). Since mGrK and hGrK both cleave SET and hnRNP K, it is unclear why mGrK and hGrK do not induce cell death in tumor cells under the conditions that we tested. Additional research is required to reconcile the different effects that cleavage of SET and hnRNP by granzymes apparently may have in cells.

Our preliminary results indicate that mGrK and hGrK are not cytotoxic, and these granzymes may thus have alternative or additional roles in vivo. Levels of soluble GrK are elevated in the circulation of patients with infections (16, 29-31), indicating that GrK may have extracellular functions during inflammation. Indeed, mGrK releases mature IL-1β from mouse macrophages activated with LPS (6). hGrK induces IL-6 and IL-8 secretion from human lung fibroblasts (15), and hGrK synergistically potentiates LPS-induced cytokine release from human monocytes (16). Furthermore, mGrK and hGrK protect against influenza virus infection (6, 17). We are currently comparing the anti-viral and cytokine-inducing functions of hGrK and mGrK in vitro, which will teach us whether we should proceed to develop conventional (GrK knockout) mouse models to study these intriguing immune-modulatory functions of GrK.

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ELEVATED GRANZYME M-EXPRESSING LYMPHOCYTES DURING CYTOMEGALOVIRUS LATENCY AND REACTIVATION AFTER ALLOGENEIC STEM CELL TRANSPLANTATION

Niels Bovenschen[1,], Sanne N.M. Spijkers[2], Annette C. Wensink[1], Ingrid M.M. Schellens[2,3], Robert van Domselaar[1], and Debbie van Baarle[2,3]

1 Department of Pathology, University Medical Center Utrecht, 3584 CX, Utrecht, The Netherlands

2 Department of Immunology, University Medical Center Utrecht, 3584 EA, Utrecht, The Netherlands

3 Department of Internal Medicine and Infectious Diseases, University Medical Center Utrecht, 3584 EA, Utrecht, The Netherlands

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Abstract

Granzymes are serine proteases involved in the clearance of tumor cells and virally inHuman cytomegalovirus (HCMV) reactivation can cause serious complications in allogeneic stem cell transplantation (SCT) patients. HCMV is controlled by cytotoxic lymphocytes that release antiviral granzymes. Recently, we have demonstrated that granzyme M (GrM) inhibits HCMV replication in vitro, however the physiological role of GrM and its cellular distribution during HCMV infection remains unknown. Here, we examined GrM expression in lymphocyte populations during HCMV infection. The percentage of GrM-expressing effector-memory CD4+ T-cells was higher in HCMV latently-infected healthy individuals compared to uninfected individuals. SCT recipients had higher percentages of GrM-expressing CD4+ T, CD8+ T, γδT, and NKT cells. Despite lower total T-cell numbers, HCMV reactivation in SCT patients specifically associated with higher percentages of GrM-expressing CD4+ (total and central-memory) T-cells. GrM was elevated in plasma during HCMV reactivation, pointing to extracellular perforin-independent functions of GrM. We conclude that GrM may be important in regulating HCMV latency and reactivation in SCT patients.

Abbreviations: HCMV, human cytomegalovirus; SCT, stem cell transplantation; GrM, granzyme M; GrB, granzyme B.

Introduction

Human cytomegalovirus (HCMV) is a widespread β-herpesvirus that causes lifelong asymptomatic infections in humans (1, 2). However, HCMV infections can lead to severe disease in immunocompromised individuals. Particularly, reactivation from latency can cause severe complications in allogeneic hematopoietic stem cell transplant (SCT) recipients due to an impaired immune system caused by conditioning regimens and T-cell depletion therapy in combination with immunosuppressive drugs (3, 4). Differences in T-cell reconstitution and the antiviral immune response of individual patients likely discriminate between efficient immunological control of HCMV infections and reactivation.

HCMV infections are generally controlled by cytotoxic lymphocytes, including cytotoxic T lymphocytes, NK cells, γδT-cells, and NKT-cells (1). This is illustrated by strong virus-specific CD4+ and/or CD8+ T-cell responses that are directed to a broad range of viral epitopes with marked hierarchies of immunodominance (5-7). Cytotoxic lymphocytes exert their antiviral functions predominantly through releasing interferon-γ (IFN-γ) and the granule-exocytosis pathway (8-11). The latter pathway is characterized by release of the pore-forming protein perforin and a family of homologous serine proteases, called granzymes. Perforin allows the entry of granzymes into the target cell where granzymes can mediate their antiviral effects by cleaving host cell or viral proteins resulting in clearance of the virus or blockade of its replication. In humans, five granzymes (GrA, GrB, GrH, GrK, and GrM) have been identified with distinct substrate specificities and antiviral pathways (12). Besides pro-apoptotic effects of granzymes, we have recently shown that GrM can also inhibit HCMV replication in the absence of cell death in vitro, mediated through cleavage of viral protein pp71 and host cell heterogeneous nuclear ribonucleoprotein K (13, 14).

It has been demonstrated that GrB-positive HCMV-specific CD8+ and CD4+ (effector) T lymphocytes emerge after primary HCMV infection and are maintained during latency, suggesting a role for GrB in HCMV infections in vivo (15-19). Although GrM-deficient mice are more susceptible to murine CMV infections (20), the physiological relevance of GrM as well as its (cellular) distribution during HCMV infections in humans remains unknown. This knowledge is of importance to further uncover the effector immune response against HCMV. In the present study, we addressed the question how GrM expression is distributed among cytotoxic lymphocyte subsets and in plasma during HCMV latency and reactivation in vivo. To this end, we used HCMV-(un)infected healthy individuals as well as patients that received allogeneic SCT, which are ideal to discriminate between HCMV latency and reactivation.

We conclude that GrM is expressed by lymphocyte populations that play a role in HCMV immunity, suggesting that GrM is important in regulating HCMV latency in healthy individuals and in controlling HCMV reactivation in SCT patients. Furthermore, GrM levels were elevated in plasma during HCMV reactivation, pointing to a previously unrecognized extracellular role of GrM in controlling HCMV infection.

Methods

PATIENT AND TRANSPLANTATION CHARACTERISTICS

Forty patients receiving allogeneic SCT were prospectively followed up for 12 weeks after SCT and the characteristics have previously been summarized (21). For this study, we used a selection of 31 of these 40 patients based on the availability of patient material (Table 1), which were weekly analyzed for HCMV-reactivation and lymphocyte subsets. The underlying hematological disease consisted for the majority of acute lymphatic leukemia, acute myeloid leukemia, multiple myeloma and non-Hodgkin's lymphoma. Patients received an allogeneic SCT from either a related (n = 9) or an unrelated (n = 22) donor. The stem cell source was mostly peripheral blood, and for most patients a nonmyeloablative conditioning regimen was used. In vivo T-cell depletion consisting of ATG was added to the conditioning regimen for patients receiving grafts from unrelated or HLA-mismatched donors. Whole blood samples were routinely drawn weekly from all patients to determine HCMV DNA loads. Plasma was removed for HCMV determination. Whole blood was used to determine absolute CD4+ and CD8+ T-cell counts and the leftover blood was used to isolate peripheral blood mononuclear cells (PBMCs). PBMCs stored in liquid nitrogen were used for this study. In total, 14 of 31 patients had evidence of a HCMV reactivation. Of those patients, 6 had a minor reactivation (denoted as +; peak viral load between 50-1000 copies/mL) and 8 had a major reactivation (denoted as ++; peak viral load > 1000 copies/mL). Written informed consent was obtained from all patients, in accordance with the Declaration of Helsinki (Medical ethical committee of the UMCU nr 05174).

HCMV STATUS AND MONITORING

HCMV monitoring was based on a real-time TaqMan HCMV DNA PCR assay in ethylenediaminetetraacetic acid (EDTA)-treated plasma, which was prospectively performed weekly for all patients until 4 months after transplantation. Patients were treated preemptively with valganciclovir (900 mg twice daily) when the HCMV DNA load exceeded 500 copies/mL. Valaciclovir was given to all patients prophylactically (500 mg twice daily). Viral reactivation and/or infection was defined as an HCMV load exceeding the detection limit of 50 copies/mL in plasma (21). Because no plasma or serum was available from healthy controls, HCMV status was determined by analysis of the presence of a specific T-cell response by IFN-γ Elispot assays and 12 day expansion assays. IFN-γ Elispot assays were performed as previously described (22). Twelve day expansion assays were performed as previously described (23), except cells were stimulated with HCMV peptide pools.

GRM MEASUREMENTS AND ABSOLUTE T-CELL COUNT DETERMINATION

Frozen PBMCs were thawed in RPMI supplemented with 20% fetal calf serum (FCS) and pen-strep, washed in RPMI supplemented with 10% FCS and pen-strep, counted, and washed in PBS supplemented with 0.5% bovine serum albumin (BSA) and 0.1% sodiumazide. 1x106 cells were used for flowcytometric assessment of GrM and GrB expression within different lymphocyte subsets. Cells were incubated with one of three cell-surface marker combinations to determine GrM and GrB protein levels in different cell subsets; 1. CD3-PerCP, CD56-APC (Biolegend), CD8-AmCyan (V500) and CD16-Pacific Blue (BD); 2. CD3-PerCP, CD8-V500, CD27-PE-Cy7 (eF780) (eBioscience) and CD45RO-APC-Cy7 (BD); 3. CD3-Pacific Blue (eF450) (eBioscience), CD8-V500, TCR γδ-APC (BD) and TCR Vδ2-PerCP (Biolegend). Cells were subsequently permeabilized and lysed for intracellular granzyme staining. After permeabilization, cells were incubated with Alexa Fluor 488-conjugated anti-GrM (clone 4B2G4), generated as described previously (24), and GrB-PE (Sanquin). Samples were measured on an LSR II FACS machine and analyzed using FACSdiva software. In almost all samples at least 200,000 events were acquired. Intracellular perforin staining was performed as previously described (21). Absolute T-cell numbers per milliliter of whole blood were determined as previously described (21). GrM plasma levels were measured by enzyme-linked immunosorbent assay according to manufacturers' instructions (USCN Life Science Inc.).

STATISTICAL ANALYSIS

Median percentages of granzyme-positive cells were determined and all data were considered non-Gaussian distributed. The Mann-Whitney U test was used to compare granzyme protein levels between individual cell subsets. Spearman was used to calculate correlations. $P<0.05$ was considered statistically significant.

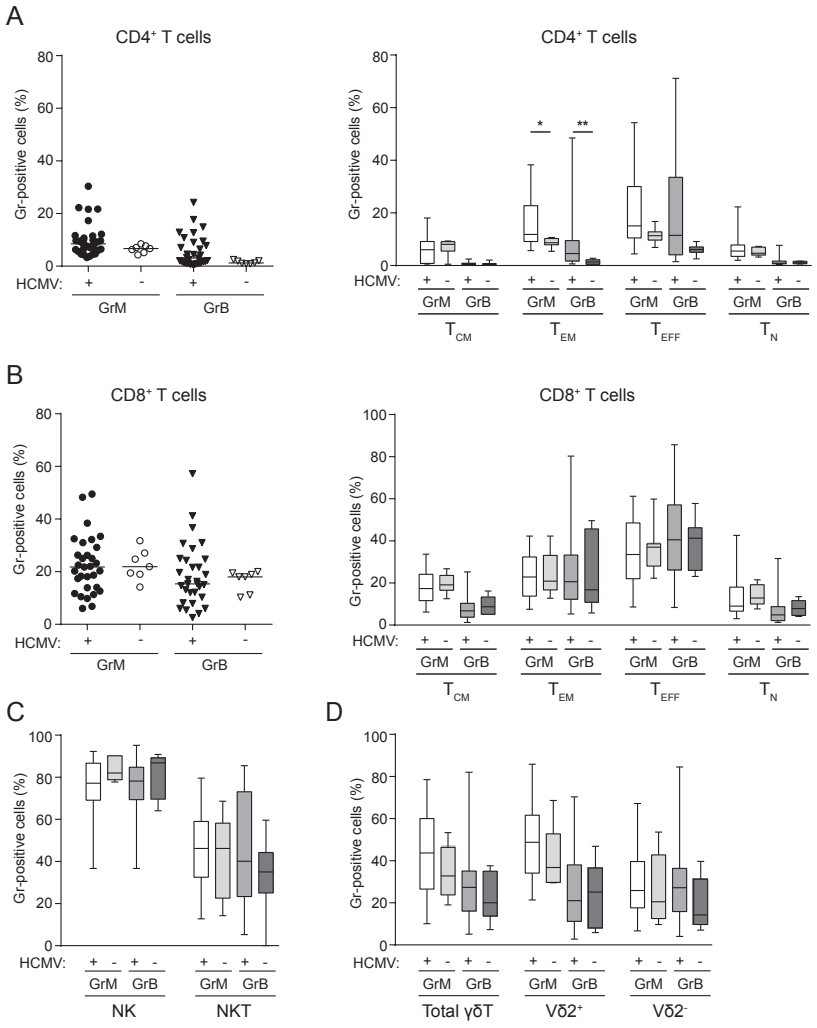

Figure 1.
GrM and GrB expression in lymphocytes during HCMV latency.

Median percentages of GrM- and GrB-positive cells were determined in lymphocyte subsets of HCMV latently-infected (n = 31, +) and uninfected (n = 7, -) healthy individuals. (A) Median percentages of GrM- and GrB-positive T-cells in total CD4+ and CD8+ T-cells, (B) CD4+ and CD8+ T-cell phenotypic subsets, (C) NK and NKT-cells, and (D) γδT-cell subsets. In box plots, the horizontal line in the middle of each box indicates the median; the top and bottom borders of the box mark the 75th and 25th percentiles, respectively; and the whiskers above and below the box mark the range. (TCM, central memory T-cells; TEM, effector memory T-cells; TEFF, effector T-cells; TN, naive T-cells) *$p < 0.05$, **$p < 0.01$

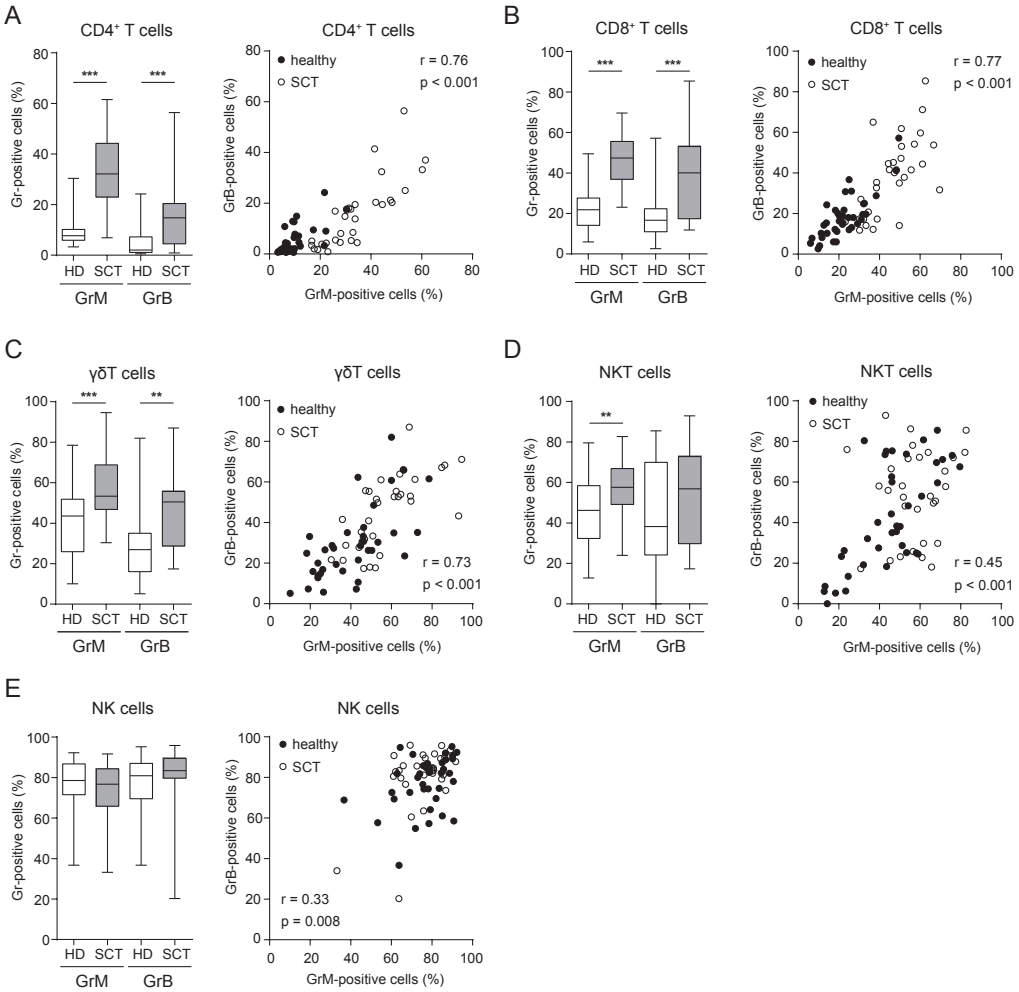

Figure 2.

