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Cytotoxic lymphocytes; instigators of dramatic target cell death☆

Nigel J. Waterhouse*,[1], Chris J.P. Clarke[1], Karin A. Sedelies, Michele W. Teng, Joseph A. Trapani*

Peter MacCallum Cancer Centre, Locked Bag 1, A'Beckett St, Melbourne, Vic. 8006, Australia

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Abstract

Most mammalian cells are constantly threatened by viral infection and oncogenic transformation. To maintain healthy function of organs and tissues it is critical that afflicted cells are efficiently detected and removed. Cytotoxic lymphocytes (CL) are chiefly responsible for efficiently seeking out and eliminating damaged or infected cells. It is known that CLs must specifically recognize and bind to their targets, but the molecular events that occur within the target cell that lead to its death are still poorly understood. The two main processes initiated by CLs to induce target cell death are mediated by ligation of surface receptors or release of toxic proteins from secretory granules (granule exocytosis) of the CL. Here we review some of the key findings that have defined our knowledge of the granule exocytosis-mediated pathways to CL-mediated killing and discuss recent insights that challenge conventional views in the important area of CL effector function.

Keywords: Cytotoxic; Lymphocytes; Granzyme; Apoptosis; Mitochondria; Cell death

1. Introduction

Tissues are sculpted from many cells working co-operatively with their neighbours. Throughout life, developmental, homeostatic and immunological mechanisms rigorously regulate the number of cells in any tissue while protecting the body from disease. Each day, many billions of cells are created in the adult and as a consequence many billions of cells must also die. Each cell contains internal monitoring programs that determine whether it has become defective or reached the end of its useful life and these programs are capable of activating pathways that lead to cell death. If this mechanism fails and even one cell evades the death signal and continues to divide, cancer may result. Cell death is also used as a mechanism of host defence against viral pathogens. Viruses enter cells via surface receptors and hijack the cell's metabolic machinery to proliferate, eventually escaping to infect neighbouring cells. Many viruses can spread rapidly and cause widespread tissue damage while some such as Human papilloma viruses have oncogenic poten-

Box 1.

The path of the righteous cell is beset on all sides by those infected by virus and the tyranny of transformed cells. Blessed is the cytotoxic lymphocyte who, in the name of defense and homeostasis, shepherds the weak through the valley of darkness, for he is truly his brother's keeper and the helper of damaged tissue. And I will strike down upon thee with great vengeance and furious anger those who attempt to poison and destroy my tissue. And you will know my name is Immunity when I lay my vengeance upon you. Ezekiel 25:17/Pulp Fiction 1993 (Adapted Draft).

When the original version was recited by Jules in Pulp Fiction, the outcome was deadly. Several shots were fired and the target was killed without mercy. With similar zeal, cytotoxic lymphocytes search out and with a volley of granzymes, destroy any cell that is dangerous

Abbreviations: CL, cytotoxic lymphocyte; MOMP, mitochondrial outer membrane permeabilization; SMAC, second mitochondrial activator of caspases; IAP, inhibitor of apoptosis protein

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* Corresponding authors. Tel.: +61 3 96563725; fax: +61 3 96561411.
E-mail addresses: nigel.waterhouse@petermac.org (N.J. Waterhouse), joe.trapani@petermac.org (J.A. Trapani).

[1] These authors contributed equally to this manuscript.

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to the surrounding cells. Whether viewed as blood thirsty serial killers or hired guns, CL are extremely efficient at their job given the number of cells that can be infected by virus, the rapidity with which virus replicates and the number of cells that die in the body in any given day. How they kill is of some debate, however like Jules they seem to fire multiple shots. Whether all are on target, where they hit or whether all shots are required is unclear.

tial. Therefore the efficient removal of infected cells is a very important process. As the majority of the viral life cycle occurs inside a cell, death of the infected cell is the most efficient, and sometimes the only, way to eliminate viral infections (Box 1).

In the initial stages, host defence against a viral infection is mediated by an 'innate' immune response in which cytokines such as interferon γ or TNFα stem the ability of the virus to proliferate. Natural killer cells, a subset of cytotoxic lymphocytes (CL) (Box 2),

Box 2.

