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Granzyme D Is a Novel Murine Mast Cell Protease That Is Highly Induced by Multiple Pathways of Mast Cell Activation

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Granzymes are serine proteases known mostly for their role in the induction of apoptosis. Granzymes A and B have been extensively studied, but relatively little is known about granzymes C to G and K to M. T cells, lymphohematopoietic stromal cells, and granulated metrial gland cells express granzyme D, but the function of granzyme D is unknown. Here we show that granzyme D is expressed by murine mast cells and that its level of expression correlates positively with the extent of mast cell maturation. Coculture of mast cells with live, Gram-positive bacteria caused a profound, Toll-like receptor 2 (TLR2)-dependent induction of granzyme D expression. Granzyme D expression was also induced by isolated bacterial cell wall components, including lipopolysaccharide (LPS) and peptidoglycan, and by stem cell factor, IgE receptor cross-linking, and calcium ionophore stimulation. Granzyme D was released into the medium in response to mast cell activation. Granzyme D induction was dependent on protein kinase C and nuclear factor of activated T cells (NFAT). Together, these findings identify granzyme D as a novel murine mast cell protease and implicate granzyme D in settings where mast cells are activated, such as bacterial infection and allergy.

Mast cells (MCs) are best known for their role in allergic reactions such as anaphylaxis and asthma. More recent studies have shown that mast cells also have a critical role in host defense against pathogens (1–3), in cancer, and in autoimmune diseases (4–6). Mast cells can be activated through cross-linking of the high-affinity IgE receptor, resulting in degranulation as well as production of *de novo*-synthesized mediators (7). In addition, they can be activated through various pattern recognition receptors, including, among others, Toll-like receptors (TLRs) (8, 9), and complement receptors. Mast cells produce a wide range of mediators, including proteases (chymase, tryptase, and carboxypeptidase A3), histamine, serotonin, serglycin proteoglycan, and numerous cytokines and chemokines (10, 11).

Granzymes are a family of serine proteases that are expressed primarily by cytotoxic T lymphocytes (CTLs) (12). Humans express five granzymes, granzymes A, B, H, K, and M, while 10 granzymes have been identified in mice, granzymes A to G and K to M (13). Granzymes are involved in defense against viral infection and toward transformed cells by inducing apoptosis in target cells. Many other, nonapoptotic functions of granzymes have also been identified, including modification of the extracellular matrix, degradation of viral products, receptor activation, and induction of cytokine production (13–15). Moreover, it was recently reported that granzyme A- and M-deficient mice show increased susceptibility to lipopolysaccharide (LPS)-induced toxicity, implicating granzymes as regulators of inflammation (16, 17).

Relatively little is known about granzyme D. It has been reported to be expressed in lymphohematopoietic stromal cells, where it was suggested to have a role in stromal cell-lymphocyte interactions (18). Furthermore, Allen and Nilsen-Hamilton reported previously that granzyme D is developmentally regulated during pregnancy together with granzymes E, F, and G in granulated metrial gland cells and is upregulated by interleukin-2 (IL-2) and IL-15 (19).

In a previous study, we investigated the global effects of live *Streptococcus equi* on the gene expression pattern in mast cells (20). *S. equi* is a Gram-positive serological group C streptococcus that causes severe upper respiratory tract infections in horses (21). *S. equi* is also pathogenic for rodents (22). We found that coculture of mast cells with live bacteria induced a profound induction of numerous inflammatory cytokines and chemokines as well as of several transcription factors and signaling molecules. In addition, the gene array data suggested that granzyme D may be expressed. Here we show that granzyme D is indeed expressed by mast cells and that its expression is dramatically induced by coculture with live bacteria and also by isolated bacterial cell wall products, stem cell factor (SCF), and IgE receptor cross-linking. Furthermore, we show that granzyme D induction is dependent on protein kinase C (PKC) and on the transcription factor nuclear factor of activated T cells (NFAT). Together, this study identifies granzyme D as a novel mast cell protease and implicates granzyme D in settings where mast cells are activated.

MATERIALS AND METHODS

BMMCs. Bone marrow-derived mast cells (BMMCs) from wild-type (WT) C57BL/6, TLR2$^{-/-}$, and TLR4$^{-/-}$ mice were prepared and cultured as described previously (23). For generation of BMMCs with a connective tissue mast cell (CTMC)-like phenotype, 30% WEHI-3B conditioned medium (which contains IL-3) or 5 ng/ml IL-3 (Peprotech, Rocky Hill, NJ) and 25 ng/ml SCF (Peprotech) were added. For generation of mucosal mast cell (MMC)-like BMMCs, 5 ng/ml IL-3, 25 ng/ml SCF, 5 ng/ml IL-9, and 1 ng/ml transforming growth factor β (TGF-β) were added.

PCMCs. Peritoneal cell-derived mast cells (PCMCs) were established according to a protocol described previously by Malbec et al. (24). The PCMC population was of a homogenous mast cell phenotype, as judged

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TABLE 1 Primers used for qPCR

Target, direction[b]	Sequence	Amplicon size (bp)	Efficiency[a]
Hprt, fw	5′-GAT TAG CGA TGA TGA ACC AGG TTA-3′	133	1.09
Hprt, rev	5′-GAC ATC TCG AGC AAG TCT TTC AGT-3′		
Granzyme D, fw	5′-AGC TGG AGC AGA GGA GAT CA-3′	172	1.12
Granzyme D, rev	5′-TTG GAC AGA GCT GTT TTT GC-3′		
Granzyme B, fw	5′-GCC AAT GGA ACA CCT CTT CT-3′	147	1.09
Granzyme B, rev	5′-GAT AAC ATT CTC GGG GCA CT-3′		
mMCP-6, fw	5′-CAT TGA TAA TGA CGA GCC TCT CC-3′	116	1.11
mMCP-6, rev	5′-CAT CTC CCG TGT AGA GGC CAG-3′		
Perforin, fw	5′-ATG AGC AGA GCC CGG TGG CA-3′	127	1.04
Perforin, rev	5′-GTC GTG GGC AGC AGT CCT GG-3′		

[a] Efficiency = $10^{(-1/\text{slope})} - 1$.
[b] fw, forward; rev, reverse.

by morphological criteria, expression of cell surface c-kit and high-affinity IgE receptor, and expression of mast cell granule proteases (25).

