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Review

Mechanisms of granule-dependent killing

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Cytotoxic T lymphocyte and natural killer cell-initiated cell death is one of the primary mechanisms used by higher organisms to eliminate viruses and transformed cells. In this context, target cell death is rapid and efficient and initiated via two main pathways, involving either the ligation of death receptors or through the granule-exocytosis pathway. The granule-exocytosis pathway has attracted much attention over the past 10 years and consequently, a mechanism for granule-dependent killing has become reasonably well established. In the granule-dependent pathway, several proteolytic enzymes called granzymes are delivered to the target cell, promoting the activation of a family of death-inducing proteases called caspases. If caspases are inhibited by viral proteins or are inactivated through mutation, granzyme-mediated proteolysis of other cellular substrates ensures the timely death of infected or transformed cells. Here, we examine the findings that have shaped our current understanding of the mechanics of granule-dependent killing and discuss recent insights that have clarified some long-standing discrepancies in the granzyme literature.
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Apoptosis is an innate mechanism of programmed cell death that enables multicellular organisms to eliminate unwanted cells and constitutes the most common form of cell death in the body that occurs during development, tissue remodelling, cell homeostasis and during the response of the immune system to viral infection and cellular transformation.[1] Internal surveillance systems constantly monitor the intracellular environment, determining when a cell has reached the end of its useful life or has become defective in some way. In response to a variety of cues, a conserved program of cell death is initiated, culminating in the orderly dismantling of the cell.[2,3] Proximal in this process and key players in the molecular control of apoptosis are proteins of the BCL-2 family, which orchestrate the downstream activation of caspases.[4,5] Activated caspases then cleave a diverse cohort of protein substrates resulting in the rapid and efficient death of the cell.[3] However, this surveillance mechanism can be corrupted by mutation, in some cases leading to cellular transformation. In addition, many viruses employ strategies to sabotage the cell death programme of infected cells,[6,7] as this appears to be the main defensive strategy utilized by the host to destroy the invader. In such situations, the cell-mediated immune response is necessary for the removal of tumorigenic and virus infected cells.

Cytotoxic T lymphocytes (CTL) are initially activated in the peripheral lymphoid organs by recognizing foreign antigen presented on the surface of an antigen-presenting cell, usually a dendritic cell. This recognition depends on the presence, in the antigen-presenting cell, of class I major histocompatibility complex (MHC) proteins, which bind intracellular antigenic peptides and carry them to the cell surface where they are presented along with a co-stimulatory signal, to the CTL. Once activated, CTLs can recognize the same class I MHC-peptide complex on the surface of a virus-infected cell. Upon encounter of specific antigen for the first time, T cells are induced to clonally expand in order to combat the infection and this can take several days. The 'memory' of this first antigen encounter is facilitated by the production of memory T cells, which ensures a much faster and more aggressive response when the antigen is encountered for the second time. Natural killer (NK) cells play a pivotal role in the innate immune system.[8] Unlike T cells, they do not require pre-activation by dendritic cells and are thus important in the early stages of infection, before the T-cell response is fully underway. Although this is the case in humans, a recent study has highlighted differences in perforin and granzyme expression between human and murine NK cells.[9] Human NK cells constitutively express perforin and granzymes A and B.[9] However, whereas resting murine NK cells contain plentiful granzyme A but little perforin or granzyme B, upon activation, the levels of perforin and granzyme B within murine NK cells substantially increase with a commensurate increase in cytotoxicity.[9] The differences in the regulation of perforin and granzyme expression between mouse and man suggests that there may be substantial differences between the immune systems of different species and that care must be

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Abbreviations: CAD, caspase-activated DNase; cSMAC, central supramolecular activation complex; CTL, cytotoxic T lymphocyte; DNA-PK, DNA-dependent protein kinase; FHL, familial hemophagocytic lymphohistiocytosis; ICAD, inhibitor of caspase-activated DNase; MCMV, murine cytomegalovirus; MHC, major histocompatibility complex; MTOC, microtubule organizing centre; NK, natural killer; NUMA, nuclear mitotic apparatus protein; PI-9, protease inhibitor-9; ROS, reactive oxygen species; Treg, T regulatory
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taken when extrapolating mouse data to humans, a point which will be returned to later in the review. NK cells can recognize and kill virally infected or tumor cells and can rapidly produce cytokines and chemokines, which have immune stimulatory and antimicrobial effects.[10] NK cell activation has been studied extensively in recent years and is discussed elsewhere in this issue.[11]

CTLs can kill their targets through one of two main pathways. CTLs expressing ligands of the tumor necrosis factor superfamily on their cell surface can kill target cells expressing the corresponding receptors, whereas NK cells appear to rely predominantly on the granule-dependent pathway. The second cell death pathway involving the perforin-mediated trafficking of a family of proteases, called granzymes, from CTL/NK cells to the target cell cytosol (Figure 1), is the focus of this review.

Granule-Dependent Killing

A major mechanism of CTL/NK cell-mediated killing involves the delivery of secretary lysosomes, or granules, into the immunological synapse between the effector and its target.[12,13] In CTLs, the granules are synthesized only after the cell has encountered specific antigen and has become activated,[12] whereas in NK cells, cytotoxic granules are formed during development. Therefore, NK cells are equipped to respond rapidly to the threat of tumorigenicity or infection.[14] The major granule components are perforin and granzymes, which, upon delivery to the target, combine to engineer rapid cell death with killing time as short as 20 min.[15] Recognition of a target by a CTL cell triggers rapid polarization of the microtubule organizing center (MTOC) toward the immunological synapse,[16–18] with granules moving swiftly along microtubules in the direction of the plasma membrane.[19]