Correlation between GrM and GrB expression in lymphocyte subsets of healthy individuals and SCT patients.

GrM and GrB expression was analyzed in lymphocyte subsets of both healthy donors (n = 38) and SCT patients (n = 31) throughout 12 weeks follow-up. Mean percentages of granzyme-positive cells are depicted in both box plots and scatter plots. In scatter plots, the median percentages of GrM-positive cells were plotted against the median percentages of GrB-positive cells for (A) CD4+ T-cells (healthy donors, r = 0.6884, p < 0.0001; SCT patients, r = 0.8725, p < 0.0001), (B) CD8+ T-cells (healthy donors, r = 0.7255, p < 0.0001; SCT patients, r = 0.6668, p < 0.0001), (C) NK cells (healthy donors, r = 0.3734, p = 0.0209; SCT patients, r = 0.3180, p = 0.0813), (D) NKT-cells (healthy donors, r = 0.6115, p < 0.0001; SCT patients, r = 0.07210, p = 0.7050), and (E) γδT-cells (healthy donors, r = 0.6921, p < 0.0001; SCT patients, r = 0.7048, p < 0.0001). In box plots, the horizontal line in the middle of each box indicates the median; the top and bottom borders of the box mark the 75th and 25th percentiles, respectively; and the whiskers above and below the box mark the range. **p < 0.01, ***p < 0.001

Results

ANALYSIS OF GRANZYME PROTEIN LEVELS IN LYMPHOCYTE SUBSETS

To assess the role of HCMV latency and reactivation on GrM and GrB protein levels in different lymphocyte populations, we identified αβT cells, γδT cells and NKT cells within the CD3+ lymphocyte population by flow cytometry. αβT cells were first divided into CD4+ (CD3+CD8-) and CD8+ (CD3+CD8+) T cells, and further subdivided into the naive (TN) (CD27+CD45RO-), effector (TEFF) (CD27-CD45RO-), effector memory (TEM) (CD27+CD45RO+), and central memory (TCM) (CD27+CD45RO+) T cell phenotypic subsets (see supplementary Figure 1A). γδT cell subsets (CD3+Vδ2- and CD3+Vδ2+) and NKT cells (CD3+CD8+CD56+) were also identified (see supplementary Figures 1B-C). Within the CD3- lymphocyte population, NK cells were defined as CD3-CD16+CD56+ (see supplementary Figure 1B). In each lymphocyte subset, intracellular GrM and GrB protein levels were analyzed (see supplementary Figures 1D-F).

HIGHER PERCENTAGES OF GRM- AND GRB-POSITIVE EFFECTOR MEMORY CD4+ T-CELLS IN HCMV LATENTLY-INFECTED HEALTHY INDIVIDUALS

To examine the long-term effects of HCMV infection on GrM and GrB levels in lymphocytes, we assessed granzyme protein levels in lymphocyte populations of 31 HCMV latently-infected healthy individuals and 7 uninfected healthy individuals. No significant differences in the percentage of GrM- and GrB-positive cells could be observed within total CD4+ (Figure 1A) and total CD8+ (Figure 1B) T-cell populations between both groups. When phenotypic subsets within the CD4+ and CD8+ T-cell compartments were analyzed, however, we observed significantly higher percentages of GrM- and GrB-positive CD4+ TEM-cells (Figure 1A). Percentages of granzyme-positive cells within CD8+ T-cell subsets did not differ between latently-infected and uninfected individuals (Figure 1B), nor were there any differences observed within the NK and NKT-cell populations (Figure 1C), or γδT-cell subsets (Figure 1D). These data point to a protective role of GrM- and GrB-positive CD4+ TEM cells during HCMV latency.

HIGHER PERCENTAGES OF GRANZYME-POSITIVE CD4+ T, CD8+ T, NKT, AND γδT-CELLS IN SCT PATIENTS

GrM and GrB protein levels were measured weekly in lymphocytes of SCT patients after transplantation (n=31) and median percentages of granzyme-positive lymphocytes throughout a 12 week follow-up period were determined in order to avoid fluctuations based on variable onset of reactivation and T-cell reconstitution. Percentages of both GrM- and GrB-positive cells were higher within the CD4+ T-cell (Figure 2A), CD8+ T-cell (Figure 2B)

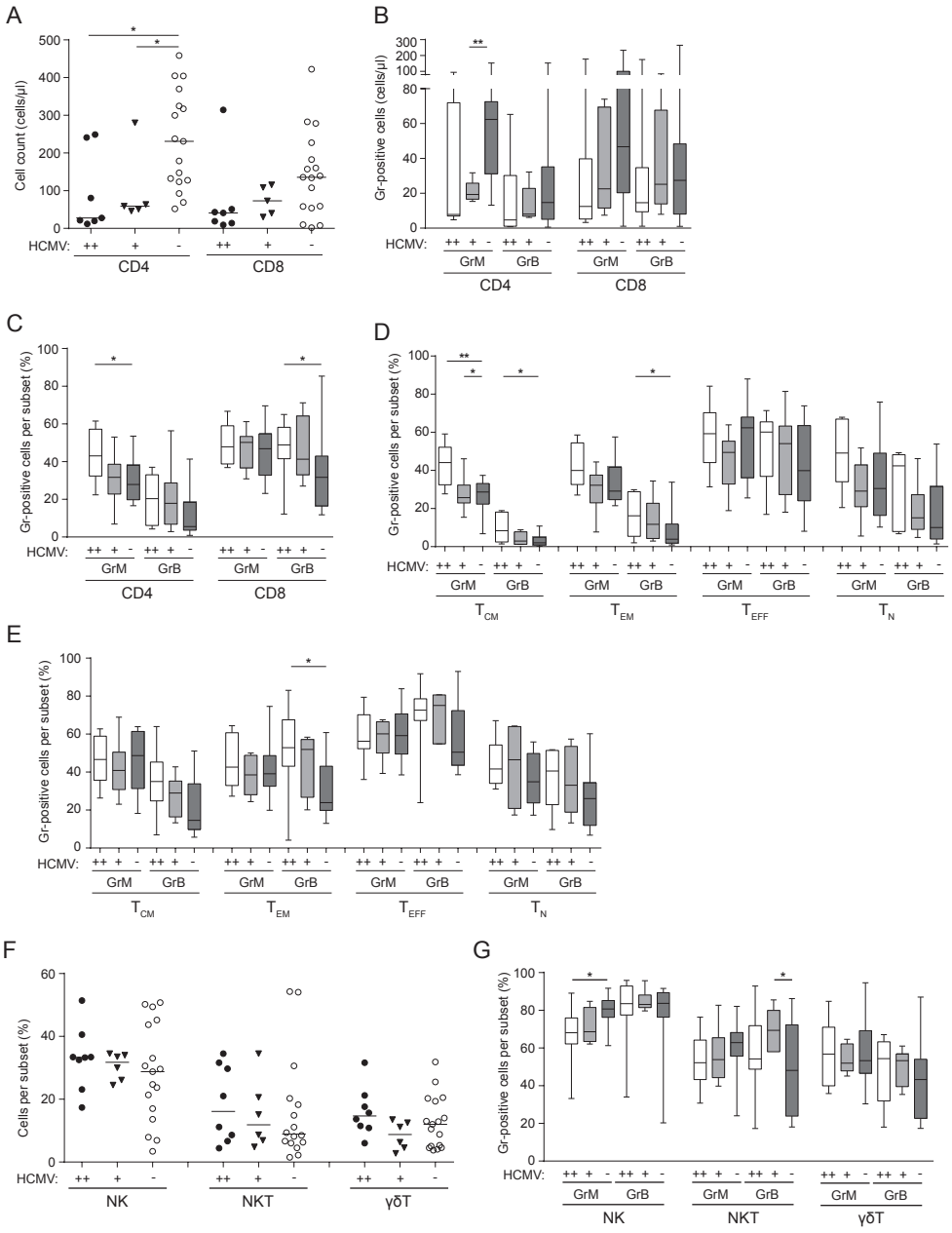
A
Cell count (cells/µl)
HCMV:
CD4
CD8
B
Gr-positive cells (cells/µl)
GrM
GrB
C
Gr-positive cells per subset (%)
D
T_{CM}
T_{EM}
T_{EFF}
T_N
E
F
Cells per subset (%)
NK
NKT
γδT
G

Figure 3.
Association between granzyme expression in lymphocyte subsets and HCMV reactivations in SCT patients.

In total, 14 of 31 patients had evidence of a HCMV reactivation. Of those patients, 6 had a minor reactivation (peak viral load between 50-1000 copies/mL) and 8 had a major reactivation (peak viral load > 1000 copies/mL). GrM and GrB expression was analyzed in lymphocyte subsets of SCT patients that experienced major (n = 8, ++), minor (n = 6, +), or no (n = 17, -) HCMV reactivation throughout 12 week follow-up period. (A) Median absolute numbers of total CD4+ and CD8+ T-cells or (B) GrM- and GrB-positive CD4+ and CD8+ T-cells. (C) Median percentages of GrM- and GrB-positive cells within the total CD4+ or CD8+ T-cell population. (D) Median percentages of GrM- and GrB-positive cells within CD4+ T-cell phenotypic subsets. (E) Median percentages of GrM- and GrB-positive cells within CD8+ T-cell phenotypic subsets. (F) Median percentages of NK cells within the total CD3- lymphocyte population, and NKT and γδT-cells within the total CD3+ lymphocyte population. (G) Median percentages of GrM- and GrB-positive cells within the total NK, NKT and γδT-cell population. In box plots, the horizontal line in the middle of each box indicates the median; the top and bottom borders of the box mark the 75th and 25th percentiles, respectively; and the whiskers above and below the box mark the range. (TCM, central memory T-cells; TEM, effector memory T-cells; TEFF, effector T-cells; TN, naive T-cells) *$p < 0.05$, **$p < 0.01$

and γδT-cell (Figure 2E) populations of SCT patients compared to healthy individuals. Within the NKT-cell population, only the percentage of GrM-positive, but not GrB-positive, cells was higher in SCT patients compared to healthy individuals (Figure 2D). No differences were observed within the NK cell population between SCT patients and healthy individuals (Figure 2C). In all lymphocyte populations, there was a significant correlation between the percentages of GrM- and GrB-positive lymphocytes. These data indicate that shortly after SCT transplantation percentages of granzyme-positive cells within the CD4+ T-cell, CD8+ T-cell, NKT-cell, and γδT-cell compartments are increased.

GRM- AND GRB-POSITIVE LYMPHOCYTES ARE ASSOCIATED WITH HCMV REACTIVATION

To determine whether GrM and GrB are associated with HCMV reactivation, median numbers of total cells and granzyme-positive cells per lymphocyte subset throughout follow-up were determined and correlated to HCMV reactivation. Absolute numbers of CD4+ T-cells were significantly lower in SCT patients with minor (+) and major (++) HCMV reactivation compared to patients with no reactivation (-) (Figure 3A), and as a result there was a significant lower number of total GrM-positive CD4+ T-cells (Figure 3B). We further analyzed differences in the percentages of granzyme-expressing lymphocytes throughout follow-up and correlated this to minor or major HCMV reactivation (Figure 3C). Interestingly, the percentage of GrM-positive CD4+ T-cells was significantly higher in patients with major

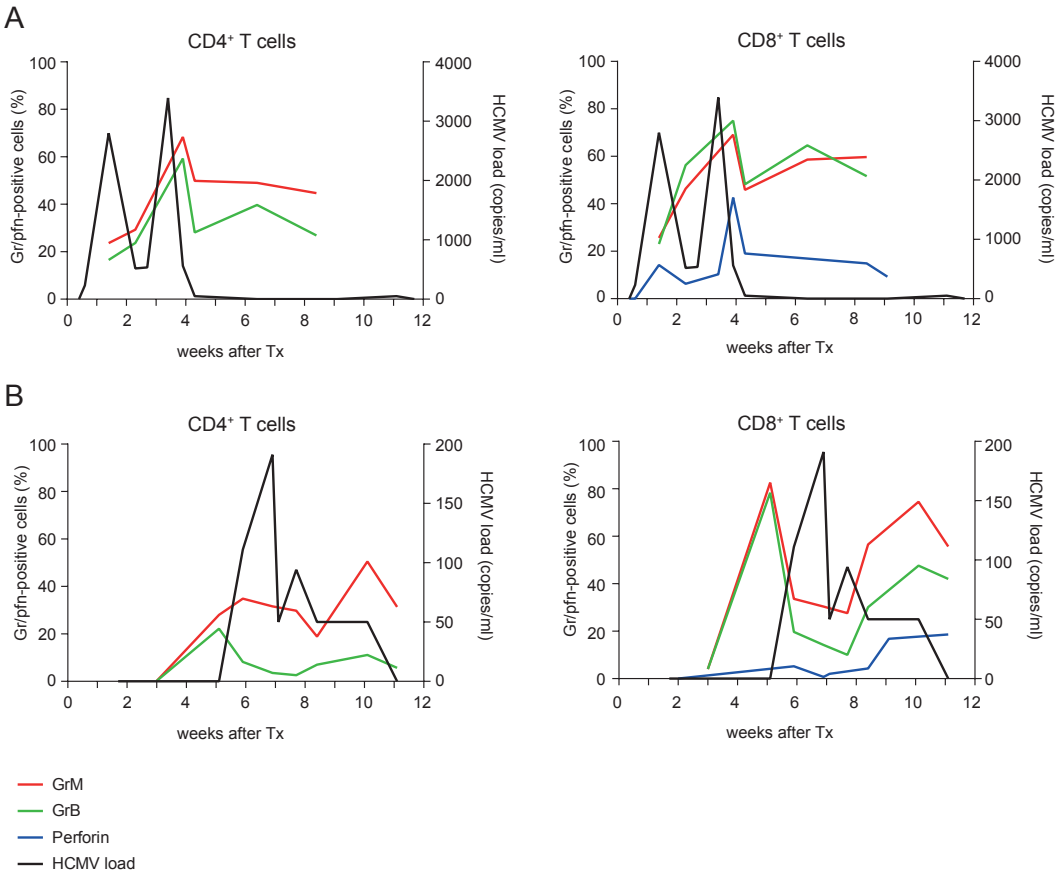

Figure 4.
Longitudinal analysis of granzyme expression in lymphocytes of individual SCT patients with HCMV reactivation.

