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Granzyme A activates another way to die

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Acknowledgements
I am indebted to the members of my laboratory, past and present, who have worked to unravel the cell death pathway activated by GzmA. This work was supported by NIH grants AI045587 and AI063430.

Immunological Reviews 2010
Vol. 235: 93–104

Immunological Reviews
0105-2896

Summary: Granzyme A (GzmA) is the most abundant serine protease in killer cell cytotoxic granules. GzmA activates a novel programed cell death pathway that begins in the mitochondrion, where cleavage of NDUFS3 in electron transport complex I disrupts mitochondrial metabolism and generates reactive oxygen species (ROS). ROS drives the endoplasmic reticulum-associated SET complex into the nucleus, where it activates single-stranded DNA damage. GzmA also targets other important nuclear proteins for degradation, including histones, the lamins that maintain the nuclear envelope, and several key DNA damage repair proteins (Ku70, PARP-1). Cells that are resistant to the caspases or GzmB by overexpressing bcl-2 family anti-apoptotic proteins or caspase or GzmB protease inhibitors are sensitive to GzmA. By activating multiple cell death pathways, killer cells provide better protection against a variety of intracellular pathogens and tumors. GzmA also has proinflammatory activity; it activates pro-interleukin-1β and may also have other pro-inflammatory effects that remain to be elucidated.

Keywords: *granzyme A, cytotoxic T cell, SET complex, NDUFS3; NM23-H1, TREX1*

Introduction

Release of the contents of cytotoxic granules by cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells into the immunologic synapse formed between the killer cell and its target cell is important for immune elimination of viruses, intracellular bacteria, and tumors (1–6). Cytotoxic granules contain perforin (PFN), a pore-forming protein, and a group of cell death-inducing serine proteases called granzymes (Gzm; for granule enzyme) (7–15). PFN delivers the Gzms into the target cell, where they activate distinct cell death pathways. There are five human Gzms and 10 mouse Gzms, expressed from three gene clusters: GzmA and GzmK, both tryptases that cleave after Arg or Lys basic residues, on chromosome 5 (human) and 13 (mouse); GzmB and GzmH on chromosome 14 (human) and their mouse counterparts (GzmB and GzmC) also on chromosome 14; and GzmM, which cleaves after Met or Leu, on chromosome 19 (human) and chromosome 10 (mouse). A GzmA homolog was the first immune cytotoxic cell protease to appear during evolution (in bony fish) (16, 17). When all types of killer cells are

considered, overall GzmA is the most abundant Gzm, as it is widely expressed in both $CD8^+$ CTLs and NK cells. GzmB is also widely expressed in $CD8^+$ killer cells, and GzmM is highly expressed in NK cells. The other tryptase GzmK that is encoded next to GzmA is the closest GzmA homolog and shares many of the same substrates (18–21). GzmA knockout mice retain GzmK, which may partially compensate for the loss of GzmA (22). Residual GzmK expression in GzmA knockout mice may have led to erroneous underestimates of the importance of GzmA in cellular immunity to viruses and cancer. It is still unclear whether GzmK has distinct functions from GzmA.

Expression of individual Gzms and PFN vary in different clonal populations and depends on how they are activated (17, 23, 24). Most circulating $CD8^+$ T lymphocytes that express any Gzm express both GzmA and GzmB, but some cells are positive for only one Gzm. Individual $CD8^+$ T cells show unexpected diversity in expression of cytotoxic effector molecules (25–27). During *in vitro* activation of mouse naive lymphocytes, GzmA and GzmC expression is consistently delayed compared with cytolytic activity and PFN and GzmB expression (27). However, when mouse CTLs are activated *in vivo* by influenza virus infection, most antigen-specific $tetramer^+$ $CD8^+$ T cells in the lung 1-week after infection express both GzmA and GzmB, and about 1/3 also express PFN. Moreover, there is no difference in the induction *in vivo* of GzmA, GzmB, or PFN.

GzmA activates caspase-independent programed cell death that morphologically resembles apoptosis but has unique substrates and mediators (see below; *Table 1*). GzmB activates caspase cell death pathways by initiating effector caspase cleavage and by directly cleaving some key caspase pathway substrates, such as bid and ICAD (28–38). However, CTL granule-mediated cytolysis is unimpaired by blocking the caspase pathway or overexpressing bcl-2 (39–41). GzmA activates a unique, parallel cell death pathway that does not involve the caspases (42–56). Only a few substrates, PARP-1 and lamin B, are common to both GzmA and GzmB (32, 45, 55). Although most of the literature about GzmA has focused on its role in cell death, the first GzmA substrate identified was the proenzyme pro-interleukin (IL)-1β (57). GzmA activates this key proinflammatory cytokine, suggesting an important role for GzmA in inflammation. In the past year, the relative importance of GzmA in inflammation versus cell death has been a matter of some discussion (see below) (58).

