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Granulysin Delivered by Cytotoxic Cells Damages Endoplasmic Reticulum and Activates Caspase-7 in Target Cells

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Abstract

Granulysin is a human cytolytic molecule present in cytotoxic granules with perforin and granzymes. Recombinant 9-kDa granulysin kills a variety of microbes, including bacteria, yeast, fungi, and parasites, and induces apoptosis in tumor cells by causing intracellular calcium overload, mitochondrial damage, and activation of downstream caspases. Reasoning that granulysin delivered by cytotoxic cells may work in concert with other molecules, we crossed granulysin transgenic (*GNLY*^{+/-}) mice onto perforin (*perf*^{-/-}) or granzyme B (*gzmb*^{-/-}) deficient mice to examine granulysin-mediated killing in a more physiologic whole-cell system. Splenocytes from these animals were activated in vitro with IL-15 to generate cytolytic T cells and NK cells. Cytotoxic cells expressing granulysin require perforin, but not granzyme B, to cause apoptosis of targets. Whereas granzyme B induces mitochondrial damage and activates caspases-3 and -9 in targets, cytotoxic cell-delivered granulysin induces endoplasmic reticulum stress and activates caspase-7 with no effect on mitochondria or caspases-3 and -9. In addition, recombinant granulysin and cell-delivered granulysin activate distinct apoptotic pathways in target cells. These findings suggest that cytotoxic cells have evolved multiple nonredundant cell death pathways, enabling host defense to counteract escape mechanisms employed by pathogens or tumor cells.

Cytotoxic cells play a central role in the elimination of cells infected with viruses or other pathogens, tumor surveillance, and transplant rejection (1). Target cell apoptosis results from activation of conserved death machinery by either engagement of death receptors or granule exocytosis (2–8). Cytotoxic cell granules contain several components with distinct activities that activate proenzymes, perturb membranes, protect cytotoxic cells from their own cytolytic mechanisms, or initiate apoptosis in the target cell (1). Perforin, which disrupts membranes, and granzymes, a family of serine proteases, are among the most abundant proteins in cytotoxic cell granules. The importance of perforin and granzymes to cell-mediated cytotoxicity was confirmed using gene-targeted mice: Cytotoxic cells from *perf*^{-/-} or *gzmb*^{-/-} mice exhibit major defects in killing targets in vitro, and in vivo these animals are more susceptible to tumors and to some viral and intracellular bacterial infections (3, 4, 6, 9,

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Disclosures

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10). Granzyme B initiates apoptosis via two pathways: either by direct activation of executioner caspases (caspase-3 and caspase-7) (11, 12) and/or by cleavage of Bid (13, 14).

Granulysin, another component of cytotoxic cell granules, is expressed constitutively by NK cells and by CTLs 3–5 d after activation (15). Granulysin is synthesized as a 15-kDa molecule that is cleaved at both the amino and the carboxyl termini to a 9-kDa form (16). Recombinant 9-kDa granulysin is broadly cytolytic against tumors and microbes, including Gram-positive and Gram-negative bacteria, yeast, fungi, and parasites (17). Granulysin has been implicated in a variety of human diseases, and expression of granulysin correlates with good clinical outcomes in cancer and infections (18–20). Granulysin homologs are found in humans, cows, pigs, and horses, but not in mice or rats (21, 22). To test the physiologic role of granulysin, we used a bacterial artificial chromosome to generate *GNLY* transgenic mice (23). These animals have normal development and phenotype, and their lymphocytes express granulysin in a manner similar to human T and NK cells.

In a series of studies, we described the mechanism by which recombinant 9-kDa granulysin causes apoptosis in Jurkat tumor cells (24–28). Positively charged granulysin interacts with the Jurkat cell surface on the basis of electrostatic charge (25) and then rapidly enters the cell, presumably by a scissoring action, as implicated by the crystal structure (26). There is a rapid increase in intracellular calcium and a decrease in intracellular potassium (27), resulting in mitochondrial depolarization and release of both cytochrome *c* and apoptosis-inducing factor (AIF) (27). Cytochrome *c* release activates caspase-3, which, together with AIF (28), induces endonuclease activation and apoptosis (27). Of note, experiments using recombinant 9-kDa granulysin may not accurately mimic the conditions of cytotoxic granule delivery by NK cells or CTLs in that 1) a relatively high (micromolar) concentration of recombinant granulysin is required to induce lysis, and 2) other molecules such as perforin, calreticulin, and granzymes are not present. To study the mechanism of granulysin-induced cell death under more physiologic conditions; we crossed *GNLY*^{+/-} mice onto *perf*^{-/-} and *gzmb*^{-/-} mice and used in vitro propagated cytotoxic NK cells from these strains to lyse target cells. In accordance with previous reports (3, 29), NK cells lacking either granzyme B or perforin are defective in their ability to kill target cells. We found that NK cells expressing granulysin but lacking perforin are unable to kill target cells. Lysis by NK cells expressing granulysin but lacking granzyme B is similar to that of wild-type (WT) NK cells. Surprisingly, granulysin delivered by NK cells does not cause mitochondrial damage or activate either caspase-3 or caspase-9 in target cells, whereas recombinant 9-kDa granulysin activates these pathways. NK cells expressing granulysin cause both endoplasmic reticulum (ER) stress and caspase-7 activation in target cells, whereas 9-kDa granulysin does not. Thus, NK cell-delivered granulysin and recombinant granulysin induce target cell death through distinct pathways.

Materials and Methods

Mouse strains and cell lines

C57BL/6 perforin knockout mice (*perf*^{-/-}) and C57BL/6 granzyme B knockout mice (*gzmb*^{-/-}) were purchased from The Jackson Laboratory, Bar Harbor, ME. C57BL/6 granulysin transgenic mice (*GNLY*^{+/-}) (23) were mated with *perf*^{-/-} or *gzmb*^{-/-} mice to obtain *gzmb*

-/-GNLY^{+/-}, *perf^{-/-}GNLY^{+/-}*, *gzmb^{-/-}perf^{-/-}*, and *gzmb^{-/-}perf^{-/-}GNLY^{+/-}* strains. Mice were bred at the National Cancer Institute and mice >6 wk of age were used in all experiments. The Animal Care and Use Committee of the National Cancer Institute approved all animal experiments. YAC-1 (mouse lymphoma), K562 (human erythroleukemia), and THP-1 (human monocytic cells) were maintained in RPMI 1640 supplemented with 10% heat-inactivated FBS (Hyclone, Ogden, UT), 2 mM L-glutamine, and 100 U/ml penicillin-streptomycin (complete media). EL4 and EL4.F15 cells (30) were maintained in MEM with 10% FBS and 10 mM 2-mercaptoethanol (2-ME).

Activation of splenocytes and granulysin expression

Single-cell suspensions of splenocytes were cultured at 2.5×10^6 cells/ml in 24-well plates in complete medium supplemented with 25 ng/ml human rIL-15 (eBioscience, San Diego, CA) and 0.05 mM 2-ME. Fresh IL-15 was added on 4 and 8 d of culture. Granulysin expression was analyzed by intracellular staining and flow cytometry. The cells were labeled with fluorochrome-conjugated Abs specific for CD8 (clone 53–6.7) or NK1.1 (clone PK136) and CD3 (clone 145–2C11) (BD Biosciences, San Jose, CA), fixed and permeabilized and then stained with rabbit anti-granulysin antiserum, as described earlier (23). The flow cytometry data were analyzed with FlowJo analysis software (TreeStar, Ashland, OR).

Confocal microscopy

NK cells were isolated from IL-15-activated splenocytes by negative selection using MACS isolation kits (Miltenyi Biotec, Auburn, CA). Cells were immobilized on poly (L-lysine)-coated slides, fixed in 2% paraformaldehyde, and permeabilized (0.01% saponin and 0.1% Triton X-100). Cells were stained with rabbit anti-granulysin antiserum (1:10,000) and counterstained with anti-rabbit IgG conjugated to Alexa Fluor 488 (Molecular Probes, Carlsbad, CA) (1:1000), and fluorescent images were acquired on an LSM 510 confocal system (Carl Zeiss) using a 63× Plan-Apochromat objective.