GrM and GrB expression was analyzed in CD4+ and CD8+ T-cells of SCT patients with HCMV reactivation (n = 14) throughout 12 week follow-up period. Perforin expression in CD8+ T-cells was analyzed previously.(21) Of note, perforin expression was not measured in CD4+ T-cells. (A) Representative graphs of a SCT patient with increased percentages of granzyme-expressing T-cells that coincided with an increase in HCMV load. (B) Representative graphs of a SCT patient with decreased percentages of granzyme-expressing T-cells that coincided with an increase in HCMV load.

HCMV reactivation compared to patients without reactivation. There were no differences in the percentage of GrM-positive CD8+ T-cells between patients with or without HCMV reactivation. Unlike GrM, the percentage of GrB-positive CD8+ T-cells was significantly higher in SCT patients with major HCMV reactivation compared to patients without reactivation. Analysis of the T-cell phenotypic subsets showed that the higher percentage of GrM-positive CD4+ T-cells in SCT patients with major HCMV reactivation is mostly reflected in the central memory CD4+ T-cell pool (Figure 3D). In addition, percentages of GrB-expressing central and CD4+ TEM cells, and CD8+ TEM cells were higher in SCT patients with major HCMV reactivation (Figures 3D,E). Analysis of NK, NKT and γδT-cells showed no significant differences in the percentages of total cells (Figure 3F), but the percentage of GrM-positive NK cells was lower in SCT patients with major HCMV reactivation compared to patients without reactivation (Figure 3G). Altogether, these data show that not the absolute numbers but rather the percentages of granzyme-positive cells of several lymphocyte populations are associated with HCMV reactivation. Whereas GrB associates with CD4+ and CD8+ T-cell responses, GrM associates with CD4+ T-cells and NK cell responses toward HCMV reactivation.

DISTINCT CD4+ AND CD8+ T-CELL RESPONSES IN SCT PATIENTS WITH HCMV REACTIVATION

In our previous analysis, we assessed the median total numbers or percentages of granzyme-positive lymphocytes throughout the 12 week follow-up period. However, when we analyzed patients on an individual basis, distinct immune response patterns could be observed. Figure 4 shows representative graphs of individual patients. On the one hand, an increase in GrM- and GrB-positive CD4+ and CD8+ T-cells coincided with an increase in HCMV viral load (Figure 4A) in 8 of the 14 reactivating patients. This could indicate that the increase in granzyme-positive CD4+ and CD8+ T-cells leads to control of HCMV reactivation. On the other hand, granzyme-positive CD4+ and CD8+ T-cells were inversely correlated to HCMV viral load in 4 other reactivating patients, characterized by decreased percentages of GrM- and GrB-positive CD4+ and CD8+ T-cells when HCMV viral load is increasing (Figure 4B). This may suggest that either HCMV reactivation leads to suppression of T-cell responses or that a decrease in T-cell responses causes HCMV reactivation. As HCMV DNA load decreases later on when GrM- and GrB-positive T-cells increase, the latter may be more likely. Notably, the kinetics of GrM-expressing T lymphocytes coincided with the kinetics of GrB-positive, and to a lesser extent, perforin-expressing T lymphocytes. These data demonstrate the diversity and complexity of human T-cell responses during HCMV reactivation in SCT patients.

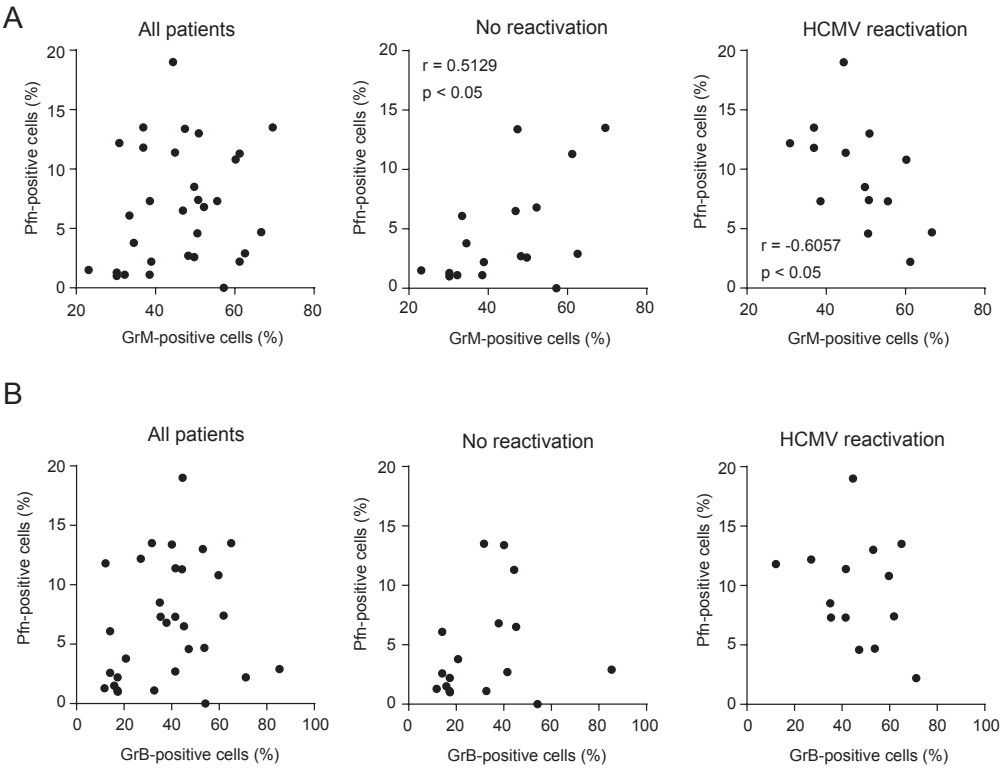

Figure 5.
Correlation between granzyme and perforin expression in CD8+ T-cells of SCT patients.

GrM and GrB expression was analyzed in CD8+ T-cells of all SCT patients (n = 31), SCT patients without HCMV reactivation (n = 17), or patients with HCMV reactivation (n = 14) throughout 12 week follow-up period. Perforin expression was analyzed previously (21). (A) The median percentages of GrM-positive CD8+ T-cells were plotted against the median percentages of perforin-positive CD8+ T-cells (All patients, r = 0.2119, p = 0.2525; No reactivation, r = 0.5219, p = 0.0353, HCMV reactivation, r = -0.6057, p = 0.0217). (B) The median percentages of GrB-positive CD8+ T-cells were plotted against the median percentages of perforin-positive CD8+ T-cells (All patients, r = 0.2454, p = 0.1833; No reactivation, r = 0.2589, p = 0.3157, HCMV reactivation, r = -0.1980, p = 0.4974).

GRM BUT NOT GRB CORRELATES WITH PERFORIN EXPRESSION IN CD8+ T-CELLS IN SCT PATIENTS

Antiviral functions of granzymes can be both dependent and independent of perforin (8). Recently, we have demonstrated that perforin expression in CD8+ T-cells correlates with HCMV reactivation in SCT patients (21). Here, we showed that granzyme-positive CD8+ T-cells also associated with HCMV reactivation (Figure 2). Therefore, we analyzed the correlation between granzyme-positive and perforin-positive CD8+ T-cells. There was no significant correlation between the percentages of GrM-positive CD8+ T-cells and the percentage of perforin-positive CD8+ T-cells when all SCT patients were analyzed (Figure 5A). However, when SCT recipients were divided into patients with or without HCMV reactivation, there was a positive correlation between the percentage of GrM-positive and perforin-positive CD8+ T-cells in SCT patients without HCMV reactivation and a negative correlation in patients with reactivation. There were no significant correlations between the percentages of GrB- and perforin-positive CD8+ T-cells in SCT patients (Figure 5B). These data suggest that GrM released by CD8+ T-cells during HCMV reactivation may predominantly exert its antiviral effects in a perforin-independent manner.

PLASMA GRM IS ELEVATED DURING HCMV REACTIVATION

If GrM has perforin-independent extracellular functions, one would expect GrM levels in plasma to be elevated. Therefore, we measured plasma GrM in three individual SCT patients with HCMV reactivation (Figure 6). Interestingly, the plasma GrM peak coincided with plasma HCMV load (Figure 6). This suggests that either the intracellular GrM pool is released from these T-cells making them less positive for intracellular GrM and/or that GrM is secreted by NK(T) cells, γδT-cells, or other cell types. These data indicate that GrM plasma levels are elevated during HCMV reactivation in SCT patients and may point to an extracellular antiviral function of GrM.

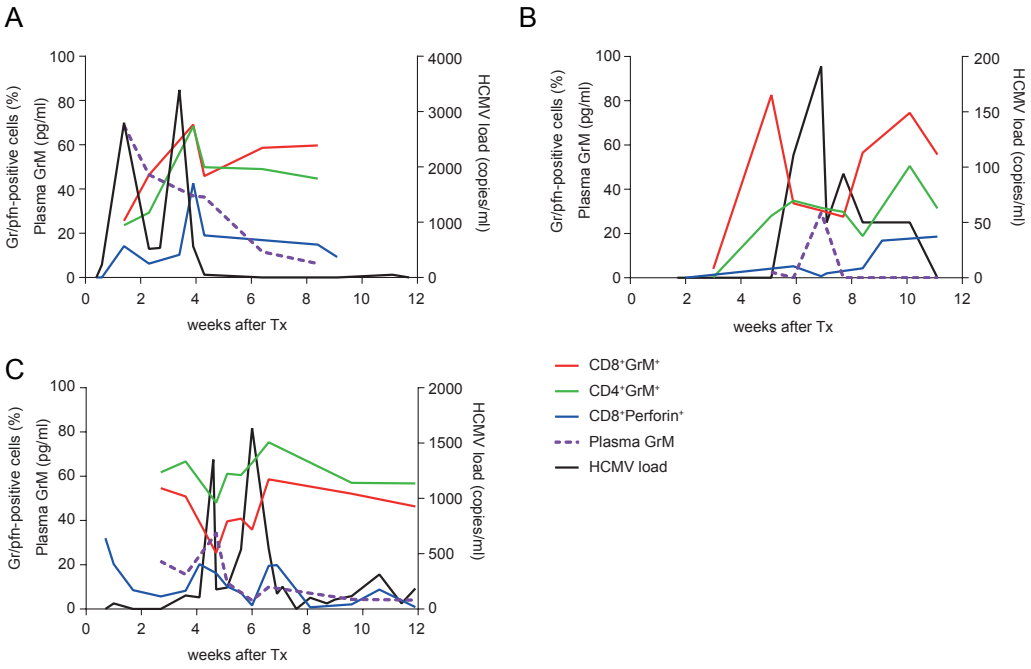

Figure 6.
Plasma GrM is elevated during HCMV reactivation.

(A-C) Three representative SCT patients with HCMV reactivation throughout 12 week follow-up period were measured for intracellular GrM expression in CD4+ and CD8+ T-cells, perforin expression in CD8+ T-cells, HCMV load, and plasma levels of GrM (pg/ml).

Discussion

HCMV infection leaves a fingerprint in the total T-cell pool characterized by high numbers of GrB-expressing CD4+ and CD8+ TEM cells during latency in HCMV-seropositive healthy individuals (16, 25, 26). In this study, we confirmed the higher numbers of GrB-positive CD4+ TEM cells during HCMV latency (Figure 1) (16). In addition, we showed for the first time that the percentage of GrM-positive CD4+ TEM cells was higher in HCMV latently-infected compared to uninfected healthy individuals (Figure 1). Thus, our data indicate that primary HCMV infection and/or latency increases the frequency of both circulating GrM- and GrB-positive CD4+ T-cells. The importance of CD4+ T-cell responses during HCMV latency is supported by several studies. HCMV latently-infected cells recruit and counteract CD4+ T-cell responses through expression of the viral IL-10-encoding gene UL111A and other immunosuppressive factors (27, 28). During latency, dominant HCMV-specific GrB-expressing CD4+ T-cell clones emerge that are poorly represented in the acute phase of HCMV infections and have immediate cytotoxic capacity towards HCMV antigen-loaded target cells (16, 17, 29). Also, it has been shown that young children have impaired HCMV-specific CD4+ T-cell responses, predominantly reduced HCMV-specific CD4+ TEM cell responses, but normal HCMV-specific CD8+ T-cell responses, which coincided with persistent HCMV replication (30). Finally, HCMV-specific IFN-γ-producing CD4+ T-cells were shown to contribute to protection from HCMV disease in HIV-infected patients (31). In this perspective, circulating granzyme-expressing (effector memory) CD4+ T-cells may contribute to the maintenance of HCMV latency.

Reactivation of HCMV from latency can cause serious disease in immunocompromised patients, for instance following allogeneic SCT (3, 4). We showed that HCMV reactivation in SCT patients associated with lower numbers of CD4+ and CD8+ T-cells (Figure 3), confirming that T-cell responses are important in preventing HCMV reactivation (1). Interestingly, percentages of granzyme-positive T-cells were associated with HCMV reactivation. Although absolute numbers of both CD4+ and CD8+ T-cells were markedly lower in SCT patients with HCMV reactivation, the percentage of GrM-positive (total and central memory) CD4+ T-cells was higher (Figure 3). Our observation that GrM-expressing central memory CD4+ T-cells are associated with HCMV reactivation is remarkable, since it is believed that these cells have no effector functions (17, 32, 33). Even though there are no noteworthy differences in the distribution of CD4+ T-cell phenotypic subsets (Figure 3), it could indicate antigen-driven stimulation and differentiation of central memory CD4+ T-cells into effector memory and/or

effector CD4+ T-cells in response to major HCMV reactivation. Alternatively, GrM may play an extracellular perforin-independent immuno-regulatory role following cellular secretion. This is consistent with our observations that there was a negative correlation in patients with detectable HCMV reactivation between GrM- and perforin-positive CD8+ T-cells (Figure 5), and that GrM levels in plasma were elevated during HCMV reactivation in 3 out of 3 patients that were measured (Figure 6). Higher percentages of GrB-positive CD4+ and CD8+ T-cells in SCT patients with HCMV reactivation (Figure 3) is in agreement with previous studies that identified the emergence of GrB-positive HCMV-specific CD4+ and CD8+ TEM cells after primary HCMV infection in renal transplant recipients (15, 16, 19). Interestingly, percentages of GrM-positive NK cells were lower in SCT patients with HCMV reactivation (Figure 3). Altogether, these associations could point to a two-step model in which 1) HCMV reactivation is triggered by inadequate NK, CD4+ and CD8+ T-cell responses in the initial phase, and 2) secondary T-cell responses with increased granzyme-positive CD4+ and CD8+ T-cells exert antiviral activities to control HCMV infection. However, longitudinal analysis of individual SCT patients showed that T-cell responses greatly differ between patients (Figure 4), which is most likely the result of various variable transplantation-related factors. This complicates the establishment of a general model for initiation and immune-regulated control of HCMV reactivation in SCT patients and emphasizes the complexity of the interplay between the host immune response and virus.

We showed differences in the median percentages of granzyme-expressing cells within the total CD4+ and CD8+ T-cell population as well as in the phenotypic subsets between SCT patients with and without HCMV reactivation. Since we analyzed total T-cell populations, differences could be more pronounced within the HCMV-specific T-cell compartment. Unfortunately, analysis of HCMV-specific T-cells in large patient cohorts is complicated by, amongst others, the high variety of HLA-specific HCMV epitopes between individuals (5-7). Studying immune responses during HCMV reactivation in SCT patients provides valuable information to our understanding how the immune system controls HCMV infections and might lead to new antiviral therapies. Cellular immunotherapy is a promising approach to treat HCMV reactivation in SCT patients (34). Adoptive transfer of donor-derived HCMV-specific T-cells has already been proven to be a safe and effective treatment for HCMV infection (35-40). It would be worthwhile to investigate whether increasing granzyme protein levels within these HCMV-specific T-cells could provide improved protection to HCMV reactivation.