GzmA and GzmB both independently activate programed cell death when delivered into target cells by PFN. The individual Gzms, including some (or possibly all) of the orphan enzymes, each independently activate distinct parallel and non-overlapping programs of cell death (15). Lymphokine-activated killer cells isolated from mice deficient in GzmA or the GzmB cluster have comparable cytolytic activity (*Fig. 1*). While only one molecule (PFN) effectively delivers the Gzms into target cells, each Gzm can trigger cell death. Mice knocked out for either GzmA or the GzmB cluster are both unimpaired in their ability to defend against most viruses and experimental tumor inoculation. These experiments highlight the functional redundancy of the Gzms. However, target cells may be selectively resistant to one or another Gzm, i.e. by bcl-2 overexpression or by expression of viral serpins. Requirements for an individual Gzm have been shown by specific immune challenges. For example, GzmA-deficient mice are more susceptible to the pox virus ectromelia (59), and GzmB-deficient mice have a markedly attenuated incidence of graft versus host disease (GvHD) (60). The redundancy in Gzms may provide better protection against the diversity of pathogens we encounter, some of which have developed strategies for evading the action of individual Gzms (59, 61–67). GzmAxGzmB cluster-deficient mice are immunocompromised but not as profoundly as PFN knockout mice, presumably

***Table 1.* Validated intracellular GzmA substrates**

Substrate	Cellular location	Functional effect of cleavage	Reference
Pro-IL-1β	Cytosol	Activation of cytokine	57
NDUFS3	Mitochondrion	Disrupts electron transport, generates ROS, disrupts mitochondrial potential	56
SET	ER-associated and nucleus	Activates GzmA-mediated DNA degradation by disinhibiting NM23-H1	42, 47
APE1	ER-associated and nucleus	Inhibits base excision repair and reduction of oxidized transcription factors	49
HMGB2	ER-associated and nucleus	Inhibits DNA binding	48
Histone H1	Nucleus	Decondenses chromatin	46
Core histones	Nucleus	Decondenses chromatin	46
Lamin A, B, C	Nucleus	Disrupts nuclear envelope	45
Ku70	Nucleus	Inhibits double-strand break DNA repair	53
PARP-1	Nucleus	Inhibits addition of poly(ADP)-ribose to PARP and other substrates and interferes with DNA repair; helps maintain cellular ATP to facilitate apoptosis	55

ROS, reactive oxygen species; ER, endoplasmic reticulum.

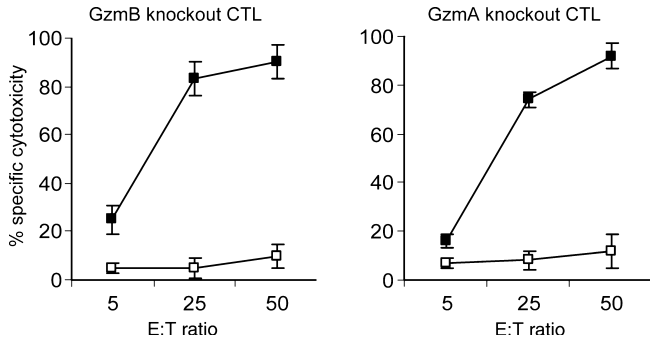

***Fig. 1.* Cytotoxic T lymphocytes derived from GzmA or GzmB knockout mouse splenocytes are comparably cytotoxic.** Phytohemagglutinin-activated splenocytes from Gzm $B^{-/-}$ mice, expressing GzmA (left), or from $GzmA^{-/-}$ mice, expressing GzmB (right), were tested for their ability to kill concancavalin-coated target cells by ^{51}Cr release assay. Both cell lines are comparable at killing, suggesting that GzmA and GzmB have similar cytolytic potency when released from intact cells.