Flow-based killing assay

Target cells (YAC-1, EL4, EL4.F15, K562, or THP-1) were washed two times with PBS and labeled with 100 nM CFSE (Molecular Probes) for 15 min at 37°C. The reaction was stopped by addition of RPMI 1640 supplemented with 10% FBS. Cells were washed twice with PBS supplemented with 2% FBS, then resuspended in complete medium supplemented with 0.05 mM 2-ME and 25 ng/ml IL-15. Effector cells were mixed with 10^4 labeled target cells in 50 µl medium in 96-well plates and incubated at 37°C for 3–4 h at different E:T ratios. A total of 1 µg/ml 7-aminoactinomycin D (7-AAD) (Molecular Probes) was added to each well immediately before flow cytometry. Cell death (7-AAD incorporation and cell shrinkage) was analyzed by flow cytometry after gating on CFSE-positive target cells, as described earlier (23). Percentage of specific lysis was calculated by the following formula: % specific lysis = [(% experimental lysis 2 % spontaneous lysis)/100 – % spontaneous lysis] × 100.

In vitro assays using recombinant granulysin

Recombinant granulysin was expressed and purified as described previously (16). Cells were seeded in 96-well plates at a density of $4-5 \times 10^5$ /ml (100 ml/well) in medium containing 0.1% FCS. Cells were cultured for 2 h in the presence of granulysin (25 μ M). Mitochondrial membrane depolarization, reactive oxygen species (ROS) generation, and ER membrane integrity were measured as described below.

Mitochondrial membrane perturbation and ROS generation

Target cells were labeled with 100 nM DDAO-SE [7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one)-succinimidyl ester] (Molecular Probes) for 15 min at 37°C, washed, and then mixed with effector cells at an E:T ratio of 10:1 for 2 h. For ROS detection, cells were incubated with 2 μ M hydroxyethidine (2-HE) (Molecular Probes); for mitochondrial membrane depolarization, cells were incubated with 10 nM DiOC₆(3) (3,3-dihexyloxycarbocyanin) (Molecular Probes) for 15 min. Fluorescence was measured using two-color flow cytometry after gating on DDAO-SE⁺ target cells. The gates were set using target cells alone.

Detection of activated caspases

Effector and target cells were incubated for 2–3 h at a 10:1 E:T ratio, washed with PBS/1% BSA at room temperature, fixed, and permeabilized with the BD Cytotfix/Cytoperm Kit (BD Biosciences) and then stained with either FITC-labeled mAb against the active form of caspase-3 (clone C92605; BD Pharmingen), caspase-7 (Cell Signaling Technology, Danvers, MA), caspase-9 (Cell Signaling Technology), or anti-m12D341 (rabbit serum specific for the active form of caspase-12, a kind gift from Dr. T. Momoi, National Institute of Neuroscience, Tokyo, Japan). In the case of anti-caspase-7, -9, or -12 Abs, cells were washed with perm/wash buffer and further stained with PE-conjugated anti-rabbit IgG (Invitrogen). After two washes with perm/wash buffer, cells were resuspended in 1% paraformaldehyde/PBS and analyzed by two-color flow cytometry.

Cytochrome c release

Cytochrome *c* release was quantified by flow cytometry as described (31). Briefly, effector and target cells were incubated at an E:T ratio of 10:1 for 2 h and then were mildly permeabilized with 25 μ g/ml digitonin in 100 mM KCl on ice for 5 min. Cells were washed once with cold PBS, fixed in 4% paraformaldehyde, permeabilized with 0.05% saponin plus 3% BSA, and then incubated with the FITC-conjugated anti-cytochrome *c* mAb 6H2.B4 (BD Pharmingen). Cells were resuspended in 100 μ l 4% paraformaldehyde in PBS and analyzed by FACS.

TUNEL assay

Effector cells were incubated at 37°C with 10^4 DDAO-SE-labeled target cells in 50 μ l medium in 96-well plates. The cell mixtures were fixed in 2% paraformaldehyde and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice before staining with the In Situ Cell Death Detection Kit, Fluorescein (Roche Applied Sciences, Indianapolis, IN), following the manufacturer's protocol. Stained cells were analyzed by

flow cytometry. Target cells were preincubated with 40 μ M pan-caspase inhibitor Quinoline-Val-Asp-CH₂-OPh (Q-VD-OPH) (MP Biomedicals, Salon, OH) for 1 h before the addition of effector cells.

Disruption of the ER

Effectors and targets were incubated for 1 h at various E:T ratios followed by incubation with 0.2 μ g/ml brefeldin A conjugated to BODIPY 558/568 (BFA BODIPY) (Molecular Probes) for another 30 min (32). Cells were washed twice with PBS and analyzed by flow cytometry.

Results

Granulysin delivered by cytotoxic cells requires perforin, but not granzyme B, for cytolysis of targets

To examine the mechanism of action of granulysin in cytolysis by cytotoxic cells, we used *GNLY* transgenic mice previously generated in our laboratory (23). Because granzyme B and perforin are both present in cytolytic granules with granulysin, the interaction of these three effector molecules was evaluated by crossing *GNLY*^{+/-} mice with *gzmb*^{-/-}, *perf*^{-/-}, and *gzmb*^{-/-}*perf*^{-/-} mice. Splenocytes from these mice were activated in vitro using IL-15, and expression of granulysin was assessed by intracellular staining and flow cytometry (Fig. 1A) (23). As shown earlier (23), IL-15 induces granulysin expression in NK1.1⁺ NK cells. By day 7, ~50% of NK1.1⁺ CD3⁻ cells express granulysin, but only 2% of CD8⁺ cells express granulysin (Fig. 1B). Splenocytes from *GNLY*^{+/-} animals lacking either perforin or granzyme B showed similar expression of granulysin (data not shown). Using confocal microscopy, we observed that granulysin is localized in the granules of NK cells (Fig. 1C) and colocalizes with granzyme B in these cytolytic granules (Supplemental Fig. 1). The presence or absence of granzyme B and/or perforin did not affect localization of granulysin in NK cells (Fig. 1C).

In vitro cytotoxicity assays were used to interrogate apoptotic pathways activated by granulysin. Splenocytes from WT, *GNLY*^{+/-}, *gzmb*^{-/-}, *gzmb*^{-/-}*GNLY*^{+/-}, *perf*^{-/-}, *perf*^{-/-}*GNLY*^{+/-}, *gzmb*^{-/-}*perf*^{-/-}, and *gzmb*^{-/-}*perf*^{-/-}*GNLY*^{+/-} cultured with IL-15 for 7–10 d were used as cytotoxic cells. CFSE-labeled YAC-1 cells were used as target cells and assayed for incorporation of 7-AAD, which measures apoptosis/late cell death. Cytotoxicity was assayed over a range of E:T ratios and at different time points. As shown previously (23), there is no difference between *GNLY*^{+/-} and WT NK cells in apoptosis of YAC-1 targets (Fig. 2A–C). We also used EL4 and Fas-resistant EL4.F15 cells (30) to determine the role of Fas-mediated signaling on apoptotic pathways activated through granulysin. We found that cytotoxic cells from *GNLY*^{+/-} mice showed better killing of both EL4 and EL4.F15 cells, compared with cytotoxic cells from WT mice, but there was no effect of defective Fas signaling on cell death caused by either WT or *GNLY*^{+/-} NK cells (Supplemental Fig. 2A, 2B). In the absence of perforin, cell death was not observed over a range of E:T ratios (Fig. 2A). Although NK cells lacking granzyme B had reduced cytolytic capacity, compared with WT and *GNLY*^{+/-} effectors (Fig. 2B, 2C), NK cells from *gzmb*^{-/-}*GNLY*^{+/-} mice showed cytolytic activity equivalent to that of WT and *GNLY*^{+/-} NK cells.

