CYTOTOXIC T LYMPHOCYTES: ALL ROADS LEAD TO DEATH

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Cytotoxic T lymphocytes (CTLs) provide potent defences against virus infection and intracellular pathogens. However, CTLs have a dark side — their lytic machinery can be directed against self-tissues in autoimmune disorders, transplanted cells during graft rejection and host tissues to cause graft-versus-host disease, which is one of the most serious diseases related to CTL function. Although this duplicitous behaviour might seem contradictory, both beneficial and detrimental effects are the result of the same effector proteins. So, an understanding of the mechanisms that are used by CTLs to destroy targets and a knowledge of pathogen immune-evasion strategies will provide vital information for the design of new therapies.

MICROTUBULE-ORGANIZING CENTRE
A region of the cell from which microtubules grow. Motor proteins that are associated with the microtubles are responsible for the directed movement of organelles in the cytoplasm.

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The survival of a multicellular organism is absolutely dependent on a functioning immune system to protect against a myriad of pathogens. Recent studies of the key cytotoxic effector cells — cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells — have revealed that the mechanisms that are involved in the destruction of target cells are multifaceted. Initially, it was believed that simple membrane damage would suffice, but it is now clear that more-subtle and complex pathways are involved, which result in apoptotic or necrotic death. It is preferable for the host that cells die by apoptosis — which is a 'silent' or non-inflammatory form of death — but necrotic death of individual cells is still better than death of the entire organism. In each of the cell-death pathways, there are many potential effectors and alternative routes of action. Only now are we starting to unravel the complexities that are involved.

Early microscopic studies of CTLs led to the view that interactions with target cells were transient and that the effectors could deliver a death signal to multiple targets sequentially. It was then discovered that the MICROTUBULE-ORGANIZING CENTRE, Golgi apparatus and lytic granules of a CTL were rapidly oriented towards the contact area in conjugates with target cells[1–3], and that acid phosphatase — which is normally found in granules — was deposited at the interface of CTL–target conjugates[4]. Combining these seminal observations with preliminary information on potential cytolytic effector molecules, the granule exocytosis model of killing was formulated[5]. According to the original description, this mechanism involves a rearrangement of the effector-cell cytoplasm that results in granule exocytosis and the delivery of the key effector molecule, the pore-forming protein perforin. This model remains valid, but it now incorporates other key granule proteins that make an important contribution to the mechanism of target-cell destruction.

As more CTL lines were analysed, it became clear that calcium-dependent granule exocytosis is not the only cell-death pathway that is used. We now know that CTLs can kill through the FAS (CD95; a member of the tumour-necrosis factor receptor family of death receptors) pathway, which requires neither calcium nor perforin[6–8]. It seems, however, that CTLs use the granule pathway most often to destroy target cells in culture. Only when this pathway is compromised are significant levels of FAS-mediated killing observed. In part, this might be due to the fact that the effector cells come pre-loaded with cytolytic proteins in granules and, so, killing through this pathway occurs more rapidly. The situation *in vivo* is undoubtedly more complicated and, often, results are paradoxical. A recent review on effector lymphocytes in autoimmunity highlights these issues and underlines that the relative importance of FAS and perforin remains unclear[9]. For example, perforin-deficient non-obese diabetic (NOD) mice (an animal model of

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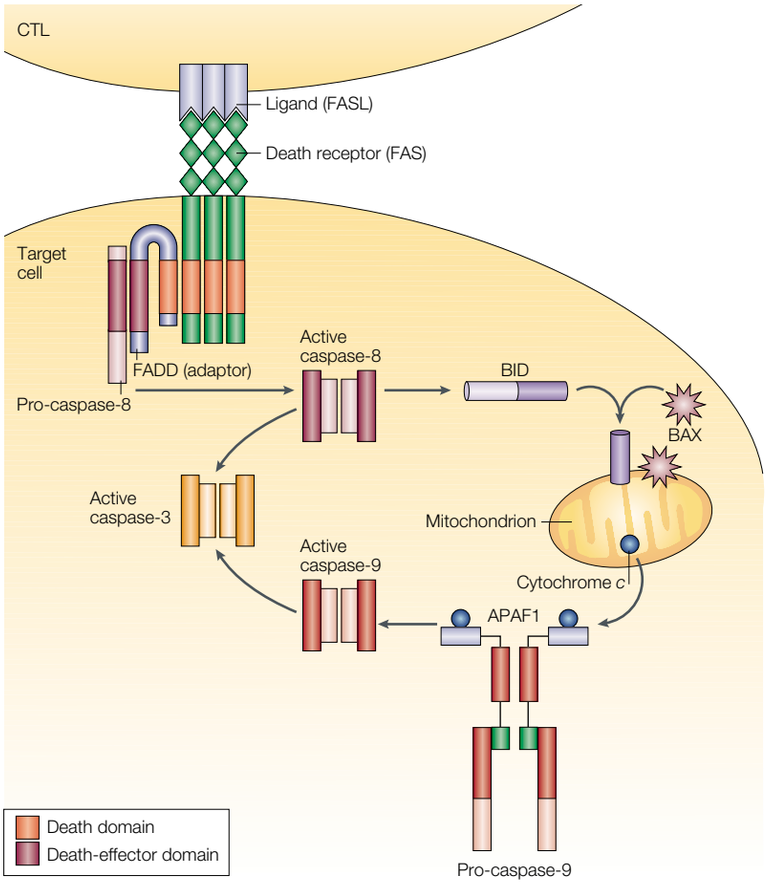