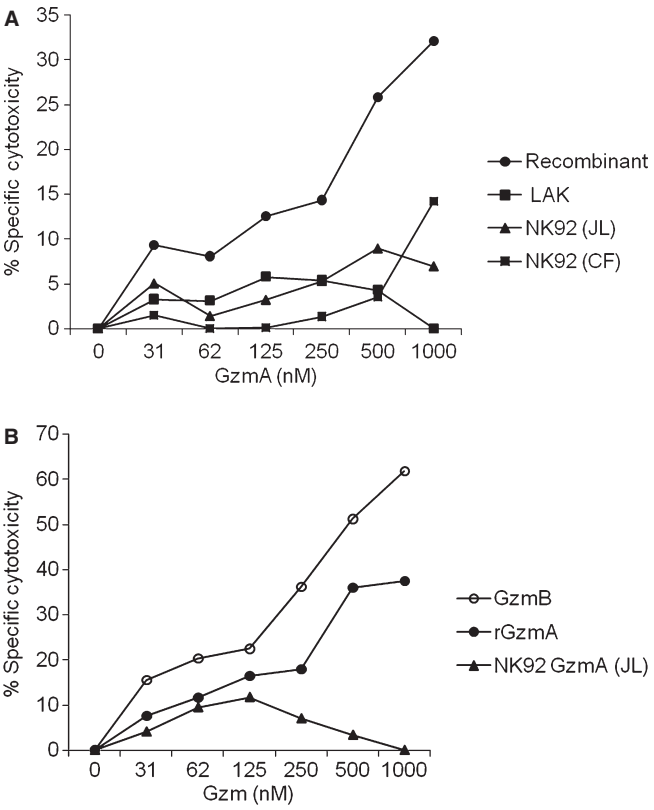

***Fig. 2.* Recombinant GzmA is more active than purified native GzmA.** (A) Cytotoxicity of recombinant GzmA, purified in bacteria, was compared with that of GzmA purified from human natural killer cells by our laboratory (JL) or Froelich (CF) and to GzmA purified from lymphokine activated T cells (LAK). (B) Recombinant GzmA (rGzmA) and NK cell GzmA were compared with GzmB. The recombinant GzmA is active at 250 nM, similar to GzmB, while the purified proteins have much less activity. This could be as a result of incomplete processing of the native purified protein in cells, although the reason for the apparent discrepancy needs to be investigated. Figure modified from (74), reprinted with permission.

because the other 'orphan' Gzms also provide immune protection. CTLs from GzmAxGzmB cluster-deficient mice retain the ability to kill target cells. However, they appear to induce cell death that is morphologically distinct from either PFN-mediated necrosis or CTL-mediated apoptosis (51, 68–70). Nonetheless, GzmAxGzmB doubly deficient mice have a more pronounced phenotype than GzmB knockout mice in several *in vivo* tests of killer cell function including GvHD (50) and tumor clearance (71). Moreover, NK cell cytotoxicity is more compromised in mice deficient in both GzmA and the GzmB cluster than in just the GzmB cluster (72). These *in vivo* studies highlight the importance of GzmA cytotoxic function.

Despite the abundant *in vivo* and cellular evidence for the equal importance of GzmA and GzmB in immune elimination of pathogens and tumors, GzmB has been much more widely studied than GzmA, largely because it activates the caspase pathway, which is so important in developmental cell death. However, new evidence (73) (see below) suggests that the cell death pathway initiated by GzmA may also be activated in non-immune neuronal cell death, especially during ischemia and seizures. The idea that GzmA may be less important than GzmB in inducing cell death has been resurrected in a recent study (58). When cytolytic effects of purified GzmA and GzmB from human NK cells are compared, GzmA is much less cytotoxic than GzmB, requiring micromolar concentrations of GzmA for activity. We confirmed that finding (74). However, when we compared the cytolytic activity of purified NK cell GzmA with recombinant GzmA, we found that the purified protein is barely active in killer cell assays, while the recombinant enzyme expressed in bacteria was cytolytic at high nanomolar concentrations and has comparable activity to GzmB (74) (*Fig.* 2). The Ley group (50 and T. Ley, personal communication) also found similarly high cytolytic activity of recombinant GzmA expressed in yeast. Further work is required to understand why the purified NK cell GzmA has such low activity. One possibility is that much of the purified native material contains proenzyme that has not yet been activated or that the enzyme preparation contains an inhibitor or has been inactivated in cells.

Gzm proteins were previously thought to be expressed uniquely only in killer cells, NK cells, and CTLs, which could be either $CD8^+$ or cytolytic T-helper 1 (Th1) $CD4^+$ cells (13). Naive T cells lack Gzm proteins, which are induced after antigen recognition. However, this occurs only when T cells are fully activated and requires a costimulatory signal. The signal that most consistently induces GzmA, GzmB, and PFN is IL-2 (75). In mice IL-2 regulates PFN and Gzm expression directly

and independently of its effect on $CD8^+$ T-cell survival and proliferation (76, 77). However, mice genetically deficient in IL-2 can elicit a CTL response against many viruses, tumors, and allografts (78, 79). Nonetheless, cytotoxicity is impaired under certain conditions (80). The other common γ chain (γ_c)-dependent cytokines may substitute for IL-2 in knockout mice. Other cytokines implicated in regulating Gzms and PFN are the IL-6/IL-12 family (81, 82). Little is known about what transcriptional factors regulate GzmA expression. GzmB has recently been shown to be expressed without PFN in a variety of non-cytolytic cell (especially during inflammation), including mast cells, basophils, B cells, T-regulatory cells, and cells in the reproductive system, suggesting diverse functions beyond inducing cell death (15). However, there is still no good evidence that GzmA is expressed in these cell populations. GzmA expression has not been as well studied. GzmB may be more widely expressed because the GzmB cluster is juxtaposed to myeloid cell protease genes, whose expression may enhance transcription of the GzmB gene.