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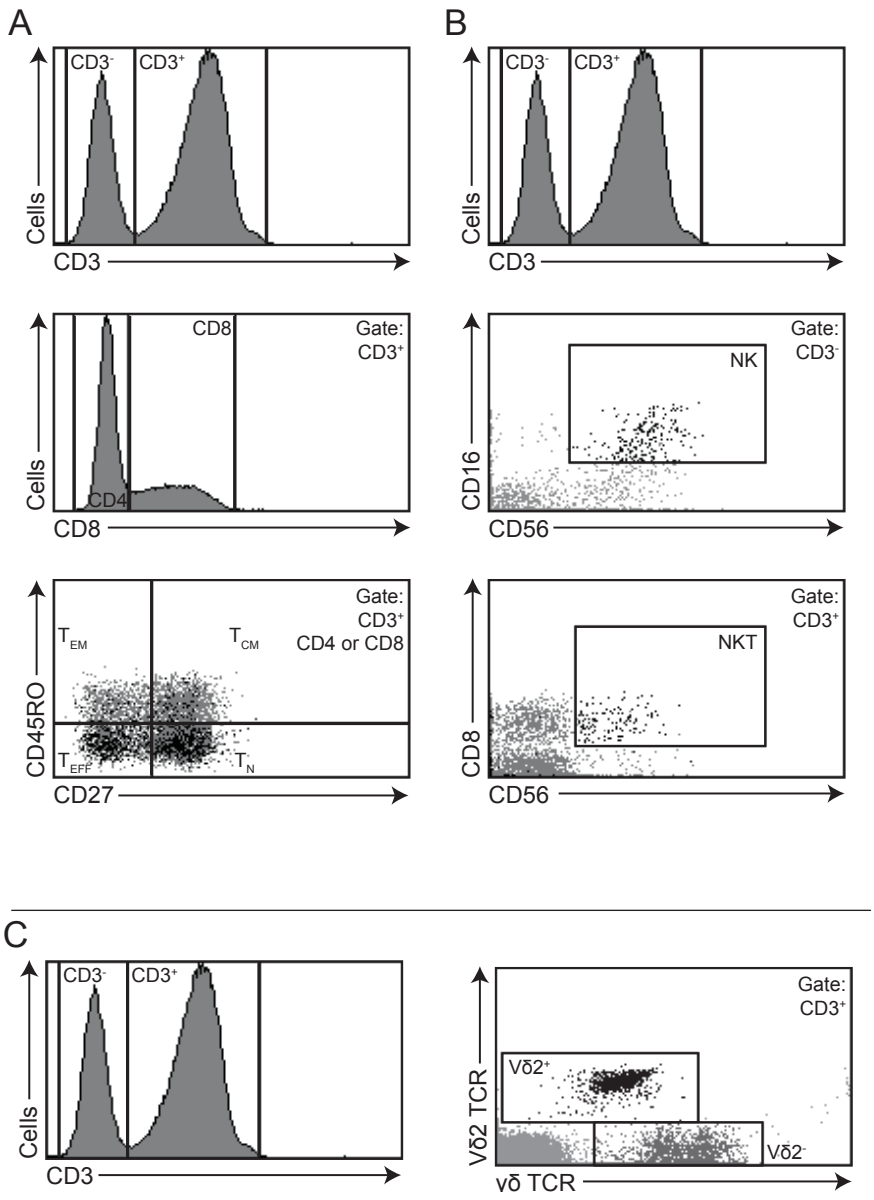
Patient Characteristics

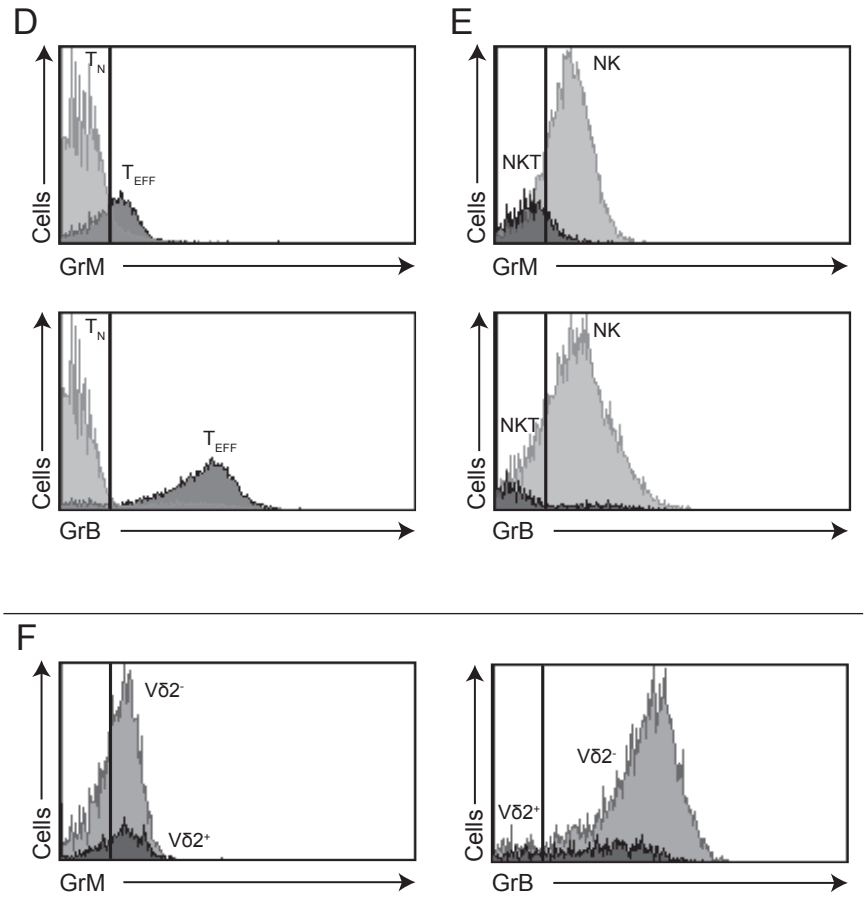
Characteristic	All	HCMV reactivation
No of patients	31	14 (45.2)
Sex		
Male	17	8 (47.1)
Female	14	6 (42.9)
Median age, years (range)	51.6 (21.6-65.9)	54.4 (23.8-65.9)
Stem cell source		
Cord blood	3	2 (33.3)
Peripheral blood	27	11 (40.7)
Bone marrow	1	1 (100)
Donor		
Related	9	3 (33.3)
Unrelated	22	11 (50.0)
HLA mismatch		
Yes	8	5 (62.5)
No	23	9 (39.1)
HCMV serological status (R/D)		
R^+/D^+	10	8 (80.0)
R^+/D^-	12	5 (41.7)
R^-/D^+	2	1 (50.0)
R^-/D^-	7	0 (0)

NOTE. Data are no. (%) of patients, unless otherwise indicated.

HCMV, human cytomegalovirus; R/D, recipient/donor.

Supplementary Figure 1





Supplementary figure 1.
Analysis of intracellular GrM and GrB expression in lymphocyte subsets. (A).

Gating strategy of CD4+ and CD8+ T cells, and the phenotypic subsets within these populations. (B). Gating strategy of the NKT and NK cell subsets. (C) Gating strategy of Vδ2+ and Vδ2- ΓΔT cells. (D) Representative plots of GrM and GrB expression within the CD3+ (CD4+ and CD8+) T cells. The grey histogram represents naive T cells and the black histogram represents effector T cells. (E) Representative plots of GrM and GrB expression within NK cells (grey histogram) and NKT cells (black histogram). (F) Representative plots of GrM and GrB expression in both γδT cell subsets. The grey histogram represents Vδ2- γδT cells and the black histogram represents the Vδ2+ γδT cells. (TCM, central memory T cells; TEM, effector memory T cells; TEFF, effector T cells; TN, naive T cells)

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7

GENERAL DISCUSSION

General discussion

Granzymes have since long been known as effector molecules of cytotoxic cells that induce apoptosis in tumor cells and virally infected cells (1, 2). In addition, it has been known for several decades that levels of soluble granzymes are elevated in patients suffering from various inflammatory disorders (3). Compared to healthy controls, increased granzyme levels have, amongst others, been measured in serum, plasma or broncheo-alveolar lavage fluid (BALF) of patients with bacterial, viral or parasitic infections and in patients with rheumatoid arthritis (4-14).

Whether the release of soluble granzymes under inflammatory conditions has functional consequences is still not fully understood. The rise in granzyme levels may reflect aspecific leakage as a consequence of increased activity of cytotoxic lymphocytes. This would imply that the released granzymes have no extracellular functions, but merely escape inadvertently during intracellular induction of apoptosis in target cells. Alternatively, however, the release may be targeted, and granzymes may possess additional immunomodulatory of inflammatory functions. Indeed, as reviewed in chapter 1, a number of such novel functions of in particular soluble granzymes have been revealed of late (15-22). Soluble granzymes are involved in the release of proinflammatory cytokines from macrophages (16, 18, 20), monocytes (15, 20, 21) and fibroblasts (15, 17, 22). Granzymes cleave and activate proinflammatory cytokines (19, 23-25) and regulate cytokine release in mouse models of endotoxemia and infection (26, 27). Furthermore, granzyme expression has been detected in a number of cell types that are not traditionally considered to possess cytotoxic activity. These include B cells (28-30) and dendritic cells (31-35). Granzyme expression may confer cytotoxicity to these cells, especially in combination with perforin expression. However, granzymes may also fulfill additional functions besides cytotoxicity, thus contributing to the immunoregulatory potential of granzyme-expressing cells. In the current thesis, novel immunomodulatory functions of GrK, GrA, and GrB are identified. These findings will be summarized and discussed below.

GRK HAS A PROINFLAMMATORY FUNCTION INDEPENDENT OF ITS CATALYTIC ACTIVITY

Granzymes are serine proteases, and their role in apoptosis and inhibition of viral replication depends entirely on their catalytic activity (36-39). Therefore, most research on the role of granzymes in inflammation has until now focused on granzyme functions connected to catalytic activity. For instance, GrA and GrB cleave pro-forms of several cytokines, thus activating these cytokines or enhancing their biological activity (19, 23-25).

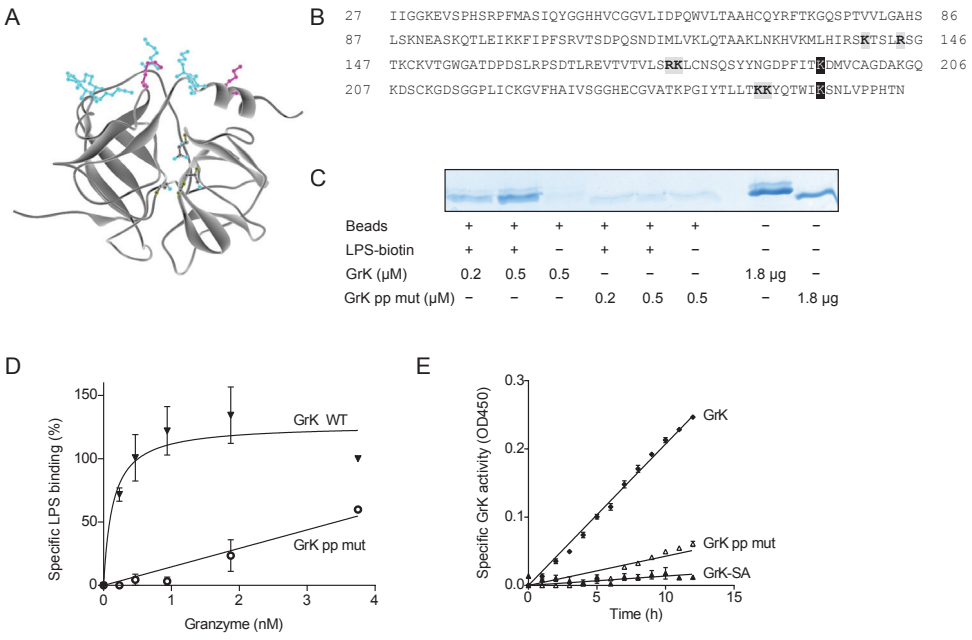

Figure 1.
GrK positive patch mutant binding to LPS is diminished.

A) Tertiary structure of hGrK. The positive patch is visible at the top of the molecule. Amino acids that were mutated to create the positive patch mutant are depicted in light blue. Non-mutated amino acids are depicted in purple. The catalytic center (visible in the middle of the molecule) is depicted in yellow and light blue. B) Amino acid sequence of hGrK. Amino acids constituting the positive patch are boxed in grey (mutated amino acids) or black (non-mutated amino acids, letters in white). C) Binding GrK pp mut to LPS is compromised compared to binding of WT GrK. LPS-coated beads were incubated with WT GrK or GrK pp mut. Bound protein was analysed by SDS-PAGE and total protein staining. Granzymes directly from stock were used as a control. Data are representative of two independent experiments. D) Binding GrK pp mut to LPS is compromised compared to binding of WT GrK. Biotinylated granzymes were incubated on immobilized LPS and binding was visualized using Streptavidin-HRP. Specific binding (in % of maximal WT GrK binding) is depicted. Data are represented as average ± range (n=2 per datapoint) and are representative of two independent experiments. E) The catalytic activity of GrK pp mut is reduced compared to that of WT GrK. Equimolar concentrations of WT GrK and GrK pp mut were incubated with the small chromogenic substrate Ac-Lys-pNA and hydrolysis was measured for 12 h. Catalytically inactive GrK-SA was used as a control. Data were corrected for background binding obtained with buffer only, and are representative of at least three independent experiments. GrK pp mut cleavage of Ac-Lys-pNA was ~20% of that observed for WT GrK (slopes: GrK WT 0.021; GrK pp mut 0.0043; GrK-SA 0.0014).

Furthermore, granzyme-induced cytokine release and cytokine cleavage is fully dependent on granzyme catalytic activity (17-21). In chapter 2, however, we show – for the first time – a proinflammatory function of GrK that is not related to its catalytic activity. We find that GrK synergistically potentiates proinflammatory cytokine responses from human monocytes induced by live Gram-negative bacteria and by the important Gram-negative bacterial cell wall component lipopolysaccharide (LPS). Unexpectedly, similar results were obtained using a catalytically inactive GrK mutant, in which the serine in the catalytic center has been replaced with an alanine (GrK-Serine-Alanine or GrK-SA). The finding that GrK has a function not dependent on its proteolytic properties is not without precedent in the literature. Azurocidin, also known as Heparin-binding protein (HBP) or Cationic protein of 37 kDa (CAP37), is a proteolytically inactive serine protease released by neutrophils that shares substantial homology with granzymes. The ability to cleave substrates has been lost due to mutation of two out of the three amino acids constituting the catalytic center (40). Azurocidin dose-dependently enhances LPS-induced cytokine release from monocytes (41, 42). This property of azurocidin, together with our data on the catalytically inactive mutant of GrK (15), implies that serine protease catalytic activity is not a prerequisite for these types of serine proteases to enhance LPS-induced cytokine responses in human monocytes.

GRK MAY BIND TO LPS VIA ITS PUTATIVE HEPARIN-BINDING SITE

Rather than being dependent on its catalytic activity, the effect of GrK on the LPS-induced cytokine release from human monocytes may be due to a molecular interaction between GrK and LPS. In chapter 2, we demonstrate that both recombinant and native GrK bind to LPS. This binding is independent of GrK catalytic activity, since the catalytically inactive GrK-SA mutant binds to LPS with similar efficiency as active GrK. GrK liberates individual LPS molecules from micelles and promotes complex formation between LPS and CD14. Since individual LPS molecules more easily engage in complexes with CD14, and thus are more effective in activating monocytes than LPS micelles (43-45), we propose that GrK lowers the threshold for monocyte activation by LPS.