Both albinism and immune deficiencies are symptomatic of a number of genetic disorders associated with the dysfunction of CTLs and melanocytes, both of which contain secretory lysosomes. Secretory lysosome function is impaired in these individuals and the study of such disorders has greatly advanced our understanding of the mechanisms of cytotoxic granule secretion. Individuals with Hermansky–Pudlak syndrome type 2 lack the cytosolic adapter protein 3 (AP-3), which has been shown to be important for granule movement along microtubules and because of this, CTLs from these individuals show a substantial loss of cytotoxic activity.[20] When the granules move toward the interface between effector and target, Rab27a, missing in Griscelli syndrome, is thought to be required for movement of the granules from the MTOC to the plasma membrane.[21–23] The 400 kDa protein, Lyst, lacking in Chediak–Higashi syndrome, has been implicated in the fusion of granules with the plasma membrane.[24,25] A more recent study has highlighted the importance of Munc13-4, mutated in familial hemophagocytic lymphohistiocytosis subtype 3 (FHL3), in the priming of granules, which precedes membrane fusion.[26,27]

After the cytotoxic granules have been polarized at the cell membrane, they are secreted into the immunological synapse in an ordered sequence of events. Target-effector cell contact triggers the accumulation of talin and adhesion molecules at the contact site, which combine to form a circular structure that binds and stabilizes the polarized MTOC.[28,29] This structure surrounds a central supramolecular activation complex (cSMAC) through which the polarized granules pass before secretion.[28] The final step i n the process of granule delivery is accomplished when the

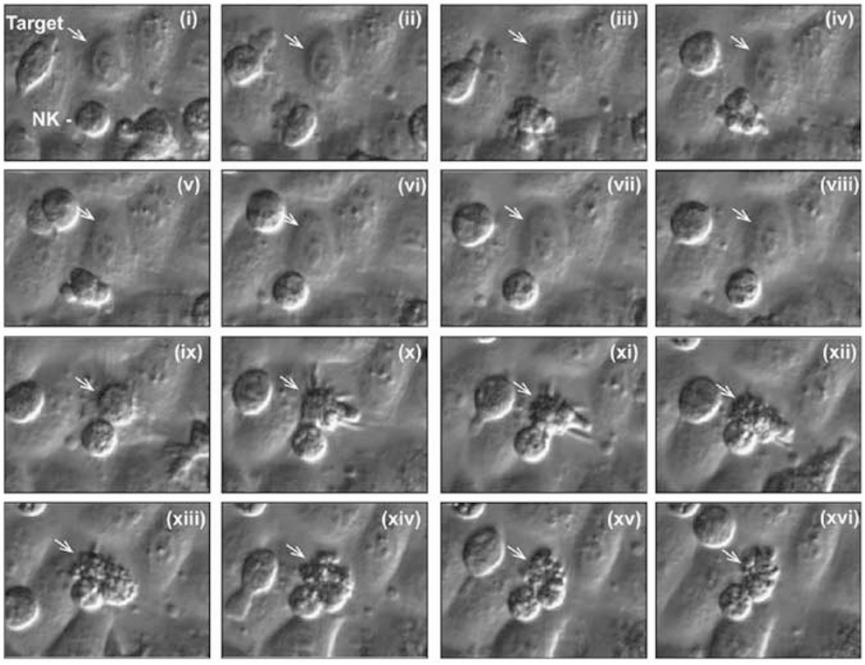

Figure 1 Time course of NK-mediated killing. The NK cell line, YT, was used to kill HeLa cell targets and images were recorded at 1-min intervals. The frames shown represent 20-min intervals between the initial encounter of the YT cell and its HeLa target. Thus, the time elapsed from frame (i) to frame (xvi) is 300 min

centrosome interacts with the plasma membrane at the cSMAC and delivers the cytotoxic granules into a cleft between the cells, formed by indentation of the target cell membrane.[30] The result of all of this is the rapid delivery of cytotoxic granules to a precisely defined point between the effector and target, with the CTL/NK cell free to flee the scene and kill again. Interestingly, membrane fusion, independent of granule exocytosis, has been shown to occur between the killer and its victim, forming a very tight seal between the two cells through which CTLs can acquire target membrane proteins as the cells part company.[28,31] MHC class I, among other proteins, may be ripped off the target as the effector moves away and, as a result, this may render the CTL susceptible to fratricidal killing by other CTLs, thus establishing a self-regulating mechanism for downregulating the immune response.[28,31]

Perforin Facilitates Entry of Granzymes into the Target Cell

The pore-forming protein perforin was originally purified from CTL granules in 1985,[32] and since then, its precise function has been the focus of much debate. On the one hand, perforin is thought to form pores in the target cell membrane through which granzymes may pass directly into the cytosol. However, it has also been proposed that granzymes are taken up into target cell endosomes, where perforin is required for their release into the cytosol. Whatever the exact role of perforin, studies with knockout mice and with individuals suffering from genetic disorders in which perforin function has been abrogated have highlighted the crucial role this protein plays in lymphocyte-mediated cytotoxicity.[33,34] Firstly, the generation of perforin-deficient mice has demonstrated the absolute requirement of this protein for granzyme-dependent killing.[35–37] Perforin deficiency also results in an increased susceptibility to tumor formation and infection by a number of different pathogens.[35,38–43] Although mice lacking perforin remain healthy when kept in a clean environment, half of all perforin-deficient animals develop spontaneous B-cell lymphomas within their lifetime, highlighting the critical role perforin-dependent killing plays in tumor surveillance.[40]

In humans, perforin is mutated in 30% of all patients suffering from FHL, with perforin inactivation associated with the FHL 2 subtype.[34] FHL is an autosomal recessive disorder resulting in uncontrolled T and NK cell activation and proliferation, and hemophagocytosis (erythrocyte phagocytosis resulting from uncontrolled activation of macrophages, which results from excessive T-cell activation).[44] Here, the abrogation of CTL/NK cell cytotoxic function caused by perforin mutation is thought to impair downregulation of the immune response after pathogen clearance, suggesting a critical role for perforin/granzyme-mediated killing in immune homeostasis.[34,43,45] Perforin-deficient mice are generally kept in a sanitary environment, free from pathogen challenge, and this has been proposed as a reason for the difference between the mouse and human perforin-deficiency phenotype. Indeed, perforin-deficient mice have been shown to develop an FHL-like syndrome after infection with certain pathogens, indicating that initial immune challenge is required for the FHL phenotype to manifest.[46,47]