Cytotoxic lymphocytes are a specific group of immune cells with the power to form conjugates with and kill target cells. This group of cells is divided into cytotoxic T lymphocytes (CTL), natural killer cells (NK) and natural killer T (NKT) cells. While their mechanism of detecting damaged cells may be different their mode of killing by granule-mediated exocytosis to date has been thought of as very similar

participate in the innate response and act by detecting and disposing of infected cells. After several days, a more specific 'adaptive' immune response develops in which large numbers of virus-specific cytotoxic T lymphocytes are generated to target and kill the infected cells. These cells proliferate and persist in large numbers until the infection is resolved. Their number decline in response to reduction in antigen burden, however, a subset persist as a long-lived population of 'memory' cells that will provide lasting immunity to that virus.

The immune system can also recognize and destroy cells that have become transformed, a process often referred to as tumour immunosurveillance (supplementary movie 1). The physiological relevence of immunosurveillance has been the subject of a wide-ranging debate over several decades, but recent evidence strongly supports the concept. Organ transplants recipients who are treated with long-term immunosuppressive therapy have a higher relative risk of developing malignancy and this is presumed to be a consequence of the inhibitory effect of immunosuppressive drugs on T cell function [1,2]. Animal models predict that the ability of CLs to kill tumour cells plays an important role in immunosurveillance as loss of key effectors of CL-mediated cell death, such as perforin, increases the incidence of chemically induced and spontaneous tumours [3], and spontaneously arising lymphoma [4].

2. The signature of a serial killer

Several CL subtypes have been defined, including cytotoxic T lymphocytes, natural killer cells, natural killer T cells and γδ T cells. These cells recognize their targets in different ways, however the mechanisms by which these cells kill is believed to be similar. Initially CL were believed to lyse their targets and the terms 'cytolysis' and 'specific-lysis' are still associated with CL killing. This terminology reflects that CL-mediated killing has largely been assessed by measuring the release of radioisotopes from labeled target cells and was also consistent with in vitro experiments demonstrating that CL target cells lose membrane integrity following attack. However, the cellular and molecular events that lead to this endpoint were not characterized. The observation of DNA fragmentation in target cells [5] indicated that the signature of CL killing may be apoptosis, a form of cell death that is morphologically distinct from necrosis or lysis. In the early stages of apoptosis, the target cell shrinks and detaches from the surrounding cells, its chromatin becomes condensed and its membrane ruffled. Finally, the cell is disposed of by phagocytosis [6]. In the absence of phagocytosis, the dying cell progresses to ''secondary necrosis'' and it loses its plasma membrane integrity at this time. Thus, although assays commonly evaluate the effects of ''lysis'', the signature of death appears to be apoptotic (supplementary movie 2).

Apoptosis is orchestrated by a family of proteases called caspases (Box 3),

Box 3.

Caspases are a group of proteases with a preference for cleavage at the C-terminus of certain specific aspartic acid residues. They are present in the cells as inactive zymogens which are activated by cleavage at the C-terminal aspartic acid. The specificity for and activation by cleavage at specific aspartic acid residues suggests that autocatalysis or cross activation in a catalytic cascade are important processes in caspase activation

that cleave specific cellular substrates [7]. CL can induce caspase activation in their targets via one of two distinct contact-dependent mechanisms. Upon detection of a target, the CL forms an immune synapse and ligates death receptors on the target cell. This triggers the formation of a death inducing signaling complex (DISC) which recruits and activates caspases directly [8]. Upon formation of the immune synapse, the CL also polarize their granules to the

site of the synapse and release the granule contents [9]. Granule toxins are taken up by the target cell where they have been shown to potently induce apoptosis [10]. The reason for the existence of two pathways leading to apoptosis is not completely resolved, however some killer cells have been reported to have a distinct preference for one or other pathway. For example, natural killer cells preferentially use the granule exocytosis pathway while $CD8^+$ cytotoxic T lymphocytes can utilize either mechanism. Further, mice deficient in death receptor killing by Fas (lpr) or FasL (gld) have lymphoproliferative disorders suggesting a major role of Fas/FasL in lymphoid homeostasis, whereas mice defective in granule-mediated killing have decreased ability to clear some viruses and diminished tumour surveillance [11]. The mechanism of caspase activation during granule-mediated apoptosis is still controversial and several pathways via which CL induce caspase independent death have also been proposed.

perforin (Table 1). Studies of CLs from gene targeted animals have determined that the only protein absolutely required for granule-induced killing is perforin. Perforin-deficient mice are born and develop normally, however the CL from these mice are not able to kill via granule exocytosis [11]. As a consequence they have increased susceptibility to viral infection and a higher incidence of methylcholanthrene (MCA) induced sarcoma [3]. Consistent with these findings, CLs from a subset of familial hemophagocytic lymphohistiocytosis (FHL) patients who have mutations in the perforin gene are also unable to induce granule-mediated apoptosis [12].