(Fig. 2B, 2C). These findings suggest that target cell death by NK cells requires perforin plus either granzyme B or granulysin.

Cytotoxic cell-delivered granulysin does not mediate apoptosis through mitochondrial damage

We previously delineated the mechanisms by which recombinant 9-kDa granulysin causes cell death of Jurkat, a human tumor cell line: There is a rapid calcium influx and potassium efflux followed by mitochondrial perturbation, generation of ROS, release of cytochrome *c* and AIF, caspase activation, and apoptotic cell death (24, 25, 27, 28). To address the question of whether granulysin delivered by NK cells activates similar pathways in target cells, we monitored mitochondrial membrane depolarization and ROS generation in YAC-1 cells. Surprisingly, granulysin expression in NK cells did not affect mitochondrial membrane depolarization or ROS generation in YAC-1 target cells (Fig. 3A–D). Similar results were obtained using EL4 and EL4.F15 target cells, suggesting that Fas signaling is not involved in granulysin-induced cell death pathways (Supplemental Fig. 3A–D). In NK cells lacking granzyme B, expression of granulysin did not alter either mitochondrial membrane potential (Ψ_m) loss or ROS generation, despite the fact that *gzmb*^{-/-}*GNLY*^{+/-} NK cells had higher cytolytic activity than did *gzmb*^{-/-} NK cells (Fig. 3A–D). However, treatment of YAC-1 cells with recombinant 9-kDa granulysin resulted in Ψ_m and ROS generation, as shown earlier with Jurkat cells (25, 27, 28) (Fig. 3E). These findings indicate that recombinant 9-kDa granulysin activates apoptosis through the mitochondrial signaling pathway, whereas granulysin delivered by cytolytic granules in conjunction with perforin mediates apoptosis by a different pathway.

Granulysin delivered by NK cells mediates DNA fragmentation that is caspase dependent but does not involve caspase-3 or caspase-9

Because DNA fragmentation is a hallmark of apoptosis (33), we measured DNA fragmentation by TUNEL assay. *GNLY*^{+/-} NK cells, compared with WT cells, caused a small but significant increase in DNA fragmentation in YAC-1 targets. (Fig. 4A). We also observed that the percentage of TUNEL-positive target cells was reduced when *gzmb*^{-/-} NK cells were employed (31 to 23% for WT to *gzmb*^{-/-} effectors, respectively). *gzmb*^{-/-}*GNLY*^{+/-} NK cells caused a level of DNA fragmentation equivalent to that of WT NK cells. A pan-caspase inhibitor, Q-VD-OPH, inhibited DNA fragmentation mediated by all effectors. Therefore, both granzyme B and granulysin cause DNA fragmentation that is caspase dependent.

We previously showed that recombinant 9-kDa granulysin activates caspase-3 in Jurkat cells (25). Therefore, we examined the role of caspase-3 and its upstream initiator caspase-9 in the cell-based apoptosis assay. Both WT and *GNLY*^{+/-} NK cells caused activation of both caspase-3 and caspase-9 in YAC-1 cells, but expression of granulysin did not alter the level of activated caspase-3 or caspase-9 (Fig. 4B, 4C). NK cells lacking granzyme B were less efficient at activating both caspase-3 and caspase-9 in targets, but again, expression of granulysin did not change the amount of active caspase-3 or caspase-9 in target cells (Fig. 4B, 4C). Thus, target cell death mediated by granulysin delivered via cytotoxic cells does

not involve mitochondrial damage and its downstream signaling via caspase-9 and caspase-3.

Granulysin causes ER disruption and enhanced caspase-7 and caspase-12 activation

Because DNA fragmentation caused by NK cell-delivered granulysin involved neither caspase-3 nor caspase-9, the role of other executioner caspases was evaluated. Caspase-3 and caspase-7 are structurally similar (34), and they share many substrates, so we next tested involvement of caspase-7. YAC-1 target cells incubated with *gzm^b^{-/-}* NK cells had elevated levels of activated caspase-7, and *gzm^b^{-/-}GNLY^{+/-}* NK cells induced even more activated caspase-7 (Fig. 4D). In a 2-h assay at different E:T ratios, *gzm^b^{-/-}GNLY^{+/-}* NK caused significantly more caspase-7 activation in target cells, compared with *gzm^b^{-/-}* NK cells (Fig. 4E), indicating that cytotoxic cell-delivered granulysin activates caspase-7 rather than caspase-3 and/or caspase-9.

Although caspase-3 and caspase-7 show similar activity toward many synthetic peptide substrates, Walsh et al. (35) recently reported that caspase-3 and caspase-7 exhibit differential activity toward multiple substrate proteins, including Bid, XIAP, gelsolin, caspase-6, and the cochaperone p23. Furthermore, in CHO cells, caspase-7 localizes to the ER and physically interacts with glucose-regulated protein 78 (GRP78), an ER resident chaperone (36). During ER stress, activated caspase-7 interacts with and activates caspase-12, which is also an ER-localized caspase (37). Because the involvement of caspase-7 in ER stress has been well documented and granulysin activates caspase-7, but not caspase-3, in target cells, we evaluated ER stress pathways in target cells. In YAC-1 target cells, both *gzm^b^{-/-}* and *gzm^b^{-/-}GNLY^{+/-}* NK cells caused ER membrane damage after a 1-h incubation, and *gzm^b^{-/-}GNLY^{+/-}* effectors, compared with *gzm^b^{-/-}* effectors, caused enhanced ER disruption (Fig. 5A). When YAC-1 cells were incubated with recombinant 9-kDa granulysin for 2 h, however, no ER membrane damage was detected (Fig. 5B). Thapsigargin, which causes ER stress by inhibiting the Ca⁺⁺-dependent ATPase pump, served as a positive control for induction of ER stress. To determine the role of Fas in granulysin-mediated ER stress in target cells, we again used EL4 and Fas-resistant EL4.F15 cells. We found that *GNLY^{+/-}* effector cells induce higher ER membrane disruption than do WT effector cells (Supplemental Fig. 4A, 4C). However, ER membrane disruption was unaffected by recombinant 9-kDa granulysin (Supplemental Fig. 4B, 4D).

To further delineate the pathways activated by cytotoxic cell-delivered granulysin, we analyzed the status of caspase-12, an ER-resident protein that is involved in apoptotic pathways induced by ER stress. Caspase-12 activation was detected using an Ab specific for the cleaved form of caspase-12. Incubation of YAC-1 cells with *gzm^b^{-/-}GNLY^{+/-}* NK cells produced a higher amount of activated caspase-12 than did *gzm^b^{-/-}* NK cells (Fig. 5C), further supporting the role of cell-delivered granulysin in ER disruption.

GRP78 and C/EBP homologous protein/growth arrest DNA damage-inducible gene 153 are two other ER markers whose expression is upregulated during ER stress. Shinkai et al. (38) have reported that knockdown of GRP78 leads to enhanced cell death. Analysis of YAC-1 targets incubated with either *gzm^b^{-/-}GNLY^{+/-}* or *gzm^b^{-/-}* NK cells did not reveal any change in expression of C/EBP homologous protein/growth arrest DNA damage-inducible

gene 153 (Supplemental Fig. 5B). However, expression of GRP78 was reduced in YAC-1 cells following incubation with *gzmb*^{-/-} *GNLY*^{+/-} NK cells, compared with *gzmb*^{-/-} NK cells (Supplemental Fig. 5A).

It has been reported that cytochrome c release and activation of caspase-9 also cause ER stress (37). Although we found that the amount of activated caspase-9 in targets was similar regardless of whether the NK effector cells express granulysin (Fig. 4C), we evaluated cytochrome c release from target cells after a 2-h incubation with NK cells. Cytochrome c release was similar in YAC-1 cells incubated with either *gzmb*^{-/-} or *gzmb*^{-/-} *GNLY*^{+/-} NK cells (Fig. 5D). Thus, granulysin delivered by effector cells does not damage mitochondria but does cause ER damage.