CTLL-2 cells. CTLL-2 cells were obtained from the ATCC (ATCC TIB214). They were cultured in RPMI with Glutamax (Invitrogen) supplemented with 1 mM sodium pyruvate (Invitrogen), 10% fetal bovine serum (FBS), 60 μg/ml penicillin, 50 μg/ml streptomycin, and 10% T-STIM with concanavalin A (ConA) (BD, Franklin Lakes, NJ). Medium was changed every 3 to 4 days, and cells were kept at a concentration of 2×10^4 cells/ml.

***In vitro* exposure of BMMCs to bacteria, LPS, peptidoglycan, and SCF.** BMMCs (grown with WEHI-3B conditioned medium) and PCMCs were washed 2 times in phosphate-buffered saline (PBS) and resuspended in antibiotic-free medium (otherwise as described above) at a density of 1×10^6 cells/ml and plated into 24-well tissue plates. *S. equi* (strain 62) cells were grown overnight in Todd-Hewitt broth (Oxoid, Ltd., Basingstoke, Hampshire, UK) supplemented with 0.5% yeast extract (THB-yeast), washed 2 times in PBS, and added to a final concentration of $\sim 2.5 \times 10^7$ cells/ml at a multiplicity of infection (MOI) of 1:25. *Escherichia coli* (Novablue) cells were grown overnight in LB medium, inoculated in the morning in new LB medium, and, after ~3 h, washed 2 times in PBS and added to final concentrations of $\sim 2.5 \times 10^7$ cells/ml at an MOI of 1:25 and $\sim 1 \times 10^8$cells/ml at an MOI of 1:100. Alternatively, 1 μg/ml LPS, 50 μg/ml peptidoglycan (PGN), or 25 ng/ml SCF was added. For inhibition experiments, 1 μM PKC inhibitor Gö6976 or Gö6983, 100 nM NF-κB inhibitor [6-amino-4-(4-phenoxyphenylethylamino)quinazoline], or 5 μM NFAT inhibitor (11R-VIVIT; Calbiochem, Darmstadt, Germany) was incubated with the BMMCs 30 min before the addition of stimulus. After various time points, cells were collected by centrifugation; medium and cell fractions were frozen and stored at −20°C.

Degranulation. IgE- and A23187-mediated degranulation was performed as previously described (10).

Antibody production and purification. Rabbit antisera against the peptide CRSINDTKASARLRE (designed to be specific for granzyme D and not to cross-react with granzyme B) were produced by Genscript (Piscataway, NJ). Antisera were subsequently purified on a HiTrap NHS-activated column (GE Healthcare, Uppsala, Sweden) coupled to the granzyme D-specific peptide. Ligand coupling to the N-hydroxysuccinimide (NHS) column was done according to the manufacturer's instructions. Purification was done as follows. The column was washed with 3 ml binding buffer (75 mM Tris-HCl [pH 8.0]), 3 ml elution buffer (0.5 M NaCl, 100 mM glycine-HCl [pH 2.7]), and 10 ml binding buffer. Next, 10 ml antisera (diluted 1:1 in binding buffer) was loaded onto the column, followed by washing with 8 ml binding buffer. The column was then eluted with elution buffer (1-ml fractions) in tubes containing 50 μl neutralization buffer (1 M Tris-HCl [pH 9.0]). A total volume of 6 ml was collected. The fraction containing the largest amount of protein was then transferred into PBS (containing 0.01% sodium azide) by using a PD-10 column (GE Healthcare). The IgG fractions from the affinity-purified antibody and preimmune serum (from the same rabbit) were then purified on a protein A column (GE Healthcare), as follows: the column was washed with 10 ml binding buffer (50 mM Tris-HCl [pH 7.2]), and the sample was then loaded onto the column (diluted 1:1 with binding buffer), followed by washing with 10 ml binding buffer. The column was eluted in the same way as the NHS column and finally buffer exchanged by using PD-10 columns.

Immunoblotting. Immunoblotting using rabbit anti-mouse granzyme D (diluted 1:500), rabbit anti-mouse mast cell protease 6 (mMCP-6) (diluted 1:500), or goat anti-mouse β-actin (diluted 1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was performed as described previously (23). Briefly, samples were separated by SDS-PAGE and blotted onto polyvinylidene difluoride (PVDF) membranes, followed by blocking and incubation with primary antibodies overnight at 4°C. After washing, membranes were incubated with peroxidase-labeled anti-rabbit secondary antibody, followed by washing and development of signals by using the ECL system (GE Healthcare). For verification of antibody specificity, recombinant granzyme D (R&D Systems, Minneapolis, MN) and granzyme B (Peprotech) were used.

Immunocytochemistry. Cytospin slides were prepared from BMMCs and CTLL-2 cells. The cytospin slides were air dried and fixed in 4% PBS-buffered formalin. Heat-induced antigen retrieval was performed with PT Module buffer (Labvision [Thermo Fisher Scientific], Fremont, CA) in a Pascal decloaking chamber (DakoCytomation, Glostrup, Denmark). Slides were washed with Tris-buffered saline (TBS)–0.05% Tween 20, followed by peroxidase blocking (Dako, Glostrup, Denmark), blocking with goat serum (diluted 1:50), and addition of primary antibodies (rabbit anti-granzyme D or control antibody from preimmune serum, diluted 1:1,000 in TBS). The Envision polymer system (Dako) together with 3,3′-diaminobenzidine (DAB) was used for detection.

RNA preparation and qPCR. Real-time quantitative PCR (qPCR) using the primers specified in Table 1 was performed as previously described (23). Total RNA was isolated by using the NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany), and first-strand cDNA was synthesized by using random hexamers and the SuperScript II kit (Invitrogen, Carlsbad, CA). Real-time qPCR was performed with a final volume of 10 μl containing 200 nM both primers, iQ SYBR green Supermix (Bio-Rad), and 1 μl of cDNA, using a 7900HT Fast real-time PCR system and SDS 2.3 software (Applied Biosystems, Foster City, CA). PCR cycling conditions included a 95°C heating step for 10 min at the beginning of every run. The samples were then cycled 40 times at 95°C for 30 s (denaturation), 59°C for 20 s (annealing), and 72°C for 20 s (extension). A melting curve from

TABLE 2 Fold change in granzyme expression in mast cells in response to coculture with *S. equi* at an MOI of 25 after 4 h of incubation[a]

Gene	Fold change	Adjusted *P* value
Granzyme D (Gzmd)	42.2	0.000011
Granzyme E (Gzme)	3.58	0.005
Granzyme G (Gzmg)	2.01	0.003
Granzyme F (Gzmf)	1.36	0.22
Granzyme M (Gzmm)	1.20	0.18
Granzyme N (Gzmn)	1.16	0.39
Granzyme A (Gzma)	1.15	0.24
Granzyme K (Gzmk)	1.14	0.27
Granzyme C (Gzmc)	1.09	0.44
Granzyme B (Gzmb)	−1.27	0.085

[a] Significant adjusted *P* value of <0.05. Data are derived from reference 20.