The mechanism by which perforin contributes to the death of a target cell is somewhat controversial (Fig. 1). The structural similarities between perforin and complement component-9 suggest that its function is to form pores in the plasma membrane [13]. Destruction of the plasma membrane can cause cell death, consistent with evidence

Supplementary movie 1	Supplementary movie 2
Peripheral blood mononuclear cells (small rapid), survey HeLa cells (large static) in culture. Images were acquired every 10 s and play at 8 frames per second.	A natural killer cell targets and kills a HeLa cell. Frames were acquired every 1 min and play at 8 frames per second. The target cell is seen to round up, the plasma membrane becomes ruffled and eventually swells until the cell lyses.

3. The MO (modus operandi) of target cell death

The contents of cytotoxic granules have not been comprehensively studied, but include granule specific proteases (granzymes), other proteases and the pore forming protein

supporting a cytolytic mechanism. However, it is now known that if perforin is applied to cells at concentrations that are insufficient to cause lysis, granzymes can enter the cell and elicit a death response [14–18]. Evidence that CL can induce apoptosis and the dramatic synergy between

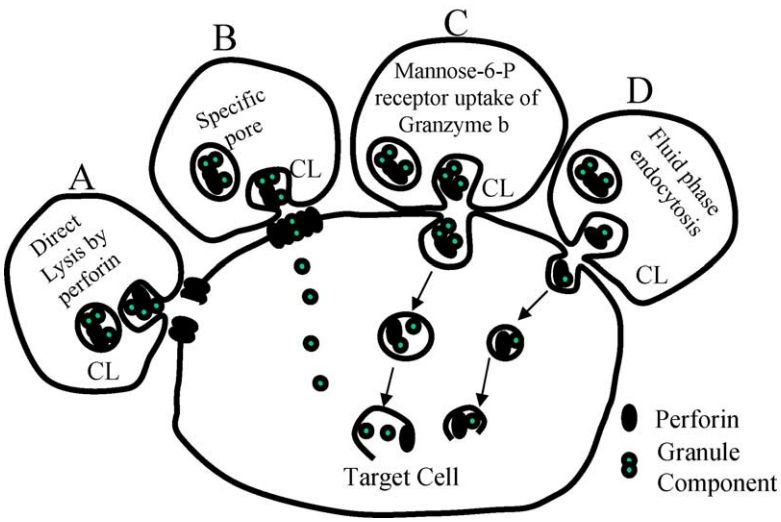

Fig. 1. Models of perforin mediated delivery of granule components. (A) Initial models predicted that perforin formed holes in the plasma membrane of the target cell, essentially inducing target cell lysis. (B) Understanding that target cell death was not lytic, a model was proposed where the pores formed by perforin allowed several of the granule components access to their target substrates within the cell. The concentration of perforin required to synergise with granzyme B to induce target cell death did not form holes of sufficient size to allow dextrans of similar size to granzyme B, entry into the target cell. (C) and (D) A further model predicts that granule components enter the cell by endocytosis (receptor-mediated or fluid phase) and that perforin releases the granule components from the endosomes.

Table 1
Components of human CL granules

Granzymes A, B, H, K, M	*Diverse proteolysis*
Perforin	*Granzyme trafficking*
Leukophysin	*Granule trafficking*
Cathepsin C, D	*Granzyme activation?*
Chondritin sulphate proteoglycans	*Sequestration*
Mannose-6-phosphate receptor	*Granzyme targeting*
Calreticulin	*Calcium binding*
Tia-1, Tia-R	*Stress monitor, mRNA Binding*
Lamp-1, Lamp-2, CD63	*Lysosomal constituent*
Granulysin	*Membrane damage*

perforin and isolated granule components led to the belief that perforin simply facilitates access of apoptosis initiating proteins to the target cell. It has not been possible to determine whether CLs deliver ''lytic'' or ''sub-lytic'' quantities of perforin in vivo, so despite the evidence of synergy between perforin and other granule components, it remains possible that perforin can also act in a lytic manner under specific circumstances.

