Cell death induced by Granzyme C

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ABSTRACT

Although the function of granzymes A and B have been defined, the functions the other highly expressed granzymes of murine cytotoxic lymphocytes (C, D, and F) have not yet been evaluated. In this report, we describe the ability of murine GzmC (which is most closely related to human granzyme H) to cause cell death. The induction of death requires its protease activity, and is characterized by the rapid externalization of phosphatidylserine, nuclear condensation and collapse, and single-stranded DNA nicking. The kinetics of these events are similar to those caused by Granzyme B, and its potency (defined on a molar basis) is also equivalent. The induction of death did not involve the activation of caspases, the cleavage of BID, or the activation of the CAD nuclease. However, Granzyme C did cause rapid mitochondrial swelling and depolarization in intact cells or in isolated mitochondria, and this mitochondrial damage was not prevented by cyclosporin A pretreatment. These results suggest that Granzyme C rapidly induces target cell death by attacking both nuclear and mitochondrial targets, and that these targets are distinct from those utilized by Granzyme B to cause classical apoptosis.
INTRODUCTION

Although a large number of granzyme (gzm) genes have been identified in the mouse (A, B, C, D, E, F, G, K, and M)\(^1\), the functions of only two of these enzymes (Gzms A and B) have been clearly defined\(^2-4\). When either GzmA or B is introduced into target cells with perforin, cell death is induced\(^5,6\). However, the critical cellular substrates for these two enzymes are clearly different. Granzyme B (GzmB) is an aspase\(^7,8\), and it is known to cleave and activate several caspases immediately upon target cell entry, including caspase-3 and -8\(^2\). GzmB also cleaves ICAD, which leads to the activation of caspase-activated DNase (CAD)\(^9,10\). Finally, GzmB is known to cleave and activate BID, which translocates to the mitochondria and facilitates the organization of a mitochondrial pore created by BAX and/or BAK, which is followed by cytochrome \(c\) (cyt \(c\)) release and apoptosome assembly\(^11-16\). GzmB can also induce rapid mitochondrial depolarization independently of BAX and BAK, in cooperation with an unknown cellular factor\(^16\). Granzyme A (GzmA), on the other hand, is a tryptase, and its known substrates include histone H\(_1\)\(^17\), nuclear lamins\(^18\); and SET, a nucleosome assembly protein that is part of a large endoplasmic reticulum-associated complex\(^19\). The precise contributions of these substrates to GzmA-induced cell death are not yet clear.

Mice have more granzyme genes than humans. While both species have Gzms A and K in tightly linked clusters\(^20\), the genes linked with GzmB are different in the two species. In the human GzmB cluster, GzmB lies upstream from granzyme H (GzmH)\(^21\), which has chymase specificity\(^22\). The mouse GzmB cluster is much more complex. The GzmB gene lies upstream from Gzms C, F, G, D, and E \((5'\rightarrow3')\)\(^23\). All of the genes are in the same transcriptional orientation, and all are expressed in cytotoxic lymphocytes to varying extents. In cytotoxic T cells activated \textit{in vitro} with PMA and ionomycin (or with one-way mixed lymphocyte reactions),
the predominant granzymes expressed are A and B. However, in lymphokine-activated killer (LAK) cell preparations and natural killer (NK) cell lines, Gzms C, D, and F are also expressed at high levels, along with A and B. The patterns of granzyme gene expression with different in vivo activation protocols have not yet been clearly defined. Even though Gzms C, D, and F can be highly expressed, their functions are unknown, and we have therefore referred to these enzymes as the “orphan” gzms.

The human orphan granzyme, GzmH, lies just downstream from GzmB, and is also maximally expressed in the LAK/NK environment. The murine orphan granzyme gene most closely related to GzmH is GzmC, which is also the first gene found downstream from GzmB in the mouse genome. In this report, we show that GzmC is capable of causing cell death. It induces many features of target cell apoptosis in a reconstituted in vitro system, but several of these features are distinct from those produced by GzmB. GzmC does not cause target cell death by activating caspases, BID, or ICAD, but it does cause direct effects on mitochondria that lead to swelling and depolarization. Therefore, GzmC is a functional protease in the cytotoxic lymphocyte repertoire, inducing target cell death with a mechanism that is different from that of granzymes A or B.
MATERIALS AND METHODS

Recombinant Granzymes and Perforin Purification

RGzmC was synthesized in *Pichia pastoris* and purified as previously described for rGzmB. Mutant rGzmCS184A was produced as described for rGzmBS183A. rGzmC production was detected by SDS-PAGE/Western blot.

Perforin was purified from the murine NK cell line 37.12 derived from an NK lymphoma in transgenic mice containing the human GzmH promoter linked to SV40-Tag. Frozen cell pellets (5 x 10^7 cells) were suspended in lysis buffer (25 mM Tris pH 7.5, 1 M NaCl, 1.0% Triton-X 100), sonicated, and dialyzed twice for 1 hr in low salt perforin buffer (25 mM Tris pH 7.5, 50 mM NaCl, 1.0 mM EDTA) at 4ºC. The lysate was centrifuged at 10,000g and loaded onto an Accell cation exchange column (Waters) for purification by affinity chromatography, eluting 1 ml fractions using a linear 0.2-0.8 M NaCl gradient. Fractions with lytic activity on sheep red blood cells were tested for tryptase and aspase activity using the colorimetric substrates BLT esterase and Box-Ala-Ala-Asp-SBzl, respectively. Granzyme-free perforin eluted at ~0.5-0.6 M NaCl. The granzyme-free fractions with perforin activity were pooled, filtered, and stored at 80ºC.

