

THE ABCS OF GRANULE-MEDIATED CYTOTOXICITY: NEW WEAPONS IN THE ARSENAL

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Granule exocytosis is the main pathway for the immune elimination of virus-infected cells and tumour cells by cytotoxic T lymphocytes and natural killer cells. After target-cell recognition, release of the cytotoxic granule contents into the immunological synapse formed between the killer cell and its target induces apoptosis. The granules contain two membrane-perturbing proteins, perforin and granulysin, and a family of serine proteases known as granzymes, complexed with the proteoglycan serglycin. In this review, I discuss recent insights into the mechanisms of granule-mediated cytotoxicity, focusing on how granzymes A, B and C and granulysin activate cell death through caspase-independent pathways.

CELL DEATH AND IMMUNITY

GRAFT-VERSUS-HOST DISEASE (GVHD). An immune response mounted against the recipient of an allograft by immunocompetent donor T cells derived from the graft. Typically, it is seen in the context of allogeneic bone-marrow transplantation.

Killer lymphocytes are key players in the effector arm of the immune response that eliminate cells infected with intracellular pathogens and transformed tumour cells¹. Cell-mediated cytotoxicity also regulates and terminates the immune response. Uncontrolled cytotoxicity can be undesirable, as in the autoimmune destruction of healthy cells, the destruction of infected and uninfected bystander cells during liver infection with non-pathogenic viruses and GRAFT-VERSUS-HOST DISEASE (GVHD). Killer cells in both adaptive and innate immunity — T cells and natural killer (NK) cells, respectively — use the same basic mechanisms for destroying their targets, although they are triggered by distinct receptors and the expression of cytolytic molecules is constitutive in NK cells, but regulated in T cells. Cytotoxic T lymphocytes (CTLs) and NK cells can destroy their targets either by releasing the contents of cytolytic granules in the granule-exocytosis pathway of cytotoxicity, or by engaging cell-surface death receptors, such as members of the tumour-necrosis factor receptor (TNFR) family, including FAS (CD95). Broadly speaking, on the basis of findings in genetically manipulated mice, human genetic diseases and *in vitro* studies, the granule-exocytosis pathway has the dominant role in eliminating virus-infected cells, protecting against other

intracellular pathogens (such as mycobacteria) and tumour surveillance^{2,3}. However, cytokine secretion also has an important role in protection from some virus infections. The death-receptor pathway, which activates the CASPASE pathway of apoptosis and has served as an important model system for understanding apoptosis, has a more important role in regulating the immune response and in some of the undesirable effects of autoimmunity, hepatitis and GVHD. This review will focus on the mechanisms of granule-mediated cytotoxicity, with special emphasis on newly described caspase-independent cell-death pathways, which are just beginning to be uncovered. Recent insights into the cell biology of granule exocytosis (how granules move to the synapse, how the immunological synapse functions in secretion and how killer cells are protected from their own death-inducing enzymes) are discussed by G. Griffiths⁴ in the companion review in *Nature Immunology*.

Granule exocytosis

Cytotoxic granules are specialized secretory lysosomes that are present only in cells with cytolytic capability⁵. When a T cell recognizes its target, through engagement of its antigen receptor (or for NK cells, an NK receptor), the area of apposition of the T cell with its

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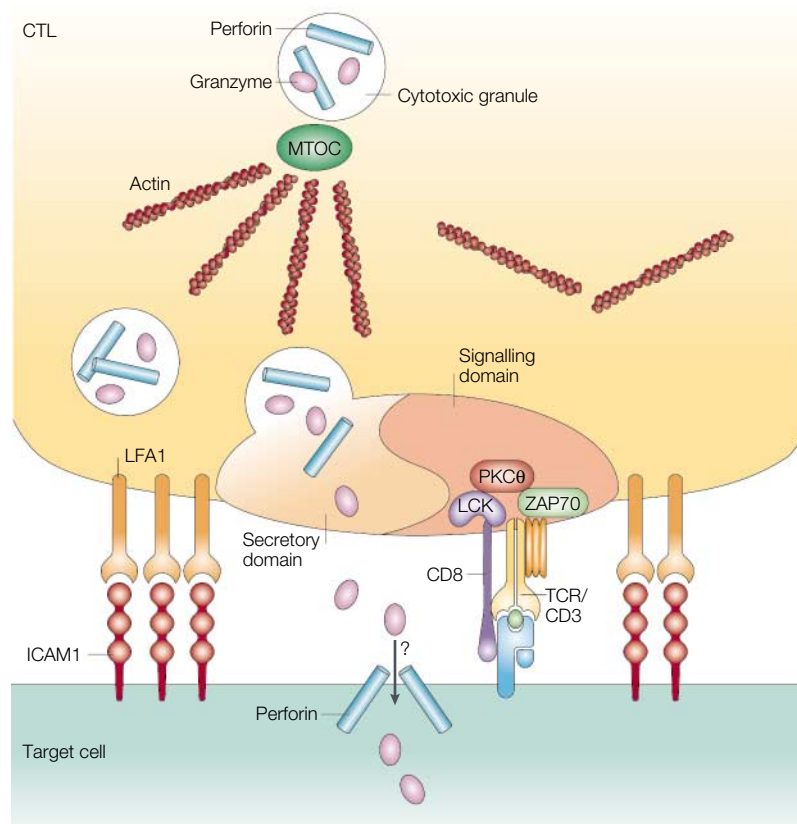


Figure 1 | Granule proteins are released through the exocytic domain of the immunological synapse. When a cytotoxic T lymphocyte (CTL) recognizes its specific target, the cytotoxic granules migrate from their dispersed locations in the cytosol towards the synapse. The movement of the granules is orchestrated by the microtubule-organizing centre (MTOC)¹⁰². In elegant studies, the synapse formed by the CTL has been shown to be divided into two domains — a signalling domain similar in structure to the synapse formed by non-cytolytic T cells, and a secretory domain to which the membrane of the cytotoxic granules fuses to deliver their cytolytic contents into the synapse^{103,104}. The formation of these two domains is similar to the organization of the neuronal synapse, where one domain is endocytic, as many of the receptor molecules are internalized after engagement, and the other domain is exocytic, allowing the release of effector molecules into the synapse¹⁰⁵. The synapse forms a tight seal that directs the cytolytic molecules to the target cell and keeps them from leaking out to damage bystander cells and tissues. However, some of the molecules do leak out, and particularly in conditions of intense T-cell activation — such as during acute Epstein–Barr virus infection, chronic HIV infection or rheumatoid arthritis — these molecules can be measured at nanomolar concentrations in the blood¹⁰⁶. How perforin delivers granzymes to the target-cell cytosol is still an enigma. ICAM1, intercellular adhesion molecule 1; LFA1, leukocyte function-associated antigen 1; PKCθ, protein kinase Cθ; TCR, T-cell receptor; ZAP70, ζ-chain associated protein kinase of 70 kDa.