4. Granzyme B, a silver bullet

Granules of CL contain several granule proteases (granzymes) that can induce death when administered to cells in combination with perforin [14]. Of these, granzyme B is the only agent that has been shown to induce caspase-dependent apoptosis [14,19,20]. Granzyme B is a 32 kDa serine protease with specificity for the C-terminus of certain specific aspartic acid residues. Granzyme B enters the cell by endocytosis, either by binding the mannose-6-phosphate receptor [21], or by constitutive fluid phase uptake [22,23]. Subsequently perforin is thought to release granzyme B from endosomes into the cytosol by an unknown mechanism. Recent evidence suggests that granzyme B is released from the CL as part of a macromolecular complex that also contains perforin and the proteoglcan serglycin [24]. Granzyme B delivered in complex with serglycin did not appear to differ in function to free granzyme B, rather it is believed that the complex may traffic more efficiently into the target cell [24,25]. This area of granzyme B biology is certain to receive closer attention in the future

Once released inside the target cell granzyme B is free to process various intracellular substrates (Fig. 2). The relative importance of these different substrates in propagating the granzyme B-initiated death signal is unclear, but several substrates are directly linked to known apoptosis pathways. A primary candidate is the pro-apoptotic Bcl-2 family member Bid [26–29]. Granzyme B cleaves Bid to generate a 15 kDa truncated protein (tBid), which translocates to the mitochondrial outer membrane where, in conjunction with other Bcl-2 family proteins (e.g. Bax and Bak) it causes mitochondrial outer membrane permeabilization (MOMP). MOMP triggers the release of several pro-apoptotic proteins including cytochrome c and SMAC/DIABLO from the mitochondrial intermembrane space [30]. Cytochrome c initiates the formation of an apoptosome complex that activates the 'initiator' caspase-9, which then proteolytically activates the 'effector' caspase-3 [31,32]. SMAC/DIABLO facilitates caspase activation by deregulating endogenous caspase inhibitors, the inhibitor of apoptosis proteins (IAPs) [33,34]. Consistent with a critical role for MOMP in granzyme B-mediated apoptosis, anti-apoptotic Bcl-2 family members (e.g. BHRF-1 and Bcl-2), which block Bid-induced MOMP, can restore the clonogenic survival of granzyme B-treated

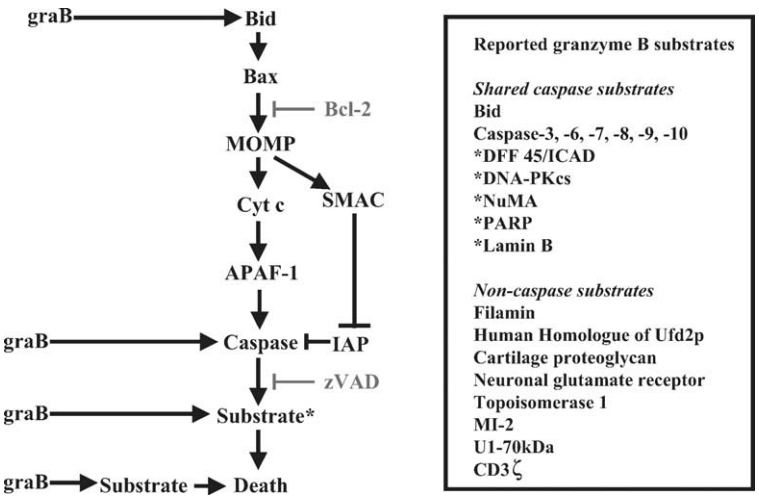

Fig. 2. Pathways to granzyme B-mediated apoptosis. Granzyme B cleaves and activates Bid, which in conjunction with Bax, induces mitochondrial outer membrane permeabilization (MOMP). Cytochrome c, released into the cytosol as a consequence of MOMP, forms a protein complex with apoptotic protease activating factor (APAF-1), which activates caspase-9 and subsequently caspase-3. SMAC, also released into the cytosol as a consequence of MOMP, deregulates inhibitor of apoptosis proteins (IAPs) allowing full caspase activation. Caspases then cleave specific substrates to orchestrate death of the target cell by apoptosis. Granzyme B has also been shown to activate caspases directly and cleave several caspase substrates*. In addition to these caspase substrates, granzyme B has also been shown to cleave several non-caspase substrates (listed).

cells [29,35]. In contrast, Bid deficient cells have been reported to be susceptible to granzyme B-mediated apoptosis by a mechanism that does not involve MOMP suggesting that granzyme B may also target other substrates [36].