We hypothesize that the binding between GrK and LPS is mediated by the putative heparin binding site of GrK (46), that resembles the heparin binding site of thrombin (46) and consists of eight arginines and lysines (Figure 1A). This putative heparin binding site has a strong positive charge and is therefore suited to bind the negatively charged LPS molecule. We tested if mutating residues within this positive patch would affect LPS binding. Residues Lysine 140 (K140), Arginine 144 (R144), R178, K179, K248 and K249 were mutated into

the neutrally charged amino acid Alanine. The mutant protein thus obtained was designated GrK positive patch mutant (GrK pp mut). After introducing mutations, GrK pp mut was expressed in *Pichia pastoris*, purified, and tested for LPS binding. Indeed, LPS binding by GrK pp mut is largely abolished (Figure 1B and C) (Wensink et al., unpublished data). GrK binding to LPS is ~30 times more effective than GrK pp mut binding to LPS, as indicated by the GrK concentration by which half-maximal binding is achieved (Figure 1C; ~0.1 nM for GrK and 3 nM for GrK pp mut). Unfortunately, the catalytic activity of the protease against a small chromogenic GrK substrate (Ac-Lys-pNA) as well as against the macromolecular substrate SET (47, 48) is also greatly compromised (Figure 1D and data not shown). The primary catalytic activity against P1-Lys (the amino acid preferred by GrK at the 1st position N-terminal of the cleavage site) does not reside in the putative heparin binding site but in the catalytic center. Thus, we suspect that the structure of GrK has changed upon introduction of the mutations in the putative heparin binding site. Therefore, we did not study this mutant further, and additional GrK mutants should be generated to study LPS binding.

A

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GrK    27   IIGGKEVSPHSRPFMASIQYGGHHVCGGVLIDPQWVLTAAHCQYRFTKGQSPTVVLGAHS   86
GrA    29   IIGGNEVTPHSRPYMVLLSLDRKTICAGALIAKDWVLTAAHCNL----NKRSQVILGAHS   88

GrK    87   LSKNEASKQTLEIKKFIPFSRVTSDPQSNDIMLVKLQTAAKLNKHVKMLHIRSK-TSLRS   146
GrA    89   ITREEPTKQIMLVKKEFPYPCYDPATREGDLKLLQLMEKAKINKYVTILHLPKKGDDVKP   148

GrK    147  GTKCKVTGWGATDPDSLRPSDTLREVTVTVLSRKLCNSQSYYNGDPFITKDMVCAGDAKG   206
GrA    149  GTMCQVAGWGRTH-NSASWSDTLREVNITIIDRKVCNDRNHYNFNPVIGMNMVCAGSLRG   208

GrK    207  QKDSCKGDSGGPLICKGVFHAIVSGGHE--CGVATKPGIYTLLTKKYQTWIKSNLVPPHTN
GrA    209  GRDSCNGDSGSPLLCEGVFRGVTSFGLENKCGDPRGPGVYILLSKKHLNWIIMTIKGAV--
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B

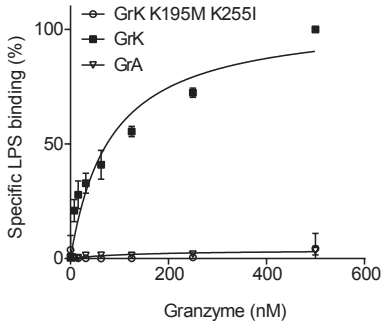

Figure 2.
GrK K195M K255I mutant does not bind to LPS.

A) Amino acid sequence comparison of hGrK and hGrA. Amino acids shared between the two proteins are boxed in grey. Amino acids constituting the positive patch are depicted in bold characters. Mutated amino acids in the GrK K195M K255I mutant are boxed in black and depicted in white characters. B) Binding of GrK K195M K255I mutant to LPS is absent compared to binding of WT GrK. Biotinylated granzymes were incubated on immobilized LPS and binding was visualized using Streptavidin-HRP. GrA was added as a negative control. Data (specific binding) are depicted as % of maximal GrK binding and are the mean ± SD of three independent experiments.

In chapter 3, we show that GrA, like GrK, synergistically potentiates LPS-induced cytokine responses from monocytes. In line with results found for GrK, this effect of GrA is independent of catalytic activity. However, in marked contrast to GrK, GrA does not bind to LPS. If LPS binding by GrK is mediated via the putative heparin binding site (46), differences in amino acid sequence in this structure could explain the difference in LPS binding between GrA and GrK. A comparison of the amino acids comprising the positive patch in GrK and GrA revealed that differences exist indeed; all amino acids are identical or similar (in charge) except two (Figure 2A). Using this knowledge, two mutations (K195M and K255I) were introduced in the positive patch of wild-type GrK, in order to change GrK amino acids into the corresponding GrA amino acids at the same position. Subsequently, this GrK>A mutant was expressed in *Pichia pastoris*, purified, and tested for LPS binding. Interestingly, binding to LPS is completely abolished in the GrK K195M K255I mutant (Figure 2B) (Wensink et al., unpublished data), indicating that the two mutated amino acids may play an essential role in LPS binding. Further study is required to ascertain whether the catalytic activity and protein structure of this GrK>A mutant are comparable to that of wild-type GrK.

Although we have hitherto not demonstrated unambiguously that the GrK putative heparin binding site (positive patch) drives the interaction with LPS, there are several clues in the literature pointing to an important role for arginines and lysines in LPS binding. Bosshart and Heinzelmann (49) demonstrate that the ability of azurocidin to stimulate LPS-induced cytokine responses in human monocytes is shared by protamines and by synthetic polypeptides rich in arginine or, to a lesser degree, lysine. Additionally, HMGB-1, a chromatin-binding protein, augments LPS-induced cytokine responses in monocytes when secreted (43, 50). This protein consists of two highly positive amino terminal HMG-box domains with a high lysine and arginine content, and a negatively charged acidic C-terminal domain (51). The synergistic effect of the protein on LPS-induced responses appears to reside in the HMG-box domains (50). In addition, there is evidence that LPS-binding by LPS-binding protein (LBP) is mediated by arginines and lysines in the LPS-binding tip of the protein (52, 53). Furthermore, apolipoprotein C1, a small protein that is uncommonly rich in lysines, binds LPS and enhances the LPS-induced TNFα response in macrophages (54, 55). Thus, the capacity to enhance LPS-induced cytokine responses is shared by several cationic proteins. This underlines the importance of the positively charged amino acids lysine and arginine in LPS binding and suggests a general mechanism. GrK positive patch mutants that do not bind to LPS will further clarify whether LPS binding is a prerequisite for GrK to potentiate LPS-induced proinflammatory cytokine responses from monocytes.

HOW DOES GRA POTENTIATE LPS-INDUCED CYTOKINE RELEASE?

If GrK binding to LPS underlies the synergistic effect of GrK and LPS on cytokine production by monocytes (chapter 2), it may be considered somewhat surprising that GrA similarly exerts this synergistic effect, since it does not bind LPS (chapter 3). At this moment, the mechanism behind the effect of GrA on the LPS-induced cytokine release from monocytes is unknown, although several possibilities are feasible. In preliminary experiments, we have studied whether GrA induces upregulation of CD14 and/or TLR4 expression on the monocyte cell surface. Monocytes purified using magnetic-activated cell sorting (MACS) were treated with GrA for 4 hours, stained with αTLR4 and αCD14 antibodies, and analyzed by flow cytometry. For CD14, three out of six donors showed a clear upregulation of CD14 (~30%) after GrA treatment. Two donors showed no difference in CD14 expression, and one donor had a lower CD14 expression than the control (data not shown). TLR4 staining was performed on 5 out of 6 donors, and four of these donors showed a decrease in TLR4 expression upon GrA treatment. One donor had a TLR4 expression similar to that of mock-treated cells (data not shown). Thus, the limited number of donors and the inconsistent results did not allow us to draw conclusions from these data. Moreover, analysis of TNFα responses to GrA with or without Gram-negative bacteria in two of the donors used for our CD14 flow cytometry experiments indicated that a synergistic effect could take place in the absence of an increase in CD14 expression (data not shown). Therefore, CD14 upregulation cannot be the only mechanism employed by GrA.

Another possibility is that GrA directly binds to CD14 and/or TLR4 and thereby stimulates LPS-induced signaling, for example by promoting complex formation between LPS and CD14. This should be studied further using native PAGE (as described for GrK in chapter 2). Finally, internalization of granzymes may also play a role in enhancement of the immune response. Since most LPS-stimulating proteins are, as mentioned above, positively charged, they are expected to bind efficiently to the negatively charged structures on the plasma membrane of monocytes, for instance proteoglycans. Once bound, they are can be internalized via (constitutive) endocytosis, pinocytosis or other internalization mechanisms. Heinzelmann et al. have shown that internalization is essential for the stimulating effect of azurocidin on LPS-induced cytokine responses (56). Furthermore, GrA binds to and is internalized by monocytes (20). These findings suggest an important role for internalization of cationic proteins in the enhancement of cytokine responses initiated by LPS. In this respect, it is interesting to note that we have found strong binding of GrK as well as GrK-SA to monocytes (Wensink et al., unpublished data) (Figure 3A). It is not known how the presence or absence

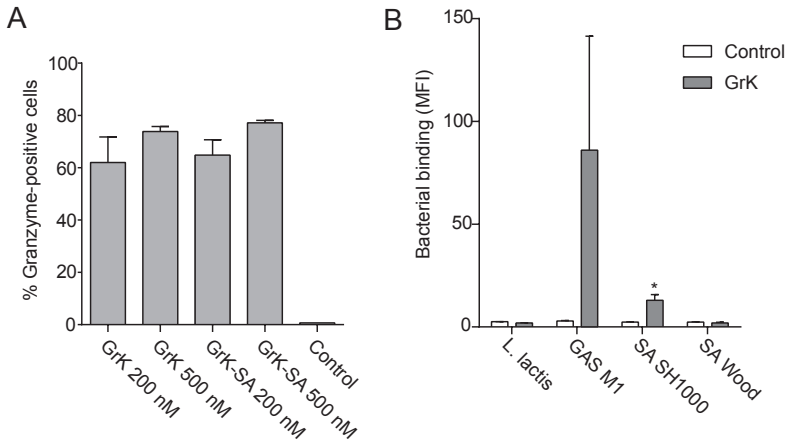

Figure 3.
GrK binds to monocytes and to Gram-positive bacteria.

A) Biotinlyated GrK and GrK-SA were incubated on primary human monocytes. Binding was detected using Streptavidin-PE and flow cytometry. No signal was detected upon incubation without granzymes. B) GrK binds to Gram-positive bacteria. Biotinylated GrK was incubated with the Gram-positive bacteria *Lactococcus lactis* (*L. lactis*), *Group A streptococcus M1* (GAS M1), *Staphylococcus aureus SH1000* (SA SH1000) and *Staphylococcus aureus Wood* (SA Wood). Binding was detected using Streptavidin-PE and flow cytometry. Data are depicted as mean ± SD from three independent experiments. *p<0.05 (unpaired t test with Welch's correction). MFI, Mean Fluorescent Intensity.

of LPS influences binding of granzymes to monocytes, and this is an interesting topic for further studies. In addition, it is unknown how granzymes bind to monocytes. No receptor for granzymes on monocytes has not been identified yet. Because LPS-enhancing properties are shared by multiple positively charged proteins (42, 43, 49, 50, 54, 55, 57, 58), it is conceivable that binding of granzymes to monocytes is aspecific instead of mediated via receptor-ligand interactions. Even granzymes that do not bind LPS may therefore encounter the endotoxin once they are bound to the cell surface of the monocyte.

HOW DO GRANZYMES BIND TO BACTERIA?

In chapter 2, we demonstrate binding of GrK to several Gram-negative bacteria. Since GrK binds to LPS (*E. coli 0111:B4*), binding of GrK to bacteria may be mediated by their LPS moieties, and differences in LPS structure between different bacterial strains could possibly

explain differences in binding intensity. Interestingly however, GrA does not bind LPS (*E. coli 0111:B4*), but readily binds to the same set of Gram-negative bacteria as GrK (chapter 3). These data can be interpreted in two ways. First, GrA does not bind to LPS from *E. coli 0111:04* but may bind other types of LPS that were not tested in our in vitro experiments. Second, the binding of at least GrA to Gram-negative bacteria is not mediated by LPS, implicating that other factors are involved. We have tested several Gram-positive bacteria for GrK binding, and found binding to 2 out of 4 tested bacterial strains (Wensink et al., unpublished data) (Figure 3B). Since Gram-positive bacteria do not express LPS, these data indicate that GrK may bind multiple bacterial components. Thus, the molecular basis of binding to Gram-negative and Gram-positive bacteria may differ. To settle these points, further studies should be undertaken to examine binding of GrA, GrK, and other granzymes to different types of LPS from different bacterial strains. Also, GrK and GrA binding to bacteria expressing truncated LPS or no LPS at all should be investigated. These studies will reveal whether or not granzymes employ a common mechanism to bind to bacteria.

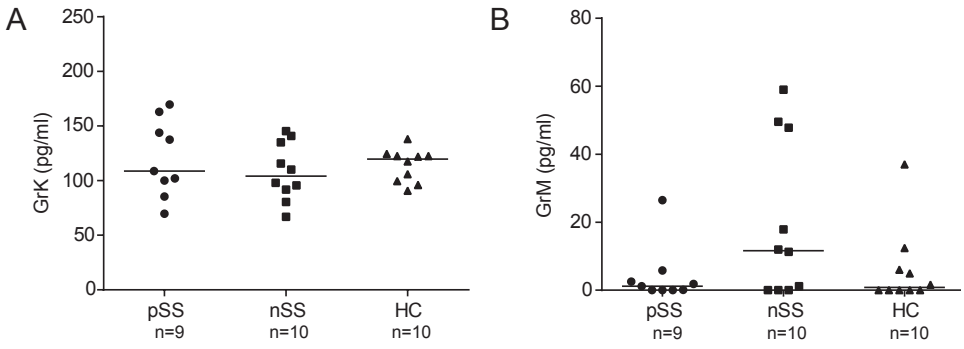

Figure 4.
GrK and GrM levels in pSS and nSS patients.

GrK (A) and GrM (B) levels in serum from pSS and nSS patients and healthy controls were measured using ELISA. Median granzyme levels are indicated.

GRANZYME EXPRESSION IN HEALTH AND DISEASE

Circulating levels of soluble GrA, GrB, GrM, and GrK are higher in patients with inflammatory diseases than in healthy controls (4-14). However, the mechanisms leading to release of soluble granzymes in these conditions are poorly defined. LPS injection results in a temporary rise in GrA and GrB levels in the serum (12), but whether other granzymes are also released in response to LPS was unknown. In chapter 4, we analyze the course of plasma levels of soluble GrK and GrM in a model of human endotoxemia. We demonstrate that soluble GrM and – to a lesser extent – GrK are transiently elevated in the serum of healthy volunteers in response to LPS. This prompted us to investigate whether granzyme release could be induced ex vivo by treating whole blood cultures with LPS. Since LPS is an important constituent of the Gram-negative bacterial cell wall, whole blood cultures were also stimulated with live Gram-negative bacteria (*Escherichia coli BL21*, *Pseudomonas aeruginosa 01* and *Neisseria meningitidis HB-1*). Interestingly, GrM release is observed in all cases, and is already seen after a few hours of stimulation. GrK release is observed only upon treatment with *Pseudomonas aeruginosa 01*, and only after 24 hrs of incubation. Thus, the release of soluble GrK is rather slow compared to that of soluble GrM. Absolute quantities of soluble GrM released in the whole blood cultures are also consistently higher than those of soluble GrK. GrM aggravates cytokine release upon LPS injection in mice (27), and GrK synergistically potentiates LPS-induced cytokine responses in human monocytes (15). However, the relative importance of GrM and GrK in the inflammatory response to LPS is unknown, and deserves further study.