CASPASES

A family of cytosolic proteases that contain a cysteine residue in the active site and that cleave their substrates after an aspartic-acid residue. They can be divided into pro-inflammatory caspases (caspases 1, 4, 5 and 11), which cleave and activate pro-inflammatory cytokines, and pro-apoptotic caspases, which cleave and activate pro-apoptotic substrates. Pro-apoptotic caspases comprise initiator caspases (caspases 2, 8 and 9), which, in turn, cleave and activate effector caspases (caspases 3, 6 and 7).

target assembles into a well-organized immunological synapse, the formation of which has been captured by video confocal microscopy^{6,7–11}. In the central region of the T-cell synapse, the T-cell receptor (TCR)–CD3 complex, CD8 and associated signalling molecules cluster and are surrounded by larger molecules, such as CD2 and leukocyte function-associated antigen 1 (LFA1), that form circumferential zones that stabilize the synapse. The synapse forms within minutes of the initial interaction of the TCR with its antigen and lasts for more than an hour until the entire TCR complex is internalized and degraded. The formation of the immunological synapse is crucially dependent on the actin cytoskeleton to move molecules in and out of the forming synapse (FIG. 1).

The cytotoxic granules contain the pore-forming protein **perforin** and a family of serine proteases known as granzymes (for granule enzyme) (BOX 1). The granzymes are processed either on route to or in the granules from inactive pro-enzymes into active enzymes by **cathepsin C** (also known as dipeptidyl peptidase I). At the acidic pH of the granules, the granzymes are inactive. Granzymes are highly specific proteases, the substrate specificity of which seems to be determined by an extended binding site, rather than by a short linear peptide around the cleavage site^{12–16}. **Granzymes A and B** are the most abundant granzymes in mice and humans and have received the most attention — in particular, granzyme B because it activates the caspase-dependent pathway of apoptosis and, similar to the caspases, it cleaves after aspartic-acid residues (in other words, it is an Aspase). Humans and rodents encode different homologous duplications of the granzyme genes in three linked chromosomal clusters. Granzyme A, a TRYPTASE, is linked to another tryptase (**granzyme K**) in both mice and humans. The granzyme-B cluster in humans also encodes a CHYMASE (**granzyme H**), as well as mast-cell chymase, and is expanded in mice to encode, in addition to granzyme B, **granzymes C, D, E, F and G**. In humans, a METASE found mostly in NK-cell granules (**granzyme M**) is linked to genes encoding neutrophil serine proteases. Not much is known about how the expression of these genes is regulated (and each gene or gene cluster is probably regulated in a different manner), but recent evidence indicates that each killer cell might express only a few of these enzymes¹⁷. Recent studies of granzymes A and C, discussed later, indicate that these enzymes are not redundant, but activate distinct pathways of cell death in target cells, providing failsafe methods to destroy tumours or pathogens that have devised ways to evade one pathway or another. Some of the granzymes might have additional functions besides inducing cell death^{18–22}.

The basic granzymes are non-covalently bound by charge to the negatively charged granule proteoglycan serglycin, which has a protein backbone of alternating Ser-Gly residues modified by side chains of chondroitin 4-sulphate glycosaminoglycan^{23,24}. Approximately 30–50 granzyme molecules bind to each ~250-kDa molecule of serglycin to produce a particle with an estimated size of ~40–200 nm in diameter, which is as big as a virus particle. Perforin, as well as other basic granule components, has also been postulated to bind to serglycin, but this interaction is likely to be weaker as perforin is less basic than the granzymes²³. The point at which granzymes dissociate from the serglycin carrier protein, and whether free or complexed granzymes enter the target cell are unknown. However, binding of granzyme B to serglycin is somewhat stronger at the acidic pH of the granules than at the neutral pH of extracellular fluid²⁴, so some free granzyme is probably available in the synapse. Most studies of granzyme-mediated cell death have used either purified native granzymes or recombinant proteins, free of serglycin. However, target-cell entry and intracellular trafficking of the serglycin–granzyme complex might differ from that of free granzymes.

Box 1 | Contents of cytotoxic granules

- Membrane-perturbing proteins — perforin and granulysin
- Granule serine proteases — granzymes A, B, C, D, E, F, G, H, K and M
- Proteoglycan matrix — serglycin
- Perforin inhibitor — calreticulin
- Lysosomal enzymes (cathepsins) with roles in granzyme processing (cathepsin C) and protecting cytotoxic T lymphocytes against perforin (cathepsin B)
- Stored T-cell effector molecules — FAS ligand and possibly β -chemokines

Other cathepsins and lysosomal proteins are also present in the granules. Granule membrane-bound cathepsin B, which localizes to the outer plasma membrane of the killer cell after granule exocytosis, might protect the CTL from destruction by its own granule enzymes released into the synapse, by proteolytic cleavage and inactivation of perforin²⁵. Human, but not rodent, granules also contain granulysin, another membrane-damaging protein homologous to antibacterial peptides such as the DEFENSINS, which can destroy bacteria, as well as tumour cells. The granules also contain the Ca^{2+} -binding protein and chaperone calreticulin, which binds to perforin and potently inhibits perforin-mediated damage^{26–29}. Calreticulin might protect CTLs from perforin polymerization in the granules (and autolysis by granzymes that might, therefore, get into the CTL cytosol), but whether it has an inhibitory role after granule exocytosis is unclear. The granules also function as a regulated storage depot for molecules with effector function in killer cells, such as FAS ligand, which can be directly transported to the cell surface as membrane-exposed proteins or for secretion³⁰. β -chemokines in killer cells are also stored in granules³¹, but whether these granules are identical to cytotoxic granules has recently been questioned (P. Henkart, personal communication).

CTL-induced caspase-independent cell death

Granzyme B, which cuts after aspartate residues (similar to the caspases), activates caspase-mediated apoptosis by cleaving caspase-3 and other caspases³². However, cell death induced by CTLs occurs in the presence of complete caspase blockade, using peptide caspase inhibitors, and in cells expressing viral caspase inhibitors, such as baculovirus p35 and the poxvirus inhibitor crmA^{33,34}. Apoptotic nuclear changes induced by CTLs are only partially blocked by caspase inhibitors. Similarly, overexpression of the anti-apoptotic protein BCL-2 in target cells has no effect on their lysis by CTLs and only partly inhibits the induction of apoptosis^{35–37}. This indicates that CTLs also activate caspase-independent cell death. Recently, caspase-independent cell-death pathways induced by three cytotoxic granule mediators — granzyme A, granzyme C and granulysin — have begun to be elucidated. Moreover, granzyme B can induce caspase-independent, as well as caspase-dependent, cell death^{33,38,39}.