Antibodies

Polyclonal rabbit anti-mouse GzmA and GzmB antibodies were previously described. The polyclonal rabbit anti-GzmC antibody was generated by immunization with a peptide (CESQFQSSYNRAN, residues 174-186) and does not react with LAK cell extracts from mice deficient for gzms B, C, D, and F. Rat anti-human perforin (Kamiya Biomedical Company, WA) was used to detect murine perforin.
**Cellular Viability Assay**

YAC-1, EL4, TA3, and P815 cell lines were cultured in K5 media \(^{46}\). Log phase target cells were harvested, washed twice, and resuspended at 2 x 10^5/ml in pre-warmed RBC buffer (modified Ca\(^{2+}\) and Mg\(^{2+}\) free Hank’s balanced salt solution [HBSS] supplemented with 0.01 M Hepes [pH 7.5] and 0.4% BSA). 0-2 \(\mu\)M recombinant gzm, 1 \(\mu\)g purified perforin, and Ca\(^{2+}\) (3.8 mM CaCl\(_2\) and 0.81 mM MgSO\(_4\) in RBC buffer) were added sequentially to 10^5 target cells in a final volume of 100 \(\mu\)l at 37°C. In some experiments, this reaction was proportionally scaled up to increase the yield of treated target cells. As negative controls for every experiment, target cells were untreated or treated with perforin, rGzmB, or rGzmC alone. To assess cell viability, 10^5 YAC-1 (H-2\(^a\)) cells were 0-1000 nM recombinant gzm and purified perforin, incubated at 37°C for 1hr, and plated in duplicate with limiting dilution in 96-well microplates that were incubated at 37°C for 14 days. As a surrogate marker for viability, 0.25 \(\mu\)g 7-AAD was added to target cells at the end of the assay, and cells were evaluated with a FACScan flow cytometer and CellQuest software (Becton-Dickinson, MA).

Fluorescein isothiocyanate (FITC)-Annexin V conjugate (Molecular Probes, OR) was used to assess phosphatidylserine externalization. 10^5 YAC-1 target cells were treated with perforin and 1 \(\mu\)M rGzms B or C and incubated for 5, 15, 30, or 60 minutes at 37°C. The cells were stained and analyzed by flow cytometry as described \(^{28}\).

Caspase activity studies were performed as previously described \(^9\). YAC-1 cells (2 x 10^5) were pretreated with 100 \(\mu\)M D-fmk or the DMSO vehicle for 30 min at 37°C. Next, cells were treated with 1 \(\mu\)M gzm plus perforin and harvested after 5, 15, 30, or 60 min of incubation at 37°C. Lysates were prepared and Ac-DEVD-AMC cleavage was measured using a Perkin Elmer HTS7000 spectrofluorimeter.
Flow-TUNEL was performed with the ApopTag Fluorescein Direct In Situ Apoptosis Detection Kit (Intergen, NY) as described\(^9\). YAC-1 cells were treated with perforin and 1 \(\mu\)M rGzms B or C for the times indicated. To assess DNA fragmentation, \(2 \times 10^5\) YAC-1 cells were loaded with rGzmB or rGzmC (1 \(\mu\)M) and incubated for 1 or 2 hrs at 37°C. Genomic DNA was extracted and analyzed by agarose gel electrophoresis as described\(^9\). Genomic DNA was also radiolabeled in the Klenow \(^{32}\)P-dATP incorporation assay as previously described\(^{47}\). Denatured DNA samples were subjected to alkaline agarose electrophoresis and the dried gel was autoradiographed.

**Immunofluorescence and Light Microscopy**

YAC-1 target cells (\(10^5\)) were treated with perforin and 1 \(\mu\)M rGzms B or C and incubated for at 37°C for 5, 15, 30, or 60 min. All of the cells (100\(\mu\)l) were immobilized onto a microscope slide with a Cytospin apparatus (Shandon, PA) at 200rpm for 3 min and air dried for 10 min. Cells were treated with modified Wright-Giemsa stain (Sigma, MO) according to the manufacturer’s protocol. Cyt c immunodetection was performed as previously described\(^{16}\).

**Electron Microscopy**

\(2 \times 10^5\) YAC-1 target cells were treated with perforin and 1 \(\mu\)M rGzms B or C for 15 min at 37°C. The cells were washed and fixed with 1% glutaraldehyde in 0.1 M sodium cacodylate buffer on ice for 30 min, and postfixed with 1% osmium tetroxide. Embedding in Polybed 812 (Polyscience, Warrington, PA) and thin sectioning on a Rechert-Joung ultra microtome were performed at the Electron Microscopy Facility (Department of Cell Biology and Physiology,
Washington University School of Medicine). Images were viewed with a Zeiss EM 902 electron microscope (Zeiss, Oberkochen, Germany).

**Recombinant BID and DFF45/ICAD Cleavage**

Recombinant BID, the rabbit polyclonal anti-BID antibody, recombinant human ICAD, and the anti-human ICAD antibody have been previously described\(^9\)^\(^{,}^4^8\). Recombinant proteins and gzms were incubated at 37°C for 30 min in 50 mM Hepes [pH 7.5], 100 mM NaCl, and 1 mM EGTA. Proteins were then separated on SDS gels and Western blot analysis was performed as described\(^1^6\).

**Swelling and Membrane Potential of Isolated Mitochondria**

Volumetric and membrane potential changes of mitochondria isolated by standard differential centrifugation from Balb/c mouse livers and incubated in experimental buffer (125 mM KCl, 10 mM Tris-MOPS, 1 mM P\(_i\), 5 mM glutamate, 2.5 mM malate, 10 µM EGTA-Tris [pH 7.4]) were performed as previously described\(^1^6\).