Recently, both perforin and granzymes have been implicated in the immunosupressive mechanism of regulatory T (Treg) cells.[48,49] These cells are normally involved in downregulating the immune response to autoantigens, however, it has also been demonstrated that Treg cells can mediate suppressive effects on immune responses to both tumor and pathogen-derived antigens.[50] This immune suppression is thought to facilitate a critical tumor immune evasion strategy and is mediated through cell contact between Treg and immune effector cells, however, the mechanisms of suppression are not clear.[50] Recent work has suggested that Treg cells can kill immune effector cells either through a perforin/granzyme-dependent pathway or through granzyme B alone, suggesting a possible role for perforin and granzymes in Treg-mediated immune suppression and thus, tumor progression.[48,49,51]

Initially, the structural and functional similarity of perforin to proteins associated with complement-mediated lysis suggested that this protein contributed to target cell death simply by damaging the cell membrane through pore formation.[52,53] However, a series of experiments in which perforin and granzymes were overexpressed in the mast cell line RBL, thus endowing them with cytotoxic potential, demonstrated that while overexpressing perforin in isolation induced target cell membrane permeabilization, it necessitated the combined expression of perforin and granzymes to induce further intracellular events associated with CTL/NK killing, such as DNA damage.[54–56] This suggested that perforin might form pores in the target cell membrane through which granzymes may pass; however, there is little experimental evidence for this at present.

It has also been proposed that granzyme B is taken into target cells in a perforin-independent manner, via a specific target cell surface receptor, the mannose-6-phosphate receptor.[57] However, this has recently been contested by a number of groups.[58–60] Transfer of granzyme B into target cell endosomes in a perforin-independent manner has also been demonstrated and, in this case, the presence of perforin was required for endosomal disruption, thereby releasing granzyme B into the target cell cytosol where it could promote apoptosis.[61,62] However, evidence for perforin-mediated pore formation in these endosomes is lacking and perforin has not been found in target cell cytosols. Recent work has suggested that the uptake of granzyme B into endosomes is predominantly charge-based, with perforin required for its subsequent release.[60,63] As all granzymes have a similar highly basic charge, a model in which the mechanism of uptake is charge-based is an attractive one. More recently, Lieberman and co-workers have proposed that perforin permeabilizes the cell membrane, facilitating granzyme uptake into large vesicles.[64]

A role for the granule proteoglycan, serglycin, in granzyme delivery has also been postulated, with the authors suggesting that serglycin binds both perforin and granzymes in a high molecular weight complex that crosses the cell membrane without pore formation.[65,66] More recently, the same group has proposed that serglycin delivers granzyme B to the target cell membrane where it undergoes electrostatic transfer from the proteoglycan to cell surface proteins.[67] However, a recent study using serglycin-deficient mice has cast doubt upon the role of this protein as a granzyme/perforin

delivery agent, instead suggesting that its main role is associated with secretory granule maturation and granzyme B storage.[68]

An important consideration is that most studies into perforin function have utilized *in vitro* systems where a sublytic concentration of perforin is used to facilitate granzyme entry. As the *in vivo* concentration of perforin secreted by effector cells is not known, protein concentrations at the immunological synapse may be much greater than those used in an *in vitro* setting and therefore may be sufficient to induce pores in the target cell membrane, although this remains unclear at present.[69] Thus, an agreed model of granzyme delivery is still some way off.

The Granzymes

The granzymes are a family of serine proteases stored within CTL/NK cell granules, with humans and rodents possessing different granzyme genes in three linked chromosomal clusters. Granzymes A, B, C, D, E, F, G, K, L, M and N are found in the mouse, while humans possess a more restricted repertoire, namely granzymes A, B, H, K and M.[70] Granzymes A and B are generally the most abundant granzymes and for this reason, most work has focused on elucidating their function. However, the first substrates have recently emerged for some of the other 'orphan' granzymes.[71–75] It should be noted that in comparison to the wealth of substrate data available for the caspases,[3] relatively few substrates have been identified for granzymes, suggesting that these proteases are comparatively specific and may cleave fewer protein targets. Table 1 provides information on the species and substrate specificity of the granzymes together with a list of known substrates.

Granzyme B

Granzyme B is a major constituent of CTL/NK cell granules, promoting apoptosis through proteolysis of a relatively small number of substrates (Figure 2).[76] Mouse knockout data have demonstrated a crucial role for this granzyme in target cell caspase activation and DNA fragmentation, with granzyme B-deficient CTLs exhibiting delayed killing, indicating that this granzyme is necessary for the fast and efficient death of the target cell.[77,78] Granzyme B is characterized as an Aspase because of its preference for substrate cleavage after aspartic acid residues, which is similar to caspases in this regard. Despite this similarity, however, granzyme B has a unique specificity, with the preferred recognition motif P4-P1 (I/V) EPD.[79] In addition, extended substrate specificity has been shown to be important for substrate recognition by granzyme B, with a number of residues outside the catalytic cleft playing a critical role in substrate selection.[80–82] Such extended substrate specificity implies that targets for this enzyme are highly restricted and this is borne out by observations that caspase-3 cleaves in the region of 10 times more cellular substrates than granzyme B.[3,83] There appears to be two main pathways to granzyme B-induced killing, one involving direct activation of caspases and the other mediated through granzyme B-initiated promotion of mitochondrial permeabilization.