Caspase-independent death by granzyme B

Granzyme B not only cleaves caspase-3 and some of the other caspases to unleash the caspase cascade, but also activates cell death and apoptosis in the presence of complete caspase inhibition using short peptide fluoromethylketones, which do not inactivate granzyme B. Cell death (measured by a chromium-release assay) induced by granzyme B is completely unimpaired by blocking caspase activity^{33,38–40}. So far, all known non-caspase targets of granzyme-B-mediated cleavage are actually downstream caspase substrates, with granzyme B substituting for the blocked initiator or effector caspases. One important direct target of granzyme-B-mediated cleavage is BID (BH3-interacting domain death agonist), which, when cleaved, destroys the integrity of the mitochondrial outer membrane⁴¹ and thereby causes the release from the intermembrane space of the pro-apoptotic molecules cytochrome *c*, endonuclease G (ENDOG) and HtrA2/OMI (a recently identified serine protease that inhibits the inhibitor of apoptosis protein (IAP), is upregulated in response to heat shock and is homologous to the bacterial heat-shock protein and chaperone Htr)^{42–45} (D. Martinvalet and J.L., unpublished observations) (FIG. 2 and BOX 2). However, apoptosis-inducing factor (AIF) is not released⁴⁵. Granzyme B cuts BID at a location distinct from that cleaved by caspase-8 (at Asp75 rather than Asp59), but presumably both cleaved BID molecules function in a similar manner. (Despite earlier results from three laboratories indicating that BID is a direct physiological substrate of granzyme B, a recent study questions whether granzyme-B-mediated cleavage occurs directly and independently of caspase activation under physiological conditions⁴⁶. Resolving this issue will require further experimentation.) Mitochondrial amplification of the caspase pathway is essential for apoptosis in some cell types. In addition to BID-mediated mitochondrial damage, which involves the BCL-2-family members BAX (BCL-2-associated X protein) and BAK (BCL-2 antagonist/killer)^{43,47} and can be inhibited by BCL-2 (REF. 48), granzyme B also directly disrupts the mitochondrial transmembrane potential in a caspase- and BID-independent manner^{45,49}. In fact, fibroblasts from mice genetically deficient in Bid or doubly deficient in Bax and Bak still have disrupted mitochondrial transmembrane potential after treatment with granzyme B and perforin, but do not release cytochrome *c*⁵⁰. Therefore, there are at least two caspase-independent mitochondrial effects of granzyme B, one of which requires BID and the other of which involves direct actions of granzyme B without cytosolic mediators (BOX 2).

Granzyme B can also directly unleash the caspase-activated DNase (CAD) by directly cleaving its inhibitor ICAD^{40,51}. Also, the mitochondrial protein ENDOG, released by the action of granzyme-B-cleaved BID, can induce oligonucleosomal DNA damage⁵². Therefore, granzyme B independently and directly activates two routes to DNA damage, even when caspase activation is blocked.

In addition to targeting mitochondria and DNA degradation directly, granzyme B also directly cleaves

TRYPTASE

An enzyme that (similar to trypsin) cuts after basic amino acids such as lysine and arginine.

CHYMASE

A protease that (similar to chymotrypsin) cuts after hydrophobic amino acids.

METASE

A protease that cuts after methionine residues.

DEFENSINS

Small basic peptides produced by immune cells that are microbicidal and work by damaging bacterial membranes.

several downstream caspase substrates. These include the sensor of DNA damage to initiate repair, poly (ADP ribose) polymerase (PARP); the catalytic subunit of DNA-dependent protein kinase (DNA-PK_{cs}), which is involved in repairing double-stranded DNA breaks; the nuclear mitotic apparatus protein (NUMA_s); and the nuclear-envelope intermediate-filament protein lamin B^{53–55}. DNA-PK_{cs} and NUMA are cut at sites distinct from those used by the caspases, whereas lamin B is cut at the site used by caspase-6.

Granzyme A

Granzyme A has long been thought to activate a slow cell-death pathway, because the release of oligonucleosomal DNA fragments in cells treated with granzyme A and perforin cannot be detected until ~16 hours after

treatment, a timescale that is similar to the slow death pathway that is initiated by ligating cell-surface death receptors^{56,57}. However, experiments in which granzyme A and B independently and synergistically induce apoptosis in a perforin-dependent manner⁵⁸. Moreover, cell death induced by recombinant granzyme A delivered into cells with perforin — as measured by microscopy, chromium-release assay or trypan-blue inclusion — occurs within minutes, as rapidly as that induced by granzyme B at comparable concentrations of recombinant enzymes³⁹ (P. J. Beresford and J.L., unpublished observations). Although CTLs from granzyme-B-deficient mice induce the release of oligonucleosomal DNA into culture supernatants more slowly than do