**Release of Mitochondrial Matrix Entrapped Dyes**

10 mg/ml mitochondria were loaded with 10 µM calcein-AM (acetoxymethyl ester) or rhod-2-AM for 30 min at 25°C in isolation buffer in the dark, washed and resuspended in isolation buffer. Mitochondrial matrix esterases cleave the acetoxymethyl moiety, thereby generating the fluorescent calcein and rhod-2 that remain entrapped into the mitochondrial matrix\(^4^9^,^5^0\). Calcein and rhod-2 have a M.W. of 622.54 and 869.06, respectively. 0.5 mg/ml of loaded mitochondria incubated in experimental buffer were treated as described in Figure
Legend 6. After 30 min, mitochondria were sedimented by centrifugation at 14000xg for 3 min. Calcein and rhod2 fluorescence were measured in the pellet and in the supernatant at 25°C in a Perkin Elmer LS50B spectrofluorimeter, with excitation and emission wavelengths set at 488±5 and 540±5 nm for calcein, and 540±5 and 580±5 nm for rhod-2, respectively.

**Real-time Mitochondrial Membrane Potential Imaging in situ**

Real time $\Delta \psi_m$ imaging of wild-type MEFs loaded with 10 nM TMRM (tetramethylrhodamine methyl ester) in the presence of the multi-drug resistance P-glycoprotein inhibitor verapamil was performed exactly as previously described $^{16}$. Images were stored and analyzed, and data were normalized as previously described $^{16}$. 

RESULTS

GzmC Can Induce Cell Death

Recombinant mature GzmC (rGzmC) and attenuated GzmC (rGzmC-S184A, an active site serine to alanine point mutant) were expressed in *Pichia pastoris* and purified by cation exchange chromatography\(^{26}\). The comparable GzmB mutant, rGzmB-S183A, has attenuated proteolytic activity against a tetrapeptide substrate (Boc-Ala-Ala-Asp-SBzl) and its protein substrates caspase-3 and ICAD\(^9\). Perforin was partially purified from the adherent NK cell line 37.12\(^{25}\). The electrophoretic mobility and abundance of each purified enzyme was verified on silver stained SDS-PAGE gels (Fig 1A). The identity of perforin and the recombinant granzymes was confirmed by Western blot analysis. The partially purified perforin used in these studies does not contain detectable quantities of granzyme A, B, or C (Fig 1B). Since neither peptide nor protein substrates of murine rGzmC has been identified as yet, no enzymatic assay for this protein is available. For this reason, all experiments comparing GzmB and C were performed using equimolar amounts of these proteins.

A plating assay was performed to evaluate the clonigenic potential of cells treated with gzms and perforin. Purified perforin efficiently traffics recombinant granzymes into target cells\(^9\). YAC1 target cells were treated with purified perforin plus each recombinant gzm for 1 hr at 37\(^\circ\)C. Subsequently, the cells were plated by limiting dilution and cultured for 2 weeks to allow for the outgrowth of individual cells, so that clonigenic potential could be quantitated. Similar reductions in clonogenic potential were observed in target cells treated with perforin plus 50 nM rGzmB or C. In contrast, cells treated with perforin only or with each gzm by itself caused no
reduction in clonogenic potential. Gzm protease activity was required for the induction of target cell death, since the attenuated mutant granzymes (rGzmB-S183A and rGzmC-S184A) did not alter the clonogenic potential of treated cells (Figure 2A). Since these mutations attenuate the activities of the proteases but do not eliminate them, high doses (>500 nM) of the mutant enzymes also induce cell death, but less efficiently (data not shown).

Target cells loaded with rGzmB or C exhibited a dose-dependent loss of membrane integrity that was quantifiable with flow cytometry. The cell impermeable molecule 7-amino-actinomycin D (7-AAD) is a cytometric probe for cells with membrane damage. Flow cytometric analyses of YAC1 target cells are illustrated in dot plots comparing forward scatter (X-axis) and 7-AAD staining (Y-axis). Untreated cells or those treated with perforin or granzymes alone are mostly large and viable (i.e. forward scatter\textsuperscript{hi}/7-AAD\textsuperscript{lo}), and are found in the lower right quadrant of the dot plot (Figure 2B, top row). YAC1 target cells treated with rGzmB and perforin became smaller in size and 7-AAD\textsuperscript{hi}. Comparable changes occurred with rGzmC, and equivalent enzyme concentrations caused similar effects. Both rGzmB and C are potent inducers of cell death, since delivery of perforin and 50 nM or 100 nM of either rGzmB or C results in fewer than 10% or 1% viable target cells, respectively (Figure 2C, right panel). In contrast, treatment of YAC1 cells with perforin and 1 or 3 µM of purified human cathepsin G, neutrophil elastase, or proteinase 3 did not cause any changes in 7-AAD positivity (S. Raptis and TJ Ley, unpublished).

Membrane damage measured with flow cytometry was also directly compared with clonogenic efficiency. As noted previously, the percent of 7-AAD\textsuperscript{lo} YAC1 cells declined significantly after the addition of perforin and rGzm B or C (Figure 2C, left...
The loss of membrane integrity after one hour of treatment correlated strongly with reduced clonogenic efficiency. However, at very low concentrations of granzymes, 7-AAD staining underestimated the loss of clonogenic efficiency, perhaps because some 7-AAD low cells were committed to die, but had not yet experienced membrane changes. Similar data were acquired using three other target cell lines (EL4, P815, TA3) and cultured murine embryonic fibroblasts (MEFs, data not shown). Combinations of rGzms B and C were also delivered to YAC-1 cells by fixing the dose of one (50 nM) and increasing the other (50-1000 nM). The 7-AAD changes induced by the two proteases were additive, suggesting that rGzmB and C activate independent pathways (data not shown).