Table 1 A list of currently known granzymes, with details of species expression, specificity and a non-exhaustive list of substrates

Granzyme	Species	Specificity	Substrates
B	Human/ Mouse	Asp/Glu	BID[a] Caspse-3, -7, -8[a] ICAD α-Tubulin Rock II MCL-1 Lamin B PARP DNA-PK NuMa MI-2 Topoisomerase 1 Cartilage proteoglycan U1-70kDa Neuronal glutamate receptor Human homolog of Ufd2p
A	Human/ Mouse	Arg/Lys	SET Ape1 Histone H1 HMG2 Core Histone Ku70 Lamins A-C
K	Human/ Mouse	Arg/Lys	BID SET
M	Human/ Mouse	Met/Leu	DFF45/ICAD PI-9
H	Mouse	Phe/Leu (predicted)	Adenovirus DBP Adenovirus 100 K AP
C	Mouse	Asn/Ser (predicted)	Unknown
D	Mouse	Phe/Leu (predicted)	Unknown
E	Mouse	Phe/Leu (predicted)	Unknown
F	Mouse	Phe/Leu (predicted)	Unknown
G	Mouse	Phe/Leu (predicted)	Unknown

[a]BID was recently found to be a very poor substrate for mouse granzyme B, whereas mouse caspase-8 is not cleaved by granzyme B from either species.[106]

Granzyme B Directly Cleaves and Activates Caspases

Like the caspases, granzyme B cleaves its substrates after aspartic acid residues, so it is not surprising that several caspases have been proposed to be direct granzyme B substrates including caspase-3, -6, -7, -8, -9 and -10.[84–92] However, recent work suggests that caspase-2, -6 and -9 are cleaved indirectly by granzyme B-activated caspase-3.[83] Caspase activation provides granzyme B with a direct means of activating the target cell death machinery and, as such, would be predicted to promote a rapid onset of apoptosis. As granzyme B is the only granzyme known to process and activate caspases, it is likely that the delay in killing kinetics observed in granzyme B-deficient effector cells results from a failure to activate these enzymes, either directly or indirectly.[77]

Granzyme B can Promote Caspase-Independent Cell Death by Triggering Mitochondrial Permeabilization

While caspases are believed to enhance granzyme B-mediated cell death, their requirement is not absolute. Many

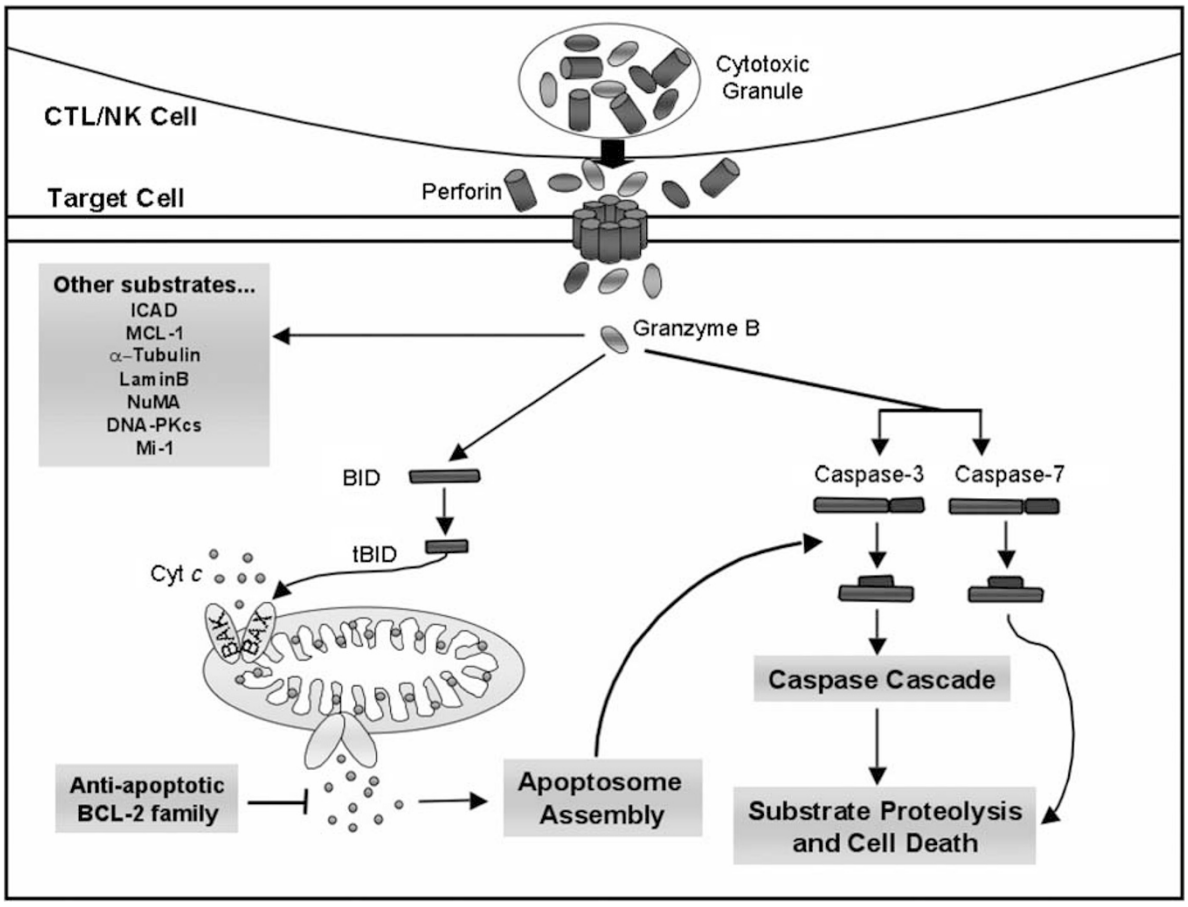

Figure 2 Pathways to granzyme B-mediated cell death. Granzyme B, together with other granzymes, enters the target cell by a perforin-dependent mechanism, the precise details of which are still unclear. Once in the target cell cytosol, granzyme B promotes death through two main pathways, either through BID-dependent mitochondrial permeabilization or through direct caspase processing and activation. Granzyme B-mediated proteolysis of the BH3-only protein BID exposes a myristolyation signal in this protein, targeting it to mitochondria where it induces the oligomerization of BAX and/or BAK in the outer mitochondrial membrane. This facilitates cytochrome *c* release into the cytosol, assembly of the apoptosome, with subsequent caspase-9 activation and the ensuing caspase cascade. Note that antiapoptotic BCL-2 family members can inhibit cytochrome *c* release and thus, block this pathway. Granzyme B can also directly process the effector caspases-3 and -7, although whether this granzyme can fully activate caspase-–3 and -7 has been the subject of debate. Direct activation of the latter effector caspases would lead to a caspase activation cascade and cleavage of a myriad of death substrates resulting in the efficient death of the target cell. Granzyme B cleaves DFF45/ICAD, the inhibitor of a DNase, DFF40/CAD, which when released from inhibition mediates the internucleosomal degradation of DNA that is synonymous with this mode of killing. This granzyme has also been shown to cleave a variety of other proteins implicated in the maintenance of nuclear integrity (Lamin B), protection against cell death (MCL-1), DNA repair (DNA-PK_{cs}), microtubule dynamics (α-tubulin) and a host of autoantigens (NUMA, U1-70kD, Mi-2)