60°C to 90°C was generated at the end of every run. Primer efficiency was determined by performing qPCR with the following dilutions of cDNA: 1:1, 1:10, and 1:100. Hypoxanthine guanine phosphoribosyl transferase (Hprt) was used as a housekeeping gene.

Statistical analysis. Data are shown as means ± standard errors of the means (SEM). Statistical analyses were performed by using GraphPad Prism 4.0c (GraphPad Software) and an unpaired Student *t* test for two-tailed distributions (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

RESULTS

Granzyme D was identified as one of the most upregulated genes by using Affymetrix gene chip microarray analysis comparing control bone marrow-derived mast cells (BMMCs) with BMMCs cocultured with *S. equi*. As shown in Table 2 (results derived from original data reported previously [20]), out of all of the granzymes, coculture with *S. equi* had the most profound effect on the granzyme D gene, which was upregulated 42 times. Granzymes E and G were also slightly upregulated, but all other granzymes were unaffected. An examination of additional serine protease genes covered by the Affymetrix gene chip revealed only small effects, including modest inductions of the Mcpt8 (coding for mouse mast cell protease 8 [mMCP-8]) (~2.5-fold), Mcpt1 (coding for mMCP-1) (~1.8-fold), and Mcpt4 (coding for mMCP-4) (~1.6-fold) genes (data not shown). Thus, among the family of serine proteases, granzyme D expression is selectively induced in mast cells upon coculture with live Gram-positive streptococci.

To verify the expression of granzyme D in mast cells, qPCR was performed by using the primers specified in Table 1. Indeed, granzyme D expression in BMMCs was verified (Table 3), and the dramatic induction of the granzyme D gene upon coculture with live streptococci was also verified (Fig. 1A). Since granzyme B has been shown to be expressed by mast cells (26), it was also of interest to monitor the expression of granzyme B in response to coculture with live streptococci. However, in contrast to granzyme D, coculture with bacteria did not cause a significant induction of the granzyme B gene (Fig. 1B). However, the granzyme B expression level was considerably higher than that of granzyme D in nonstimulated cells, and it is notable that the basal granzyme B expression level was in the same range in BMMCs as in CTLL-2 cells (a CTL-like cell line) (Table 3).

Bacteria can activate cells through different pattern recognition receptors, including the TLRs, of which TLR4 is the main receptor for LPS present on the surface of Gram-negative bacteria and TLR2 is a main receptor for PGN present on Gram-positive bacteria (27). Notably, multiple TLRs, including TLR2 and TLR4,

TABLE 3 Expression of the granzyme D, granzyme B, and perforin genes in CTLL-2 and BMMCs[a]

	Expression level	
Gene	CTLL-2 cells	BMMCs
Granzyme D	101.50	0.02
Granzyme B	28.00	33.80
Perforin	6.60	ND

[a] All values are relative to values for the housekeeping gene Hprt (Hprt expression is set as 1). ND, nondetectable.

are expressed by mast cells (8, 9), and we therefore hypothesized that granzyme D induction could be mediated by any of these TLRs. In particular, we hypothesized that granzyme D induction may be blunted in TLR2-deficient cells, since *S. equi* is a Gram-positive pathogen. Indeed, BMMCs deficient in TLR2 showed a significantly lower level of induction of the granzyme D gene than WT controls, and there was a also trend of decreased granzyme D induction in TLR4$^{-/-}$ BMMCs (Fig. 1A). In contrast, the absence of TLR2 or TLR4 did not significantly affect the expression of granzyme B either under baseline conditions or in response to live streptococci (Fig. 1B).

For comparison, we also examined whether granzyme D expression was induced in response to Gram-negative bacteria (*E. coli*). As shown in Fig. 1C, granzyme D was upregulated in cocultures of mast cells and *E. coli*, but the extent of upregulation was limited compared to the upregulation in response to *S. equi*. It was also seen that granzyme B, although not being induced by *S. equi*, was significantly upregulated by *E. coli* (Fig. 1D).

To study if the effects of live bacteria on granzyme D and B expression were reflected by similar effects on gene regulation in response to purified cell wall components of Gram-positive and -negative bacteria, respectively, we examined the effects of PGN and LPS. These experiments revealed a significant induction of granzyme D in response to LPS (Fig. 1E) and a significant although quite small induction in response to PGN (Fig. 1G). Granzyme B was not significantly induced in response to either LPS or PGN, although there was a trend of granzyme B induction in response to LPS (Fig. 1F and H).

To assess whether granzyme D is also expressed by terminally differentiated MCs, we investigated if granzyme D is expressed by peritoneal cell-derived mast cells (PCMCs), an emerging *ex vivo* system for studies of mast cell function (24). Indeed, granzyme D was expressed by PCMCs, and similar to BMMCs, the expression of granzyme D was strongly upregulated after coculture of PCMCs with live *S. equi* cells (Fig. 1I).

Next, we assessed whether granzyme D expression correlates with mast cell maturation by investigating the expression of granzyme D during development of bone marrow cells into mast cells. In mice, mast cells are classified as either connective tissue-type mast cells (CTMCs) or mucosal-type mast cells (MMCs), with the different subpopulations expressing different sets of mast cell-specific proteases (7, 28). In order to investigate whether granzyme D expression is selectively expressed by any of these mast cell subtypes, bone marrow cells were developed into both CTMC-like and MMC-like BMMCs. To accomplish this, different sets of cytokines were used. To generate CTMC-like BMMCs, either WEHI-3B conditioned medium (containing IL-3) or purified IL-3 plus SCF were used (29). For generation of MMC-like