Features of GzmC-Induced Death

The membrane phospholipid phosphatidylserine (PS) translocates from the inner to the outer leaflet of the plasma membrane in the early phase of apoptosis and precedes the loss of membrane integrity. Cells that are Annexin V$^{hi}$ and 7-AAD$^{lo}$ indicate PS externalization in cells that are beginning to undergo apoptosis$^{28}$; a significant percentage of YAC1 cells treated with perforin and rGzmB or C at 37°C for 5 minutes exhibited this staining pattern. At later time points, the proportion of cells at a later stage of apoptosis (i.e. double positive) was substantially expanded; these changes did not occur in untreated cells, or cells treated with rGzmB or C alone. However, perforin alone caused some cells to become Annexin V$^{hi}$ and/or 7-AAD$^{hi}$, probably because of the limited membrane damage caused by this protein (Figure 3A). Nuclear condensation is a morphologic hallmark of apoptosis. Wright-Giemsa staining of YAC1 cells treated with
perforin and rGzmB demonstrated GzmB-dependent nuclear collapse and fragmentation. The appearance of the condensed nuclei differed for rGzmC however, since nuclear collapse was not accompanied by fragmentation in most cells (Figure 3A). Cells treated with perforin alone, rGzmB alone, or rGzmC alone were identical in appearance to untreated cells (Figure 3B and data not shown). Low magnification (2,850X) transmission electron microscopy revealed chromatin condensation induced by rGzmB or C. Nuclear condensation and chromatin clumping was not detected in untreated cells, nor those treated with perforin alone or granzymes alone. Cytoplasmic vacuolization and disruption also occurred in cells treated with perforin plus either GzmB or C (Figure 3C).

**GzmC induces DNA nicking during the induction of cell death**

We next wished to determine whether rGzmC-mediated death involves DNA damage, another hallmark of apoptosis. Flow-TUNEL quantifies terminal deoxynucleotidyl terminase (TdT) catalyzed incorporation of fluorescein-labeled nucleotides into the free 3’-OH DNA ends at the single cell level. As shown in Figure 4A, nearly 50% of YAC1 cells treated with perforin plus rGzmC were TUNEL positive after 5 minutes, increasing to 75% at 60 minutes. rGzmB and C both induced rapid TUNEL positivity with equal efficiency, but rGzmB treated cells were slightly more TUNEL positive at 30 and 60 minutes. Minimal TdT labeling occurred in cells exposed to perforin or granzymes only.

To assess whether rGzmC-induced DNA nicking was associated with oligonucleosomal fragmentation, we next evaluated target cell genomic DNA on agarose gels. Oligonucleosomal DNA laddering was induced by perforin plus rGzm B (Fig 4B),
but failed to occur in target cells treated with perforin plus rGzmC for 1 or 2 hours. The same samples were analyzed by flow cytometry to assure that cellular death had occurred: few 7-AADlo target cells persisted following treatment with perforin and either rGzm B or C, as expected (Figure 4B, percentages of 7-AADlo cells shown below each lane/treatment condition). These data suggested that GzmC-induced death is associated with single-stranded DNA nicking, not double-stranded cleavage. Nicked DNA can be radiolabeled with 32P-dATP using the Klenow polymerase; single-stranded nicked fragments can then be resolved with denaturing alkaline gel electrophoresis and autoradiography, as shown in Figure 4C. Extensive DNA nicking was revealed in cells treated with perforin plus rGzmC. The double-strand DNA breaks created by GzmB-activated CAD were also labeled, as predicted. DNA extracted from untreated cells, or cells treated with perforin or gzms only, was not extensively nicked (Figure 4C).

**GzmC causes cell death without activating caspases, BID, or ICAD**

Freshly prepared protein extracts derived from YAC1 cells treated with perforin plus rGzmB transiently generated significant DEVD’ase activity (measuring caspases-2, -3, and –7) (Figure 5A, top panel). High concentrations (>25 µM) of D-fmk, a broad-spectrum fluoromethylketone conjugated peptide inhibitor, completely inhibit caspase activity induced by rGzmB ⁹. When target cells were pretreated with 100 µM D-fmk, GzmB-induced DEVD’ase activity was not detected (Figure 5A top panel). At every time point, samples were removed for 7-AAD based cytometric analyses to estimate the target cell viability. GzmB caused a rapid reduction in the percentage of viable (i.e. 7-AADlo) target cells regardless of caspase inhibition (Figure 5A, lower panel). In contrast, there was no measurable DEVD’ase activity produced in
cells treated with perforin plus rGzmC (Figure 4B, top panel). Similarly, perforin plus rGzmC caused a rapid reduction in cell viability regardless of whether cells were pretreated with D-fmk (Figure B, lower panel). Beyond 60 minutes, advanced target cell destruction makes analysis of caspase activity impossible.

rGzmB efficiently cleaves p22 BID to p15 tBID, a key effector of the mitochondrial apoptotic pathway. rGzmC does not cleave recombinant p22 BID under the same conditions (Figure 5C). rGzmB directly processes the 45 KDa recombinant human (rhICAD) into its p30 form, but rhICAD was not cleaved with rGzmC under similar conditions (Figure 5D). Western blot analyses of proteins extracted from YAC1 cells treated with perforin and rGzm B or C verified that rGzmB rapidly processes caspase-3, BID, and ICAD, but that rGzmC does not (data not shown).