groups have shown that granzyme B-mediated cell death is not prevented by artificially blocking caspase activity using specific inhibitors of these proteases.[93–95] However, in these circumstances, death occurs with dramatically delayed kinetics, further highlighting the role caspases play in promoting apoptosis by granzyme B.[96] This caspase-independent mode of killing strongly suggested that granzyme B could activate other cell death pathways or cleave other substrates that may orchestrate the death of the target cell.

In many apoptosis pathways, apoptotic signals converge on mitochondria where they promote the oligomerization of the BCL-2 family members BAX and/or BAK in the outer mitochondrial membrane.[97,98] BAX/BAK oligomerization promotes mitochondrial permeabilization with the resulting escape of mitochondrial intermembrane space proteins including cytochrome *c*.[99] This is a defining event in many forms of apoptosis as cytochrome *c* release into the cytosol facilitates the formation of a complex, known as the apoptosome, between APAF-1 and caspase-9.[100,101] Assembly of the apoptosome results in caspase-9 activation followed by a downstream caspase cascade, which ensures the rapid death of the cell.[102,103] The antiapoptotic protein BCL-2 acts at mitochondria to inhibit cell death by heterodimerizing with and inhibiting the death promoters BAX and BAK, thereby blocking the efflux of cytochrome *c* and other mitochondrial intermembrane space proteins.[104] The observation that BCL-2 overexpression could rescue cells from many of the hallmarks of granzyme B-mediated apoptosis raised the intriguing possibility that mitochondrial permeabilization represented a pivotal step in this process.[105–107] Because caspase inhibitors were ineffective at preventing granzyme B-mediated cytochrome *c* release,[105] whereas the overexpression of BCL-2 was sufficient to abrogate killing,[107] this strongly suggested that granzyme B utilized a caspase-independent,

mitochondrial-mediated cell death pathway. The BH3-only protein BID was subsequently identified as a substrate for this granzyme. In a manner, analogous to caspase-8-mediated proteolysis of BID in the death receptor pathway, processing of BID by granzyme B is thought to expose a myristolyation sequence, targeting the latter protein to mitochondria where it can promote the oligomerization of BAX and/or BAK in the outer mitochondrial membrane.[108–111] The resulting cytochrome *c* release, followed by assembly of the apoptosome and the ensuing caspase activation cascade ensures rapid cell death. However, if caspases are inactivated, granzyme B can still kill cells, albeit with slower kinetics, and this is likely as a direct result of BID-mediated mitochondrial permeabilization followed by a rapid decline in ATP synthesis.

Interestingly, granzyme B has also been proposed to promote a loss of mitochondrial transmembrane potential (an event which normally follows mitochondrial permeabilization) in a caspase and BID-independent manner.[109,112] In addition, Ley and co-workers used BID-deficient and BAX/BAK doubly-deficient mouse fibroblasts to show that granzyme B-induced loss of mitochondrial transmembrane potential and cell death could proceed independently of caspases, BID and BAX/BAK and without cytochrome *c* release.[95] This implies a pathway through which granzyme B can bypass caspases, BID, BAX and BAK, to kill the target cell. However, up to 2 μM recombinant granzyme B was used in this study and such enzyme concentrations may not be achievable *in vivo*.[95] Interestingly, in a recent paper by Waterhouse *et al.*,[113] the authors used concentrations of granzyme B in the low nanomolar range to demonstrate that loss of mitochondrial transmembrane potential in response to granzyme B is indeed caspase-dependent, indicating that at low concentrations, granzyme B may rely on caspases for this phenomenon.

Granzyme B Cleaves DFF45/ICAD and Other Caspase Substrates

The observation that granzyme B could promote DNA fragmentation in cells lacking functional caspases suggested that this granzyme might directly activate nucleases and in this regard, DFF45/inhibitor of caspase-activated DNase (ICAD) (also cleaved by caspase-3) has been identified as a granzyme B substrate.[114,115] DFF45/ICAD acts as an inhibitor of a DNase (DFF40/caspase-activated DNase (CAD)) and upon proteolytic cleavage of this protein by granzyme B, CAD is free to mediate the internucleosomal degradation of DNA that is one of the hallmarks of apoptosis induced by CTL/NK cells.[114–117] Studies with ICAD-deficient cells revealed that loss of this protein confers a partial resistance to granzyme B-induced DNA fragmentation and cell death.[114]

α-Tubulin has recently been identified as a granzyme B substrate,[118,119] with proteolysis of the protein demonstrated in its C-terminus, a region associated with the regulation of microtubule polymerization.[120] Indeed, treatment of purified α-tubulin with granzyme B enhanced microtubule polymerization rates *in vitro*, whereas the killing of HeLa cell targets with NK cell effectors dramatically altered microtubule morphology, suggesting that granzyme B targeting of α-tubulin may disrupt the microtubule network and thus constrain the mitotic potential of tumor cells.[118] As many viruses utilize the host cell microtubule network to enter and exit cells, prompt disruption of target cell microtubules by granzyme B may limit both viral replication and the spread of mature virus to neighboring cells.