It is known that neutralizing antibodies against pro-inflammatory cytokines inhibit granzyme release in whole blood cultures in response to LPS (12). This knowledge, in combination with the finding that granzymes A, B and K synergistically potentiate proinflammatory cytokine release in response to LPS (15), implies that granzymes and proinflammatory cytokines engage in a positive feedback loop in response to LPS. This feedback loop may ensure prolonged release of both granzymes and proinflammatory cytokines by mononuclear cells in response to LPS. The involvement of proinflammatory cytokines further suggests that activation of (innate) immune cells is required for granzyme release.

In chapter 5, we investigate granzyme levels in serum and synovial fluid from patients with rheumatoid arthritis (RA) and psoriatic arthritis (PsA). Rheumatoid arthritis (RA) is a chronic, systemic auto-immune disorder characterized by inflammation and hyperplasia of the synovial lining of the joints (59). Hyperplastic synovial tissue may invade the underlying cartilage and bone, resulting in tissue destruction. This local inflammatory process in the

joints is often accompanied by a systemic inflammation, the pathogenesis of which is not fully understood, although genetic and environmental factors and the production of auto-antibodies play a role (59). In RA, there is extensive infiltration of the inflamed tissue by mononuclear cells, and these cells contribute to tissue destruction by the production of inflammatory and cytotoxic mediators (59). GrA and GrB-expressing cells are present in the synovial tissue, and levels of these granzymes are elevated in synovial fluid (SF) and serum of RA patients (13, 14, 60-62). Information on levels of other granzymes in this disease, however, is lacking. We demonstrate that GrM, but not GrK, is elevated in SF from RA patients, compared to SF from osteoarthritis (OA) patients. Levels of soluble GrM or GrK are not elevated in SF from PsA patients, and serum levels of GrK and GrM in RA and PsA patients in the same range of those of healthy controls. This finding suggests selective production of GrM in RA joints. At this moment, the precise role of GrM in RA joints remains unclear. Interestingly, GrM levels in RA synovial fluid correlate with levels of several proinflammatory cytokines. This may reflect a role for GrM in the release of these cytokines, and/or a role of cytokines in the production of GrM in the rheumatoid joint. Which stimuli induce release of GrM in RA is unknown and deserves further study.

In addition, we determined GrK and GrM levels in primary Sjögren's syndrome (pSS), another chronic auto-immune disease. In pSS, continued inflammation of salivary and lacrimal glands results in xerostomia (dry mouth syndrome) and keratoconjunctivitis sicca (dryness, burning and irritation of the eyes caused by dry eyes) (63). Why inflammatory reactions persist in these exocrine glands is incompletely understood, but like in RA, there also is profound production of auto-antibodies and excessive lymphocyte infiltration of the inflamed tissue (63). Non-Sjögren's sicca (nSS) patients experience similar symptoms as pSS patients but do not have evidence of an underlying auto-immune disease (64). Therefore, these patients are often used as a control next to pSS patients (64). GrK and GrM levels in serum from pSS (n=9) and nSS (n=10) patients were similar to levels in healthy controls (Figure 4A and B). In some nSS patients GrM serum levels were higher than levels in healthy controls. However, no clinical or demographic differences between nSS patients with normal or elevated GrM were found. GrA expression has been reported in lacrimal and salivary glands in pSS patients (65), suggesting that GrA plays a role in pSS disease progression. Furthermore, auto-antibodies from the serum of pSS patients detect cleavage fragments of auto-antigens that may have been generated by GrB (66). In contrast, neither GrK nor GrM are upregulated in the serum of pSS or nSS patients, arguing against a role for GrK or GrM in these disorders.

In chapter 7, we demonstrate elevated levels of GrM during inflammation. In patients suffering from a human cytomegalovirus (HCMV) infection due to reactivation of the virus after allogeneic stem cell transplantation, a temporary rise in circulating GrM levels was found. To our knowledge, this is the first report demonstrating elevated granzyme levels in the blood upon HCMV reactivation. However, increased levels of soluble GrA and GrB upon primary HCMV infection have been reported in the literature (67, 68). Taken together, these data indicate that granzymes play an important role in the control of HCMV infection or reactivation, possibly using extracellular mechanisms.

CAN MOUSE MODELS BE USED TO STUDY GRANZYME FUNCTION?

Mouse models are often employed when studying granzyme functions, but it is mostly unknown whether results obtained with murine granzymes are relevant for human disease. Since there are ten murine granzymes as opposed to five human granzymes (69, 70), differences can be expected on the mere basis of granzyme numbers alone. Furthermore, the macromolecular substrate specificities and immunomodulatory functions of human and murine granzymes may have diverged during evolution. Indeed, marked differences between human and mouse GrA, GrB and GrM have been demonstrated (71-76). In chapter 6, the substrate specificities of human and murine GrK (hGrK and mGrK) are mapped. Using a 2D DIGE protease proteomic approach, we find that hGrK and mGrK have a partially overlapping but certainly not identical degradome. About 40% of substrates are shared, but the remainder of the substrates are unique for hGrK or mGrK. This indicates that caution is required when interpreting data obtained with mGrK. We determined the number of macromolecular substrates cleaved by hGrK and mGrK at a given granzyme concentration, but did not identify which proteins are degraded. This should be the subject of further research. However, using a direct approach based on known hGrK substrates (47, 48, 77), we did observe cleavage of the human proteins hnRNP K and SET by both hGrK and mGrK.

The cytotoxic potential of some granzymes, particularly GrA and GrK, is currently subject of debate (18, 20, 78). In our hands, as is described in chapter 6, both hGrK and mGrK are not cytotoxic when delivered intracellularly to human and murine tumor cell lines. This is in contrast to results published by Fan and coworkers, who report extensive apoptosis in target cells treated with GrK (48, 79-82), but consistent with the observation that mGrK is not cytotoxic (18). We did not observe cytotoxicity of hGrK in the experiments described in this thesis, neither in previous experiments (Bovenschen, de Poot et al., unpublished data). In addition, we and others have proposed alternative functions for hGrK (15, 17). Therefore, the contribution of hGrK to the killing and elimination of aberrant cells remains unclear.

Currently, it is unclear whether mGrK binds LPS and, like hGrK, potentiates LPS-induced cytokine responses in mouse monocytes or macrophages. This is currently being studied in our group. The outcome of these experiments may determine whether convential (non-humanized) mouse models of endotoxemia can be used to study GrK functions in mouse models for sepsis and infections. In conclusion, extrapolating results obtained with mGrK or in murine models to the human situation may be inappropriate. In our view, it is important to carefully compare the activity of murine granzymes to that of their human homologues. In cases where the substrate specificities and functions of mouse and human granzyme homologues differ from each other, results obtained with murine granzymes should always be confirmed using their human counterparts.

THERAPEUTIC APPLICATIONS AND FUTURE PERSPECTIVES

The results described in this thesis indicate that granzymes are involved in inflammatory processes, including the innate immune response to LPS and Gram-negative bacteria. Targeting granzymes may therefore may therefore constitute a treatment option for sepsis and other inflammatory-infectious disorders. Importantly, not all granzymes appear to modulate the immune response to LPS in the same manner. Indeed, our data suggest that granzymes have evolved to augment inflammatory responses to LPS in complementary and possibly overlapping manners. This functional redundancy can be regarded as a failsafe mechanism in case one of the granzymes is inhibited, and underlines the importance of granzymes in promoting inflammatory processes. However, it may complicate targeting a single granzyme as an anti-inflammatory therapy.

To develop effective anti-inflammatory therapies based on blocking granzyme functions, the potential of other granzymes to promote LPS-induced cytokine responses must be elucidated first. At this point, it is unclear whether GrB, GrH and GrM bind to LPS, bacteria or monocytes. It is important to investigate whether GrH and GrM augment LPS-induced cytokine responses in monocytes, and to identify the mechanism(s) by which GrA and GrB exert their effects. Before developing therapeutic interventions, it is furthermore essential to substantiate the physiological relevance of the in vitro effects that we have observed. A first clue for this comes from the observation that mice injected with GrK and LPS have significantly higher levels of proinflammatory cytokines in their serum than mice injected with LPS (chapter 2). However, these mice were injected with human GrK, while their endogenous granzymes were still present. Also, the dose of granzyme that was applied was based on in vitro experiments and may differ from the physiological situation. To study the physiological relevance of human granzymes in the immune response to LPS, pan-granzyme-deficient

RAG-deficient x common γ-chain deficient mice that lack mature T cells and NK cells could be injected with human PBMCs in which granzymes have been knocked out one by one. Endotoxemia may then be induced by treatment with LPS or bacteria, after which the relative importance of each granzyme in the immune response to LPS could be evaluated.

If all human granzymes employ different mechanisms to stimulate cytokine responses, it may be difficult to design therapies that block all these functions. A possible drawback of inhibiting only one out of five granzymes is the functional redundancy mentioned above as it implies that granzyme functions will be taken over by other granzymes. Therefore, it may be useful to carefully study which granzymes are released in which disorders, so that therapy can be adapted based on the granzyme expression profile. When multiple granzymes are expressed, it would be interesting to see whether these granzymes share intracellular signaling pathways to induce cytokine release, so that these signaling pathways can be inhibited. Furthermore, blocking extracellular activities of granzymes before intracellular signaling pathways are activated may be an interesting strategy. As mentioned earlier, GrK binds to LPS and at least GrK and GrA display binding to monocytes, and this may contribute to their potential to boost LPS-induced cytokine release. Therefore, when searching for therapeutic opportunities to inhibit this effect of granzymes, inhibiting the binding of granzymes to LPS or to monocytes may proof to be essential. In the future, inhibiting granzyme effects on the release of pro-inflammatory cytokines may provide a valuable tool to dampen inflammatory reactions in sepsis and possibly in other diseases.

ACKNOWLEDGEMENTS

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NEDERLANDSE SAMENVATTING

De meeste mensen worden niet vaak ziek, behalve de incidentele verkoudheid of griep. Dit lijkt een klein wonder, als je bedenkt hoe vaak we in aanraking komen met virussen, bacteriën en andere ziekteverwekkers. Om nog maar te zwijgen van normale lichaamscellen die zich proberen te ontwikkelen tot tumorcel. Afgezien van hygiëne, voeding en andere leefomstandigheden, is onze goede gezondheid te danken aan ons immuunsysteem, dat in staat is om bedreigingen in de meeste gevallen tijdig en effectief op te ruimen.

AANGEBOREN EN AANGEPASTE IMMUNITEIT

Ons immuunsysteem wordt voor studiedoeleinden vaak gesplitst in twee 'armen': de aangeboren immuniteit en de aangepaste immuniteit. De aangeboren immuniteit bestaat uit een cellulaire component (verschillende typen witte bloedcellen) en een humorale component (een groep eiwitten in het bloed, genaamd het complement systeem). Deze tak van ons immuunsysteem is zeer goed in staat lichaamsvreemde stoffen te herkennen en daarop te reageren. Lichaamsvreemde stoffen zijn bijvoorbeeld stoffen die door bacteriën worden gemaakt en aan de buitenkant van de bacterie zitten. De aanwezigheid van een lichaamsvreemde stof in het lichaam wordt door ons immuunsysteem gezien als een teken van de aanwezigheid van een ziekteverwekker, en dus als een bedreiging. Als een lichaamsvreemde stof wordt herkend door de aangeboren immuniteit vindt een algemene, niet-specifieke immuunrespons plaats met als doel de lichaamsvreemde stof op te ruimen. Deze immuunrespons is snel, maar slaagt er niet altijd in om de ziekteverwekker voldoende op te ruimen.

De aangeboren en aangepaste immuniteit zijn geen gescheiden systemen, maar beïnvloeden elkaar op grote schaal. Door de aangeboren immuunreactie wordt de aangepaste immuniteit dan ook geactiveerd. Deze bestaat eveneens uit een cellulaire component van witte bloedcellen, te weten T cellen en B cellen, die specifiek gericht zijn tegen één lichaamsvreemde stof. Daarnaast produceren B cellen na activatie grote hoeveelheden antistoffen, dit is de humorale kant van de aangepaste immuniteit. De aangepaste immuunreactie komt iets trager op gang dan de aangeboren, maar is vaak zeer doeltreffend. Een ander voordeel van de aangepaste afweer is dat T cellen en B cellen een geheugenfunctie hebben. Van de T cellen en B cellen die één specifieke lichaamsvreemde stof herkennen blijven na het opruimen van een infectie kleine hoeveelheden in het lichaam achter. De volgende keer dat het lichaam in aanraking komt met dezelfde vreemde stof, bijvoorbeeld door infectie met dezelfde bacterie, kan de aangepaste immuniteit daardoor veel sneller reageren.

Om met elkaar te communiceren, maken cellen uit zowel de aangeboren als de aangepaste immuniteit gebruik van een uitgebreid repertoire aan signaalstoffen, genaamd cytokines. Dit zijn eiwitten die worden uitgescheiden door de ene cel om de andere cel te activeren of juist te remmen. Als een cel van de aangeboren afweer bijvoorbeeld een bacteriële stof waarneemt, zal hij hier onder andere op reageren door het uitscheiden van cytokines. Deze signaalstoffen activeren andere immuuncellen en bevorderen daardoor de ontsteking. Het op gang brengen van een ontstekingsreactie is nodig om een ziekteverwekker op te ruimen. Er bestaan ook cytokines die juist ontstekingsremmend werken en de ontsteking een halt toeroepen op het moment dat de ziekteverwekker is opgeruimd. Een juiste balans tussen ontstekingsbevorderende en ontstekingsremmende cytokines is heel belangrijk. Bij een doorslaande balans in de richting van activatie kan schade ontstaan aan organen en kan een patiënt zelfs komen te overlijden. Dit kan bijvoorbeeld het geval zijn als een bacterie in het bloed terechtkomt en daar sepsis (bloedvergiftiging) veroorzaakt.