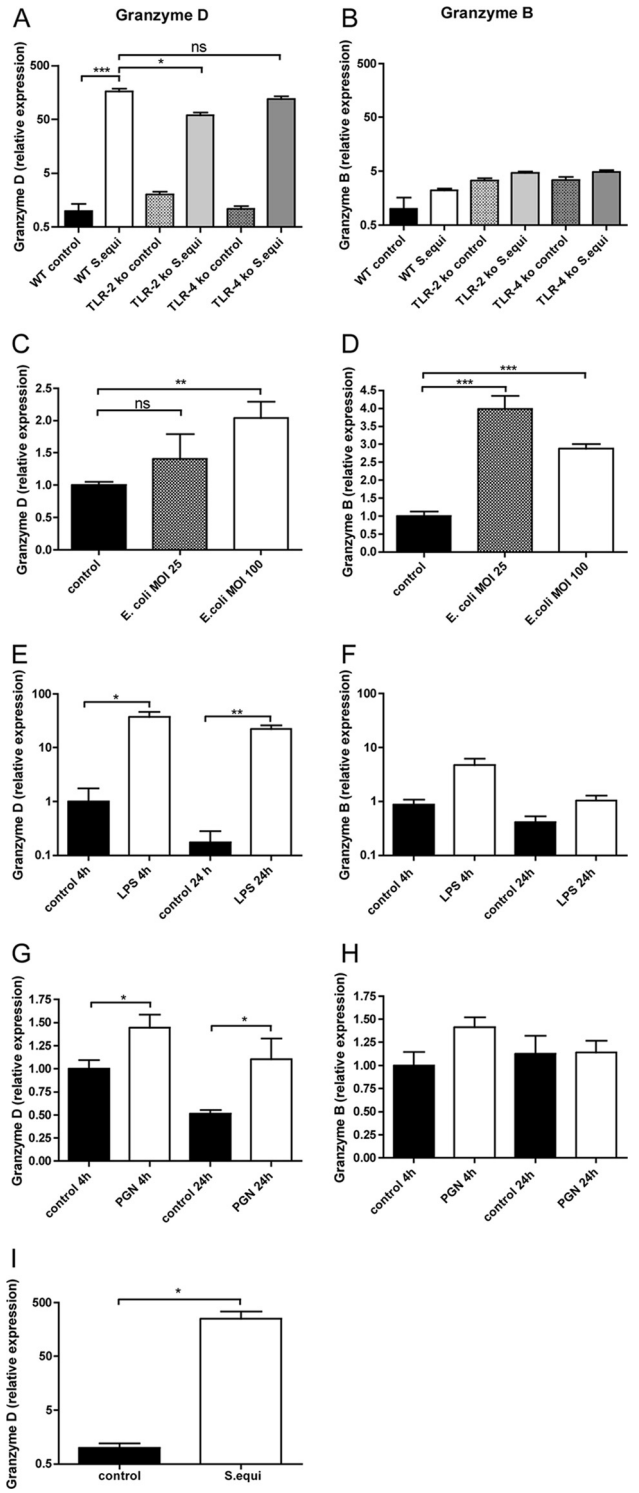

FIG 1 Gram-positive streptococci (*S. equi*) and Gram-negative bacteria (*E. coli*) induce the expression of granzyme D in mast cells. WT, TLR2$^{-/-}$, and TLR4$^{-/-}$ BMMCs (A to H) or PCMCs (I) (10^6 cells/ml) were cocultured for 4 h with live *S. equi* cells (MOI = 25) (A, B, and I) or live *E. coli* cells (MOI = 25 or 100) (C and D). BMMCs (10^6 cells/ml) were incubated with 10 μg/ml LPS (E and F) or 50 μg/ml PGN (G and H). At the time points indicated, total RNA was extracted and analyzed by qPCR for levels of mRNA coding for granzyme D (A, C, E, G, and I) or granzyme B (B, D, F, and H), as indicated. All values are relative to values for nonstimulated control cells (4 h). Shown are means ± SEM (n = 3 to 4). ko, knockout. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

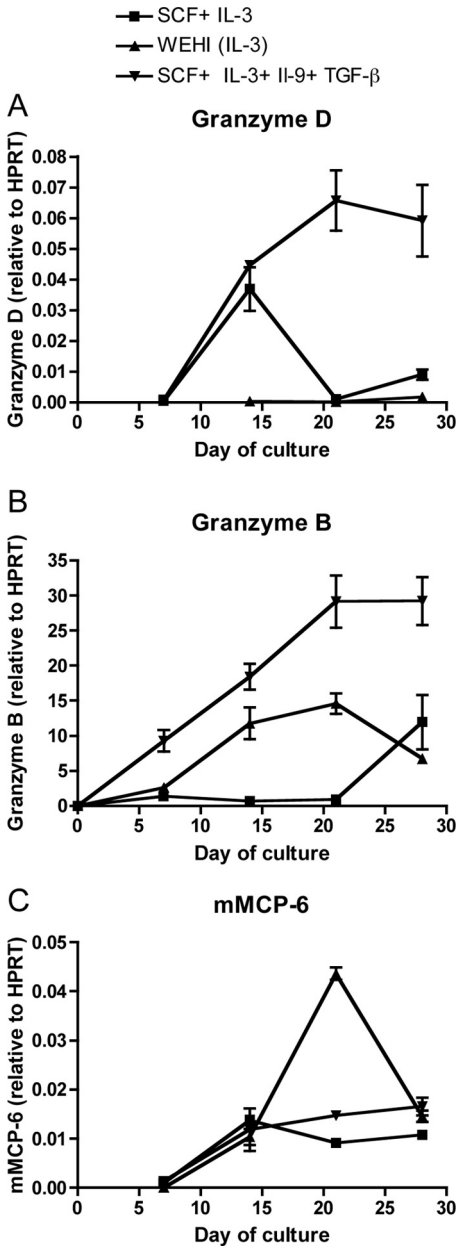

FIG 2 Relative expression levels of granzyme D (A), granzyme B (B), and mMCP-6 (C) during BMMC maturation from bone marrow precursors. CTMC-like BMMCs were developed by incubation of bone marrow precursors in medium supplemented with either WEHI-3B conditioned medium or additives of IL-3 plus SCF, as indicated. MMC-like BMMCs were developed in the presence of SCF plus IL-3, IL-9, and TGF-β. All values are relative to values for the culture with SCF plus IL-3 at day 28. Shown are means ± SEM (n = 3). The results shown are representative of 3 individual experiments.