The cell biology of GzmA biosynthesis and storage in killer cells and delivery to target cells

During Gzm protein synthesis, processing, and storage, several mechanisms ensure that the Gzms are not active within the killer cell to protect it from self-destruction. All the Gzms are synthesized as inactive precursor molecules. The precursors contain a signal sequence that directs them to the endoplasmic reticulum (ER) and an N-terminal dipeptide that must be removed to activate the protease. In the Golgi, a mannose-6-phosphate (M6P) tag, a sorting signal for lysosomal transport, is added to direct the Gzms to the cytotoxic granules, which are specialized electron dense secretory lysosomes. The N-terminal dipeptide is only removed in the cytotoxic granule by cathepsin C (dipeptidyl peptidase I) (83). However, both mice and humans genetically deficient in cathepsin C have only partially reduced Gzm activity and modestly reduced antiviral immunity (84, 85), suggesting that an alternate enzyme may activate the pro-enzyme. In fact, IL-2 treatment stimulates dipeptide removal in cells from patients with Papillon-Lefevre syndrome, who lack functional cathepsin C (86). Gzm proteolytic activity is negligible at the acidic pH of the granule. In the granule the Gzms, which are very basic (pI = 9–11), and PFN are bound to the serglycin proteoglycan, which probably also keeps them inactive (87).

When the killer cell is activated by conjugation with a target cell destined for elimination, cytotoxic granules move toward the immune synapse, and the cytotoxic granule membrane fuses with the killer cell plasma membrane, releasing the granule contents into the immune synapse (88). Gzms likely dissociate from serglycin before they enter target cells (89). The highly basic Gzms bind to the negatively charged target cell membrane by electrostatic interactions (90–92) and also by specific receptors, such as the cation-independent M6P receptor (93). However, specific receptors are not required for binding and cytotoxicity (90, 94, 95). The lack of a requirement for a receptor eliminates one potential mechanism for escaping immune surveillance.

During CTL activation, the concentration of GzmA at the immune synapse is estimated to be roughly 8 μM (74). This estimate was calculated based on the GzmA yield from killer cells (approximately 20 $\mu g/10^9$ cells) (96), and conservative estimates that approximately one-tenth of CTL granule contents are released into a single synapse with a volume of $<5\ \mu m^3$. Therefore, GzmA synapse concentrations are approximately $2 \times 10^9\ \mu g/5\ \mu m^3$ or approximately 8 μM, a concentration that is more than adequate for inducing cytotoxicity, which is activated at 250 nM GzmA. Although most Gzms are released into the immune synapse, the seal may not be completely tight or some enzyme might be directly secreted instead of being directed to cytolytic granules. During chronic inflammation small amounts of GzmA leak out into extracellular fluids, where it can have other biologic effects (see below). In rheumatoid arthritis joints or the blood of acquired immunodeficiency syndrome (AIDS) patients, GzmA can reach low nanomolar extracellular levels [versus <1 pM in normal blood (97) and μM concentrations in the immune synapse].

Gzm entry into the target cell cytosol is mediated by PFN, but how PFN delivers Gzms into the target cell is still not completely worked out (14). Although PFN multimerizes in the target cell plasma membrane to form pores (98, 99), the original model of Gzm entry through plasma membrane pores (7, 8) is probably not correct (100). That model would predict that Gzms are taken up directly into the target cell cytosol. However, Gzms and PFN are first jointly endocytosed in a clathrin and dynamin-dependent manner into early endosomal antigen (EEA)-1-staining early endosomes (101–103) and are then released into the cytosol about 5–10 min later (103, 104). At sublytic PFN concentrations that do not cause necrosis but deliver Gzms, the PFN pores in the plasma membrane cause a transient calcium influx but may be too small to allow Gzms through. The rise in intracellular calcium is sensed by all cells as a sign of plasma membrane damage. This triggers a stereotypic cell membrane repair response, sometimes called cellular 'wound healing', which has at least two features: (i) fusion of subcellular organelle membranes (lysosomes and endosomes) to the damaged plasma membrane to patch the leak

and (ii) accelerated membrane endocytosis to remove the damaged membrane (103, 105, 106). PFN triggers both of these events, which are required to permit the target cell to avoid necrotic death and undergo apoptosis (103, 104). Gzms are then released from endosomes by an unknown mechanism, which likely involves PFN pore formation in the endosomal membrane. Although some key Gzm targets are cytosolic (i.e. bid and ICAD for GzmB), other important targets are in other membrane-bound cellular compartments, including the nucleus and mitochondrion (see below) (*Table 1*). GzmA rapidly translocates from the cytosol and concentrates in the nucleus (49, 107, 108), where key substrates are cleaved (SET, Ape1, lamins, histones, Ku70, PARP-1). Using immunoelectron microscopy and flow cytometry analysis of mitochondria incubated with fluorescently tagged GzmA, we found that GzmA crosses the double mitochondrial membrane to enter the mitochondrial matrix, where it initiates mitochondrial damage (56). This occurs rapidly as soon as GzmA gets into the cytosol. GzmA does not have a mitochondrial import signal, so a specific mitochondrial import mechanism is required. Mitochondrial import of GzmA is an active process that requires an intact mitochondrial transmembrane potential and may be mediated by GzmA binding to mitochondrial chaperone heat shock proteins (56, 109).