Granulysin-mediated cell death in human targets

Although the availability of *GNLY*^{+/-} mice allowed us to use cytotoxic cells from these animals to study the role of granulysin in cell-mediated apoptosis, granulysin is normally expressed in human, but not in mouse, NK cells. Therefore, the observed mechanistic differences between NK cell-mediated lysis of murine targets and the previously described apoptosis of human tumor cells by recombinant 9-kDa granulysin may reflect species-specific differences in the target cells. To address this, we used human K562 and THP-1 cell lines as targets for WT and *GNLY*^{+/-} effector cells. In a 3-h apoptosis assay, *GNLY*^{+/-} NK cells, compared with WT effectors, showed marked increase in both K562 and THP-1 target cell death. (Fig. 6A, 6B). Perforin is required for apoptosis of human target cells because WT and *GNLY*^{+/-} NK cells, but not *perf*^{-/-} or *perf*^{-/-} *GNLY*^{+/-} NK cells, caused apoptosis of K562 and THP-1 target cells (Supplemental Fig. 6A, 6B). Thus, cytotoxic cell-delivered granulysin requires perforin for apoptosis of both human and mouse target cells.

We next analyzed DNA fragmentation of human targets, using WT and *GNLY*^{+/-} effector cells. *GNLY*^{+/-} NK cells were much more effective at inducing DNA fragmentation in human targets than were WT NK cells (Fig. 6C, 6D), suggesting that some species-specific differences do exist. The pan-caspase inhibitor QVD-OPH almost completely inhibited DNA fragmentation in both target cells.

The role of ER stress in human target cell killing was also assessed. *GNLY*^{+/-} NK cells induced a higher level of ER disruption in both K562 and THP-1 targets than did WT NK cells (Fig. 6E, 6F). Taken together, these findings indicate that the presence of perforin is critical for cell-delivered granulysin-facilitated cell death in either human or mouse target cells. Furthermore, apoptosis mediated by cell-delivered granulysin involves ER stress, DNA fragmentation, and apoptosis in both mouse and human target cells (Fig. 7).

Discussion

Granulysin is expressed by activated CTLs and NK cells and is present in cytolytic granules with perforin and granzymes (17). To date, our understanding of the apoptotic pathways activated by granulysin comes from in vitro assays using recombinant 9-kDa granulysin protein (24, 25, 27, 28). In the current study, we investigated the apoptotic pathways activated by granulysin delivered to target cells by effector cells. We crossed mice

expressing granulysin with mice lacking perforin or granzyme B and activated splenocytes from these animals to produce in vitro activated NK cells. In the absence of perforin, no lysis of target cells was observed. However, granulysin could substitute for granzyme B to cause target cell death. Investigation of intracellular events following effector–target cell interaction revealed that NK cell-delivered granulysin and recombinant granulysin activate distinct pathways resulting in apoptosis: Recombinant granulysin activates apoptosis through mitochondrial damage and caspase-3, whereas cell-delivered granulysin induces cell death through ER stress and caspase-7.

The granulysin transgenic mice used for this work were generated using a bacterial artificial chromosome containing the human *GNLY* gene and its 5′ and 3′ flanking regions (23). Granulysin is expressed in leukocytes from transgenic mice with kinetics similar to what is found in PBMCs from humans: Granulysin is constitutively expressed in NK cells and, following activation through the TCR, appears in T lymphocytes 8–10 d after activation. This is significantly later than perforin and granzyme B, which are detectable by 2 d after activation (23).

CTLs and NK cells mediate target cell lysis by two independent pathways: exocytosis of preformed granules and ligation of death receptors on target cells with their respective ligands on effector cells (2–8). Both of these pathways lead to target cell apoptosis and membrane disruption. Membrane disruption is chiefly mediated by perforin, whereas both granzymes A and B are crucial for granule-mediated nucleolysis, with granzyme B being the main contributor (39). We found that IL-15-activated NK cells from *perf*^{-/-} splenocytes did not induce apoptosis or DNA fragmentation regardless of whether they express granzyme B and/or granulysin. In contrast, our group previously reported that recombinant 9-kDa granulysin is able to enter and cause death of both murine and human tumor cells (16, 27). Those earlier experiments used 50–100 μM recombinant granulysin, implying that at high concentrations, granulysin can cause membrane damage and enter cells as predicted by its crystal structure (26). Although we do not know the actual concentration of granulysin in cytolytic granules, the concentration may not be high enough to cause membranolysis, thus requiring synergy with perforin.

Recombinant 9-kDa granulysin (50 μM) causes apoptosis of mammalian cells through a well-defined series of events: An initial increase in cytosolic Ca²⁺ and a decrease in K⁺ trigger disruption of mitochondria and ROS production, which results in release of cytochrome *c* and AIF, leading to activation of caspase-3 and DNA fragmentation (24, 25, 27, 28). However, cytotoxic cell-delivered granulysin does not mediate additional mitochondrial damage, regardless of the presence of granzyme B. These findings indicate that, in the absence of other cytolytic molecules, micromolar granulysin is able to cause ionic imbalance and mitochondrial depolarization, but when granulysin is delivered in cytolytic granules, mitochondria are not the primary target.

NK cells expressing granulysin (in the presence or absence of granzyme B) mediate increased DNA fragmentation, as measured by TUNEL assay, and this DNA fragmentation is almost completely blocked by a pan-caspase inhibitor. Previously, we reported that recombinant 9-kDa granulysin activates caspase-3 (25). Effector cells from WT or *GNLY*^{+/-}

animals activated both caspase-3 and caspase-9 with similar efficiency, as measured by Abs specific for the cleaved forms of each caspase. These data again suggest that under different experimental conditions, granulysin activates different apoptotic pathways. A similar dichotomy was reported for granzyme B. Addition of purified murine granzyme B to cells activates apoptosis through caspase-3 (11, 40), whereas delivery of granzyme B by cytotoxic cells activates both caspase-3 and Bid cleavage. However, in target cells lacking Bid, murine granzyme B⁺ CTLs did not elicit any cytochrome *c* release, whereas in targets lacking caspase-3, murine granzyme B⁺ CTLs caused normal cell death. These findings indicate that 1) exogenous granzyme B activates different pathways than does granzyme B delivered by CTLs, and 2) as yet undetermined pathways are involved when granzyme B is delivered through CTL granules (41).

We observed that NK cells lacking granzyme B were less efficient than WT NK cell at activating both caspase-3 and caspase-9, but there was no change in caspase-3 or caspase-9 activation when NK cells from *gzmb*^{-/-}*GNLY*^{+/-} animals were tested, indicating again that granulysin does not activate caspase-3 or caspase-9. Pardo et al. (42) reported that ex vivo virus-immune CD8⁺ cells from *gzmb*^{-/-} animals do not induce activation of caspase-3 or caspase-9 in target cells.

Granulysin delivered by effector cells causes an increase in DNA fragmentation that is blocked by pan-caspase inhibitors but does not involve mitochondrial damage or activation of caspase-3. Therefore, we sought to identify alternative apoptotic pathways activated by granulysin. Caspase-7 is another executioner caspase that is closely related to caspase-3, with highly similar structure and function (34). We observed that NK cells expressing granulysin in the absence of granzyme B mediated enhanced activation of caspase-7, implicating it in granulysin-induced cell-mediated apoptosis. It has been reported that caspase-7 is localized to the ER along with other ER chaperones and is involved in ER stress. In the ER, GRP78 forms a complex with caspase-7 and caspase-12, preventing release of caspase-12 from the ER. In ER stress, caspase-7 associates and activates caspase-12, resulting in increased cell death (36, 37). We found that *gzmb*^{-/-}*GNLY*^{+/-} NK cells induce more ER stress and cleavage of caspase-7 than do *gzmb*^{-/-} effectors. We also observed enhanced activation of caspase-12 in YAC-1 cells after incubation with *gzmb*^{-/-}*GNLY*^{+/-} NK cells. GRP78 blocks caspase-7 activation, and reduced expression of GRP78 leads to enhanced cell death under ER stress conditions (36, 38). We also detected reduced expression of GRP78 and enhanced expression of activated caspase-7 in targets incubated with *gzmb*^{-/-}*GNLY*^{+/-} NK cells. The results obtained with the cell-based cytotoxicity assay suggest that granulysin triggers ER but not mitochondrial stress, resulting in the activation of the caspase-7 pathway, leading to DNA fragmentation and cell death. In contrast, recombinant 9-kDa granulysin causes mitochondrial but not ER damage, indicating that the context of granulysin delivery is central to the apoptotic pathways activated.