BMMCs, recombinant IL-3 plus SCF, IL-9, and TGF-β were used (30). As shown in Fig. 2A, granzyme D expression was undetectable in bone marrow cells (day 0) but was strongly induced during late stages (from about day 14) of mast cell development. The granzyme D expression level was higher in MMC-like BMMCs than in either of the two types of CTMC-like BMMCs. Notably, CTMC-like cells grown in the presence of SCF plus IL-3 expressed higher levels of granzyme D than did CTMC-like BMMCs devel-

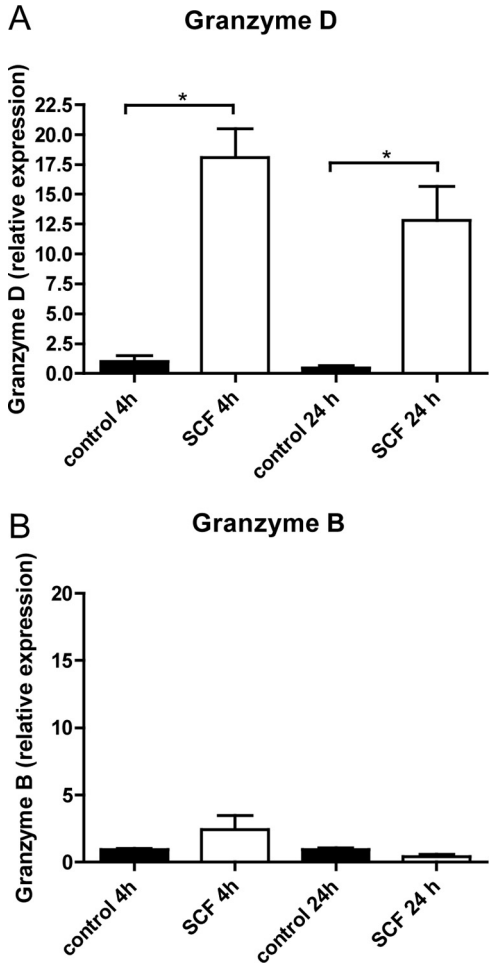

FIG 3 Relative expression levels of granzymes D and B in response to SCF. BMMCs (10^6 cells/ml) were incubated with 25 ng/ml SCF. At the time points indicated, total RNA was extracted and analyzed by qPCR for levels of mRNA coding for granzyme D (A) or granzyme B (B). All values are relative to values for nonstimulated control cells (4 h). Shown are means ± SEM ($n = 3$). The results shown are representative of 2 individual experiments. *, $P < 0.05$.

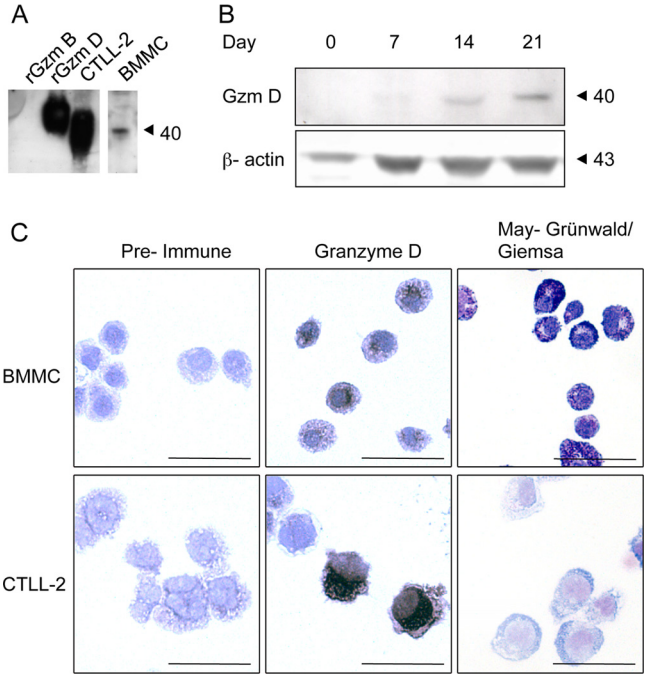

FIG 4 Granzyme D protein in BMMCs. Granzyme D-specific rabbit antisera were generated as described in Materials and Methods. (A) Western blot analysis showing that the granzyme D-specific antibody detects recombinant granzyme D (rGzm D) (0.5 μg protein loaded) but does not cross-react with recombinant granzyme B (rGzm B) (0.5 μg protein loaded). The antibody also recognized similar-sized proteins in both CTTL-2 cells and BMMCs. Note that recombinant granzyme D, due to a His tag for purification, migrates slower than granzyme D in CTLL-2 cells and BMMCs. (B) Western blot showing an increased level of granzyme D protein during BMMC development from days 0 to 21. β-Actin was used as a loading control. (C) Immunohistochemical staining of BMMCs and CTLL-2 cells with the granzyme D-specific antibody, using antibody from preimmune antiserum as a negative control. Cells were also stained with May-Grünwald/Giemsa stain. The results shown are representative of 3 individual experiments.

oped in the presence of WEHI-3B conditioned medium (containing IL-3 but low levels of SCF), raising the possibility that granzyme D expression is induced by SCF. Indeed, addition of SCF to BMMCs (developed in the presence of WEHI-3B conditioned medium) caused a profound induction of granzyme D expression, whereas no significant effect on granzyme B expression was seen (Fig. 3A and B). Notably, SCF is the main growth factor for mast cells (31), and these data thus suggest that granzyme D is associated with maturation of bone marrow precursor cells into mature mast cells.

Granzyme B expression also correlated positively with mast cell development, and similar to granzyme D, the granzyme B expression level was higher in MMC-like BMMCs than in either of the two types of CTMC-like BMMCs (Fig. 2B). Notably, granzyme B expression was induced at earlier stages of mast cell development than seen for granzyme D, with granzyme B expression being detectable from approximately day 7 and onwards (Fig. 2B). For comparison, the expression of mMCP-6 during mast cell development was monitored. In agreement with mMCP-6 being a marker of the CTMC-like phenotype (32), the mMCP-6 gene expression level was high in late-stage cultures (from day ~14) of CTMC-like BMMCs. However, in agreement with previous findings (33), mMCP-6 gene expression was also clearly detectable in BMMCs of the MMC-like phenotype (Fig. 2C).

In CTLs, granzymes are located in cytotoxic granules and are delivered into target cells by a perforin-mediated mechanism (14). If granzyme D would have an analogous function in mast cells as in CTLs, it may be expected that granzyme D is coexpressed with perforin. However, perforin expression was undetectable in mast cells, whereas robust expression of perforin was detected in CTLL-2 cells (Table 3).