In addition to targeting the BH3-only protein, BID, granzyme B has also been shown to cleave the antiapoptotic BCL-2 family member, MCL-1.[121] It has been suggested that granzyme B-mediated proteolysis of MCL-1 liberates the proapoptotic BH3-only protein, BIM, enabling BIM to promote cytochrome *c* release and cell death.[122] The importance of BIM for granzyme B-dependent killing is supported by data showing that knockdown of this protein in breast carcinoma cells leads to a dramatic reduction in their susceptibility to killing by purified granzyme B and adenovirus. However, it remains to be demonstrated that this pathway is widely utilized in diverse target cell types.[122]

Granzyme B directly targets several other downstream caspase substrates. These include, among others, poly (ADPribose) polymerase (PARP); the catalytic subunit of DNA-dependent protein kinase (DNA-PK), which is involved in repairing double-stranded DNA breaks; the nuclear mitotic apparatus protein (NUMA); the nuclear envelope intermediate filament protein lamin B; Filamin; cartilage proteoglycan and a variety of autoantigens.[123–128] However, the significance of these proteolytic events for cell fate, or for the development of autoimmune disease, remains unclear.

The Preferred Killing Mechanism of Granzyme B is Species-Dependent

There has been much debate regarding the preferred killing pathway of granzyme B. Several studies have suggested that BID is the preferred substrate for this protease and that this pathway is the primary route to granzyme B-mediated apoptosis,[105,129–131] whereas others have argued that BID proteolysis in this context occurs secondary to caspase activation.[132,133] Regarding the importance of BID, robust caspase-3 activation by granzyme B was shown to be dependent on a BID-mediated mitochondrial positive-feedback loop,[129,130] whereas BID-null cells were found to be resistant to granzyme B-initiated apoptosis.[131] However, the most convincing evidence in favor of BID as the primary target of granzyme B comes from the observation that BCL-2 can block granzyme B-induced apoptosis, whereas caspase inhibitors merely delay death, thus placing BID proteolysis by granzyme B upstream of caspase activation.[105,113]

However, recent studies have shown that although BID is efficiently cleaved by human granzyme B, murine granzyme B cleaves this protein very poorly by comparison.[96,134,135] These observations have significant implications as many investigators use human granzymes on murine cells and vice versa, a practice that has led to some confusion concerning the role of BID in granzyme B-dependent killing. Although human and murine granzyme B share 69% identity, these granzymes differ in residues important for extended substrate recognition. Furthermore, human and murine granzyme B were shown to exhibit divergent substrate preferences with regard to the proteolysis of several important substrates including BID and DFF45/ICAD.[96] In particular, although human granzyme B efficiently processed both caspases and BID, of human or mouse origin, mouse granzyme B did not

cleave BID in either species, exhibiting a marked preference toward caspases.[96,135] This suggests that mouse granzyme B directly activates caspases to promote apoptosis in a BID-independent manner, whereas human granzyme B may kill via either pathway. Indeed, the demonstration that caspase inhibitors rescued cells from mouse granzyme B, but failed to do so when the human enzyme was used, strongly suggests that mouse granzyme B relies heavily upon direct caspase activation to kill target cells.[96]

As mouse granules contain many more granzymes than their human counterparts, it is also possible that mouse granzymes possess a greater degree of functional redundancy, with granzyme B playing a less important role in the mouse. Thus, care should be taken when working with purified or recombinant granzymes as mixing granzyme B from one species with cells of another can produce misleading results.

Granzyme A

Early work with granzyme A indicated that this granzyme can induce a slower form of cell death than granzyme B,[136] and this is associated with the release of oligonucleotide fragments from target cells.[137,138] Indeed, CTLs deficient in granzyme B were later shown to kill targets with markedly slower kinetics than wild-type cells, suggesting that the other granzymes can promote cell death, albeit with reduced efficiency.[77] Cells targeted with perforin and granzyme A undergo cell death characterized by single-strand DNA nicks instead of the double-strand breaks associated with granzyme B.[139] In addition, granzyme A-mediated killing was not abrogated by the addition of caspase inhibitors or by BCL-2 overexpression, further distinguishing its mode of action from that of granzyme B.[139,140]

Substrates of granzyme A have been slow to emerge, but it is now known that this granzyme targets components of a high molecular weight endoplasmic reticulum-associated complex called the SET complex, which contains three granzyme A substrates (SET, HMGB2 and APE1) together with the tumor suppressor proteins pp32 and NM23-H1 (Figure 3).[140–144] Shortly after target cell entry, granzyme A moves to the nucleus where it activates the DNase, NM23-H1, by proteolysis and inactivation of its inhibitor SET,[140] in a manner similar to granzyme B-mediated release of the Dnase, CAD, from its inhibitor, ICAD. NM23-H1 is then free to promote the single-strand DNA nicks associated with granzyme A-mediated killing, with the help of a more recently identified SET complex constituent, the 3′–5′ exonuclease, TREX1.[145] After NM23-H1 cuts a strand of DNA, TREX1 stops the DNA ends from re-annealing by removing bases from the free 3′ end, thus preventing DNA repair. The importance of TREX1 to NM23-H1-mediated DNA damage and granzyme A-induced killing was highlighted by the inhibition of DNA damage and cell death induced by granzyme A/perforin in target cells deficient in TREX1.[145]