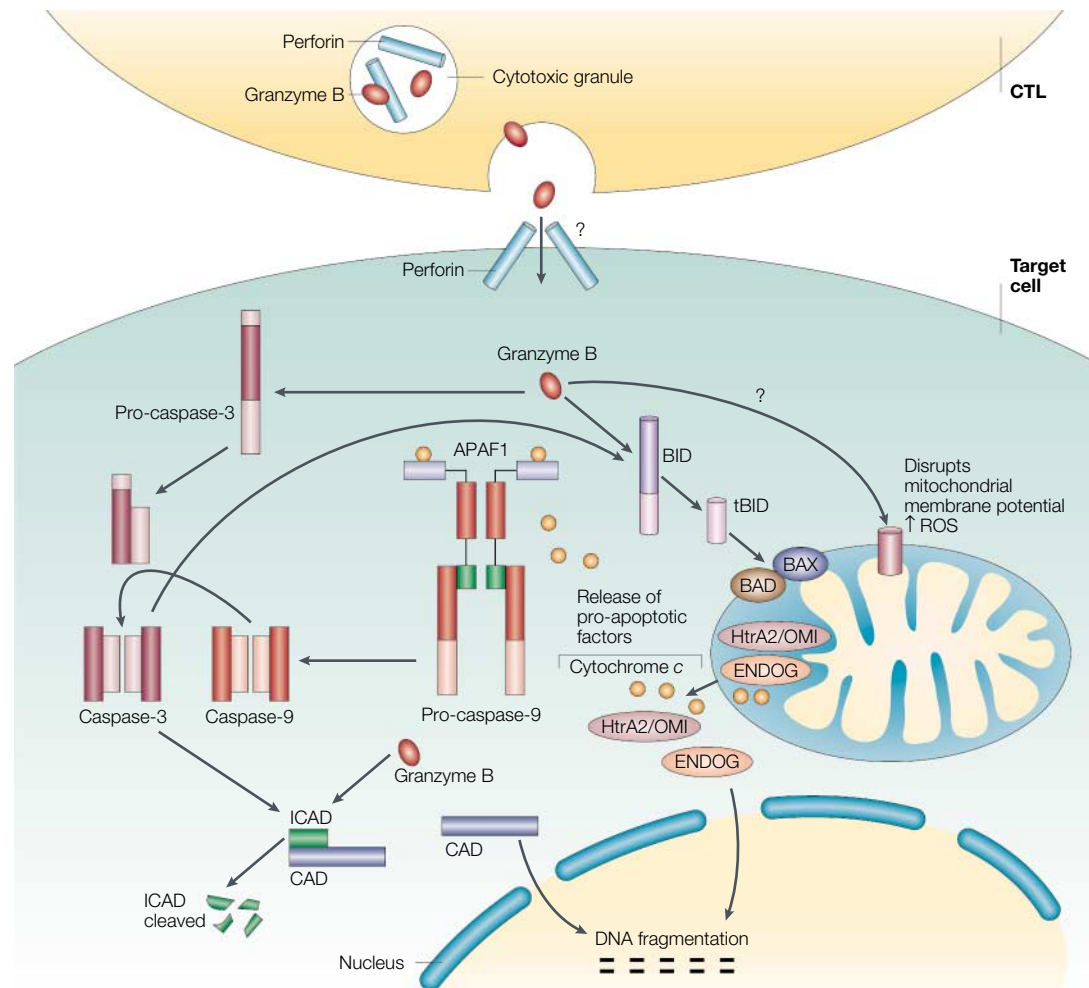
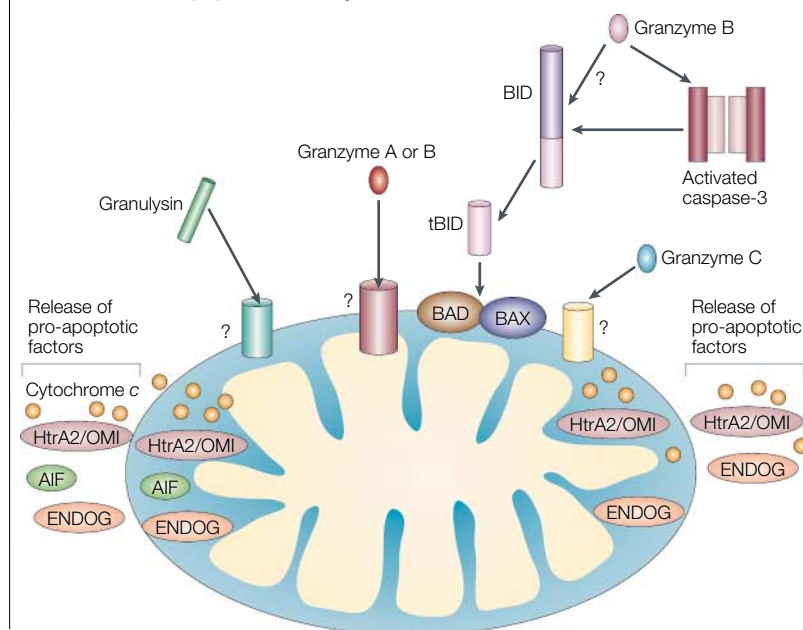


Figure 2 | Granzyme B activates caspase-independent mitochondrial and nuclear cell-death pathways. Granzyme B enters the target-cell cytosol by a poorly understood mechanism involving perforin. Two early targets of granzyme B are BID (BH3-interacting domain death agonist) and pro-caspase-3. Truncated BID (tBID) disrupts the outer mitochondrial membrane to cause release of the pro-apoptotic factors cytochrome c (which is crucial for activating pro-caspase-9), HtrA2/OMI (which blocks inhibitors of apoptosis) and endonuclease G (ENDOG; which activates DNA damage). Cytochrome c that is released from the intermembrane space binds to APAF1, which recruits pro-caspase-9 to form the apoptosome. In the apoptosome, caspase-9 is autocatalytically activated and in turn can proteolytically activate caspase-3. Partially activated pro-caspase-3 can also dimerize and activate itself. Granzyme B also disrupts the mitochondrial transmembrane potential through an unknown mechanism and directly cleaves ICAD (inhibitor of CAD) to free CAD (caspase-activated DNase) to cause DNA fragmentation. APAF1, apoptotic protease-activating factor 1; BAD, BCL-2 antagonist of cell death; BAX, BCL-2-associated X protein; CTL, cytotoxic T lymphocyte; ROS, reactive oxygen species.

Box 2 | Multiple pathways of mitochondrial damage

Cytotoxic granule components damage mitochondria by two distinct mechanisms — release of pro-apoptotic factors from the intermembrane space and disruption of the mitochondrial transmembrane potential. Granzyme B does the former by activating caspases to produce truncated (t)BID (BH3-interacting domain death agonist) or by directly cleaving BID. The physiological relevance of direct cleavage has been questioned recently⁴⁶. Truncated BID activates BAX (BCL-2-associated X protein) and BAD (BCL-2 antagonist of cell death) to form a pore in the outer mitochondrial membrane. Granulysin directly disrupts the mitochondrial outer membrane in the same manner that it disrupts bacterial membranes when it acts as a microbicide. It is not known how granzymes A, B and C disrupt the mitochondrial transmembrane potential. Granzyme C also causes the release of pro-apoptotic molecules from the intermembrane space, possibly when the swollen mitochondria burst and disrupt the integrity of the outer membrane⁶⁵. AIF, apoptosis-inducing factor; ENDOG, endonuclease G.



CTLs from wild-type mice, they are unimpaired in cytotoxicity⁵⁹. Granzyme-B-deficient mice are as resistant to viruses and tumour challenge as wild-type mice, whereas granzyme-A-deficient mice are highly susceptible to infection with the mouse poxvirus ectromelia and are less able to contain the interneuronal spread of herpes simplex virus infection^{59–62}. CTLs deficient in both granzyme A and granzyme B (produced from mutant mice that also did not efficiently express other genes in the granzyme-B cluster⁶³) have a profound defect in cytotoxicity, similar to that of CTLs from mice deficient in perforin⁶⁴. Taken together, these results indicate that granzyme A induces a distinct cell-death pathway from granzyme B, which is important for immune protection.