To study the expression of granzyme D in mast cells at the protein level, antibodies toward a unique peptide of granzyme D were generated (see Materials and Methods). As shown in Fig. 4A, the anti-granzyme D antibody detected an ~40-kDa band in CTLL-2 cells. A band with the same migration velocity was seen in lysates of BMMCs, verifying that granzyme D is indeed expressed by mast cells (Fig. 4A). Moreover, as shown in Fig. 4A, the antibody reacted with recombinant granzyme D but showed no cross-reactivity with recombinant granzyme B. Note that recombinant granzyme D, due to the presence of a His tag for purification, migrates somewhat slower than the corresponding antigen in

CTLL-2 cells. In accordance with the increased levels of granzyme D mRNA expression during mast cell differentiation (Fig. 2A), the levels of granzyme D protein showed a progressive increase as a function of increasing maturation (Fig. 4B).

The expression of granzyme D at the protein level was also verified by immunohistochemical analysis, showing predominantly perinuclear staining of BMMCs for granzyme D (Fig. 4C). As a positive control, CTLL-2 cells were strongly stained by the granzyme D antibody (Fig. 4C), whereas IgG (protein A purified) from preimmune serum gave negligible staining. As judged by May-Grünwald/Giemsa staining, the studied BMMC population was homogenous, with essentially all cells showing a mast cell morphology (Fig. 4C).

Many of the known functions of mast cells, such as their role in anaphylactic reactions, involve binding of multivalent antigen to IgE molecules bound to the high-affinity IgE receptor on the mast cell surface, thereby causing IgE receptor cross-linking and degranulation (34). To investigate whether granzyme D expression is induced under these circumstances, granzyme D expression in response to IgE receptor cross-linking was evaluated. As shown in Fig. 5A, IgE receptor cross-linking caused a dramatic upregulation of granzyme D expression. In contrast, granzyme B expression was unaffected by IgE receptor cross-linking (Fig. 5B). IgE receptor cross-linking leads to a rapid increase in the cytosolic calcium concentration, and to directly address the role of calcium in granzyme D induction, we stimulated the BMMCs with a calcium ionophore. As shown in Fig. 5C, the calcium ionophore A23187 caused a dramatic upregulation (almost 30,000-fold) of granzyme D expression, whereas only a modest (~4-fold) induction of granzyme B was seen (Fig. 5D). Also, in PCMCs, granzyme D expression was upregulated in response to the calcium ionophore (Fig. 5E).

To investigate if mast cell activation influences the levels of granzyme D protein, BMMCs were stimulated with the calcium ionophore, followed by Western blotting using the granzyme D-specific antibody. As shown in Fig. 5F, mast cell activation caused a decrease in the amount cell-associated granzyme D 4 h after addition of activating stimulus, compatible with the release of granzyme D from preformed pools. After 24 h, intracellular levels of granzyme D protein were higher in activated than in nonactivated cells, in agreement with calcium ionophore-stimulated granzyme D expression. Moreover, granzyme D protein accumulated in the cell supernatant following calcium ionophore stimulation, suggesting that granzyme D is secreted by activated mast cells. Granzyme D was also released by mast cells in response to coculture with live bacteria (data not shown). For comparison, the effects of mast cell activation on the levels of mMCP-6, a tryptase stored in secretory granules (28), was examined. In the absence of an activating agent, cell-associated mMCP-6 protein was clearly detected (Fig. 5F, bottom). When the cells were activated by calcium ionophore stimulation, a reduction in the level of cell-associated mMCP-6 was evident, and this was accompanied by a corresponding appearance of mMCP-6 protein in the culture medium.

To elucidate the signaling pathways involved in the upregulation of granzyme D, we used inhibitors of PKCs, NF-κB, and NFAT. As shown in Fig. 6A, the PKC inhibitor Gö6976 (PKCα/β1 and PKD1 inhibitor) (35, 36) totally blocked the upregulation of granzyme D induced by IgE receptor cross-linking. In contrast, the PKC inhibitor Gö6983 (PKCα/β/γ/δ inhibitor) (35, 36) potentiated granzyme D upregulation, indicating a role for Gö6983-sensitive PKC in suppressing granzyme D expression. NF-κB blockade did not cause significant inhibition of granzyme D expression in response to either IgE receptor cross-linking (Fig. 6A) or LPS (Fig. 6C) but partially inhibited the granzyme D upregulation in response to SCF (Fig. 6B) and PGN (Fig. 6D). Inhibition of the NFAT pathway, on the other hand, almost completely abrogated granzyme D upregulation in response to IgE receptor cross-linking (Fig. 6A), SCF (Fig. 6B), and LPS (Fig. 6C) and partially blocked induction in response to PGN (Fig. 6D). Hence, granzyme D induction is strongly dependent on Gö6976-sensitive PKC and on the NFAT pathway but is less dependent on signaling through NF-κB.

DISCUSSION

It has been established for many years that granzymes stored in the lytic granules of CTLs, in particular granzymes A and B, have the ability to induce apoptosis after perforin-mediated delivery into the cytosol of target cells. However, more recent studies have shown that granzymes may have a number of other functions, including matrix remodeling, degradation of viral products, receptor activation, and induction of cytokine production (13–15, 37). Moreover, recent studies have shown that mice lacking granzyme A or M show partial resistance to LPS-induced toxicity (16, 17).

Here we show that mast cells express granzyme D. At first glance, this may seem to have some discrepancy with findings reported previously by Pardo et al., who did not detect granzyme D expression in mast cells (26). However, it is important to note that Pardo et al. developed mast cells in the presence of IL-3 alone. In agreement with data reported by Pardo et al., we show that mast cells grown in IL-3 only (developed in WEHI-3B conditioned medium) express low levels of granzyme D. On the other hand, when mast cells were grown in the presence of IL-3 together with SCF, the latter being a mast cell growth factor, robust granzyme D expression was detected. The present report thus suggests that granzyme D expression accompanies the SCF-dependent differentiation of bone marrow precursors into mature BMMCs. Granzyme D may thereby be classified as a novel mast cell serine protease. However, in contrast to the mast cell chymases and tryptases, granzyme D expression is not unique to mast cells.

Granzyme D expression was markedly induced when BMMCs were developed in the presence of TGF-β and IL-9, in addition to IL-3 plus SCF. As shown previously, culture of bone marrow cells in the presence of SCF/IL-3/TGF-β/IL-9 results in BMMCs of an MMC-like phenotype, as indicated by the expression of typical MMC markers (30). Hence, the elevated expression level of granzyme D in SCF/IL-3/TGF-β/IL-9-developed mast cells, as opposed to cultures developed in SCF/IL-3 only, suggests that high basal levels of granzyme D expression may be a characteristic of mast cells of the mucosal type.