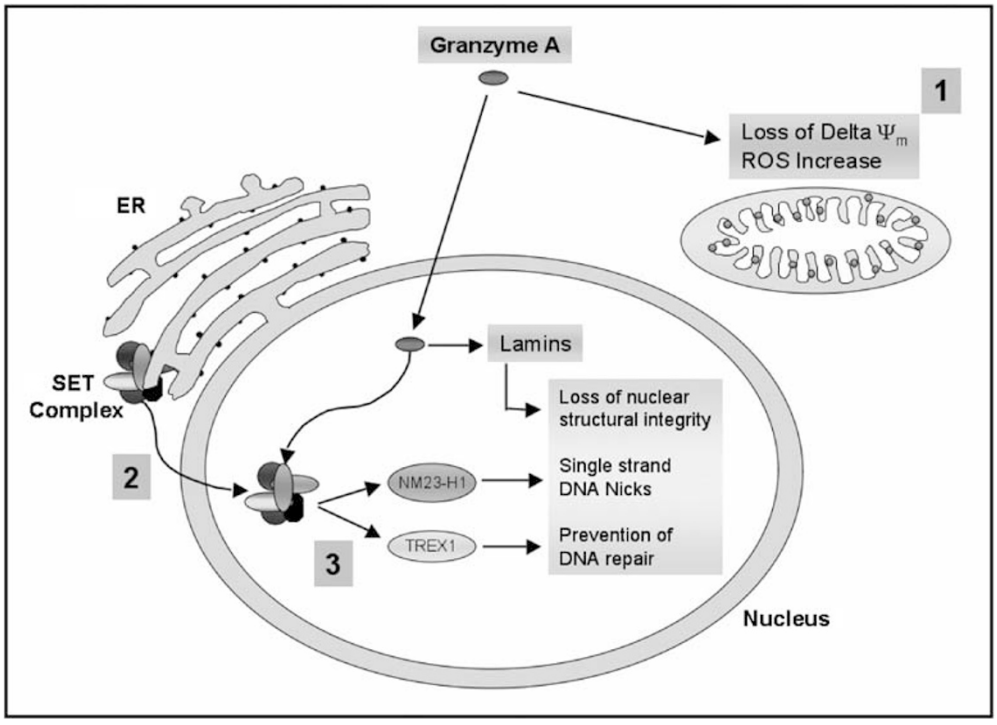

Figure 3 Granzyme A promotes cell death by targeting the nucleus. Upon entry into the target cell, granzyme A facilitates a decrease in mitochondrial transmembrane potential (deltaψ_m) and an increase in ROS, through a poorly understood mechanism (1). This ROS increase leads to the translocation of the SET complex to the nucleus where it may be involved in the transcription of genes associated with the response to oxidative stress (2). Granzyme A enters the nucleus and targets three members of the SET complex for proteolysis; the nucleosome assembly protein, SET, the high mobility group protein 2 (HMG2) and the base-excision repair enzyme apurinic/apyrimidinic endonuclease 1 (APE 1) (3). Proteolysis of SET releases inhibition of the DNase NM23-HI, resulting in the single-strand DNA nicks most commonly associated with granzyme A-mediated cell death. Degradation of the SET complex may also free the 3′–5′ exonuclease, TREX1, which may stop DNA ends from re-annealing by removing bases from the free 3′ end, thus preventing possible DNA repair. Granzyme A also weakens the structural integrity of the nucleus by targeting Lamins A–C for proteolysis

A problem with the above interpretation of granzyme A-mediated cell death has been that the SET complex normally resides in the endoplasmic reticulum, whereas granzyme A cleaves its substrates in the nucleus. The complex was thus proposed to translocate to the nucleus after granzyme A enters the target cell.[144] Although the precise cellular role of the SET complex requires clarification, it has been proposed that this complex translocates to the nucleus under conditions of oxidative stress, where it activates the expression of genes involved in the oxidative stress response.[138] Recent work has now shown that shortly after gaining entry to the target cell, granzyme A induces oxidative stress by promoting mitochondrial depolarization and a rapid increase in reactive oxygen species (ROS), which in turn targets the SET complex to the nucleus where NM23-H1 can be activated by granzyme A.[146]

The Orphan Granzymes

As mentioned earlier, granzymes other than granzyme A and B have been termed 'orphan' granzymes. The phenotype of granzyme A/B knockout mice appears to indicate that granzymes A and B are the most important death-inducing granzymes.[78] As a result, less work has been devoted to the orphan granzymes and their function remains somewhat obscure. Recently, however, the emergence of the first substrates for these proteases has revived interest in this area.

In humans, granzyme H is most closely related to granzyme B and a recent study has demonstrated a novel mechanism of granzyme H-mediated killing.[147] The authors used recombinant granzyme H to show that this granzyme induces a caspase and BID-independent form of cell death characterized by chromosomal condensation and nuclear fragmentation together with mitochondrial depolarization and ROS production.[147] Granzyme C has been shown to induce mitochondrial depolarization and cytochrome *c* release, promoting death in a caspase-dependent manner.[148] An earlier study demonstrated the release of ROS and cell death by granzyme K, which suggested it may kill in a manner similar to granzyme A.[112] Indeed, a recent report has identified the granzyme A target, SET, as a granzyme K substrate.[73] In a manner analogous to granzyme A-mediated DNA degradation, proteolysis of SET by granzyme K was shown to release its inhibition of the DNase NM23-H1 resulting in single-strand DNA nicks and cell death.[73] The recent demonstration of BID proteolysis by recombinant granzyme K has raised the intriguing possibility that this granzyme may kill by targeting mitochondria in a manner similar to granzyme B and also by mimicking the nuclear degradation pathway favored by granzyme A.[74] However, the dependence of granzyme K-mediated killing on the accumulation of ROS in this study questions the significance of BID proteolysis in this context.[74]

Granzyme M was originally shown to promote a predominantly necrotic form of cell death without the requirement for either caspases or mitochondria,[71] and more recently, the first substrates for this protease have been identified.[72,149] In a manner similar to granzyme B, granzyme M has been shown to activate the DNase CAD through proteolysis of its inhibitor ICAD, leading to DNA fragmentation.[72] However, the functional relevance of this is debatable, as cell death was not reported by the authors.[72] Granzyme M has also been shown to bind to and inactivate protease inhibitor-9 (PI-9), an endogenous inhibitor of granzyme B,[149] suggesting that this granzyme may play a role in lowering the threshold for granzyme B-mediated cell death in target cells expressing high levels of PI-9.

The suggestion that granzymes may work in concert with one another was recently offered credence by the demonstration that granzyme H can cleave the adenovirus inhibitor 100 K assembly protein, which is an inhibitor of granzyme B, thus relieving viral-mediated granzyme B inhibition.[75] In addition, granzyme H also cleaved the adenovirus DNA-binding protein, a component critical for viral DNA replication.[75] This clearly demonstrates the functional advantage of possessing a range of different granzymes and also, the role that granzymes may subserve outside of directly inducing cell death.

Lessons from Granzyme-Deficient Animals

As mentioned above, perforin-deficient animals have been produced by a number of groups.[35–37,150] As perforin is required for the trafficking of granzymes, its deficiency results in the complete loss of all granzyme activity and consequently, perforin deficiency has a much more severe phenotype than the loss of individual granzymes. However, knockout studies have demonstrated the importance of granzymes in a number of settings.