Loading recombinant granzyme A with perforin induces a new form of cell death, which has many features of apoptosis, including chromatin condensation, nuclear fragmentation, externalization of phosphatidyl serine in the cell membrane and loss of the mitochondrial transmembrane potential^{39,55} (D. Martinvalet and J.L., unpublished observations) (TABLE 1). However, this pathway is completely independent of the caspases³⁹. After granzyme-A loading with perforin, caspases are not

cleaved or activated, and downstream caspase substrates are by and large untouched. Cytochrome *c* is not released from mitochondria, although the mitochondrial membrane is depolarized and mitochondria become dysfunctional, as shown by the increased level of reactive oxygen species (D. Martinvalet and J.L., unpublished observations). Moreover, granzyme-A-induced cell death is not inhibited by the overexpression of BCL-2. The molecular basis for mitochondrial disruption by granzyme A (and for the caspase-independent mitochondrial disruption induced by granzymes B and C^{49,50,65}) is unknown (BOX 2).

Granzyme A induces a new type of DNA damage. CAD activated by granzyme B and the caspases makes blunt double-stranded cuts to produce oligonucleosomal DNA fragments of ~200 base pairs in length. In the granzyme-A-mediated pathway, however, DNA is damaged by single-stranded nicks, which can be labelled by KLENOW POLYMERASE, but not by TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE (TdT), and can be visualized on denaturing alkaline agarose gels, but not on conventional agarose gels. Moreover, because the DNA fragments are large, DNA is not released into culture supernatants, explaining why, according to this assay, DNA damage is slow. Also, DNA is less damaged by granzyme A than by an equivalent amount of granzyme B (Klenow labelling is reduced by a factor of approximately one log). Whether this pathway is termed apoptosis is largely a matter of semantics — the cells have the morphological features of apoptosis, but caspases are not involved, and DNA is damaged, but not to oligonucleosomal fragments.

Granzyme A targets the SET complex. During the induction of cell death, granzyme A targets a newly identified 270–420 kDa endoplasmic reticulum-associated complex, known as the SET complex, which contains two tumour-suppressor proteins (pp32 and NM23-H1) and three granzyme-A substrates (the nucleosome-assembly protein SET; the DNA-binding protein high-mobility group protein 2, HMG2; and the rate-limiting base-excision repair enzyme apurinic/apyrimidinic endonuclease 1, APE1)^{16,66–69} (FIG. 3). Granzyme A destroys the known functions of these substrates. Although the normal function of the SET complex is unknown, on the basis of the functions of its components, it has been proposed to facilitate the activation of transcription and transcription-related DNA repair in response to oxidative stress⁶⁹. Indeed, the proteins in this complex translocate rapidly to the nucleus in response to an increase in the level of reactive oxygen species and after granzyme-A loading with perforin. The SET complex holds the key to the single-stranded DNA damage induced by granzyme A. The granzyme-A-induced DNase (GAAD) is NM23-H1, which had been shown previously to nick DNA⁷⁰, and SET is its inhibitor (IGAAD)⁶⁷. In a manner reminiscent of the activation of CAD by cleavage of its inhibitor ICAD, NM23-H1 is activated by granzyme-A-mediated cleavage of SET. After granzyme-A loading or CTL-mediated attack, SET and NM23-H1 translocate to the nucleus and SET is degraded, allowing NM23-H1 to nick chromosomal DNA. The *in vivo* relevance of the SET complex in

KLENOW POLYMERASE

The large fragment of *Escherichia coli* DNA polymerase I produced after cleavage with subtilisin. The Klenow fragment has 5'→3' polymerase activity and 3'→5' exonuclease activity, but no 5'→3' exonuclease activity. It is used to end-label free 3' recessed ends of DNA.

TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE

(TdT). An enzyme expressed during lymphocyte development that adds nucleotides to the free 3' end of DNA breaks. It is used to assay apoptosis by catalysing the addition of radiolabelled or biotinylated nucleotides to sites of DNA damage.

Table 1 | Features of cell-death pathways induced by granzymes

	Granzyme A	Granzyme B	Granzyme C
Common features			
Rapid loss of membrane integrity	Yes	Yes	Yes
Annexin-V staining	Yes	Yes	Yes
Chromatin condensation	Yes	Yes	Yes
DNA damage	Yes	Extensive	Yes
Mitochondrial depolarization	Yes	Yes	Yes
Caspase activation	No	Yes	No
DNA damage			
Oligonucleosomal DNA fragmentation	No	Yes	No
Single-stranded DNA nicks	Yes	No	Yes
TdT labelling	No	Yes	Yes
Klenow labelling	Yes	Yes	Yes
Mitochondrial damage			
Inhibition by BCL-2 overexpression	No	Yes	N.D.
Cytochrome <i>c</i> release	No	Yes	Yes
Mitochondrial swelling	N.D.	Yes	Extensive

N.D., not determined; TdT, terminal deoxynucleotidyl transferase.

granzyme-A-induced cell death has been shown by the resistance to granzyme-A-mediated cell death and DNA damage of target cells with silenced NM23-H1 expression, and the enhanced sensitivity to cell death of target cells with silenced expression of SET or APE1.

Granzyme A also targets other important nuclear proteins (FIG. 4). During granzyme-A loading with perforin, the linker histone H1 is completely degraded and the tails are cut from the core histones⁷¹. This opens up chromatin and enhances DNA fragmentation by exogenous nucleases in intact nuclei. This might contribute to the synergy observed for DNA damage mediated by granzymes A and B. In addition, granzyme A cuts all of the lamins, the main structural components of the inner nuclear envelope⁵⁵. Lamin B is also a substrate of granzyme B, and lamins A and B are proteolysed by the caspases. The fact that these diverse cell-death pathways all target lamins indicates that disrupting the nuclear envelope is a key event in apoptosis.

After loading with perforin, granzymes A and B both become concentrated within minutes in the nucleus of target cells^{72–74}. It is unclear how the granzymes get into the nucleus, because the granzyme-A dimer is probably too big to get in efficiently by diffusion and the nuclear localization of granzyme B is so rapid (with a half-life of ~2 minutes⁷⁴) that diffusion seems too slow. Disruption of the nuclear envelope might be required, but the proteolysis of the lamins by the granzymes (although detectable within 15 minutes) might not occur to a significant extent until after the granzymes have entered the nucleus.