Granzyme D expression was undetectable in bone marrow precursors and in 7-day-old cultures but was induced after about 2 weeks of culture, suggesting that granzyme D is a late-stage differentiation marker for BMMCs. In contrast, granzyme B expression was detected at earlier stages of mast cell maturation. In a previous report, Garcia-Sanz et al. investigated granzyme expression in different cell types. They used two mast cell lines, ABFTL1 and P815, but did not detect granzyme D expression in either of these cell lines (12). However, a likely explanation for the lack of granzyme

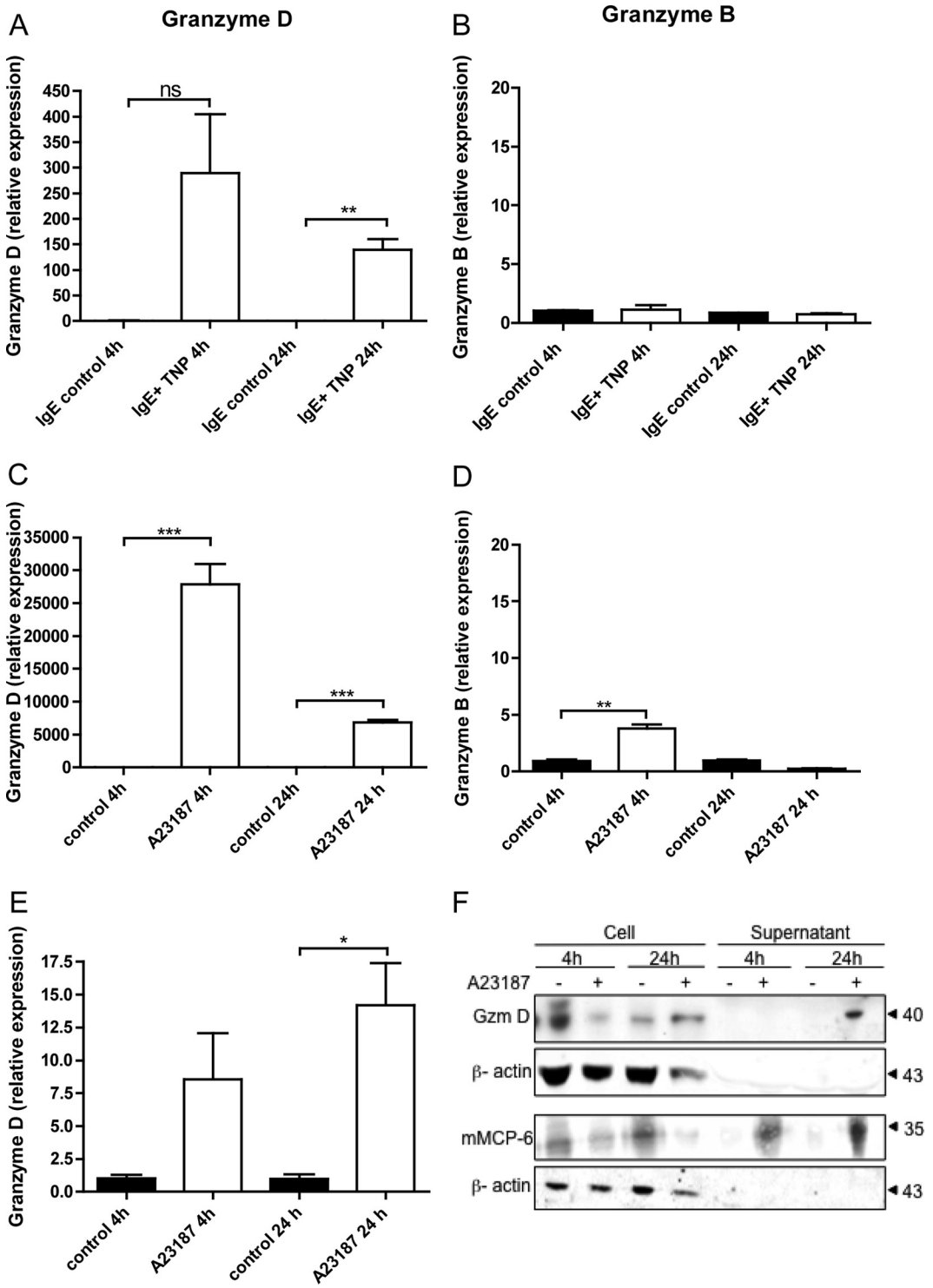

FIG 5 Relative expression levels of granzymes D and B in response to IgE receptor cross-linking and stimulation by calcium ionophore. (A and B) BMMCs (10^6 cells/ml) were incubated with α-trinitrophenyl (α-TNP) IgE overnight. Cells were then either left untreated (IgE control) or treated with TNP-ovalbumin (OVA) (IgE + TNP) to induce IgE receptor cross-linking. At the time points indicated, total RNA was extracted and analyzed by qPCR for levels of mRNA coding for granzyme D (A) or granzyme B (B). ns, not significant. (C to E) BMMCs (C and D) or PCMCs (E) (10^6 cells/ml) were treated with 2 μM calcium ionophore A23187. At the time points indicated, total RNA was extracted and analyzed by qPCR for the content of mRNA coding for granzyme D (C and E) or granzyme B (D). All values are relative to values for nonstimulated control cells (4 h). Shown are means ± SEM ($n = 3$). (F) Western blot showing the effect of calcium ionophore (A23187) (2 μM) stimulation of BMMCs on the levels of cell-associated and released (supernatant) granzyme D and mMCP-6. β-Actin was used as a loading control. The results shown are representative of 3 individual experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