Ley and co-workers first produced mice deficient in granzyme B by using a PGK-neo cassette inserted into the granzyme B gene.[77] However, it was subsequently discovered that these animals were also markedly deficient for granzyme C, D and F, which together with granzyme B form the granzyme B gene cluster. Until recently, all granzyme B knockout data utilized cells from these animals and the observed effects could have been partly attributable to these other granzymes. Recently, however, Ley and co-workers removed the cassette, specifically targeting the granzyme B gene alone and found that results published using cells from the original mice were essentially identical for CTLs deficient in granzyme B only.[151] In addition to reaffirming the original granzyme B knockout data, this work suggested that the orphan granzymes C, D and F play only a minor role in CTL/NK cell-mediated killing.[151] The most pronounced defects resulting from the loss of granzyme B are a profound attenuation of DNA fragmentation, a complete inhibition of caspase activation and a general slowing of the normal cell death kinetics.[72,78] Granzyme B-deficient effectors can still kill target cells, however, likely through other granzymes (although the role of granzymes C, D and F may be relatively minor as mentioned above) and this highlights the impressive redundancy associated with this mode of killing.

Granzymes A and B have been shown to be important for clearance of the poxvirus pathogen, ectromelia, with granzyme A/B doubly deficient animals displaying a more profound effect than single knockouts.[152] These granzymes were observed to be crucial for the survival of mice infected with the intracellular protozoan Trypanosoma cruzi.[153] A recent study has indicated that granzyme B may play an important role in mouse cytomegalovirus (MCMV) clearance.[9] The

authors used an *in vivo* model of MCMV infection to show that murine NK cells deficient in granzyme B are defective with regard to viral clearance. At high viral doses, this results in survival levels similar to that of the perforin knockout, suggesting that under these conditions, granzyme B may be required for MCMV clearance.[9] An earlier study assessed susceptibility to MCMV in mice lacking peforin or granzyme A/B.[43] Viral titers increased substantially in both perforin-deficient and granzyme A/B-deficient mice, however, granzyme A/B-deficient mice eventually survived the infection, whereas perforin-deficient animals did not, suggesting that granzyme B is not required for overcoming MCMV infection.[43] However, the discrepancy between these papers may relate to differences in the MCMV doses used.[9,43] In the latter study, the recovery of granzyme A/B-deficient mice from infection suggests that orphan granzymes may play a role in MCMV clearance. Indeed, granzyme M-deficient mice have recently been generated, and although NK cells from these animals showed no cytotoxic defect, a delay in the clearance of MCMV suggests that granzyme M may play a role in the eradication of some viral infections.[154]

With regard to the tumorigenic function of CTL/NK cells, granzyme deficiency has produced conflicting results and, to date, an increased susceptibility of granzyme B-deficient mice to lymphoma has not been reported. In addition, granzyme A/B-deficient animals were shown to retain potent *in vivo* antitumor activity, suggesting that these granzymes are not required for tumor clearance.[155] However, recent work performed in a similar manner showed that mice deficient for granzymes A and B displayed an uncontrolled tumor growth with kinetics similar to that of perforin-deficient animals.[156] It should be noted however that although perforin-deficient mice develop spontaneous lymphoma, granzyme A/B-deficient mice do not, indicating that the growth of tumors in granzyme A/B-deficient mice may be restricted to experimental tumors.[40] Overall, the results indicate that granzymes A and B are both important for viral clearance and may also be associated with protection against tumor growth in certain circumstances.

In a recent paper by Waterhouse *et al.*,[157] the authors investigate the killing mechanism of the orphan granzymes. CTLs deficient in granzyme A/B but expressing normal levels of mRNA for granzyme K and, to a lesser extent, granzyme C (other orphan granzymes were not screened for) were utilized to show that these granzymes may induce a predominantly necrotic phenotype in target cells.[157]

Inhibitors of Granzyme Function

Viruses have co-evolved with their hosts for millennia, so it is no surprise that many of them have developed tactics to subvert the host's efforts at eradication. By preventing or even delaying host cell apoptosis, viruses offer themselves valuable time with which to replicate and spread to other cells. In this regard, a number of viruses encode direct granzyme B inhibitors. BHRF, showing functional similarity to BCL-2, is produced by the Epstein–Barr virus and blocks granzyme B-induced apoptosis.[107] The adenovirus L4 100 kDa protein forms a stable complex with granzyme B, thus inhibiting its proteolytic activity.[158] Granzyme H-mediated proteolysis of this inhibitor, with the subsequent re-activation of granzyme B, demonstrates the impressive cooperation involved in granzyme-mediated killing.[74]

As perforin and granzymes are secreted between effector and target, it has been postulated that some of their toxic constituents may 'leak' back into the CTL/NK cell, and because of this the effector might need protection against this eventuality.[159,160] Interestingly, the serpin PI-9 has been linked with a protective function in immune effector cells.[159,161] PI-9 acts as a pseudo substrate of granzyme B, forming an irreversible, SDS-stable, complex with the enzyme and potently inhibiting its function.[162] In light of this, recent work has demonstrated an important role for PI-9 in protecting CTLs from endogenous granzymes released by the breakdown of cytotoxic granules.[161]

Concluding Remarks

The abnormalities associated with loss of the granule-dependent killing pathway, in both mice and humans, underscore the importance of this mode of cell death in fighting disease and in the regulation of the immune system. Recent work has greatly advanced our understanding of perforin and granzyme action, although many puzzles remain. The precise mechanism of perforin-mediated granzyme delivery is unclear, with many conflicting reports serving only to contradict one another. Much new data are now available for the orphan granzymes, suggesting that some of these enzymes may play a more important role in immune function than previously considered. This may be especially true in the mouse where granzyme B seems to play a more restricted role than in humans.[96] In addition, non-apoptotic roles for granzymes are beginning to emerge,[84,118,163] which suggests that granzymes may have a broader role in the immune system than is currently appreciated and that other granzyme functions await discovery.

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