Figure 1 | **Engagement of FAS (CD95) on a target cell with CTL-expressed FAS ligand (FASL; CD178) results in apoptotic death.** Stimulation of the FAS receptor results in recruitment of the initiator caspase, caspase-8, through interaction with the adaptor molecule FAS-associated death domain protein (FADD) by means of death domains and death-effector domains. This results in the activation of caspase-8. Caspase-8 has different effects in different cells of the TYPE I/II SYSTEM. In type I cells, it is able to activate other members of the caspase family (such as caspase-3) directly[68]. By contrast, in type II cells, caspase-8 activation results in cleavage of the proapoptotic BCL2-family member BID, and the translocation of BID and BAX to the mitochondria. Once inserted into the mitochondrial membrane, BID and BAX induce the release of mitochondrial cytochrome *c*, which results in the activation of caspase-9 through interaction with the adaptor molecule apoptotic protease-activating factor 1 (APAF1). Then, caspase-9 is able to activate caspase-3. CTL, cytotoxic T lymphocyte.

human insulin-dependent diabetes mellitus) have a reduced incidence of disease compared with wild-type NOD mice[10], and in transgenic models of viral antigen expression in pancreatic β-cells, the perforin pathway is most important for cell killing[11]. By contrast, NOD *LPR* and *GLD* MICE do not develop diabetes[12,13], and CTL clones seem to kill β-cells exclusively by means of the Fas pathway[14]. Perhaps, both pathways are important at different stages of diabetes. Others have suggested that FAS signalling is more important for lymphocyte homeostasis[15].

These two death-inducing strategies that are used by CTLs account for most of the contact-dependent destruction of targets. However, CTLs also produce several cytokines, such as tumour-necrosis factor (TNF) and interferon-γ (IFN-γ), that have cytotoxic action when secreted in the vicinity of target cells. The focus of this review is the granule-mediated mechanism.

LPR MICE
These are naturally occurring mutants that bear a deletion of the *Fas* gene.

GLD MICE
These mice have a naturally occurring mutation of Fas ligand that causes a generalized lymphoproliferative disease.

CASPASES
A family of cysteine proteinases that are involved in the initiation and effector stages of apoptosis.

Cytotoxic T lymphocytes: dual death initiators

Cytotoxic cells of the immune system are of two main types: CTLs and NK cells. Over the past several years, it has become increasingly apparent that these two types of cell have common effector mechanisms. Both NK cells and CTLs induce cell death by means of granule- and FAS-mediated pathways, which trigger the inherent apoptotic response. The FAS pathway has provided a model for how apoptosis is triggered through death receptors (FIG. 1), and similar signalling pathways through FAS-associated death domain protein (FADD) and CASPASE activation occur for other death receptors, such as TNF-related apoptosis-inducing ligand (TRAIL). For further information, see REFS 16,17.

Although the granule-exocytosis pathway was discovered some years before the FAS–FAS ligand (FASL; CD178) pathway, the molecular signals that link the granule pathway to cell death remain enigmatic. A main focus of research on granule-mediated mechanisms has been to understand the relationship between granule exocytosis from CTLs and the induction of apoptosis in target cells. The granules of cytolytic cells contain various proteins, some of which are now known to have a defined role in inducing target-cell death. Included among these are the pore-forming protein perforin and a family of serine proteinases that are known as granzymes (TABLE 1). Although perforin alone has been shown to mediate membrane damage *in vitro*, it is the combined action of perforin and granzymes that is necessary for the induction of apoptosis *in vivo*.

Granzyme delivery: death knocks at the door

Perforin is found exclusively in the cytoplasmic granules of cytolytic cells, and it has a pivotal role in granule-mediated killing induced by CTLs and NK cells. Cytolytic cells from perforin-deficient mice have obvious defects in the ability to induce both membrane damage (as measured by a chromium-release assay) and apoptosis[18–20] (TABLE 2). Also, perforin-knockout mice are more susceptible to tumours and infection[18,21–23]. However, the exact role of perforin during granule-mediated cell death is under review at present.

Perforin was regarded initially as being simply a pore-forming molecule that induced death by damaging the target-cell membrane. Evidence for this was based on the membraneolytic property of perforin and electron-microsocopic studies that showed the formation of poly-perforin plasma-membrane-specific pores[24–26]. Although perforin is essential for granule-mediated cell death, perforin alone is unable to induce all of the features that are associated with CTL-mediated killing. Indeed, the physiological relevance of perforin-induced chromium release has been questioned recently in a GRAFT-VERSUS-HOST DISEASE model of CTL function[27]. Not surprisingly, other granule components are essential for many of the characteristic morphological and biochemical features of CTL function, which indicates that cell killing is a concerted effort that requires the presence of other granule proteins, as well as perforin. These observations led to the hypothesis that perforin channels in the plasma membrane facilitate the entry of other granule proteins, including the