The effector cells used in our study contain murine perforin and granzymes along with human granulysin, giving rise to potential issues of species-specific substrates. Previously, we showed that recombinant 9-kDa granulysin causes apoptosis in both mouse and human targets with similar efficiency (16, 25). Recently Rosen and colleagues (43) investigated the substrate specificities of human and mouse granzyme B. The primary sequence of these two

molecules is well conserved, but the substrate specificities differ significantly. Human and mouse granzyme B cleave species-specific procaspase-3 more efficiently than the unmatched substrate. Moreover, human granzyme B efficiently cleaves human or mouse Bid, but neither human nor mouse Bid is cleaved by mouse granzyme B. To begin to address potential species-specific differences in our system, we used the human lymphoid cell lines K562 and THP-1 as targets for the mouse NK cells. In support of our findings using murine target cells, we found that murine NK cells expressing granulysin caused significantly more apoptosis, DNA fragmentation, and ER stress than did NK cells without granulysin. Similar to mouse targets, DNA fragmentation in human target cells induced by effector cells (with or without granulysin) is largely caspase dependent.

In conclusion, to our knowledge, this study is the first investigation of the intracellular mechanisms by which granulysin induces cell death during the cytotoxic cell–target cell interaction. Granulysin activates an apoptosis pathway that involves ER stress and caspase-7, a series of events that are distinct from those activated by recombinant 9-kDa granulysin (Fig. 7). In contrast, granzyme B activates a pathway that involves mitochondrial damage and caspase-3 and caspase-9 activation (Fig. 7). Activation of multiple nonredundant pathways by different molecules delivered by cytolytic effector cells may enable the host defense system to counteract the multiple escape mechanisms employed by pathogens or tumor cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used in this article:

7-AAD	7-aminoactinomycin D
AIF	apoptosis-inducing factor
BFA BODIPY	brefeldin A conjugated to BODIPY 558/568
DDAO-SE	7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one)-succinimidyl ester
DiOC₆(3)	3,3-dihexyloxycarbocyanin
ER	endoplasmic reticulum
GRP78	glucose-regulated protein 78
2-HE	hydroxyethidine
Ψ_m	mitochondrial membrane potential

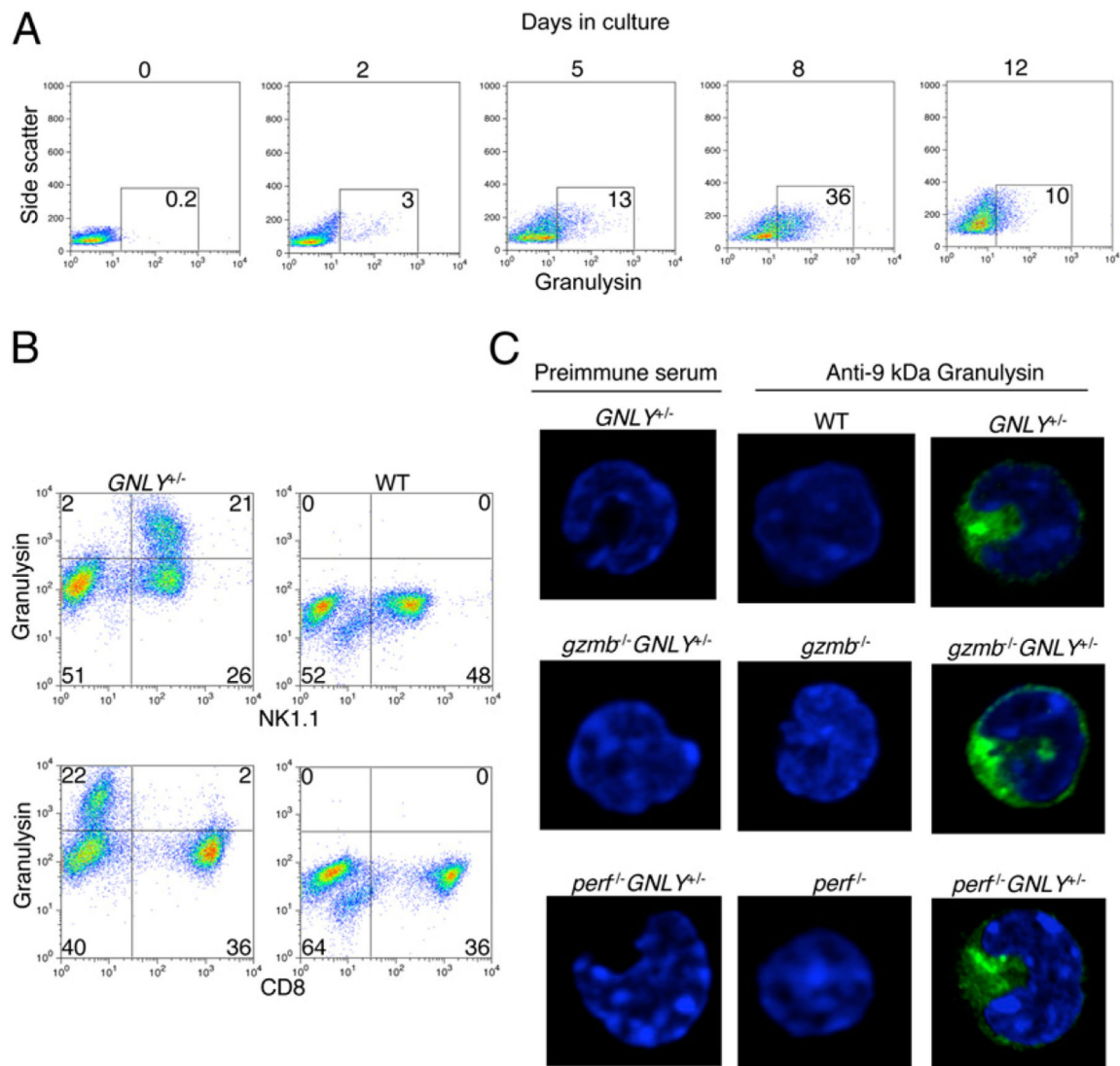
2-ME	2-mercaptoethanol
Q-VD-OPH	Quinoline-Val-Asp-CH ₂ -OPh
ROS	reactive oxygen species
WT	wild-type