GzmA structure

The structure of GzmA is similar to that of trypsin. Human GzmA has been crystallized with high resolution (110, 111). Gzm activation by dipeptide removal is likely accompanied by a radical conformational change, as a GzmA mAb does not recognize pro-GzmA (42). GzmA differs from the other Gzms in forming a covalent homodimer. Dimerization creates an extended site for substrate binding that confers a high degree of specificity to GzmA for its substrates. In particular, because of the extended exosite, GzmA substrates do not share a common cleavage site peptide sequence; moreover, when the preferred cleavage site is mutated, alternate nearby basic residues may be cleaved instead (53). Unlike trypsin, the proteolytic activity of GzmA is highly selective. When nuclear lysates are incubated with trypsin overnight, most proteins are degraded. However, after incubation with a similar concentration of GzmA overnight, most protein bands remain unchanged (112). This selectivity has enabled identification of physiologically relevant GzmA substrates by comparing changes in protein abundance of the entire proteome of intact organelles treated or not with GzmA (56, D. Jensen and J. Lieberman, manuscript in preparation).

GzmA inhibitors

The regulation of proteolytic enzymes in tissues by endogenous inhibitors is critical to maintain homeostasis and prevent undesirable damage. Although Gzm trafficking within CTLs minimizes leakage of active enzyme out of granules, any stray molecules in the cytoplasm could cause cell death (113). During granule exocytosis, some Gzms might inadvertently re-enter the effector cells. Because CTLs typically kill several targets in succession without harming themselves, an important question is how do CTLs protect themselves from their own cytotoxic molecules? One protective mechanism is externalization of a cytotoxic granule membrane protein (cathepsin B), capable of proteolytically inactivating PFN, to the killer cell plasma membrane during granule fusion (114). Cathepsin B protects the killer cell membrane from any PFN redirected to the CTL side of the synapse. However, killer cells from cathepsin B knockout mice survive encounters with target cells unharmed, suggesting that other protective mechanisms exist (115). One other way is expression of serpins by killer cells (116). Serpins are a large protease inhibitor family that inactivate their targets either by covalently and irreversibly binding to the enzyme active site or by forming extremely tight noncovalent complexes (117–119). No intracellular GzmA inhibitors or serpins are known. However, some extracelular trypsin inhibitors also inhibit GzmA. GzmA is bound and irreversibly inhibited in the circulation by two trypsin inhibitors, α-2 macroglobulin and anti-thrombin III (120). GzmA complexed to proteoglycans is resistant to these two protease inhibitors (121). Another GzmA inhibitor is pancreatic secretory trypsin inhibitor (PSTI) (122), which is found in the blood in patients with severe inflammation and tissue destruction (123, 124). Extrapancreatic PSTI might regulate the extracellular activity of GzmA. Unlike the other two GzmA inhibitors, PSTI inhibits GzmA complexed to proteoglycans (122). It is not known whether these GzmA inhibitors are expressed in CTLs. No viral inhibitors of GzmA are known. Synthetic Gzm inhibitors are powerful tools both for research and potentially for therapeutic applications. There are several classes of inhibitors, including isocoumarin derivatives, peptide chloromethyl ketones, and peptide phosphonates (120).

The molecular basis of GzmA-mediated cell death

GzmA induces caspase-independent cell death, morphologically indistinguishable from apoptosis. GzmA activates production of reactive oxygen species (ROS) from the mitochondria, dissipation of the mitochondrial transmembrane

potential ($\Delta\psi_m$), mitochondrial swelling and loss of cristae, displacement of phosphatidyl serine to the outer leaflet of the plasma membrane, chromatin condensation, and nuclear fragmentation (43, 49, 50, 52, 53, 56). Mitochondrial changes occur within minutes (52, 56). Phosphatidyl serine externalization (measured by annexin V staining) occurs within 30 min to 1 h. This is important, because it allows the dying cell to be taken up by macrophage scavenger systems. Therefore, the GzmA-treated cell can be engulfed and eliminated without triggering the inflammatory changes associated with necrosis. Within 2 h, the slower onset hallmarks of apoptosis appear: chromatin condensation and DNA damage. DNA is damaged by single-stranded cuts into megabase fragments much larger than the oligonucleosomal fragments generated during caspase-activated cell death (44). Because the DNA fragments are too large to be released from the nucleus, assays that measure DNA release into culture supernatants are negative until many hours later. This was incorrectly originally interpreted to mean that GzmA induces a slow, non-apoptotic death. Indeed, the caspases are not activated (43). Moreover, mitochondria are damaged without mitochondrial outer membrane permeabilization or release of pro-apoptotic mediators, such as cytochrome c, from the mitochondrial intermembrane space (51, 52, 56) Triggering mitochondrial damage is key to cell death induction as treating target cells with superoxide scavengers blocks GzmA-mediated cell death (and also blocks death by CTLs expressing all Gzms) (52).