GzmC causes mitochondrial changes in intact cells undergoing apoptosis

The mitochondria of untreated cells were in a classical condensed state with narrow cristae separated by an electron-dense matrix space, identical to the mitochondria of cells treated with perforin only or granzymes only (data not shown). Perforin plus rGzmB caused extensive cristae reorganization resembling the early morphological changes that occur after the induction of apoptosis with other mediators both in vitro and in situ (Figure 6A). Perforin plus rGzmC induced profound swelling of YAC1 mitochondria with cavitation and loss of cristae structure, and outer membrane (OM) rupture.

The appearance of these striking morphologic changes prompted us to investigate mitochondrial function during GzmC-mediated apoptosis. We assessed mitochondrial
membrane potential ($\Delta \psi_m$) changes with real-time imaging, using with the potentiometric dye tetramethylrhodamine methyl ester (TMRM). Treatment of MEFs with perforin plus rGzmC induced mitochondrial depolarization, while perforin alone had no effect (Figure 6B). The permeability transition pore (PTP) can mediate mitochondrial swelling and depolarization during the course of apoptosis. We therefore investigated whether the PTP inhibitor cyclosporin A (CsA) was capable of inhibiting the loss of $\Delta \psi_m$ induced by rGzmC. The TMRM fluorescence decrease (i.e. depolarization) was not inhibited by CsA (Figure 6B). Quantitation of the TMRM fluorescence changes over mitochondrial regions showed that rGzmC caused a marked CsA-insensitive depolarization, approximately 10-15% greater than the depolarization induced by rGzmB in a similar experiment.

Finally, we determined that these mitochondrial changes were ultimately followed by cyt c release during the late stages of GzmC-induced death. YAC1 cells were treated with perforin plus rGzmB or C for 30 minutes and then stained for cyt c and nuclear morphology (Figure 6D). Perforin plus rGzmC triggered cyt c release from mitochondrial stores (Figure 6Dc vs. 3), while cyt c remained in the mitochondria of cells exposed to perforin alone (Figure 6Da). After release, the intensity of cyt c staining is dimmer, probably because cyt c diffuses broadly into the cytoplasm.

**GzmC Directly Induces Swelling and Membrane Depolarization in Isolated Mitochondria**

Isolated mitochondria primed with a high concentration of Ca$^{2+}$ (400 µM) undergo a sudden increase of inner mitochondrial membrane permeability due to opening of the PTP. A low concentration of Ca$^{2+}$ does not cause swelling *per se* (monitored by side scatter at 545 nm, Figure 7A). When rGzmC was added, the side scatter changes indicated that substantial swelling
developed within two minutes. “High” doses of rGzmC are required to induce these changes because very large amounts of mitochondrial protein are being treated with very small amounts of rGzmC (4 pMol rGzmC/1 mg mitochondria); these results therefore cannot be directly compared to the concentrations of rGzmC used to induce cell death. Pretreatment with the PTP inhibitors CsA or ADP plus oligomycin (not shown) did not affect rGzmC-induced swelling. In an identical experiment, the attenuated rGzmCS184A mutant did not cause detectable swelling (data not shown). Of note, rGzmB did not directly induce measurable mitochondrial swelling in a similar experiment 16, further distinguishing the mitochondrial effects of these two enzymes.

We next assessed whether swelling was associated with mitochondrial depolarization. When mitochondria were added to buffer containing the potentiometric dye rhodamine 123, fluorescence dropped as the dye was accumulated in the matrix of polarized mitochondria (Figure 7B). Addition of 40 µM Ca^{2+} resulted in the expected transient depolarization due to utilization of Δψ_m to take up Ca^{2+}. The subsequent addition of rGzmC caused a CsA-insensitive fluorescence increase (i.e. depolarization) that approached the maximum level attained with the protonophore FCCP (Figure 7B). As expected, the swelling of mitochondria incubated in saline buffers caused the release of cyt c due to outer membrane rupture, which occurred 15 minutes after the rupture caused by treatment with GzmC alone (not shown).

We next investigated the dose and Ca^{2+}-dependence of rGzmC-induced mitochondrial swelling. rGzmC caused CsA-insensitive mitochondrial swelling in a dose-dependent fashion (Figure 6C). Since Ca^{2+} concentrations ≤ 50 µM do not cause swelling per se (not shown), the Ca^{2+}-dependence of GzmC-induced swelling can be studied by priming mitochondria with increasing Ca^{2+} concentrations and measuring the swelling induced by a fixed dose of rGzmC.
GzmC-mediated swelling is Ca\textsuperscript{2+} -dependent, reaching a maximum at a Ca\textsuperscript{2+} concentration of 30 µM (Figure 7D).

Opening of the PTP is favored by mitochondrial Ca\textsuperscript{2+} accumulation. In this regard, the Ca\textsuperscript{2+} -dependence of GzmC-induced swelling resembled a characteristic feature of the PTP. However, the mitochondrial effects of GzmC were not blocked by PTP inhibitors (eg. CsA). We therefore decided to compare the size exclusion properties of the mitochondrial pore(s) generated by GzmC vs. the properties of the PTP, induced either by high dose Ca\textsuperscript{2+} or by the CsA-insensitive inducer mastoparan\textsuperscript{31}. Two fluorophores of different molecular weights, calcein (~620 Da) and rhod-2 (~860 Da), were loaded into the mitochondrial matrix. Openings of the inner mitochondrial membrane that possess exclusion sizes bigger than the dye causes their release from the mitochondrial matrix. Opening of the PTP (which has an exclusion size of ~1500 Da) by 400 µM Ca\textsuperscript{2+} consistently induced a CsA-sensitive release of both dyes. Mastoparan also caused the release of both calcein and rhod-2 from the mitochondrial matrix. However, rGzmC induced only the release of calcein, while rhod-2 was selectively retained in the mitochondrial matrix (Figure 7E). These data suggest that the pore opened by rGzmC differs from the classical PTP in its size-exclusion properties, since it only allows the flux of smaller MW solutes.
DISCUSSION