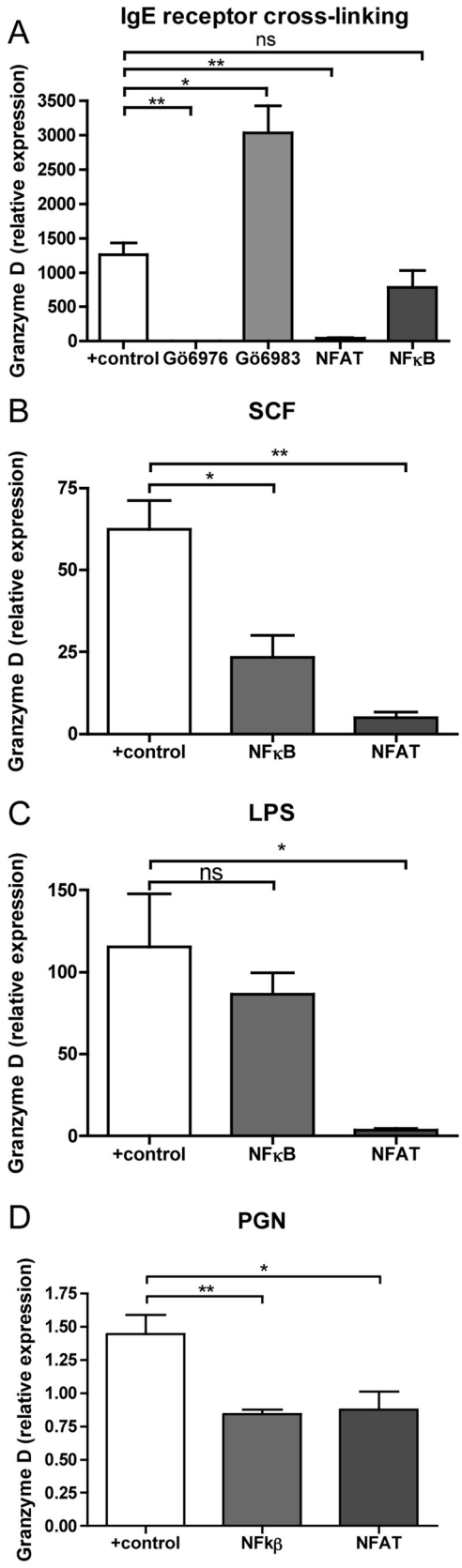

FIG 6 Granzyme D upregulation in response to different stimuli is dependent on PKC and NFAT. BMMCs (10^6 cells/ml) were activated by either IgE receptor cross-linking (A), SCF (B), LPS (C), or PGN (D). Thirty minutes before addition of stimuli, various inhibitors were added: 1 μM PKC inhibitor Gö6976, 1 μM PKC inhibitor Gö6983, 100 nM NF-κB inhibitor, or 5 μM NFAT inhibitor. Four hours after addition of mast cell-activating stimuli, total RNA was extracted and analyzed by qPCR for levels of mRNA coding for granzyme D. All values are relative to values for nonstimulated cells. +control, granzyme D expression in BMMCs activated by the indicated stimuli (without addition of inhibitor). Shown are means ± SEM ($n = 3$). *, $P < 0.05$; **, $P < 0.01$.

D expression is that both of these cell lines represent relatively undifferentiated states of mast cells. Furthermore, the detection method used by Garcia-Sanz et al., an RNase protection assay, may not have been as sensitive as the one used in this study, i.e., qPCR.

In addition to demonstrating that late-stage-differentiated BMMCs show baseline expression levels of granzyme D, we show that granzyme D expression is highly inducible by a panel of mast cell-activating agents. First, granzyme D expression was profoundly induced in response to coculture with live Gram-positive streptococci. Mast cells are strongly implicated in defense against various bacterial insults (3), but the mechanisms by which mast cells contribute to antibacterial defense are only partly understood. Based on this study, we propose that granzyme D expression and release into the surrounding milieu may contribute to the host defense against bacterial insult.

In line with the induction of granzyme D by Gram-positive bacteria, PGN, i.e., a major cell wall component of Gram-positive bacteria, was also shown to induce granzyme D expression. However, it is important to note that the extent of granzyme D induction by PGN was very moderate in comparison with the effects of live bacteria, suggesting that cell wall PGN is not the primary bacterial component responsible for the strong inducing effect of live *S. equi*. Furthermore, we show that the extent of granzyme D induction in response to live Gram-positive streptococci was reduced in mast cells lacking TLR2, TLR2 being a major pattern recognition receptor for PGN (27). We also show that granzyme D is induced by Gram-negative bacteria (*E. coli*), although the effect of *E. coli* on granzyme D expression was much lower than the effect of *S. equi*. It was also seen that LPS, a major cell wall component of Gram-negative bacteria, induces granzyme D expression. Together, our data thus indicate that the induction of granzyme D is a general event occurring in response to multiple types of bacteria and in response to multiple bacterium-derived compounds.

In addition to being induced by components related to the innate immune system, we show that granzyme D is induced in response to IgE receptor cross-linking, suggesting that granzyme D induction also occurs by adaptive immune mechanisms. Mast cell activation through the IgE receptor pathway has been shown to induce numerous proinflammatory compounds, such as various cytokines and chemokines (38), but the expression of serine proteases of the chymase and tryptase type does not appear to be influenced by IgE receptor cross-linking (39). Hence, to the best of our knowledge, this report is the first to demonstrate a robust upregulation of any serine protease in response to IgE receptor cross-linking.

To gain further insight into the mechanism of granzyme D upregulation, we investigated the roles of various signaling pathways. IgE receptor engagement is known to cause a pronounced cytosolic calcium influx from both intracellular and extracellular

sources (34). In line with a role of calcium signaling in granzyme D induction, calcium ionophore stimulation caused a dramatic upregulation of granzyme D expression.

Using inhibitors, we show that the upregulation of granzyme D is dependent on a Gö6976-sensitive PKC but also that a Gö6983-sensitive PKC represses expression. In agreement with these findings, mast cells deficient in PKCβI (Gö6976 sensitive) show impaired cytokine production in response to IgE receptor cross-linking (40, 41). Also in line with the present findings, PKCδ (Gö6983 sensitive) has been shown to downregulate mast cell degranulation (42). We also show that granzyme D induction in response to IgE receptor cross-linking is strongly dependent on NFAT, in agreement with previous findings demonstrating that IgE-mediated induction of tumor necrosis factor alpha (TNF-α) and IL-13 in mast cells is dependent on the NFAT pathway (43). Notably, granzyme D induction in response to both SCF and LPS was also strongly dependent on the NFAT pathway.

In summary, the present report implicates granzyme D as a novel mast cell serine protease that is strongly upregulated by multiple regimens of mast cell activation. Granzyme D may thus be released under various circumstances in which mast cells are activated and may therefore have an impact on any pathological condition in which mast cells contribute. In view of the known proapoptotic function of other granzyme family members, it is tempting to speculate that granzyme D exocytosed by mast cells may have a role in inducing cell death. Alternatively, in analogy with the reported role of granzyme B in extracellular matrix remodeling (37), exocytosed granzyme D may take part in such processes. However, these possibilities remain to be experimentally evaluated, e.g., through the development of granzyme D-deficient animals.

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