When GzmA enters a target cell, its first key act is to cripple mitochondrial electron transport and disrupt the mitochondrial potential (*Fig.* 3). In the mitochondrial matrix, GzmA cleaves NDUFS3, a component of electron transport complex (ETC) complex I to interfere with mitochondrial redox function, ATP generation, and maintenance of $\Delta\psi_m$ and to generate superoxide anion (52, 56). GzmA-mediated mitochondrial changes are partially inhibited by cyclosporine A and bongkrekic acid, two inhibitors of the mitochondrial permeability transition (PT) pore, suggesting a potential role for the PT pore in GzmA-induced mitochondrial damage. The importance of ROS generation and NDUFS3 cleavage and disruption of ETC complex I was underscored by inhibition of GzmA-mediated ROS generation and cell death by two complex I inhibitors, rotenone and piericidin A. Of note, pseudo ρ^o cells, which are deficient in mitochondrial DNA and consequently are deficient in complex I function and aerobic metabolism, do not generate ROS after treatment with GzmA and PFN and are resistant to GzmA-mediated cell death. As many tumor cells rely on glycolysis rather than aerobic metabolism (the Warburg effect), they may be relatively resistant to GzmA.

The superoxide generated by damaged mitochondria drives an ER-associated oxidative stress response complex, called the SET complex, which plays a critical role in GzmA-induced nuclear damage, into the nucleus (44, 52) (*Fig.* 4). The SET complex contains three nucleases [the base excision repair (BER) endonuclease Ape1, an endonuclease NM23-H1, and a 5′–3′ exonuclease Trex1], the chromatin modifying proteins SET and pp32, which are also inhibitors of PP2A, and a DNA binding protein that recognizes distorted DNA, HMGB2 (42, 44, 47–49, 54). One of the functions of the complex is to repair abasic sites in DNA, the dominant type of oxidative DNA damage. GzmA, which traffics to the nucleus by an unknown mechanism, converts this DNA repair complex into an engine for DNA destruction by cleaving SET, an inhibitor of the endonuclease NM23-H1 (47). This allows NM23-H1 to nick DNA; the exonuclease Trex1 in the SET complex then chews up DNA further at the break, making it difficult for the target cell to mend the damage (54). At the same time, GzmA cleaves and inactivates HMGB2 and Ape1 in the SET complex (48, 49). This disrupts BER as well as the other known function of APE1, reducing oxidized transcription factors, such as FOS, JUN, and NF-κB, involved in the immediate early repair response (125–129). The oxidized transcription factors are unable to bind to DNA and activate gene transcription.

Much of GzmA's action appears to be focused in the nucleus where GzmA concentrates (107). Within the nucleus, GzmA opens up chromatin by cleaving the linker histone H1 and removing the tails from the core histones, making DNA more accessible to any nuclease (45). Like the caspases and GzmB, GzmA also disrupts the nuclear envelope by cleaving lamins (46). Substrates shared by multiple cell death pathways may be critical for a cell to undergo programed cell death. Whether a cell lives or dies after an apoptotic stimulus depends to some extent on its ability to repair DNA damage. In addition to knocking out BER by cleaving APE1, GzmA also interferes with DNA repair more generally by cleaving and inactivating Ku70, important in double-strand break repair by non-homologous end joining (53), and PARP-1, an early sensor of both single and double-stranded DNA damage (55). The Ku complex binds to the ends of DSB and recruits DSB repair proteins, preventing these reactive ends from initiating dangerous chromosomal translocations. GzmA cleavage disrupts Ku70 binding to DSB (53). Cells with silenced Ku70 are more sensitive to GzmA, while cells overexpressing Ku70 are relatively resistant. It is not clear why this should be the case, as the GzmA programed cell death pathway does not involve DSB. This suggests that Ku70 might have other unknown functions in cell death. Although Ku70 is not targeted by the caspases,

DNA-PK$_{cs}$, another Ku complex component, is cleaved by GzmB and caspase 3.

GzmA cleaves PARP-1 after K498 to separate its DNA binding domain from its catalytic domain, which adds poly(ADP) ribose (PAR) to proteins, including itself (55). PARylation of PARP-1 recruits DNA repair factors to sites of both single-stranded and double-stranded DNA damage. PARP-1 is a well known target of GzmB and the caspases (32, 130, 131). Cells with silenced PARP-1 or treated with a PARP-1 inhibitor are hypersensitive to GzmA and cells over-expressing K498A PARP-1 are relatively resistant. GzmA not only interferes with PARP-1 activity, but the N-terminal fragment produced after GzmA PARP-1 cleavage also binds to DNA and acts as a dominant negative to interfere with the DNA repair function of uncleaved PARP-1. PARP-1 is the only known common substrate, other than lamin B, shared with the caspase pathway. In fact, cells overexpressing the non-cleavable mutant PARP-1 that die are more likely to die by necrosis than apoptosis. This finding can be explained by cellular ATP depletion, as PARP-1 uses ATP to generate PAR. As apoptosis requires cellular ATP, if PARP-1 were not inactivated, ATP depletion would push cells to a more inflammatory necrotic death. Therefore, inactivating PARP-1 may be a feature of all forms of programed cell death.