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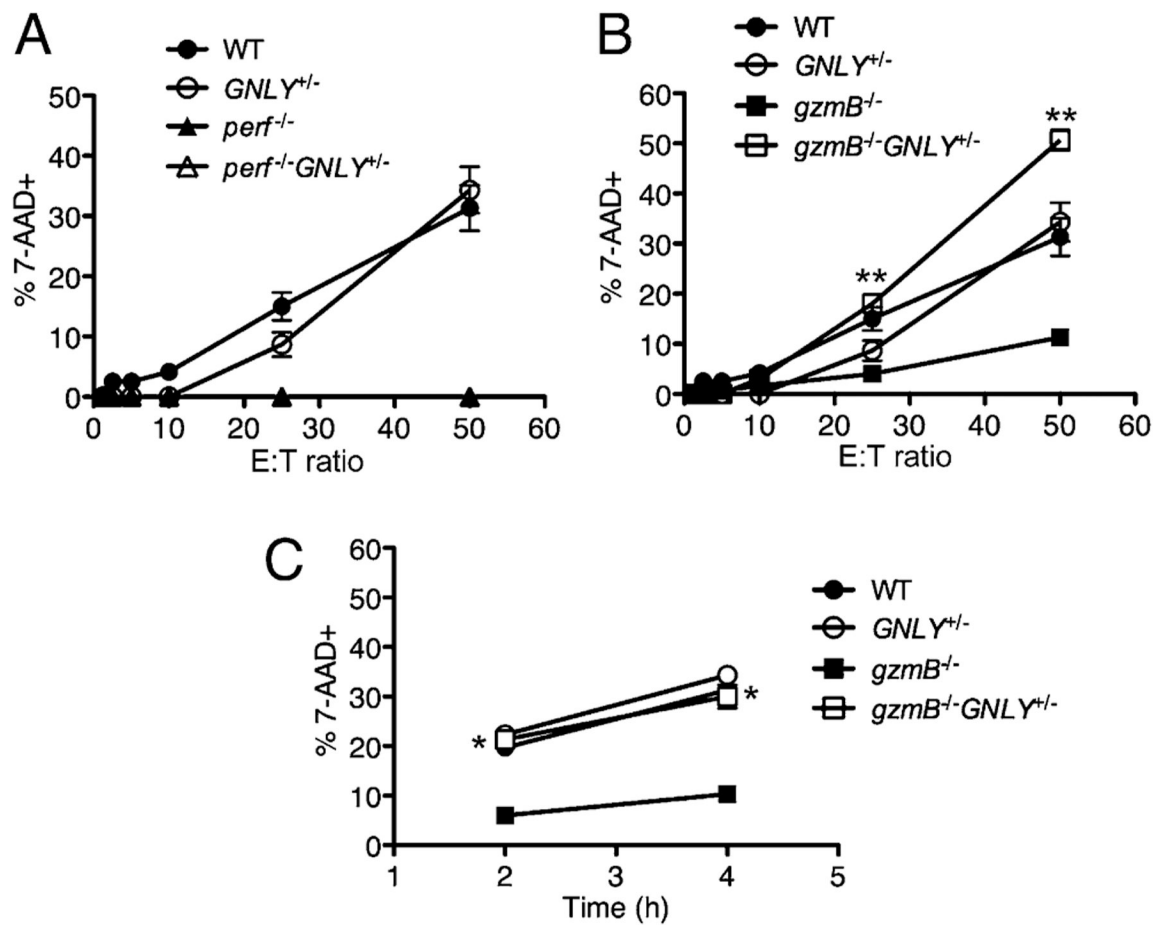
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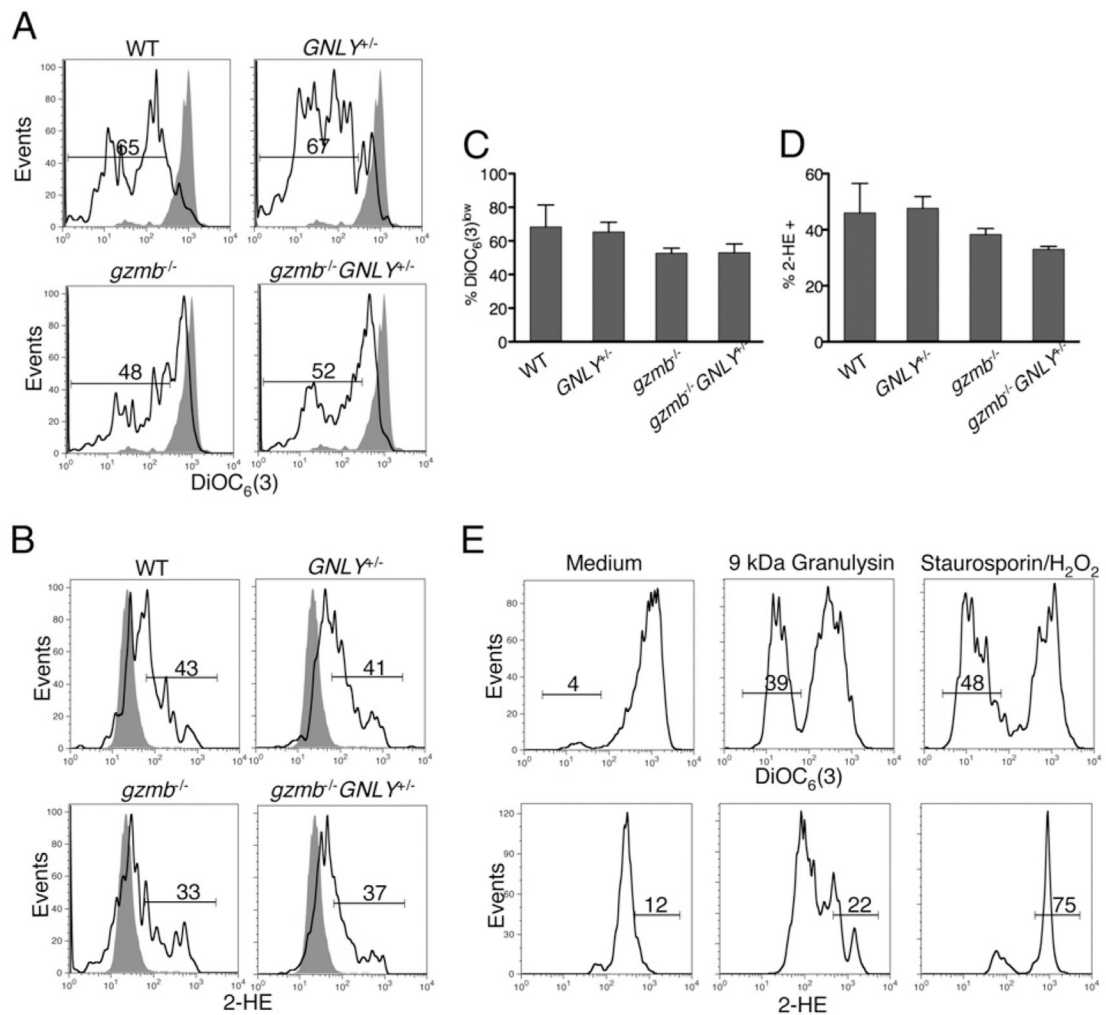
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**FIGURE 1.**

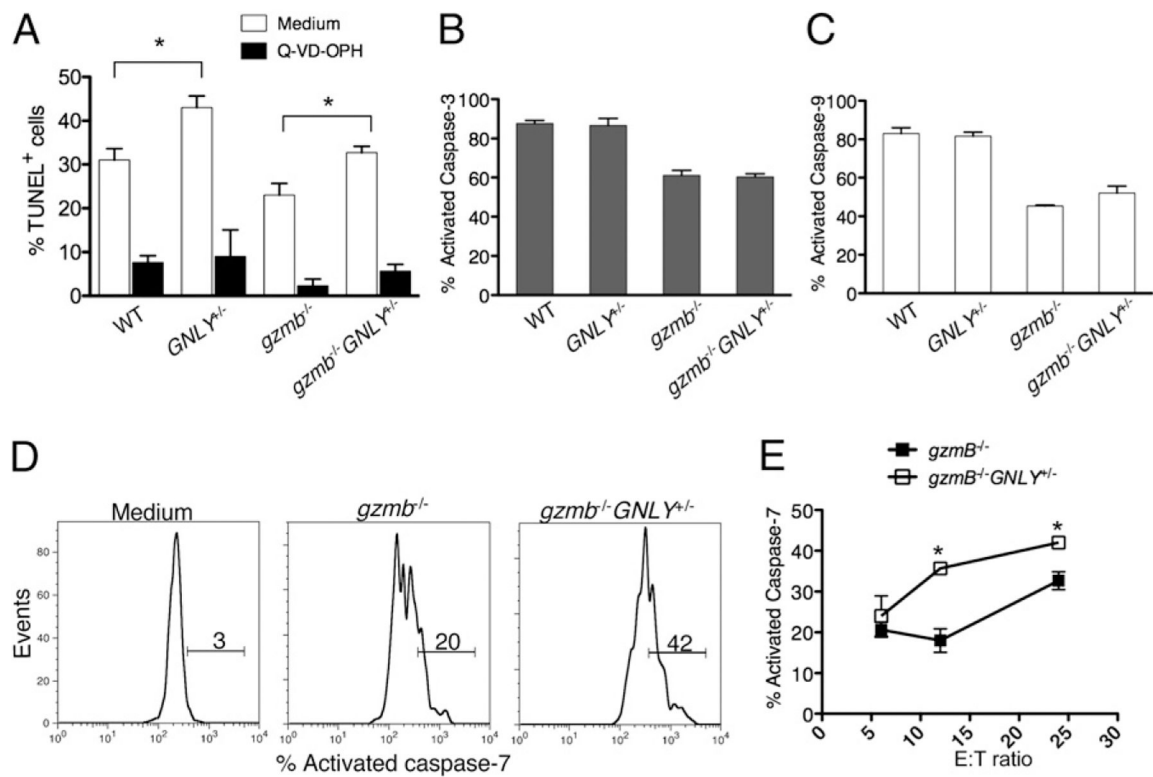
Granulysin expression in murine NK cells. **A**, Intracellular staining and flow cytometry analysis of granulysin expression kinetics in IL-15-activated splenocytes from *GNLY*^{+/-} mice over 12 d of culture. **B**, Expression of granulysin in NK1.1⁺ and CD8⁺ cells after 7 d of culture of *GNLY*^{+/-} and WT splenocytes with IL-15. **C**, Confocal microscopy of granulysin expression in IL-15-activated NK cells. NK cells were isolated from IL-15-activated splenocytes by negative selection using magnetic beads (Miltenyi). Nuclei are stained with DAPI shown in blue; cells were stained with either preimmune serum (*left panel*) or anti-9-kDa granulysin Ab (green; *center and right panels*). Data are representative of three independent experiments.