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Table 1 | **Granzymes**

Protease	Peptide cleavage-site specificity	Species
Granzyme A	Arg/Lys	Mouse, rat, human
Granzyme B	Asp/Glu	Mouse, rat, human
Granzyme C	Asn/Ser	Mouse, rat
Granzyme D	Phe/Leu	Mouse
Granzyme E	Phe/Leu	Mouse
Granzyme F	Phe/Leu	Mouse, rat
Granzyme G	Phe/Leu	Mouse
Granzyme H	Phe	Human
Granzyme J	Unknown	Rat
Granzyme K	Arg/Lys	Mouse, rat, human
Granzyme M	Met/Leu	Mouse, rat, human

granzymes, into the target cell (FIG. 2a). Indeed, direct microscopic evidence for the secretion of granzymes into the intercellular cleft of the IMMUNOLOGICAL SYNAPSE of a CTL–target conjugate has been published recently[28]. So, the granzymes are well placed to pass through a perforin channel that is formed in the target-cell membrane.

Recently, however, granzymes A, B and H have been shown to enter target cells in the absence of perforin[29–33]. These observations raised obvious questions about the generality of the widely accepted perforin-pore model. In the absence of perforin, internalized granzymes are retained in endosome-like vesicles, and they are not associated with any cellular toxicity or apoptosis[32]. Treatment with perforin after internalization of granzyme B results in the morphological and biochemical features that are associated with apoptosis, which indicates that perforin has a role in the release of target-cell compartmentalized granzymes[30–33]. Additionally, at low doses, perforin does not form membrane channels that are large enough to allow the entry of granzyme B[34], and the function of perforin can be replaced by either a replication-deficient strain of adenovirus or membranolytic agents[30,34]. These observations led to the hypothesis that granzymes can be internalized by receptor-mediated endocytosis, and they further imply a role for perforin in mediating the release of granzymes from intracellular vesicles (FIG. 2b).

TYPE I/II SYSTEM
A classification of cells on the basis of their sensitivity to FAS-mediated killing. Type I cells recruit caspase-8, which results in the subsequent cleavage of caspase-3. Type II cells activate caspase-3 through a mitochondria-dependent step.

GRAFT-VERSUS-HOST DISEASE
The immune reaction against a graft recipient that is mounted by immune-competent cells of a graft.

IMMUNOLOGICAL SYNAPSE
A distinct region formed at the contact zone between the cytotoxic T lymphocyte and target cell due to the specific reorganization of cell-surface membrane proteins.

Table 2 | **Effector-deficient cytotoxic T cells**

Deficient effector	Phenotype	References
Perforin	All aspects of granular killing impaired; Fas-mediated killing normal	18–20,107
Granzyme A	Granular killing normal; Fas-mediated killing normal	80
Granzyme B	Rapid DNA fragmentation impaired; late DNA fragmentation occurs; Fas-mediated killing normal	69,70
Granzyme A and granzyme B	Rapid DNA fragmentation impaired; late DNA fragmentation impaired; deficient in graft-versus-host disease *in vivo*; Fas-mediated killing normal	27,84
Dipeptidyl peptidase	Rapid DNA fragmentation impaired; late DNA fragmentation impaired; Fas-mediated killing normal	108

One member of the granzyme family, granzyme B, has been shown recently to enter target cells by receptor-mediated endocytosis[35]. Early experiments showed that granzyme B could bind to the surface of cells in a saturable fashion, which indicated the presence of an undetermined receptor[30]. More recently, the mannose-6-phosphate receptor has been shown to bind and internalize granzyme B, but a second agent, such as perforin or adenovirus, is required for the release of granzyme B into the cytoplasm of target cells[32,35]. Importantly, cell lines that are deficient in the mannose-6-phosphate receptor are unable to bind granzyme B and are deficient in CTL-mediated DNA fragmentation[35]. These observations indicate a more refined view of cytolytic killing and, in particular, a new role for perforin. Rather than acting strictly as a plasma-membrane pore, the data indicate that perforin is necessary for the release of granzyme B from the endosomal compartment. The two pathways of granzyme-B uptake are depicted in FIG. 2a,b. These pathways are not mutually exclusive and both could occur depending on the cell type. In addition, as illustrated in FIG. 2c, granzymes that are bound to the cell surface might be internalized in response to local membrane damage induced by perforin. As the target cell attempts to repair the lesion, both perforin and granzyme would be taken up. The balance between the pathways might depend on variables such as the amount of perforin and granzymes that are delivered by the CTLs and the membrane properties of the target cell.

Granzyme B: a natural-born killer

So far, granzyme B is the only known member of the granzyme family that has a preference for proteolytic cleavage after aspartate residues. This specificity was predicted originally by molecular modelling[36], and the use of combinatorial peptide libraries indicated a preference for granzyme-B-mediated cleavage after aspartate residues at sites that have the sequence Ile/Val-Gly/Met/Glu-Xaa-Asp-Xaa-Gly (where Xaa is any amino acid)[37,38]. The recent publication of the X-ray crystal structure of granzyme B has reinforced these initial predictions and will aid in the design of inhibitors to regulate the activity of granzyme B specifically[39–41].