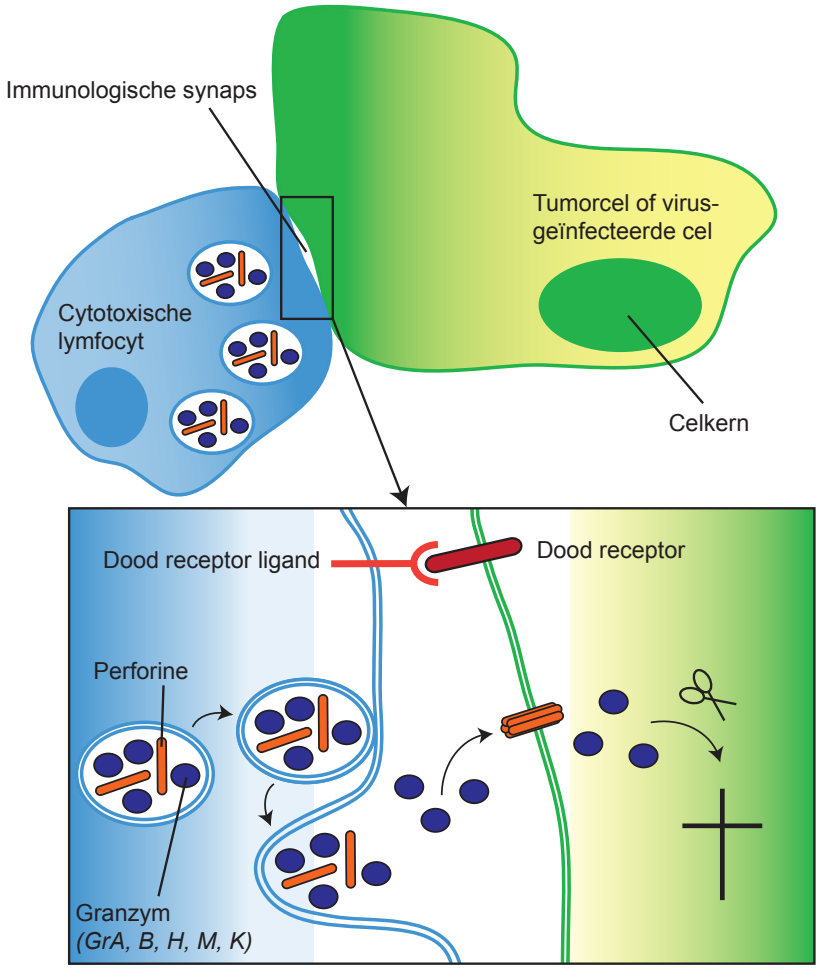
GRANZYMEN: APOPTOSE

Binnen de aangeboren immuniteit zijn verschillende typen witte bloedcellen te onderscheiden. Het gaat hierbij om mestcellen, basofielen, eosinofielen en fagocyten (monocyten/macrofagen, dendritische cellen en neutrofielen). Deze cellen zijn in staat om ziekteverwekkers rechtstreeks aan te vallen. Daarnaast zijn er Natural Killer (NK) cellen en γδ T cel receptor (TCR) cellen. Deze cellen worden tot de aangeboren immuniteit gerekend en herkennen lichaamsvreemde signalen op cellen die geïnfecteerd zijn door ziekteverwekkers. Ook herkennen ze patronen op tumorcellen die niet voorkomen op gezonde cellen. Na herkenning kunnen de geïnfecteerde cellen en tumorcellen worden gedood door de NK cellen en γδ T cellen. Binnen de aangepaste afweer herkennen we de CD4-positieve (CD4+) T cellen, CD8-positieve (CD8+) T cellen en Natural Killer T cellen (NKT cellen). Deze cellen moeten worden geactiveerd door cellen uit de aangeboren immuniteit, zoals macrofagen en dendritische cellen. Vervolgens zijn deze cellen ook in staat om geïnfecteerde cellen of tumorcellen op te ruimen. Bij elkaar genomen zijn er dus een aantal belangrijke typen witte bloedcellen die zorgen voor het doden van afwijkende cellen: NK cellen, γδ T cellen, CD4+ T cellen, CD8+ cellen en NKT cellen. Deze worden ook wel cytotoxische lymfocyten genoemd (cyto = cel, toxisch = giftig, lymfocyt = witte bloedcel), omdat ze andere cellen kunnen doden. Van CD4+ cellen is pas recent ontdekt dat zij deze eigenschap bezitten en hier is dan ook nog niet veel over bekend.

In iedere lichaamscel is er een balans tussen signalen die de cel vertellen dat hij moet blijven leven, en signalen die de cel ertoe aanzetten om een voorgeprogrammeerde vorm van celdood te ondergaan die apoptose wordt genoemd. Deze balans is essentieel; hij geeft het lichaam de mogelijkheid om gevaarlijke of overbodige cellen op een nette manier op te ruimen. Cytotoxische lymfocyten maken hiervan gebruik door in deze afwijkende cellen routes te activeren die tot apoptose leiden. Dit kan op verschillende manieren gebeuren. Ten eerste kan er in de dood-receptor route een ligand ('de sleutel') op de cytotoxische lymfocyt een binding aangaan met een dood-receptor ('het slot') aan de buitenkant van de afwijkende cel. Dit leidt binnen de doelcel tot activatie van signaalstoffen die apoptose bevorderen. Een andere belangrijke mogelijkheid is de granule-exocytose (uitscheiding van granules) route. In de cytotoxische lymfocyt liggen blaasjes (granules) opgeslagen met daarin cytotoxische eiwitten genaamd granzymen, plus het porievormende eiwit perforine. Als de cytotoxische lymfocyt een afwijkende cel herkent die moet worden opgeruimd, wordt er een hechte binding tussen de twee cellen gevormd. Tussen de twee cellen blijft dan slechts een zeer smalle kloof over, die immunologische synaps wordt genoemd (figuur 1). De blaasjes met daarin de granzymen fuseren vervolgens met de plasma membraan van de cytotoxische lymphocyt, waardoor een hoge concentratie granzymen in de immunologische synaps terechtkomt. Daarna gaan de granzymen de doelcel binnen met de hulp van het eiwit perforine, dat poriën vormt in de doelcel en de entree van de granzymen vergemakkelijkt. Eenmaal in de doelcel knippen de granzymen verschillende eiwitten in kleinere stukjes, waardoor pro-apoptotische routes worden aangezet. Hierdoor zal de doelcel doodgaan (figuur 1).

De naam granzymen is een samentrekking van 'granulair enzym'. Het eerste slaat op de plaats waar granzymen worden gevonden, het tweede op de functie van de granzymen. Het zijn enzymen die andere eiwitten knippen (doordat ze proteïnen knippen vallen granzymen binnen de enzymen in de categorie van de proteases). Als een eiwit geknipt wordt, kan de functie van het betreffende eiwit versterkt of juist geremd worden. Dit is afhankelijk van welke eiwit geknipt wordt en van de context.

In het menselijk lichaam zijn vijf granzymen geïdentificeerd: Granzym A (GrA), GrB, GrH, GrM en GrK. In muizen zijn er meer granzymen, namelijk tien. Dit verklaart deels waarom de naamgeving van de humane granzymen niet netjes op elkaar aansluit. Hoewel de aminozuursequenties van humane granzymen voor ongeveer 40% met elkaar overeenkomen, knippen zij niet allemaal dezelfde substraten (substraat = eiwit dat geknipt wordt). GrA en GrK kunnen beide knippen achter een arginine of een lysine in de aminozuursequentie van



Figuur 1. Het doden van afwijkende cellen door een cytotoxische lymfocyt.

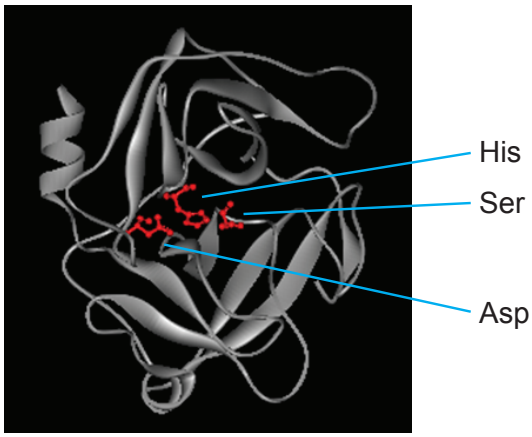
Het doden van doelcellen door cytotoxische lymfocyten gebeurt via twee verschillende routes: de dood-receptor route en de granule exocytose route. In de dood-receptor route grijpen dood-liganden op de cytotoxische lymfocyt aan op dood-receptoren aan de buitenkant van de doelcel. Dit activeert een signaleringsroute in de doelcel, die celdood tot gevolg heeft. De tweede route is de granule exocytose route, welke wordt gezien als de belangrijkste route die leidt tot celdood. Deze route maakt gebruik van blaasjes (granules) met daarin cytotoxische eiwitten die in de cytotoxische lymfocyt zijn opgeslagen. Hierin zitten onder andere granzymen en het porievormende eiwit perforine. Bij herkenning van een doelcel fuseren deze blaasjes met de membraan van de lymfocyt, waarna de inhoud van de blaasjes wordt uitgescheiden in de kleine ruimte tussen de twee cellen. De granzymen gaan dan de doelcel binnen via poriën die door perforine worden gevormd. In de doelcel knippen zij allerlei eiwitten en zetten daarmee pro-apoptotische routes aan, waardoor de doelcel doodgaat.

het substraat. GrB knipt na een asparaginezuur of een glutaminezuur, GrH na een tyrosine of fenylalanine, en GrM na een leucine of een methionine. Hoewel sommige substraten gedeeld worden door meerdere granzymen, heeft ieder granzym alles bij elkaar een unieke set substraten. Granzymen knippen niet lukraak alles wat los en vast zit, maar zijn zeer specifiek. In een cel kunnen meer dan 10.000 verschillende eiwitten voorkomen, waarvan er slechts enkele tientallen door één granzym worden geknipt.

Granzymen bestaan uit twee verschillende domeinen, met in het midden daarvan drie aminozuren die samen essentieel zijn voor de catalytische functie (knipfunctie) van het granzym (figuur 2). Dit zijn de aminozuren Histidine, Serine en Asparaginezuur, die samen het catalytische centrum van het eiwit vormen. Vanwege de aanwezigheid van de Serine in het catalytisch centrum vallen granzymen onder de serine proteases. Als een van de aminozuren in het catalytische centrum kunstmatig wordt veranderd in een ander aminozuur, verliest het granzym zijn catalytische activiteit. In dit proefschrift staan proeven beschreven die zijn uitgevoerd met GrK-Serine-Alanine (GrK-SA) en GrA-SA. Dit zijn GrK en GrA waarbij de serine in het catalytische centrum door middel van gerichte mutatie door de onderzoekers is veranderd in een alanine. Daardoor is de catalytische activiteit van de granzymen verdwenen. Deze gemuteerde granzymen, die niet meer kunnen knippen, worden in de proeven gebruikt als controle om te zien of een effect van een granzym afhankelijk is van zijn knipfunctie.

GRANZYMEN: ALTERNATIEVE OF AANVULLENDE FUNCTIES

Van alle vijf granzymen is aangetoond dat zij celdood in afwijkende cellen kunnen veroorzaken op de hierboven beschreven manier. Echter, recentelijk is er discussie ontstaan of sommige granzymen wel echt cytotoxisch zijn. Over GrA en GrK zijn data gepubliceerd die aantonen dat deze granzymen geen apoptose veroorzaken, zelfs niet bij tamelijk hoge concentraties. GrH is maar heel weinig bestudeerd. Alleen van GrB en GrM weten we met zekerheid dat ze apoptose veroorzaken bij doelcellen, hoewel de fysiologische relevantie van GrM-gemedieerde celdood nog verder moet worden onderzocht. Daarnaast is het al tientallen jaren bekend dat granzymen in verhoogde hoeveelheden aan worden getroffen in het bloed of in de longvloeistof van patiënten met verschillende infecties, waaronder bacteriële infecties, infecties met virussen en met parasieten. Ook bij mensen met reumatische artritis zijn verhoogde hoeveelheden granzymen gevonden in het bloed en in de gewrichtsvloeistof. Het extracellulair voorkomen van granzymen, zonder perforine, lijkt niet logisch aangezien granzymen de doelcel in moeten kunnen om apoptose te induceren. Zonder perforine zou dat veel te traag gaan. Daarom zoeken wetenschappers naar een verklaring voor het

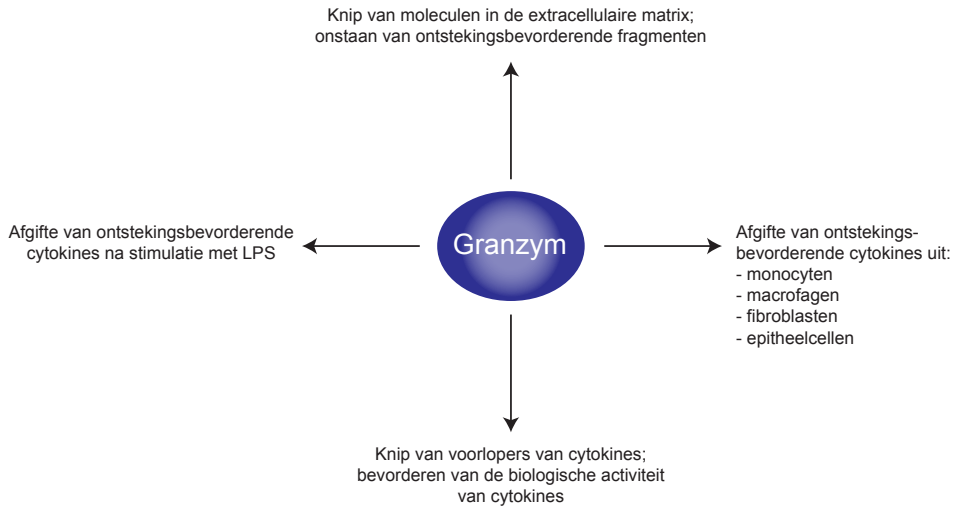


Figuur 2. Schematische structuur van een granzym.

In de afbeelding is schematisch de structuur van granzymen weergegeven. Granzymen bestaan uit twee domeinen die betrokken zijn bij substraatherkenning. Tussen de twee domeinen in ligt de catalytische triade, een groepje van drie aminozuren dat essentieel is voor de knipfunctie van het granzym. Dit groepje bestaat uit een histidine, een serine en een asparaginezuur. Als een van deze aminozuren kunstmatig wordt veranderd in een ander aminozuur verliest het granzym zijn catalytische activiteit (knipfunctie). De meeste granzymen zijn monomeren, alleen GrA is een dimeer. Dit houdt in dat het bestaat uit twee keer de in de afbeelding weergegeven eiwitstructuur.

voorkomen van 'vrij' granzym in de circulatie. Een mogelijke verklaring is dat granzymen weglekken uit de immunologische synaps terwijl cytotoxische cellen doelcellen doden, en in het bloed verder geen functie hebben. Maar het zou ook kunnen dat granzymen aanvullende functies hebben in ontstekingsprocessen, en dat de verhoogde hoeveelheden granzymen in de circulatie van patiënten geen toeval is. Inderdaad zijn de laatste jaren verschillende nieuwe functies van granzymen ontdekt. Deze functies worden uitgebreid bediscussieerd in hoofdstuk 1. Granzymen kunnen bijvoorbeeld verschillende substraten in de extracellulaire matrix rondom cellen knippen, waardoor er kleine fragmenten ontstaan die normaalgesproken niet voorkomen. Deze fragmenten kunnen immuuncellen aantrekken en daardoor ontstekingsprocessen bevorderen. Daarnaast is van sommige granzymen bekend dat ze cytokines of de voorlopers daarvan kunnen knippen en op die manier de biologische activiteit van het cytokine kunnen verhogen. GrB knipt bijvoorbeeld de voorloper van het cytokine Interleukine 1α (IL-1α). Het knipfragment wat daarbij ontstaat, heeft een veel grotere biologische activiteit dan zijn voorloper. Hierdoor kan GrB een bijdrage leveren aan ontstekingsprocessen.

Om de functies van granzymen beter in kaart te brengen, zijn door onderzoekers voor verschillende granzymen (A, B en M) knockout muizen gemaakt, muizen die het betreffende granzym niet kunnen maken. Deze muizen zijn vervolgens ingespoten met een stof die veel voorkomt op bepaalde bacteriën, namelijk lipopolysaccharide (LPS). Dit is een veelgebruikte manier om bloedvergiftiging (een septische shock) na te bootsen. Als de granzym knockout muizen worden ingespoten met LPS, overleven GrA en GrM knockout muizen langer dan wildtype muizen (de data voor GrB knockout muizen zijn niet eenduidig). Hieruit blijkt dat granzymen een rol spelen bij de afweerreactie tegen LPS. Inderdaad produceren GrM



Figuur 3. Schematisch overzicht van ontstekingsbevorderende functies van granzymen.