Several studies have also indicated that, in addition to activating cell death, granzyme A might have a pro-inflammatory effect by acting on extracellular proteins or cell-surface receptors. For example, granzyme A

activates the pro-inflammatory cytokine interleukin-1 β (IL-1 β) by cleaving its pro-peptide¹⁸. Other reports indicate that granzyme A might proteolytically activate macrophages to secrete cytokines¹⁹. It can also cause neurite retraction on astrocytes and inhibit thrombin-induced platelet aggregation by cleaving the thrombin receptor^{20,21}. One study indicates another anticoagulant effect of granzyme A through activating pro-urokinase to activate plasminogen²². Other papers indicate possible roles in degrading extracellular matrix proteins.

Granzyme C

In mice, an expanded cluster of genes downstream from the gene encoding granzyme B on chromosome 14 encodes the 'orphan' granzymes C, G, D, F and E. These neglected granule components are not highly expressed after antigenic stimulation, but they are turned on in some cytotoxic cells stimulated in the presence of large amounts of IL-2 or might be constitutively expressed by some NK cells. So far, no functional importance of the orphan granzymes has been shown. However, preliminary results from the Ley laboratory evaluating GVHD induced by lymphocytes from granzyme-B cluster-deficient mice (which do not express granzymes B, C, D or F) compared with lymphocytes deficient in expression of only granzyme B indicate that the downstream orphan granzymes might be physiologically relevant⁶⁵ (T. Ley, personal communication). A recent study⁶⁵ shows that granzyme C is a potent inducer of yet another cell-death pathway, distinct from that induced by either granzyme A or granzyme B (TABLE 1). Equimolar concentrations of granzymes B and C delivered with perforin comparably and equally rapidly induce cell death. Similar to the other granzymes, granzyme C induces cell-membrane damage, ANNEXIN-V STAINING, loss of the mitochondrial membrane potential and chromatin condensation. Similar to granzyme A, granzyme C causes caspase-independent cell death with single-stranded DNA nicks, rather than oligonucleosomal DNA fragmentation (TABLE 1). However, whereas granzyme-A-induced nicks are radiolabelled by Klenow polymerase, but not TdT, granzyme-C-induced nicks are labelled by both enzymes.

The mitochondrial changes caused by granzyme C are particularly marked, with impressive mitochondrial swelling, cytochrome *c* release and opening of a mitochondrial pore that might be distinct from the permeability transition pore (PTP), because it is not blocked by inhibitors of the PTP such as cyclosporine A. Cytochrome *c* is released within 30 minutes of granzyme-C loading, probably as a consequence of mitochondrial rupture and loss of the integrity of the outer membrane as a result of the swelling that accompanies the disruption of the mitochondrial transmembrane potential. Unexpectedly, although cytochrome *c* is released, the caspases are not activated. The enzymatic specificity of granzyme C is unknown; surprisingly, it does not cut even small peptide substrates. Nevertheless, mutation of the presumed active site of granzyme C (Ser195) completely abrogates all of its effects, indicating that proteolysis is crucial to its function.

ANNEXIN-V STAINING
Annexin V binds to phosphatidyl serine, which is normally located on the inner leaflet of the plasma membrane, but which flips to the outer layer during apoptosis. Annexin-V staining is often used as an indicator of apoptosis.

Beyond the ABCs: the other orphan granzymes

Whereas descriptions of the cell-death pathways activated by granzymes A and C are just beginning to emerge, the other orphan granzymes have been truly neglected. Granzyme K, encoded by a gene duplication of granzyme A, is a tryptase-like granzyme A and is expressed in granzyme-A-deficient mice. Although recombinant granzyme K has been produced by several groups^{75,76}, little is known about its activities. In one report, purified native granzyme K induced caspase-independent cell death without apoptotic nuclear morphology, but with disruption of the mitochondrial potential and mitochondrial dysfunction, as measured by the generation of reactive oxygen species⁴⁹. Granzyme H, the other human granzyme in the granzyme-B cluster, is a chymase; although recombinant granzyme H has

been available for a few years, there are no reports that it induces cell death⁷⁷. In fact, a purified rat granzyme with chymase activity actually inhibits perforin-mediated haemolytic activity⁷⁸. Nothing is known about the other orphan granzymes in the mouse granzyme-B cluster. Granzyme M, another granzyme in mice and humans that cleaves preferentially after methionine residues⁷⁹, is not expressed by conventional CD4⁺ and CD8⁺ T cells, but is preferentially expressed by cells that participate in the innate immune response, including NK cells and $\gamma\delta$ T cells⁸⁰. Interestingly, most of these effector cells of innate immunity express granzyme A, but do not express granzyme B. This difference in expression might help to explain why granzyme-A-deficient mice are more immunocompromised in their ability to defend against virus infection than are granzyme-B-deficient mice. There is no evidence that granzyme M with perforin activates cell death. Therefore, it has been proposed that granzyme M might have another, non-cytolytic role in innate immunity⁸⁰.