The first biologically important substrate to be identified for granzyme B was found to be a member of the caspase family[42]. Members of the caspase family are crucial for apoptotic cell death, and they require activation by cleavage at internal aspartate residues. The cleavage of target-cell caspases by granzyme B results in the activation of the cellular apoptotic cascade (FIG. 3). Several caspases are processed by granzyme B *in vitro* and *in vivo* after granule-mediated triggering of cell death. So far, however, only caspase-3 and caspase-8 have been shown to be direct substrates for granzyme B in intact cells[43–45]. In addition, various non-caspase substrates of granzyme B have been identified, many of which have been described as antigens in autoimmune disorders[46–48]. The physiological relevance of cleavage of these proteins is unclear.

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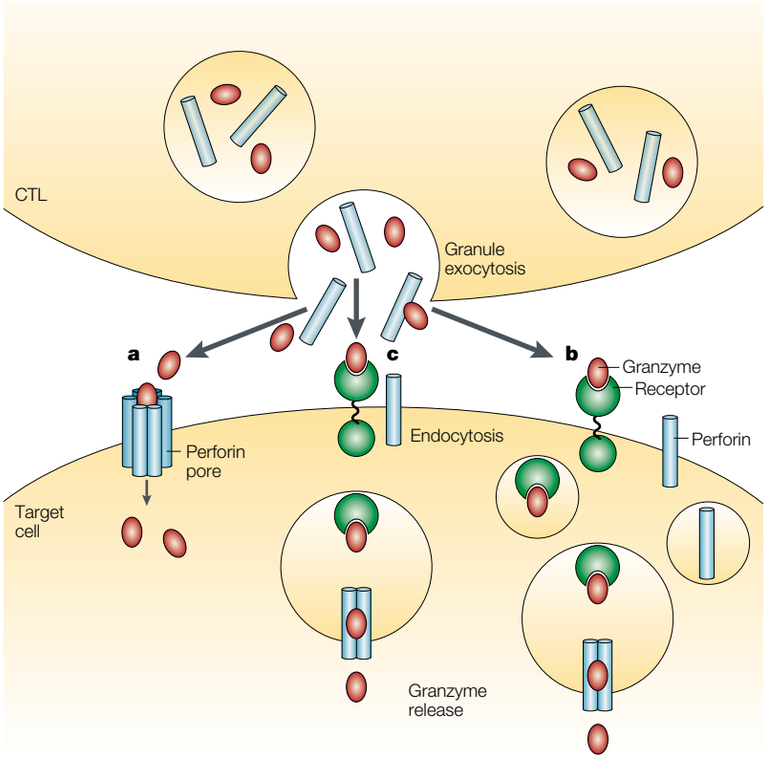

Figure 2 | **Pathways of entry for granzyme B.** On interaction of a cytotoxic T lymphocyte (CTL) with a target cell, there is a directed exocytosis of the CTL granules into the extracellular space between the two cells. **a** | The original view was that perforin polymerized to form a pore in the target-cell membrane through which granzymes could pass. **b** | More recently, the discovery of a receptor for granzyme B has indicated that granzymes might be taken up by receptor-mediated endocytosis and that perforin acts to release granzymes that are sequestered in endosomes into the cytosol of the target cell. **c** | In addition, granzymes might bind to the cell surface such that granzyme uptake is stimulated by perforin-mediated damage to the membrane .

Although granzyme B can process members of the caspase family directly, it has become clear that granzyme B can also initiate caspase-independent pathways in target cells that are crucial for apoptosis. At least one of these pathways requires granzyme-B-dependent signalling through the mitochondria. Mitochondria have a central role in the execution of apoptosis; this involves physiological alterations to mitochondria that include the disruption of electron transport and energy metabolism, the production of reactive oxygen species and the release of pro-apoptotic proteins, such as cytochrome *c*[49]. In addition to the activation of members of the caspase family, granzyme B is responsible for caspase-independent mitochondrial collapse, which results in the release of cytochrome *c*[50,51]. Granzyme-B-mediated release of cytochrome *c* is dependent on the activation of the pro-apoptotic BCL2-family member BID[52–56]. Although the same pathway is used after triggering of the FAS death receptor, granyzme-B-mediated cleavage of BID occurs at a different site from the cleavage that is mediated by caspase-8 in the FAS–FASL pathway[55–57]. In a similar manner to caspase-8-cleaved BID, granzyme-B-activated BID translocates to the mitochondria and activates BAX and BAK — two pro-apoptotic members of the BCL2 family — which results in the release of cytochrome *c*[54,58] (FIG. 3). Although the exact mechanism of cytochrome *c* release is somewhat controversial, both BAX and BAK have been implicated[59,60]. However, granzyme-B-mediated release of cytochrome *c* in Jurkat cells seems to require only BID and BAK, which indicates that the recruitment of BAX is not essential[58]. In some cases, both pro- and anti-apoptotic BCL2-family members associate with components of the mitochondrial permeability transition pore — a multi-protein complex that is found at contact sites between the inner and outer mitochondrial membranes[61–63]. Regulation of the mitochondrial permeability transition pore has been proposed as one model to account for the release of cytochrome *c*[60]. Intriguingly, granzyme B alone has been reported to trigger the opening of the mitochondrial permeability transition pore, which implicates granzyme B in another caspase-independent mode of mitochondrial collapse[52]. Recently, experiments with knockout cells have also indicated that granzyme B can disrupt mitochondria in the absence of BID and BAX[64]. However, the authors of this paper postulate that the action of granzyme B on mitochondria is indirect.