In this report, we describe the ability of murine GzmC to cause cell death. The induction of death requires its protease activity, and is characterized by the rapid externalization of phosphatidylserine, nuclear condensation, and single-stranded DNA nicking. The kinetics of these events are similar to those caused by GzmB. The induction of death did not involve the production of activated caspases, the cleavage of BID, or the activation of the CAD nuclease. However, GzmC did cause rapid mitochondrial swelling and depolarization in intact cells or in isolated mitochondria, and this mitochondrial damage was not prevented by cyclosporin A pretreatment. These results suggest that GzmC rapidly induces target cell death by attacking both nuclear and mitochondrial targets, and that these targets are distinct from those utilized by GzmB to cause classical apoptosis.

The death induced by GzmC occurs rapidly, with kinetics similar to those observed for GzmB, and the potency of this enzyme (on a molar basis) is similar to that of GzmB. The induction of cell death by GzmB and C is not a generic property of all neutral serine proteases, since three highly related neutrophil azurophil granule enzymes (neutrophil elastase, cathepsin G, and Proteinase 3) all failed to induce cell death in this in vitro system. The characteristics of the cell death induced by GzmC have many of the hallmarks of apoptosis, but the death induced by GzmC is distinct from that of GzmB in several ways: 1) Nuclear condensation is not always associated with nuclear fragmentation. 2) The DNA damage appears to be single-stranded nicking, not oligonucleosomal DNA fragmentation characteristic of CAD activation. 3) Even though GzmC induced death is associated with late cyt c release from damaged mitochondria, there is no evidence for caspase-3 activation during the early stages of apoptosis. 4) GzmB does not directly damage isolated mitochondria, while GzmC causes isolated mitochondria to rapidly
depolarize and swell. All of these differences strongly suggest that these two proteases induce cell death by attacking a different set of substrates.

The DNA damage caused by GzmC does not appear to involve the activation of CAD, since oligonucleosomal DNA fragmentation does not occur. This finding also makes it unlikely that GzmC causes DNA damage by releasing EndoG from mitochondria, since EndoG also causes oligonucleosomal DNA degradation\(^{32}\). Instead, GzmC rapidly induces single-stranded DNA nicks in target cells. At least two different nucleases could potentially account for this finding. GzmA induces single-stranded DNA nicking by cleaving and activating a single-stranded DNase within the SET complex\(^{19}\). Alternatively, mitochondria contain a caspase-independent nuclease known as apoptosis-inducing factor (AIF) that can be released from mitochondria during the induction of cell death\(^{33}\). AIF causes a unique form of nuclear condensation that is similar to that observed for GzmC, and it causes long-range DNA nicking but not oligonucleosomal DNA laddering\(^{34}\). For these reasons, it is possible that the mitochondrial release of AIF, or the activation of the SET-associated DNase (or additional nucleases not yet described) may play roles in the nuclear changes observed with GzmC-induced death. Additional experimentation will be required to identify the responsible nucleases.

GzmC can rapidly induce mitochondrial swelling and depolarization in intact cells or on isolated mitochondrial preparations. While GzmB can cause rapid mitochondrial depolarization in intact cells, it has no effect on isolated mitochondria, suggesting that it requires a cellular cofactor to cause mitochondrial damage\(^{16}\). GzmC-induced swelling was amplified by small Ca\(^{2+}\) prepulses, which is reminiscent of the Ca\(^{2+}\)-dependence of the PTP; however, PTP inhibitors did not block this swelling. This fact cannot be used to rule out a role for the PTP, since other inducers can operate in a Ca\(^{2+}\)-dependent (but CsA-insensitive) fashion\(^{31,35}\). Patch-
clamp experiments revealed that the open conformation of the PTP displays a 1.8 nS full conductance and a typical 0.9 nS subconductance state. The 1.8 nS conductance corresponds to a pore exclusion size of ~1500 Da, while the 0.9 nS conductance is predicted to correspond to a cutoff of ~750 Da. Opening of the PTP in a subconductance mode permeant to Ca\textsuperscript{2+}, but not to sucrose, has been reported in isolated mitochondria and intact cells. GzmC characteristics might reflect a selective opening of the PTP in a subconductance mode. The estimated pore size induced by GzmC, based on calcein flux (~620 Da), is compatible with the PTP operating in its half-conductance mode. On the other hand, all PTP conductances disappear in the presence of CsA. It is therefore conceivable that the GzmC-induced IM pore possesses novel features that will become more clear with a subsequent full characterization. The late release of cyt c from GzmC-treated cells may be a consequence of direct damage to the inner mitochondrial membrane caused by GzmC, followed by rupture of the outer membrane with attendant release of all mitochondrial contents. Regardless, the cyt c release was not associated with the rapid activation of caspase-3, or the activation of CAD. The dramatic drop in ATP levels that occurs after treatment with perforin and rGzmC (data not shown) correlated with the sudden mitochondrial depolarization observed in situ; this change may also limit the ability of caspase to be activated.