**FIGURE 2.**

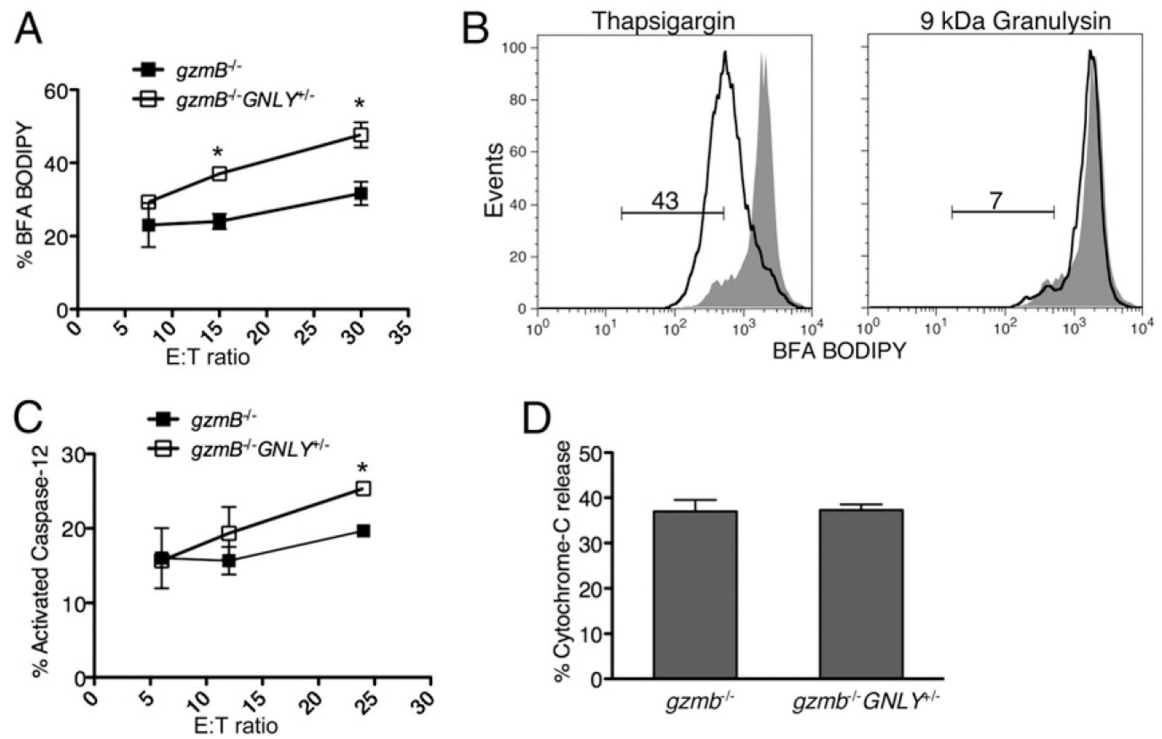
Cytotoxic cell-delivered granulysin requires perforin, but not granzyme B, for target cell apoptosis. *A* and *B*, IL-15-activated splenocytes were incubated with YAC-1 cells for 4 h at the indicated E:T ratios. *C*, Effector cells were incubated with YAC-1 cells for 2 and 4 h at 20:1 E:T ratio. 7-AAD incorporation was measured in CFSE-labeled target cells by flow cytometry. Data are representative of at least three independent experiments \pm SEM. * $p < 0.05$, ** $p < 0.005$. The p values were determined by Student t test (two-tailed, paired/unequal variances) by comparing lysis of *gzmB*^{-/-} versus *gzmB*^{-/-}*GNLY*^{+/-} effectors.

**FIGURE 3.**

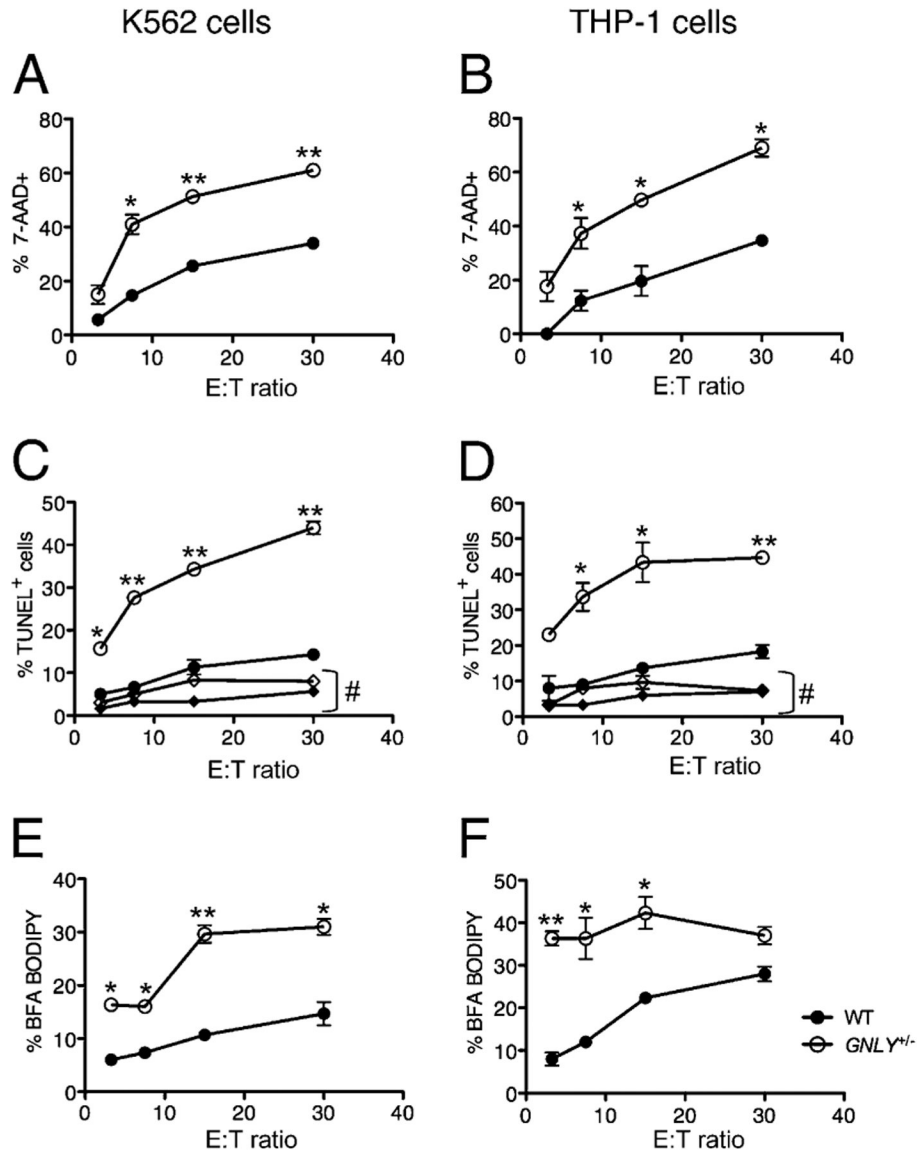
Granulysin delivered by cytotoxic cells does not affect mitochondrial polarization or ROS generation in target cells. YAC-1 cells were incubated with effectors for 2 h, and Ψ_m loss (A, C) and ROS generation (B, D) were analyzed by staining cells with $DiOC_6(3)$ or 2-HE, respectively, and analyzed by flow cytometry after gating on DDAO-SE⁺ cells. A and B, Representative flow diagrams showing Ψ_m and ROS generation in NK targets. Target cells without effectors are shown in gray and used as the negative control to set gates. C and D, Ψ_m and ROS generation in YAC-1 targets after incubation with the indicated effector cells at a 10:1 E:T ratio. E, YAC-1 cells were treated with either medium or recombinant 9-kDa granulysin (25 μ M) for 2 h, and Ψ_m and ROS generation was determined as described above. Staurosporin (1 μ M) and 2% H_2O_2 served as positive control for Ψ_m and ROS generation, respectively. $DiOC_6(3)^{low}$ or 2-HE⁺ cells are indicated by the horizontal bars, and the percent is shown by number. Data presented are the average of three independent experiments \pm SEM.