The relative importance of the caspase pathway compared with the BID pathway is unclear. BID seems to be a favoured substrate for granzyme B *in vitro*, but the substrate preference of granzyme B *in vivo* is less certain. However, the overexpression of BCL2 — an anti-apoptotic, mitochondria-localized protein — inhibits the granzyme-B-mediated release of cytochrome *c*[54–56,58,65]. Although the release of cytochrome *c* during granule-mediated apoptosis is inhibited by BCL2, not all cell lines that overexpress BCL2 are protected from granule-mediated apoptosis[66,67]. It might be that for granule-mediated killing, the relative importance of mitochondria varies depending on the cell type. This scenario is reminiscent of the type I/II system that is present in different cell types when death is triggered through FAS[68].

One of the most obvious hallmarks of apoptosis is DNA fragmentation, and this occurs during cell death induced by cytotoxic cells. The induction of rapid DNA fragmentation in target cells is severely impaired when granzyme-B-deficient CTLs are used, which implicates this proteinase in the pathway[69,70]. In target cells, DNA fragmentation can be mediated by at least two endonucleases: endonuclease G, which is released from mitochondria, and DFF40/CAD (DNA fragmentation factor 40/caspase-activated deoxynuclease)[71–73]. CAD is present normally in an inactive state, and activation requires proteolytic cleavage of the inhibitor ICAD that is bound to CAD[71,73]. After induction of the apoptotic cascade, ICAD is proteolytically cleaved and inactivated by caspase-3. Additionally, recent reports have shown that ICAD is a substrate for granzyme B[74–76]. As illustrated in FIG. 3, the cleavage of ICAD by granzyme B results in endonuclease activity[74,75]. Although granzyme B proteolytically cleaves ICAD, the inactivation of ICAD by granzyme B is disputed by one group[76]. The inactivation of ICAD by granzyme B would be a new activity for granyzme B, resulting in the induction of caspase-independent DNA fragmentation[74,75]. Although not

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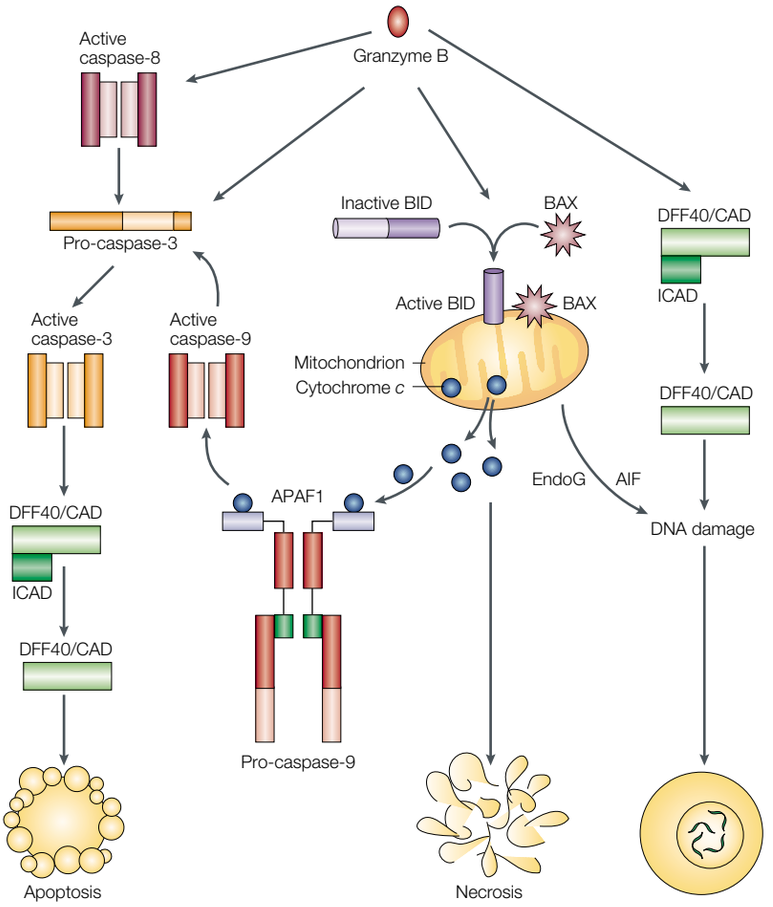

Figure 3 | **Pathways to cell death that are initiated by granzyme B.** Once released into the cytoplasm, granzyme B can initiate apoptotic cell death through the direct cleavage of pro-caspase-3 or, indirectly, through caspase-8. In addition, cleavage of BID results in its translocation, with other members of the pro-apoptotic BCL2-family such as BAX, to the mitochondria. This prompts cytochrome *c* release and the activation of caspase-9 through interaction with the adaptor molecule apoptotic protease-activating factor 1 (APAF1). Alternatively, mitochondrial dysfunction can lead to necrotic death and the release of factors such as apoptosis-inducing factor (AIF) and endonuclease G (EndoG), which mediate caspase-independent cell death. Finally, studies have shown a direct activation of DFF40/CAD (DNA fragmentation 40/caspase-activated deoxynuclease) — which damages DNA and leads to cell death — by granzyme-B-mediated proteolysis of the inhibitor ICAD.

formally proven, it is probable that granzyme-B-mediated induction of the mitochondrial apoptotic pathway will also result in the liberation of mitochondria-localized apoptosis-inducing factor (AIF) and endonuclease G, which will result in caspase-independent DNA fragmentation. This would be another granzyme-B-dependent mechanism to induce cell death.