A functional role of GzmC for cytotoxic lymphocyte-mediated target cell death has not yet been established. However, the GzmB knock-out mouse produced in our laboratory several years ago displays dramatically reduced expressed of Gzms C, D, and F in the LAK cell compartment, presumably due to a "neighborhood" effect from the retained PGK-neo cassette in the GzmB gene. We have recently removed the PGK-neo cassette from the GzmB gene using LoxP-Cre-mediated recombination, and created mice that are deficient for GzmB only (D.
Thomas, R. Behl, and T.J. Ley, unpublished). Comparisons of the cytotoxic repertoires of the lymphocytes derived from these mice are in progress. However, preliminary experiments in an established graft vs. host disease model strongly suggests that the mitigation of graft vs. host disease previously observed with GzmB cluster-deficient lymphocytes 38 may be due in part to the reduced expression of the orphan granzymes downstream from GzmB. These results support the idea that some of the orphan granzymes (perhaps including GzmC) are expressed in T cells that are activated in vivo, and that they contribute significantly to the tissue damage associated with graft vs. host disease. These results create a strong biological foundation for the continued study of the orphan granzymes in the GzmB gene cluster.

The diversity of protease specificities of the granzymes is almost certainly a fail-safe mechanism for CTL to kill target cells that express inhibitors of GzmB. Virus-infected cells 39,40 and tumor cells 41,42 are known to express serpin inhibitors of GzmB, and adenovirus expresses a decoy substrate for GzmB that is an efficient inhibitor 43. When these GzmB-specific inhibitors are present, granzymes with different specificities (like A or C), may still be able to induce target cell death by activating alternative death pathways. This adaptation may be critical for survival of the host.

The results presented in this study clearly establish GzmC as an enzyme that can cause cell death. The specificity of this enzyme and its target cell substrates are currently unknown, but a strong rationale for the continued study of this enzyme is provided by the results described here. The unique pattern of nuclear and mitochondrial damage induced by this enzyme suggests that it will prove to be an important tool for understanding an alternative apoptotic pathway. The physiologic relevance of this pathway is suggested by the GzmB cluster knockout model, but
proof of its importance awaits the development and description of the GzmC loss-of-function mouse.
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FIGURE LEGENDS

Figure 1. Purification of recombinant granzymes and NK cell-derived perforin.

(A) Recombinant gzms were produced and purified (0.5 µg), and equivalent amounts (1 µg) of rGzmA, rGzmB, rGzmBS183A, rGzmC, and rGzmCS184A were separated on an SDS-PAGE reducing gel and visualized by silver staining.

(B) Western blots. NK cell line lytstae (25 µg) and 0.5 µg of partially purified perforin, rGzmA, rGzmB, rGzmBS183A, rGzmC, and rGzmCS184A were electrophoresed on SDS-PAGE gels. Western blot analysis was performed using polyclonal rabbit anti-mouse GzmA, B, and C and the rat anti-human perforin antibody (1:2000 each).

Figure 2. Active recombinant GzmC delivered by perforin causes target cell death.

(A) Target cell viability. YAC1 cells were treated with a fixed dose of purified perforin (Pfn; 1 µg) or recombinant gzms alone as negative controls. Cells were treated with 50 nM rGzmB, rGzmBS183A, rGzmC, or rGzmCS184A plus perforin and incubated for 1 hr at 37°C. Next, samples were plated using limiting dilution in duplicate 96-well microplates and incubated for 14 days at 37°C. The percentage of total plated cells that formed clones is plotted as the mean ± SEM. This experiment was repeated 4 times with similar results.

(B) (Top row) YAC1 cells (10⁵) were treated with nothing, perforin, rGzmB, or rGzmC alone for 1 hr at 37°C. Forward scatter properties and 7-AAD staining were quantified by flow cytometry.

(Bottom rows) YAC1 cells, treated with perforin plus increasing doses of rGzmB or rGzmC for 1 hr at 37°C, were stained with 7-AAD and analyzed by flow cytometry.

(C) Dose-response. (Left panel) The percent of 7-AAD⁰ target cells, obtained from panel C, is shown for increasing doses of gzms. (Right panel) At the end of the 1 hr assay, target
cells were plated in duplicate using limiting dilution. The percentage of total plated cells that yielded clones was quantified at 14 days. This experiment was repeated 4 times with similar results.

Figure 3. Phosphatidylserine externalization and nuclear condensation occur in GzmC-induced apoptosis.
(A) Percentage of target cells with single positive annexin V$^{\text{hi}}$ (AV) vs. double-positive AV$^{\text{hi}}$/7-AAD$^{\text{hi}}$ staining. YAC1 target cells were treated with perforin and 1µM rGzmB or rGzmC at 37°C for 5, 15, 30, or 60 min. Additionally, target cells were exposed to nothing, perforin, rGzmB, or rGzmC alone. Results are representative of two independent experiments.

(B) YAC1 cells were treated with perforin alone or perforin plus 1 µM rGzmB or rGzmC for 15 min at 37°C. Cell suspensions were immobilized onto microscope slides, stained by Wright Giemsa, and visualized by light microscopy (100X magnification). Cells treated with perforin showed a normal morphological appearance. Target cells that were untreated or treated with gzms only resembled perforin treated cells (data not shown). Nuclear collapse (arrows) was observed, as well as late cell disruption (arrowheads).

(C) Transmission electron microscopy of YAC1 cells incubated with perforin and gzms as in (B) revealed chromatin condensation (arrows) and cytoplasmic disruption (bar is 2.5 µm). Perforin treated cells were identical to untreated cells or cells treated with gzms only (data not shown).

Figure 4. GzmC induces single-strand DNA nicking.
(A) Flow-TUNEL analysis was performed on YAC1 cells treated with perforin plus 1 μM rGzmB or rGzmC for the times specified. Untreated YAC1 cells and cells incubated with perforin, rGzmB, or rGzmC alone were also analyzed for FITC-TUNEL positivity.