Is the GzmA programed cell death pathway activated independently of immune effector cells?

A recent report (73) suggests that the SET complex is mobilized to cause DNA damage and apoptosis in neurons during

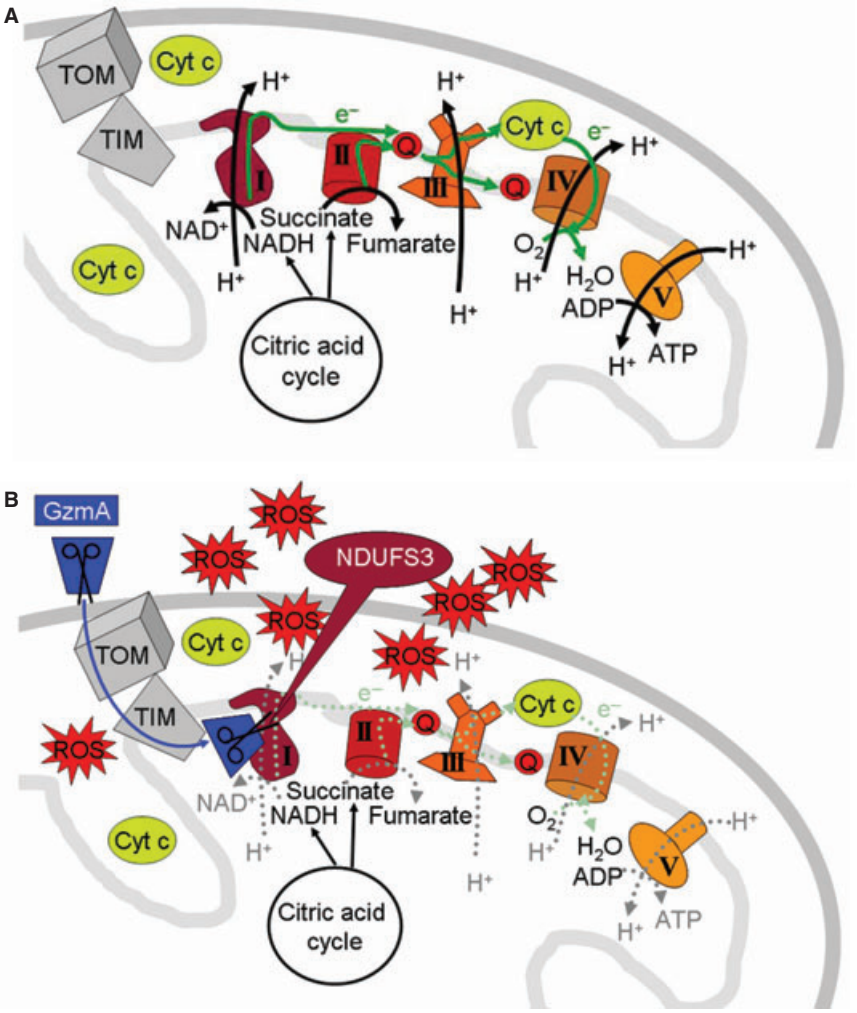

***Fig. 3.* GzmA disrupts mitochondrial electron transport by cleaving NDUFS3 in electron transport complex I.** (A) Diagram of electron transport in mitochondria depicting the five inner membrane-associated electron transport complexes. (B) GzmA wreaks havoc on mitochondrial function. GzmA is actively transported into the mitochondrial matrix by an unknown mechanism that likely involves the TOM-TIM twin translocases. Once inside, GzmA disrupts inner membrane-associated electron transport complex I by cutting NDUFS3, which is located in the neck of the stalk of complex I that protrudes into the matrix. Disrupting complex I leads to reactive oxygen species production and interferes with electron transport, the maintenance of the mitochondrial transmembrane potential, and ATP generation. Despite all these changes, the mitochondrial outer membrane remains intact, and pro-apoptotic proteins in the intermembrane space, including cytochrome c (Cyt C), are not released.