As we discuss, the ability of granzyme B to mediate cytotoxicity in many ways might enable the immune system to counter the strategies that have been evolved by pathogens to inhibit the death of infected cells. Lymphocytes seem to be protected from gratuitous damage by endogenous granzyme B by the expression of a protease inhibitor, PI9 (REF. 77). Interestingly, this same protein is expressed in some tumour cells and activated dendritic cells, in which it might be responsible for resistance to granzyme-B-mediated apoptosis[78,79].

Multiple granzymes: alternative assassins

So far, a total of 11 granzymes have been found in human and rodent cytolytic cells (TABLE 1). Of this extensive family of granzymes, only four — granzymes A, B, H and K — are present ubiquitously in human cytolytic cells. Of these, granzymes A and B are the most abundant and, at present, the best characterized.

Unlike granzyme B, granzyme A is a tryptic protease that cleaves substrates after lysine or arginine residues. Cytotoxic cells from mice that are deficient in granzyme A have normal cytotoxic responses *in vitro*[80,81], but these mice are no longer able to restrict the neuronal spread of herpes simplex virus and are unable to clear the mousepox virus ectromelia[82,83] (TABLE 2). By contrast, cytotoxic cells that are derived from granzyme-B-deficient mice are markedly attenuated in rapid target-cell DNA fragmentation[69,70]. It should be noted that these cells also lack granzymes C, D, E, F and G, and these might have a role in the phenotype. This defect, however, can be overcome by prolonged incubation times, which indicates that other granule proteins might participate in the induction of DNA fragmentation[69,70]. Mice that are deficient in both granzyme A and granzyme B are severely defective in both early and late DNA fragmentation, which indicates that granyzme A might be responsible[27,84]. In support of this idea, cells that are treated with purified granzyme A in the presence of sublytic doses of perforin undergo rapid cytolysis and have single-stranded DNA breaks that occur independently of caspase activation[85]. In addition, DNA fragmentation by granzyme B is enhanced significantly in the presence of granzyme A[85,86]. Several cellular substrates for granzyme A have been identified, including fibronectin, collagen type IV, thrombin receptor, nucleolin, interleukin-1β (IL-1β), pro-urokinase-type plasminogen activator, the nucleosome assembly protein PHAPII and, most recently, histone H1 and the nuclear lamin A/C[87,88].

The role of granzymes, other than granzymes A and B, in CTL-mediated cell death has yet to be fully explored. Granzyme K, however, has been reported to cause cell death in the presence of perforin[51]. Of the large list of granzymes that are present in rodent and human CTLs, granzyme H is the only one that is found exclusively in human cytolytic cells. Granzyme H has more than 70% amino-acid identity with granzyme B, but it has a distinct chymotrypsin-like substrate specificity[29]. Similar to granzyme B, granzyme H can also enter target cells by a perforin-independent mechanism[29]. At present, however, it is not known if the perforin-independent entry of granzyme H into target cells uses the mannose-6-phosphate receptor. Future efforts will undoubtedly clarify whether other granzymes enter by means of this receptor and will elucidate their importance in cell-mediated attack. Although other granule proteins are certain to be involved in aspects of granule-mediated cell death, the marked impairment of target-cell apoptosis by cytolytic cells from granzyme-B-deficient mice highlights the importance of granzyme B in CTL-dependent apoptosis.

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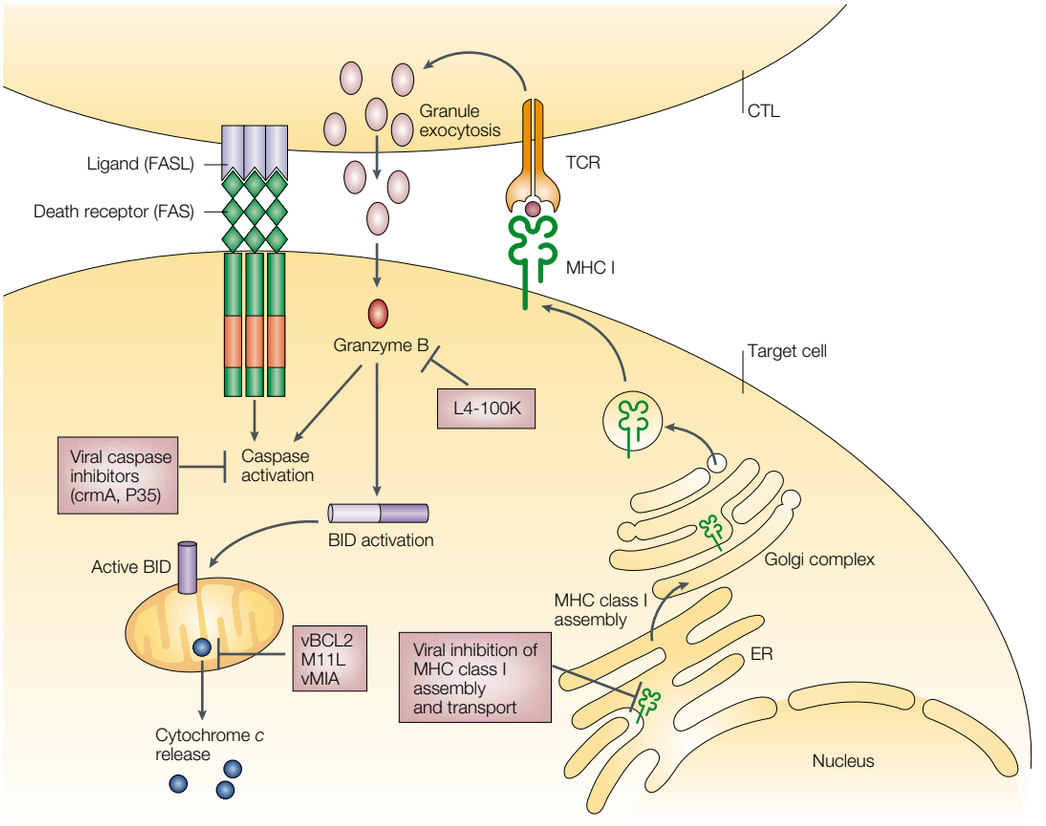