(B) YAC1 target cells were untreated or treated with perforin, rGzmB, rGzmC, perforin plus rGzmB, or perforin plus rGzmC (1 μM). Genomic DNA was harvested after incubation at 37°C for 1 or 2 hrs and visualized on an ethidium bromide stained, 2% agarose gel. To estimate cell viability in the same experiment, the percentage of 7-AADlo target cells was analyzed by flow cytometry (values for each sample are shown below each line).

(C) Genomic DNA from YAC1 target cells treated as above (panel B) were radiolabeled with 32P-dATP by the Klenow fragment of DNA polymerase I. DNA fragments were separated with denaturing alkaline gel electrophoresis and autoradiographed.

Figure 5. GzmC does not cleave or activate GzmB substrates.

(A) GzmB induced caspase-3 activity in target cells. (Top panel) YAC1 cells were pretreated with a fixed dose (100 μM) of the broad-spectrum caspase inhibitor D-fmk or the DMSO vehicle for 30 min at 37°C. Next, the cells were treated with perforin plus 1 μM rGzmB and incubated at 37°C for 5, 15, 30, or 60 min. Cellular lysates were prepared and DEVD-AMC cleavage was measured in triplicate using a spectrofluorimeter. (Bottom panel) Before target cell harvest at every time point, a fraction of each sample was stained with 7-AAD and quantified using flow cytometry to assess viability.

(B) Absence of GzmC-induced caspase-3 activity. (Top panel) As described in (A), D-fmk or DMSO pretreated YAC1 cells were treated with perforin plus 1 μM rGzmC followed
by fluorimetric analysis. (Bottom panel) Portions of the rGzmC loaded samples were stained with 7-AAD and quantified with flow cytometry.

(C) Full-length recombinant p22 BID was incubated at 37°C for 30 min alone or with increasing concentrations of active rGzmB or rGzmC. Samples were harvested and electrophoresed on reducing SDS-PAGE gels. Western blot analysis was performed with a rabbit polyclonal anti-BID antibody. Only rGzmB directly processes p22 BID to the truncated BID (tBID) form.

(D) rhICAD was processed as above in (A), and immunoblotted using a rabbit polyclonal anti-ICAD antibody. Only rGzmB processes ICAD to its p30 form.

**Figure 6.** GzmC induces target cell mitochondrial depolarization, swelling, and cyt c release in intact cells.

(A) High magnification transmission electron microscopy (22,000X). The arrows point to the mitochondria of YAC1 cells treated as described in Figure 2 (C) (bar=0.4 µm). The mitochondria of untreated YAC1 cells or cells incubated with gzms alone has the same appearance as perforin treated mitochondria (data not shown).

(B) Representative pseudocolor-coded images of TMRM fluorescence intensity in murine embryonic fibroblasts at the beginning (left panels) and at the end of the acquisition sequence (right panels). Cells were treated with perforin alone or with perforin plus 4 µM GzmC. Where indicated, cells were preincubated with 2 µM CsA for 30 min. The bar is 7 µm.

(C) Quantitation of the TMRM fluorescence changes over mitochondrial regions. Where indicated by the arrows, perforin and 4 µM rGzmC, and then 2 µM FCCP (to induce
complete depolarization) were added. Fluorescence intensity changes were quantified as described in the Experimental Procedures section.

(D) Immunofluorescence for cyt c in YAC1 cells. YAC1 target cells were treated with perforin alone (a,b), perforin plus 1 µM rGzmB (c,d), or perforin plus 1 µM rGzmC (e,f) for 30 min and immunostained for cyt c (red). Duplicate images in the lower panels (b,d,f) show nuclear staining by DAPI (blue). Note that target cells treated with perforin plus gzms display diminished cyt c staining and have apoptotic condensed nuclei. Untreated target cells and cells treated with gzms alone resembled those treated with perforin only (a,b, and data not shown).

Figure 7. GzmC directly causes mitochondrial swelling and depolarization in isolated mitochondria.

(A, B) Mitochondrial volume and membrane potential changes of purified murine liver mitochondria (MLM, 0.5 mg/ml) were monitored as described 16. In both panels, where indicated by the arrows, 40 µM Ca²⁺ and 4 µM rGzmC (black and gray traces) were added. Where noted (gray trace), 1 µM CsA was present from the beginning. In panel A, 400 µM Ca²⁺ (light gray trace) was added where indicated to induce complete swelling. In all the experiments of (B), complete depolarization was achieved by adding 400 nM FCCP where indicated.

(C) Dose dependence of GzmC-induced mitochondrial swelling. The experiment was performed as in (A), except that the indicated concentrations of rGzmC were added.
(D) Ca\textsuperscript{2+} dependence of GzmC-induced swelling. The experiment was carried out as in (A), except that the indicated Ca\textsuperscript{2+} concentrations were added prior to 4 µM rGzmC. Ca\textsuperscript{2+} concentrations of 50 µM or less did not cause detectable mitochondrial swelling.

(E) Differential release of fluorescent dyes entrapped in the mitochondrial matrix by GzmC. Mitochondria loaded with 10 µM rhod-2-AM or calcein-AM were left untreated or treated with 200 µM Ca\textsuperscript{2+}, 200 µM Ca\textsuperscript{2+} in the presence of 1 µM CsA, 2 µM mastoparan, or 4 µM rGzmC. After 30 min, mitochondria were pelleted and calcein and rhod-2 fluorescence was measured in the pellet and in the supernatant. Data are normalized for the untreated mitochondria.
Figure 1
Figure 2
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Figure 4
Figure 5
Figure 6
Figure 7
Cell death induced by Granzyme C

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