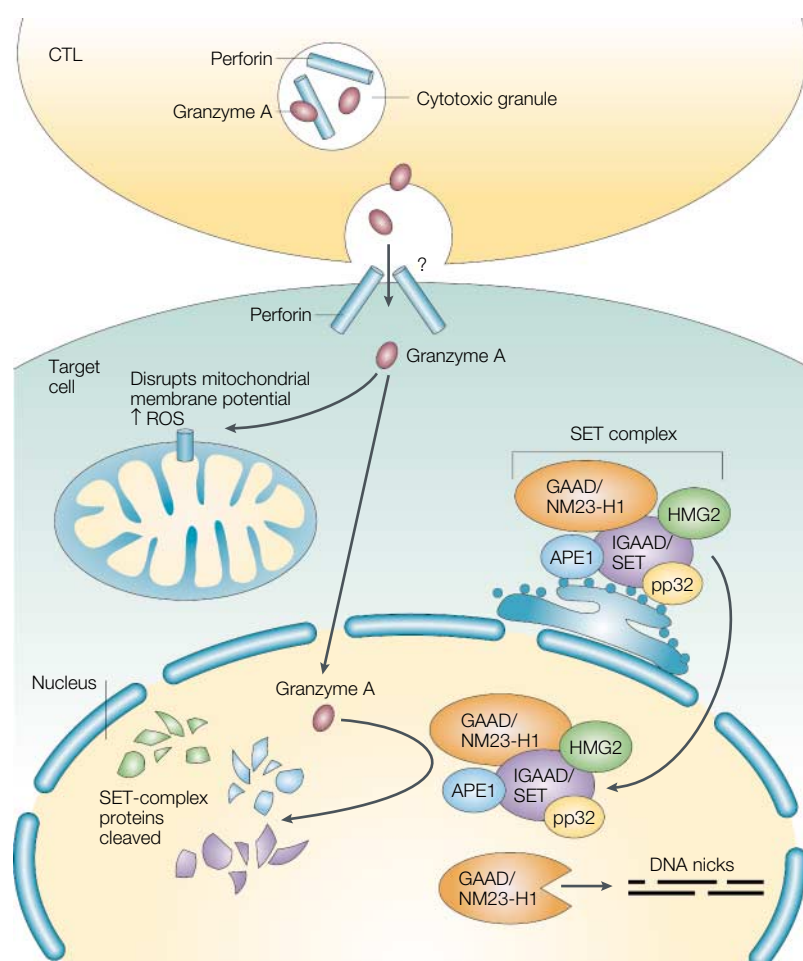


Figure 3 | Granzyme A causes caspase-independent cell death. Granzyme A (similar to granzyme B) disrupts the mitochondrial transmembrane potential by an unknown mechanism, and it also targets a newly described SET complex to cause single-stranded DNA nicks. The SET complex moves into the nucleus in response to the increased level of reactive oxygen species (ROS) generated by the action of granzyme A on mitochondria, and it might normally be involved in the repair response to oxidative stress. However, granzyme A destroys three members of the SET complex, including the nucleosome-assembly protein SET, the base-excision repair enzyme apurinic/aprimidinic endonuclease 1 (APE1) and high-mobility group protein 2 (HMG2), a DNA-binding protein that might be involved in recognizing damaged DNA. When these proteins are destroyed, the DNA-nicking protein NM23-H1 is free to cut DNA and the breaks are not repaired. NM23-H1 is the granzyme-A-activated DNase (GAAD) and SET is its inhibitor (IGAAD). CTL, cytotoxic T lymphocyte.

Membrane-disrupting molecules

Perforin: still a puzzle. Mice genetically deficient in perforin⁸¹ have severe immunodeficiency and impaired protection against viruses and tumours, because perforin is required to deliver granzymes into the cytosol of the target cell^{2,82}. Although the granzymes are redundant, as several of them can independently induce cell death, in mice at least, there is only one delivery molecule. The original model of how perforin carries out this task involves homopolymerization in the plasma membrane in a Ca²⁺-dependent manner to produce pores that act as a channel. Recently, this model has been called into question. Although, at high concentrations, perforin forms large pores that can kill a cell by necrosis in a manner reminiscent of its homologue complement, at the sublytic concentrations that are required to deliver granzymes, the pores, if formed at all, might be too small (≤ 50 nm in diameter) to allow granzymes to enter, especially when complexed with serglycin. However, the effective perforin concentration at the synapse is not known and might be quite high; whether or not pores form at the synapse is unknown. Granzyme B binds to cells and is endocytosed in the absence of perforin^{83–85}. Perforin can be added several hours after extracellular granzyme B has been washed away to initiate apoptosis. The revised hypothesis holds that although perforin is not required for granzymes to get into cells, it is required for the release of granzymes from the endocytic compartment into the cytosol and for trafficking to the nucleus. This idea is supported by the ability of non-replicating adenovirus and bacterial proteins that are known to facilitate endosomal exit (such as listeriolysin and streptolysin O) to substitute for perforin^{83,86}. These agents have become useful research tools for delivering granzymes, as recombinant active perforin has not been available. Recently, the cation-independent mannose-6-phosphate receptor (CI-MPR) has been hypothesized to be the cell-surface receptor for granzyme B (and by inference for other