Figure 4 | **Virus-encoded inhibitors of apoptosis and CTL-mediated killing.** Viruses can inhibit CTL-mediated apoptosis and necrosis by interfering with the expression of cell-surface MHC class I molecules. This can occur by means of the endocytosis of cell-surface MHC class I, retention and degradation of MHC class I in the endoplasmic reticulum (ER), or the modulation of the transporter for antigen processing that is necessary for the transport of viral peptides into the ER. Virus-encoded caspase inhibitors, such as crmA and P35, inhibit apoptosis by blocking caspase activity. In addition, virus-encoded BCL2-like proteins (vBCL2) and novel mitochondria-localized proteins, such as M11L from myxoma virus and the immediate-early glycoprotein UL37 (vMIA) from human cytomegalovirus, also inhibit apoptosis by blocking the release of cytochrome *c* from the mitochondria. The L4-100K protein of adenovirus inhibits granzyme B directly. CTL, cytotoxic T lymphocyte; TCR, T-cell receptor.

Viruses fight back

An important function of the cell-mediated immune response is the detection and elimination of pathogen-infected cells. Perhaps, the complexity of cytolytic mechanisms has evolved due to the constant struggle between the immune system and pathogenic organisms. In turn, pathogens have evolved mechanisms to subvert cell death induced by cytolytic cells. This is most evident for viruses. For a virus to replicate and disseminate in a host, manipulation of the immune system is essential, and many excellent examples of how viruses interfere with specific components of the immune system are now known[89].

The presentation of viral peptides in the context of MHC class I is the trigger for the activation of CTLs, their subsequent degranulation and target-cell death. As such, the specific loss of cell-surface MHC class I molecules is used by many viruses to evade CTL-mediated cell death[89,90]. By avoiding viral-peptide presentation by MHC class I molecules, viruses short-circuit CTL activation and exocytosis of the granule components (FIG. 4). Given the extensive list of granule components and the inherent complexity therein, the loss of cell-surface MHC class I can be considered as the ultimate 'upstream' strategy to avoid granule-mediated CTL killing. However, these cells would then become good targets for NK cells and, so, other inhibitory molecules that act downstream in the cell-death pathways would also be expected to be produced by viruses.

To further support replication in the host, viruses encode inhibitors that interfere with specific components of the apoptotic response[89,91]. For example, viruses encode caspase inhibitors — such as the *crmA* gene product from cowpox virus and P35 from baculoviruses — that block caspase activity[92,93], and the presence of *crmA* renders an infected cell resistant to the FAS-mediated branch of CTL killing[93] (FIG. 4). Not surprisingly, viruses have also established mechanisms to modulate the mitochondrial component of the apoptotic pathway, and a large number of viruses encode functional BCL2-like proteins[89,91]. More recently, new virus gene products that act at the mitochondrial checkpoint to inhibit apoptosis, but that lack homology to BCL2, have been identified in human cytomegalovirus and myxoma virus, a

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Box 1 | **Granulysin**

The granules of cytotoxic T lymphocytes (CTLs) contain a second protein, in addition to perforin, that has lytic activity — granulysin. Granulysin has homology to both *Entamoeba histolytica* amoebapores, which are able to form pores in bacterial lipid membranes, and a family of lipid-interacting proteins known as saposins, which are involved in lipid hydrolysis[98,99]. Granulysin has potent anti-microbial activity against many extracellular pathogens, including bacteria, fungi and parasites[98,99]. The combination of perforin and granulysin is also effective against intracellular *Mycobacterium tuberculosis*[100,101]. The treatment of bacteria with granulysin induces lesions on the surface of the bacteria that result in increased permeability and osmotic lysis, which leads to cell death[102]. Granulysin also induces the lysis of target cells, which is accompanied by the induction of cellular apoptosis; this adds another level of complexity to CTL-mediated cell death[103–106].