**FIGURE 4.**

Cytotoxic cell delivery of granulysin causes DNA fragmentation and activation of caspase-7. YAC-1 cells were incubated with effector cells from the indicated strains. **A**, DNA fragmentation was analyzed by TUNEL assay (10:1 E:T ratio, 4 h) in the absence (open bars) or presence (black bars) of 40 μ M pan-caspase inhibitor (Q-VD-OPH). $*p < 0.05$. **B** and **C**, Activation of caspase-3 and caspase-9 detected in target cells after a 2-h incubation at a 10:1 E:T ratio followed by staining with Abs specific for the cleaved form of each enzyme and flow cytometry. **D**, Activated caspase-7 in YAC-1 targets at a 12:1 E:T ratio after a 2-h incubation with the indicated cytotoxic cells. The horizontal bars and numbers above indicate percentage of active caspase-7⁺ cells. **E**, Caspase-7 activation at various E:T ratios after incubation with cytotoxic cells for 2 h. $*p < 0.05$. Results are the mean of three different experiments. Error bars represent SEM.

**FIGURE 5.**

Granulysin delivered by NK cells activates caspase-12 and disrupts ER. **A**, YAC-1 cells were incubated with effectors, and ER disruption was measured by BFA BODIPY staining. **B**, YAC-1 cells were incubated with either medium (gray histogram), thapsigargin (10 μ M), or 9-kDa granulysin (25 μ M), and ER disruption was measured by BFA BODIPY staining. **C** and **D**, Caspase-12 activation and cytochrome c release were determined after incubating YAC-1 cells with cytotoxic cells. * $p < 0.05$. Data presented are the mean of at least three independent experiments \pm SEM.

**FIGURE 6.**

Cytotoxic cell-delivered granulysin causes apoptosis and ER disruption in human target cells. K562 (A, C, E) and THP-1 (B, D, F) target cells were incubated with WT and *GNLY*^{+/-} effectors at various E:T ratios. A and B, Flow-based killing assay for apoptotic cells. C and D, DNA fragmentation assessed by TUNEL assay in the absence or presence (#) of pan-caspase inhibitor (Q-VD-OPH). E and F, ER stress as measured by BFA BODIPY. Results are expressed as the mean \pm SEM for three independent experiments. * $p < 0.05$, ** $p < 0.005$. The p values were determined by Student t test (two-tailed, paired/unequal variances) by comparing values of WT versus *GNLY*^{+/-} effectors.

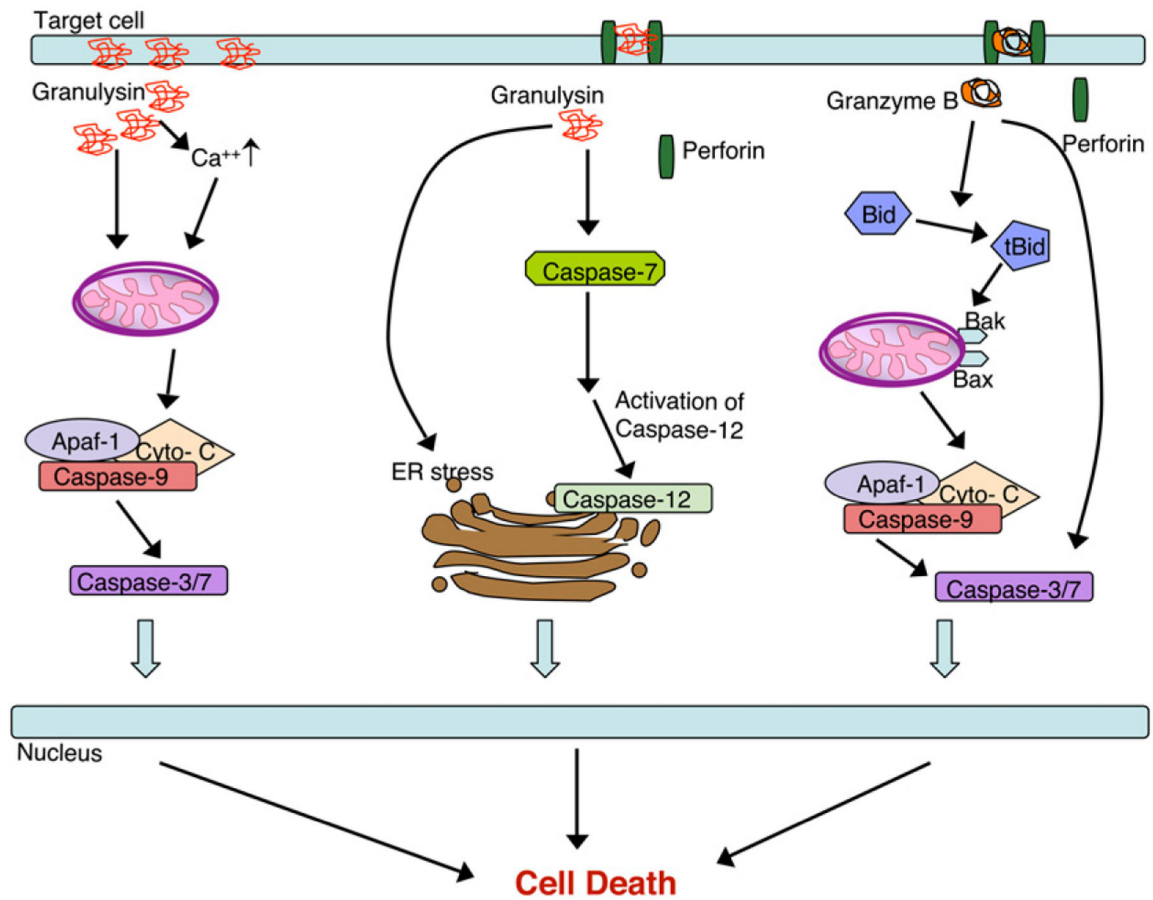


FIGURE 7. Model of apoptosis induced by granulysin or granzyme B. Recombinant 9-kDa granulysin (*left panel*), cytotoxic cell-delivered granulysin (*middle panel*), or cytotoxic cell-delivered granzyme B (*right panel*) activates different apoptotic pathways.