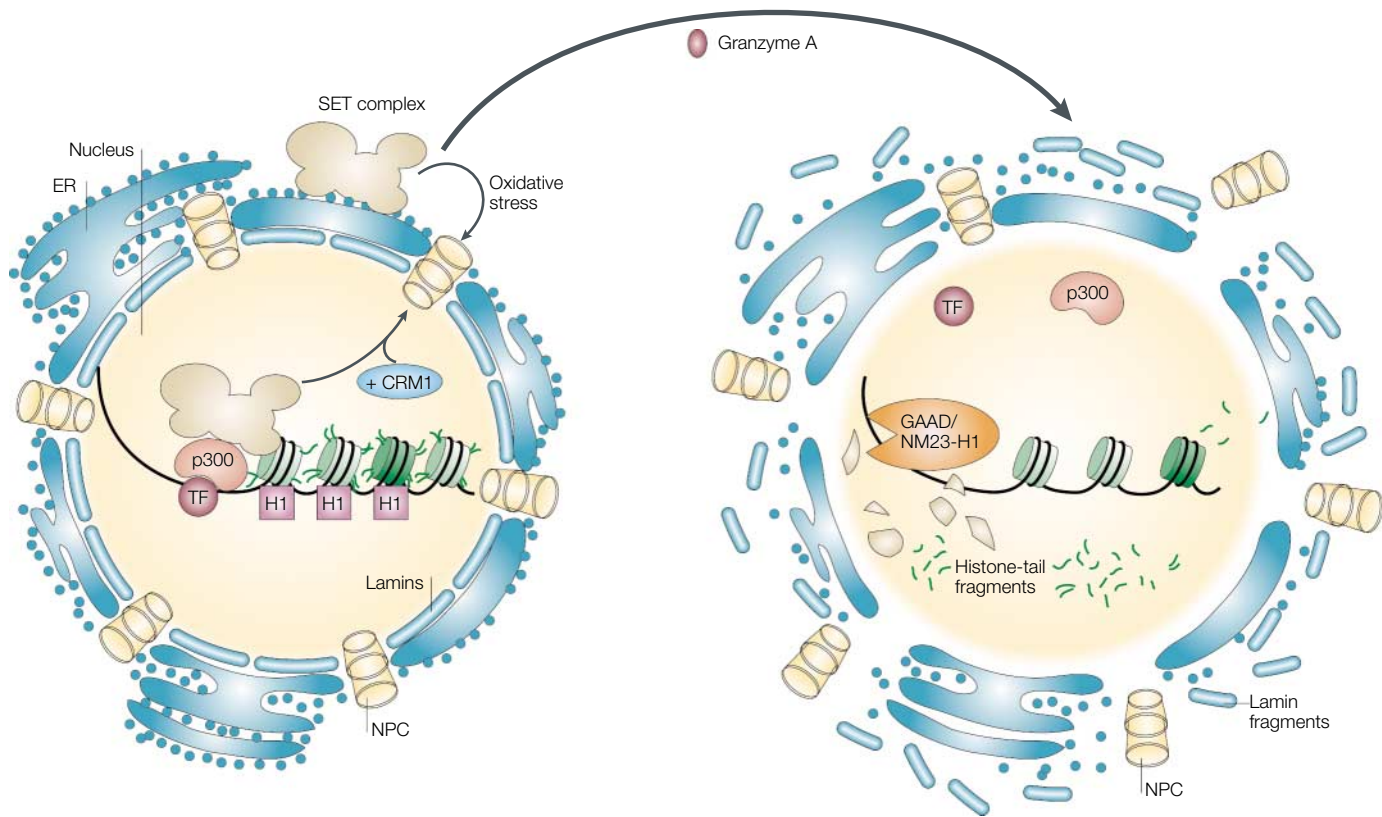


Figure 4 | **The granzyme-A bomb.** Granzyme A launches a programme of nuclear war, which not only causes DNA damage by releasing inhibition of the granzyme-A-activated DNase (GAAD) activity of NM23-H1 in the SET complex (as described in FIG. 3), but also opens up chromatin to damage by other DNases by destroying the linker histone H1 and cutting the tails from the core histones. The SET complex is postulated to be important for the repair of oxidative stress by activating the transcription of early-response genes and repairing oxidative DNA damage. In targeting the SET complex, granzyme A prevents the cell from repairing the damage it has unleashed. Granzyme A also destroys the nuclear lamins, the key intermediate-filament component of the nuclear envelope, which anchor chromatin and the nuclear-pore complexes (NPCs) to provide nuclear integrity. Lamin B is also disrupted by granzyme B. CRM1, chromosome maintenance protein 1; ER, endoplasmic reticulum; TF, transcription factor.

granzymes)⁸⁷. The same receptor is involved in targeting newly synthesized granzymes to cytotoxic granules⁵. However, most of the evidence is indirect as a direct association between granzyme B and the cell-surface receptor has not been shown. Moreover, binding to CI-MPR is unlikely to be the only route of entry, as recombinant unglycosylated granzymes produced in bacteria can also gain entry to cells and induce cell death. In addition, target cells lacking CI-MPR or that are defective in endocytosis through the clathrin-dependent pathway still take up granzyme B and are susceptible to cytotoxicity induced by granzyme B and perforin⁸⁸.

If perforin does not function by making holes in the plasma membrane, how then does it work? Perforin probably associates with its inhibitor calreticulin in the endoplasmic reticulum and needs to be activated on route to or in the granules by a cysteine protease to remove a carboxy-terminal glycosylated peptide⁸⁹. This protects intracellular membranes from damage during biosynthesis and storage. Perforin might also bind to serglycin in the granules²³. After exocytosis, perforin probably dissociates from calreticulin and serglycin⁹⁰ and polymerizes and inserts in the plasma membrane. What happens next is completely uncertain, although

the membrane barrier remains largely intact as the cell initially remains impermeable to small extracellular dyes. Perforin clearly perturbs the plasma membrane, because fluorescently labelled plasma-membrane lipids rapidly redistribute within a few minutes to other intracellular membranes, including mitochondrial and nuclear membranes⁹¹. Research into the mechanism of perforin action has been hampered by the lack of a recombinant active protein, the production of which has defied the attempts of several laboratories, and by the inability to detect perforin in target cells by fluorescence microscopy.

Granulysin. Human CTLs and NK cells, but not rodent cells, contain a second membrane-disrupting protein known as **granulysin**. Granulysin is processed from a larger precursor to a 9-kDa protein in the dense cytotoxic granules of CTLs and NK cells. Granulysin is homologous to saposin-like proteins (SAPLIP), which are involved in membrane-lipid degradation. Granulysin is an effective antimicrobial agent. It kills microorganisms by increasing the membrane permeability of Gram-positive and Gram-negative bacteria, mycobacteria and fungi^{92–97}. A recent crystal structure indicates that granulysin binds to the

bacterial membrane by a cluster of positive residues and might aggregate and then tunnel into the membrane through its hydrophobic core to cause 'molecular electro-poration'⁹⁸. To kill intracellular bacteria, the activity of granulysin might be facilitated by perforin^{92,95}.

At high concentrations in the micromolar range, granulysin also activates cell death with apoptotic nuclear features^{99–101}. It can be speculated that granulysin might provide a back-up membrane-disrupting molecule for perforin in humans, just as the various granzymes provide alternate ways to induce apoptosis; however, there is no experimental evidence to support this idea. Whether high enough concentrations are reached in the immunological synapse for granulysin to be a physiologically important mediator of mammalian cell death is unclear. A caspase-independent component of granulysin-mediated cell death remains in the presence of peptide caspase inhibitors. Similar to granzyme C, granulysin disrupts the mitochondrial outer membrane, releasing AIF and cytochrome *c*, and presumably ENDOG. The importance of mitochondria in granulysin-induced cell death is indicated by its

inhibition in BCL-2-overexpressing cells. DNA damage might occur mainly through these mitochondrial mediators. Some caspase activation might also occur, although the literature is unclear. Granulysin also increases the level of ceramide, a known inducer of caspase-dependent apoptosis, but this occurs more slowly than the other apoptotic events that are induced by granulysin.

Conclusions

CTLs and NK cells induce apoptosis by activating caspases through the Aspase granzyme B. However, they also activate new caspase-independent cell-death pathways, many of which have the morphological hallmarks of apoptosis. These alternate pathways, targeting the mitochondria and the nucleus, which are just beginning to be uncovered, are important for immune protection, especially against viruses and tumours that resist caspase-mediated cell death. These pathways potentially provide new therapeutic approaches and might also provide new probes to understand the basic biology of the life and death of cells.

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Online links

DATABASES

The following terms in this article are linked online to: **LocusLink:** <http://www.ncbi.nlm.nih.gov/LocusLink/> AIF | APE1 | BCL-2 | BAK | BAX | BID | CAD | cathepsin C | CD2 | CD3 | CD8 | CD95 | CI-MPR | DNA-PK γ | endonuclease G | granzulin | granzyme A | granzyme B | granzyme C | granzyme D | granzyme E | granzyme F | granzyme G | granzyme H | granzyme K | granzyme M | HMG2 | HtrA2/OMI | IAP | IAP | ICAD | lamin B | LFA1 | NM23-H1 | NUMA | PARP | perforin | pp32 | SET | TNFR

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