Granzymen oefenen verschillende functies uit die ontstekingen kunnen bevorderen. Tot nu toe onderscheiden we (van links naar rechts met de klok mee) de volgende functie: 1) Granzymen bevorderen de afgifte van ontstekingsbevorderende cytokines in muizen die worden ingespoten met de bacteriële stof lipopolysaccharide (LPS). Ook bevorderen humane granzymen de afgifte van proinflammatoire cytokines uit monocyten die worden gestimuleerd met lage concentraties LPS. 2) Granzymen kunnen moleculen knippen in de extracellulaire matrix, oftewel het weefsel dat zich rondom cellen bevindt. Door deze knip komen fragmenten vrij die door het immuunsysteem als afwijkend worden herkend en daardoor ontstekingen kunnen bevorderen. 3) Granzymen werken in op verschillende celtypen om de afgifte van ontstekingsbevorderende cytokines te doen toenemen. 4) Granzymen knippen voorlopers van cytokines, waardoor de biologische activiteit en werkzaamheid van deze cytokines vergroot wordt, en ontstekingen bevorderd worden.

knockout muizen minder proinflammatoire cytokines dan wild-type muizen. Het gaat hierbij onder andere om de cytokines IL-1α, IL-1β en Interferon γ (IFNγ). Blijkbaar stimuleren granzymen tijdens de afweerreactie tegen LPS het vrijkomen van cytokines. Het wachten is nu nog op GrH en GrK knockout muizen om hun rol in de immuunreactie tegen LPS in muizen te kunnen bepalen. Hoe granzymen de afgifte van cytokines bevorderen, moet nog nader onderzocht worden. Uit onderzoek met cellen van mensen en muizen is echter wel gebleken dat granzymen de afgifte van cytokines uit immuuncellen zoals monocyten en macrofagen, maar ook uit andere celtypen, kunnen veroorzaken. GrA bijvoorbeeld zorgt dat monocyten de proinflammatoire cytokines IL-1β, IL-6, IL-8 en TNFα uitscheiden. Verder zorgt GrA voor het vrijkomen van IL-6 en IL-8 uit fibroblasten en IL-8 uit epitheelcellen. Ook GrK kan afgifte van IL-6 en IL-8 uit fibroblasten veroorzaken, en GrA en GrK veroorzaken beiden uitscheiding van IL-1β uit macrofagen die met LPS zijn geactiveerd (zie figuur 3 voor een samenvatting van deze functies). Uit onderzoek is gebleken dat deze functies van granzymen afhankelijk zijn van hun catalytische activiteit: als dezelfde proeven werden uitgevoerd met inactief granzym trad er geen effect op.

In hoofdstuk 2 identificeren wij echter een functie van GrK die niet samenhangt met de catalytische activiteit van dit granzym. In dit hoofdstuk maken we gebruik van monocyten, cellen die zeer sterk reageren op de bacteriële stof LPS, en testen het effect van granzym K in samenhang met LPS. We laten zien dat behandeling van monocyten met alleen GrK géén cytokine afgifte veroorzaakt. Echter, als we GrK toevoegen in combinatie met een zeer lage concentratie LPS, zien we dat GrK de cytokine afgifte die door LPS wordt veroorzaakt meerdere malen versterkt. Er lijkt dus sprake te zijn van een synergistisch effect van LPS en GrK. Dit effect zien we ook als we levende Gramnegatieve bacteriën toevoegen (op deze bacteriën komt van nature LPS voor). Verrassend is dat het effect ook optreedt als we gebruik maken van GrK-SA, de catalytisch inactieve mutant van GrK waarin de serine uit het catalytische centrum is vervangen door een alanine. Dit betekent dat het versterkende effect van GrK onafhankelijk is van catalytische activiteit, een noviteit binnen het granzymonderzoek. In plaats daarvan lijkt het effect van GrK te berusten op binding aan LPS. We laten zien dat GrK bindt aan bacteriën en aan LPS, en ook de signaleringsroute van LPS gebruikt om zijn effect uit te oefenen. LPS is een amfifatisch molecuul, dat wil zeggen een molecuul met een hydrofiel en een hydrofoob gedeelte. In waterige oplossingen zal LPS dan ook micellen vormen (structuren waarin alle hydrofobe gedeelten naar binnen gekeerd zitten en alleen de hydrofiele gedeelten contact maken met de waterige omgeving). Deze LPS micellen zijn minder effectief in het activeren van monocyten dan losse LPS moleculen.

Wij laten in hoofdstuk 2 zien dat GrK individuele LPS moleculen uit de micel structuur kan halen. Daarbij bevordert GrK complex formatie tussen LPS en CD14. CD14 is een belangrijk molecuul in de LPS signaleringsroute. Het bevindt zich aan de buitenkant van de cel, waar het complexen met LPS kan vormen. Deze complexformatie tussen LPS en CD14 is essentieel voor activatie van de monocyt. Door de complexformatie te stimuleren verlaagt GrK de drempel voor LPS om monocyten te activeren. Dit verklaart het versterkende effect van GrK op de cytokine productie die door LPS is aangezet.

Het voor GrK gevonden effect roept de vraag op of andere granzymen soortgelijke functies hebben. In hoofdstuk 3 onderzoeken we dit voor GrA. We laten we zien dat GrA, net als GrK, de cytokine respons van monocyten versterkt die veroorzaakt is door LPS. Ook de cytokine respons die veroorzaakt wordt door bepaalde Gramnegatieve bacteriën wordt verhoogd door GrA. Dit terwijl GrA zelf geen cytokine afgifte veroorzaakt. Net als bij GrK staat het effect los van de catalytische activiteit van GrA, aangezien we het effect ook observeren met GrA-SA. In tegenstelling tot GrK bindt GrA echter niet aan LPS, en is ook niet in staat om LPS moleculen op een effectieve manier uit hun micel structuur te halen. GrA bindt wel aan dezelfde bacteriën als GrK, in elk geval binnen de set van vijf bacteriën die we getest hebben. Dit roept de vraag op of deze binding via LPS verloopt, of dat hier andere stoffen op de bacteriën bij betrokken zijn. Het mechanisme waarmee GrA de LPS-geïnduceerde cytokine respons van de monocyten versterkt is dus niet duidelijk. Het kan deels overlappen met dat van GrK, maar is er in elk geval niet identiek aan. Ook is het niet duidelijk waarom wij geen afgifte van cytokines meten wanneer we met alleen GrA behandelen, terwijl in de literatuur beschreven staat dat dit wel gebeurt. Dit zijn aanknopingspunten voor verder onderzoek.

Om de cytokine productie die door LPS veroorzaakt wordt kunnen versterken, moeten er wel granzymen vrijkomen in de circulatie op het moment dat LPS aanwezig is. In hoofdstuk 4 onderzoeken we daarom of behandeling met LPS het vrijkomen van granzymen kan veroorzaken. Uit eerder onderzoek is bekend dat GrA en GrB vrijkomen in het bloed vrijkomen als vrijwilligers worden ingespoten met kleine hoeveelheden LPS. In dit hoofdstuk laten we zien dat dat ook geldt voor GrK en met name voor GrM. Na het inspuiten van vijf vrijwilligers met LPS is er bij alle vijf een duidelijke piek in de GrM niveaus in het bloed te zien. Bij GrK is deze piek ook aanwezig, maar minder duidelijk. Vervolgens tonen we aan dat GrM ook vrijkomt als verdund bloed van gezonde vrijwilligers in het laboratorium behandeld wordt met LPS. GrK komt onder deze omstandigheden echter niet of nauwelijks vrij. Naast LPS hebben we ook bloed behandeld met levende Gramnegatieve bacteriën, waarbij GrM

snel en in ruime hoeveelheden vrijkwam. GrK kwam alleen vrij na 24 uur behandelen met de bacterie *Pseudomonas aeruginosa*. Deze bevindingen wijzen erop dat afgifte van GrM en GrK na behandelen met LPS niet op dezelfde manier gereguleerd wordt, en dat deze granzymen waarschijnlijk verschillende rollen spelen in de immuunreactie tegen LPS en levende bacteriën.

In hoofdstuk 5 bekijken we of GrK en GrM mogelijk een rol spelen bij de auto-immuunziekte reumatische artritis (RA). Uit eerder onderzoek is gebleken dat GrA en GrB in hogere concentraties in het bloed en in de gewrichtsvloeistof worden aangetroffen van mensen met RA. Hier hebben we onderzocht of dat ook het geval is voor GrK en GrM. Daarnaast hebben we gekeken naar materiaal van patiënten met psoriatische artritis (PsA), een andere vorm van gewrichtsontsteking. Het blijkt dat GrK niet verhoogd is in het serum of de gewrichtsvloeistof van de patiënten met RA of PsA. GrM is ook niet verhoogd in het serum, maar blijkt wel verhoogd te zijn in de gewrichtsvloeistof van patiënten met RA. Dit kan betekenen dat GrM lokaal afgegeven wordt bij RA en een rol speelt bij het ontwikkelen of in stand houden van gewrichtsontstekingen. Nader onderzoek moet uitwijzen wat deze rol is, al lijkt het op basis van bestaande literatuur waarschijnlijk dat GrM een rol speelt bij het vrijmaken van cytokines.

Voor onderzoek naar functies van granzymen worden vaak muismodellen gebruikt. Er wordt dan aangenomen dat de granzymen van de muis ongeveer hetzelfde doen als hun humane tegenhangers. Voor GrK was tot nu toe echter niet in kaart gebracht of dit daadwerkelijk zo is. In hoofdstuk 6 trekken we daarom een vergelijking tussen humaan en muis GrK (hGrK en mGrK). Humaan en muis GrK zijn sterk homoloog, een groot gedeelte van de aminozuursequentie komt overeen. Wij hebben door middel van een 2D DIGE benadering bekeken of beide granzymen dan ook dezelfde substraten knippen. Het blijkt echter dat hier aanzienlijke verschillen tussen zitten. Als beide granzymen worden getest op een humaan cellysaat worden in totaal 23 substraten geknipt, waarvan er 9 door beide granzymen worden geknipt. De overige 14 substraten worden of alleen door mGrK, of alleen door hGrK geknipt. Dit geeft aan dat data die met mGrK zijn verkregen niet zomaar geëxtrapoleerd kunnen worden naar humaan GrK, en andersom. Daarnaast hebben we tumorcellen van mens en muis behandeld met mGrK en hGrK om te kijken of deze granzymen celdood veroorzaken. Uit onze voorlopige data blijkt dat dit niet het geval is. Voor hGrK is dit in tegenspraak met reeds gepubliceerde literatuur van een andere onderzoeksgroep. Het is op dit moment niet duidelijk waar dit door komt.

In hoofdstuk 7 tenslotte kijken we naar wat er met het immuunsysteem gebeurt als mensen die geïnfecteerd zijn met het humaan cytomegalovirus (HCMV) een stamceltransplantatie krijgen. Het HCM virus kan na infectie niet volledig worden opgeruimd door het immuunsysteem en blijft bij besmette individuen latent aanwezig. Het virus is dan niet actief, omdat het door het immuunsysteem onderdrukt wordt. Een eenmaal besmet individu draagt echter wel zijn leven lang het virus bij zich. Bij een (stamcel) transplantatie wordt het immuunsysteem met medicijnen geremd om de kans op acceptatie van het transplantaat te vergroten. Hierdoor kan echter ook het virus reactiveren, wat in deze patiëntengroep vaak tot ernstige problemen leidt. In hoofdstuk 7 kijken we naar de reactie van het immuunsysteem op deze reactivatie. Specifiek is gekeken naar GrM en GrB gehaltes in NK cellen, NKT cellen en diverse subsets van T cellen, omdat deze betrokken zijn bij de afweer tegen het virus. In de totale patiëntengroep werden verhoogde percentages GrM- en GrB-producerende CD4+, CD8+ en γδ T cellen gevonden na stamceltransplantatie, en tevens verhoogde percentages GrM-producerende NKT cellen. In 14 van de 31 onderzochte patiënten was sprake van HCMV reactivatie na stamceltransplantatie. In deze patiënten werden, in vergelijking met patiënten zonder HCMV reactivatie, verhoogde GrM niveaus gemeten in de totale populatie CD4+ T cellen en in de subgroep central memory T (TCM) cellen. GrB was verhoogd in de totale populatie CD8+ T cellen en in de subgroepen CD4+ T effector memory (TEM) cellen en CD8+ TEM cellen. Deze bevindingen duiden op een rol van GrM en GrB bij het tegengaan van HCMV reactivatie. Daarnaast werden bij diverse patiënten hogere GrM spiegels in het bloed aangetroffen na reactivatie van het virus, wat erop duidt dat GrM ook een extracellulaire functie kan hebben bij het opruimen van het virus.

Conclusie

Uit het in dit proefschrift beschreven onderzoek blijkt dat verschillende granzymen een extracellulaire rol kunnen hebben bij verschillende ontstekingsziektes. We tonen betrokkenheid aan van extracellulaire granzymen bij reumatische aandoeningen, virusinfecties en bacteriële infecties. Zoals eerder vermeld is het van groot belang dat ons lichaam bij een infectie een adequate ontstekingsreactie op gang brengt om ziekteverwekkers op te ruimen. Echter als deze ontstekingsreactie uit de hand loopt, kan orgaanschade ontstaan en kunnen patiënten zelfs overlijden. Dit is vaak het geval bij bloedvergiftiging, dus bij de aanwezigheid van bacteriën in het bloed. Zoals aangetoond in hoofdstuk 2 en 3 verergeren granzymen de reactie van het immuunsysteem op de aanwezigheid van bacteriën of bacteriële stoffen. De werking van granzymen zou daarom in het nadeel kunnen zijn van patiënten met bloedvergiftiging. In dat geval zou het remmen van deze functie van GrK en van andere granzymen ervoor kunnen zorgen dat de immuunreactie minder heftig verloopt, waardoor de organen van de patiënt minder schade oplopen en er meer gelegenheid is om de bacteriële infectie te bestrijden. Alvorens dit getest kan worden is echter nog meer onderzoek nodig. Alleen als bekend is hoe alle vijf granzymen afzonderlijk en in samenspel de door bacteriën op gang gebrachte cytokine afgifte beïnvloeden, kan worden ingegrepen in dit systeem.

ADDENDUM

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~

About the author

Annette Wensink was born in Amstelveen on 09 November 1982. She attended the secondary schools 'Het Vlietland College' in Leiden and 'Het Antonius College' in Gouda, and graduated from the latter in 2000 (VWO). After working as an administrative employee for one year, she started to study Biological Sciences (specialization Cell Biology) at Utrecht University in 2001. From October 2003 till October 2004, she was vice-chairman on the board of the students' study association 'Utrechtse Biologen Vereniging'. She received her Bachelor of Science degree in 2006. In the same year, she commenced following the Biomolecular Sciences Masters programme at Utrecht University.

During her Master, Annette performed a nine-month internship within the research group Cellular Architecture and Dynamics from the Beta Faculty of Utrecht University, under supervision of Dr. Paul van Bergen and Henegouwen and Drs. Erik Hofman. Here, she studied the influence of gangliosides on the affinity of the EGF receptor for its ligand. She then wrote her master thesis, supervised by Dr. Elsa Regan-Klapisz from the research group Cellular Architecture and Dynamics, Beta Faculty, Utrecht University. This thesis dealt with the possible role of the Arachidonic Acid-releasing phospholipase cPLA2 in intracellular vesicle traffic. Annette next continued her practical training in the lab of the Institute for Risk Assessment Sciences at Utrecht University, under supervision of Dr. Raymond Pieters and Drs. Marise Marcondes-Rezende. In this six-month internship, she investigated the effects of probiotics on peanut allergy in mice. During her master's education, Annette furthermore co-founded the students' gymnastics association 'U-turn', and was chairman of the board for one and a half year. Annette graduated in 2008.

After two temporary jobs (as a Biology teacher and a research technician) Annette started her PhD training in 2010, at the University Medical Center Utrecht. She was supervised by Prof. Dr. Erik Hack (Department of Immunology) and Dr. Niels Bovenschen (Department of Pathology). The results obtained during her PhD research are described in this thesis.

List of publications

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