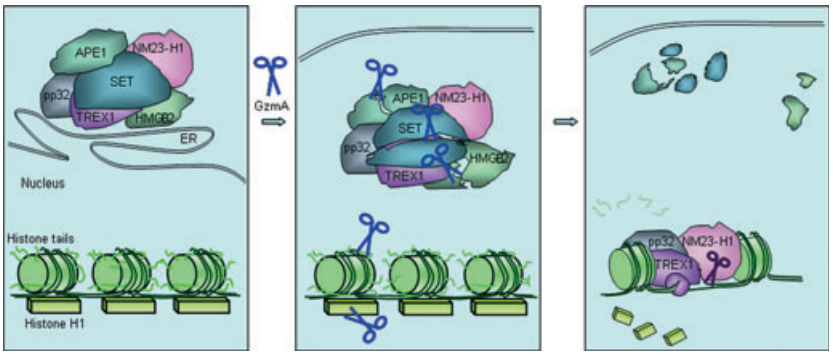

Fig. 4. The SET complex is the key to GzmA-mediated DNA damage. The SET complex, which is normally associated with the endoplasmic reticulum, translocates to the nucleus in response to the superoxide anion generated by GzmA cleavage of NDUFS3 (*Fig. 3*). GzmA also concentrates in the nucleus where many of its known substrates reside (*Table 1*). In the nucleus, GzmA cleaves three components of the SET complex: SET, HMGB2, and APE1. SET is an inhibitor of the SET complex endonuclease NM23-H1. SET cleavage activates NM23-H1 to make single-stranded DNA nicks. These are extended by the SET complex exonuclease TREX1. GzmA also degrades the linker histone H1 and removes the tails from the core histones, which opens up chromatin and makes it accessible to these nucleases. Figure adapted with permission from reference (15).

ischemia and seizure. In response to a drop in intracellular pH in neurons, a lysosomal protease, asparagine endopeptidase (AEP), is activated to cleave SET, which in turn activates NM23-H1 and TREX1 to damage DNA. Interestingly, AEP cuts SET after Asn^{175}, while GzmA cuts after the next residue (Lys^{176}). AEP KO micee are protected from brain injury. In neurons, SET is partially protected by binding the phosphoinositol 3-kinase (PI3K) enhancer PIKE-L. If this report is confirmed, understanding the GzmA pathway will facilitate understanding not only immune-mediated cell death but also neuronal cell death and potentially will provide ways of manipulating it therapeutically.

Extracellular role of GzmA

Although most research has focused on Gzm cell death-inducing properties, Gzms likely have extracellular functions in promoting inflammation and degrading extracellular matrix, potentially to allow cytotoxic cells access to target cells within tissue. During killer cell degranulation, it is unclear whether the immune synapse forms a perfectly tight gasket that completely prevents Gzms from leaking into the extracellular space. Some GzmA is detected in healthy donor serum (132). During inflammation and infection, elevated Gzm levels are found in serum and other bodily fluids. Examples include the serum of patients undergoing acute CMV infection or chronic HIV infection, the joints of rheumatoid arthritis patients, and the bronchoalveolar lavage fluid of allergen-challenged patients with asthma and patients with chronic obstructive pulmonary disease (97, 121, 132–138). Proteolysis by extracellular GzmA will be inhibited to some extent by serum and extracellular trypsin inhibitors (described above) (121), but the relative concentration of GzmA and its inhibitors, which might vary in different tissue compartments, will determine whether proteolytic effects become significant in disease settings. Although circulating Gzms might not be able to enter cells to induce cell death without a high local PFN concentration, they could proteolyze cell surface receptors or extracellular proteins to cause, as yet unappreciated, destruction, particularly if present at high concentrations at inflamed sites in the absence of natural inhibitors. The known extracellular activities of GzmA suggest a proinflammatory effect. GzmA can activate the proinflammatory cytokine IL-1β directly within cells by cleaving its propeptide (57). Although this activity could not be confirmed using native purified NK cell GzmA (58), we reproduced this result using recombinant GzmA, consistent with the hypothesis that native purified GzmA may not be fully active (74). GzmA can also proteolytically activate macrophages to secrete cytokines (139), cause neurite retraction of astrocytes, and inhibit thrombin-induced platelet aggregation by cleaving the thrombin receptor (140, 141). One study suggests another anticoagulant effect to activate pro-urokinase to activate plasminogen (142). Other older papers suggest possible roles in degrading extracellular matrix proteins (143–145). GzmA binding to basement membrane proteoglycans might protect it from inactivation by extracellular tryptase inhibitors (145).

A recent study refocuses attention of the ability of GzmA to activate monocyte production of inflammatory cytokines (58). However, this study does not describe how GzmA activates monokine production. It will be important to define whether other mechanism(s), besides IL-1β activation, are at work. The GzmA concentration required to activate proinflammatory cytokine secretion is in the low nanomolar

range, which is reached during serious infections or inflammatory states, but not in normal tissues. Therefore, it is likely that GzmA has a significant proinflammatory effect in disease settings. Because low nanomolar concentrations activate monocytes to secrete inflammatory mediators but high nanomolar concentrations are required to induce cell death, these authors concluded that inducing inflammation, rather than activating cell death, is the major physiologic role of GzmA. We do not feel this conclusion is warranted. First it does not take into consideration that GzmA concentrates in the immune synapse, achieving levels a thousand fold higher than those reached in body fluids in the most severe disease states. Only *in vivo* experiments can truly assess the relative role of a protein in disease. In the future, experiments defining GzmA-activated proinflammatory pathways and *in vivo* experiments comparing inflammation in GzmA-deficient versus wildtype mice will help sort out the importance of GzmA as a proinflammatory mediator.

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