poxvirus that causes a lethal infection in European rabbits[94,95]. The discovery of new anti-apoptotic viral proteins that act at the level of the mitochondria indicates that further study of viral anti-apoptotic mechanisms might well produce more examples. For example, it is predicted that vaccinia virus — the prototypical member of the poxvirus family — also encodes a new, unidentified mitochondrial apoptotic inhibitor[96]. As shown in FIG. 4, it is predicted that anti-apoptotic viral proteins could interfere with the granzyme-B-mediated release of cytochrome *c* and could regulate caspase activity. Additionally, recent evidence shows that the adenovirus L4-100K protein can inhibit granzyme-B-induced cell death by the direct inhibition of granzyme B[97]. Although the presence of viral apoptotic inhibitors would not be effective against non-apoptotic mechanisms of CTL-mediated killing, it is clear that the induction of apoptosis by CTLs is a main component of target-cell death. In some instances, the presence of virus-encoded apoptosis inhibitors could potentially buy the virus enough time to complete its reproductive cycle and move on.

Concluding remarks

Target-cell death induced by CTLs and NK cells can be initiated by various signals, and the functional redundancy within the range of effector molecules is reassuring. For example, multiple granzymes might act on different substrates, but each has a role in the eventual demise of the cell (BOX 1). Indeed, in normal circumstances, the granzymes probably act together to destroy the target efficiently. Arming cytolytic cells with several effector proteins also allows the immune system to circumvent blocks in the death pathway that are encoded by pathogens. In terms of the therapeutic modulation of cytolytic cells with regard to graft-versus-host disease and transplantation, however, this multiplicity is a formidable problem. To achieve immunosuppression, for example, it will be necessary to inhibit numerous pathways concurrently or to design therapeutic strategies that act at the apex of the killing cascade. In addition, we should examine the strategies that are used by viruses and other pathogens to devise functional therapeutics. The demonstration that perforin-deficient mice have a high rate of spontaneous lymphoma and are compromised in tumour clearance has led to a renewed interest in the role of CTLs/NK cells in immunosurveillance[22,23]. As noted, however, it will be important to repeat these experiments in mice with different genetic backgrounds to rule out any effect of genetic predisposition. Similar experiments in mice that are doubly deficient for granzymes A and B will also be of interest.

Undoubtedly, granzyme B and perforin form an important pathway for CTL-induced killing. It remains to be seen, however, whether the other granzymes are delivered by perforin in a similar manner to granzyme B. Indeed, the generality of the granzyme receptor-mediated endocytosis pathway must be established. It should also be pointed out that despite very persuasive evidence that granzyme B is delivered into the cytoplasm of the target cell, this has not been visualized yet in a CTL–target-cell conjugate.

Once in the cytoplasm, there are numerous potential substrates for granzymes to act on, but very few have been shown to be crucial for the induction of cell death. Cleavage of caspase-3 and BID seems to be paramount, but the relationship between these events is unclear. Whether they depend on each other might be target-cell dependent or might be influenced by the amount of granzyme that is delivered. Many of the experiments that have been used to elucidate the mechanisms of target-cell destruction are *in vitro* studies, and the assays that have been used to evaluate cell death are generally short term. *In vivo*, the 'experimental' set-up might be different: effector-to-target-cell ratios are very low; contact times between effectors and targets might be prolonged or even repetitive; and the delivery of effector proteins might be quite small in amount but concentrated in the immunological synapse. The targets might not proceed to the point of DNA fragmentation, but rather, might be phagocytosed early after caspase activation. We are just beginning to form a molecular picture of this process. What is now needed is experimental systems to investigate the validity of CTL-mediated death models *in vivo*. With the molecular blue-print in hand, we can look forward to more excitement in the next few years.

When a CTL or an NK cell encounters a target cell, it has in its arsenal numerous ways to bring about the demise of the target. Even in the granule-mediated pathway, there are alternatives, short-circuits, redundancies and synergies. The target cell has little hope faced with this onslaught because each individual component has the potential to lead to death. Overall, the emerging picture seems rather complicated, but this complexity is necessary to encompass an effective level of flexibility. To quote Oscar Wilde, "the truth is rarely pure, and never simple".

Note added in proof

While this review was in press, two relevant papers were published in which the reader might be interested. Metkar *et al.*[109] have shown that granzyme B released from CTLs exists in a multimeric complex with the proteoglycan serglycin. Russell and Ley[110] have written a comprehensive review that covers some of the same topics as this article.

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Acknowledgements

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AIF | APAF1 | BAK | BAX | BCL2 | BID | caspase-3 | caspase-8 | caspase-9 | collagen type IV | cytochrome *c* | DFF40/CAD | endonuclease G | FADD | FAS | Fas | FASL | fibronectin | granulysin | granzyme A (human) | granzyme A (mouse) | granzyme B (human) | granzyme B (mouse) | granzyme C | granzyme D | granzyme E | granzyme F | granzyme G | granzyme H | granzyme K | H1 histone | ICAD | IFN-γ | IL-1β | lamin A/C | mannose-6-phosphate receptor | nucleolin | perforin (human) | perforin (mouse) | PHAPII | PI9 | pro-urokinase-type plasminogen activator | thrombin receptor | TNF | TRAIL

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