It has alternatively been proposed that granzyme B initiates apoptosis by directly activating caspases and that MOMP occurs later, serving only to amplify the death signal [25]. Granzyme B does cleave caspases into their active subunits, albeit in cell free systems [19,20,37], and the caspase inhibitor zVAD-fmk has a protective effect on granzyme B treated cells [28]. Furthermore, granzyme B treated MCF-7 cells, that lack caspase 3, maintained their mitochondrial transmembrane potential, which is an indicator of mitochondrial health [25]. This suggests that granzyme B cannot induce mitochondrial damage in the absence of caspase-3. However, caspase inhibitors do not block cytochrome c release in granzyme B treated cells [28] and do not provide any survival advantage to such cells in long-term assays [38]. This suggests that while caspases are involved in the process of granzyme B induced apoptosis; they are dispensable for the early events that commit the cell to die. The apparent inconsistency of these studies may be explained by the ability of mitochondria to maintain their transmembrane potential after MOMP under conditions where caspase activity is blocked [39]. Together, these data point towards a two-step model with pro-apoptotic Bcl-2 family members being essential for granzyme B-induced MOMP, and subsequently activated caspases being required for the loss of mitochondrial transmembrane potential as has been demonstrated during drug induced apoptosis [40]. This model also stipulates that disruption of the mitochondrial outer membrane is the critical point in the apoptosis pathway and that following this event, cells are committed to die.

Recently, we raised the possibility that both caspases and Bid may both be physiological granzyme B substrates [30]. This hypothesis stemmed from the observation that in granzyme B-treated cells where MOMP is blocked by over-expression of Bcl-2, pro-caspase-3 was nonetheless processed to a p20 subunit, that is rendered functionally inactive by binding to IAPs, resulting in cell survival. Simulation of MOMP by adding SMAC deregulated the IAPs and the p20 subunit was auto-processed to the p17 subunit. Thus, for optimal activation of the effector caspase-3 that has potent activity requires initial processing by granzyme B, followed by auto-processing regulated by MOMP and release of SMAC.

Granzyme B has also been shown to cleave several non-caspase substrates but their importance in triggering death of cells is not yet clear. It has been proposed that the ability of granzyme B to activate the caspase activated DNase (CAD) by cleaving its inhibitor ICAD may circumvent all regulators of apoptosis [41]. This is an attractive hypothesis because it allows for CLs to target cells that are affected by mutations in these regulatory proteins, but it does not explain why over-expression of Bcl-2, which only prevents MOMP, can maintain the proliferative potential of granzyme B-treated cells. As expression of Bcl-2 does not block killing by an intact CL, this paradox may be explained by differences in the quantity of granzyme B delivered by CLs. The amount of granzyme B delivered when a CL meets its target has not been determined. Furthermore, in tissue culture based systems, cells are bathed in a pool of granzyme B and perforin, whereas CL create a discrete synapse and deliver granzyme B in a directed manner. In such circumstances granzyme B may be ''focussed'' to discrete areas of a cell at much higher concentrations than can be achieved in in vitro assays. At such concentrations, granzyme B might cleave a much broader range of substrates, bypass MOMP and evade Bcl-2. Interestingly, expression of granzyme B increases during prolonged T cell activation [42], which means that infected or malignant cells that do not succumb to initial lower doses of granzyme B that rely upon MOMP could subsequently be susceptible to higher concentrations. Alternatively, a target cell may need to be ''hit'' several times before a toxic dose of granzyme B is achieved. Such a mechanism would allow a CL to overcome the resistance of cells conferred by defects in the regulation of apoptosis.

5. A "murder" of granzymes

An alternative explanation for the inability of Bcl-2 to protect cells from CL may be that granule components, other than granzyme B, induce death by MOMP independent mechanisms. This possibility is borne out by observations that CL from granzyme B deficient mice are still effective killers, but the molecular events within their target cells do not involve rapid DNA fragmentation suggesting that these cells die by a mechanism other than classic apoptosis [43]. The most likely candidates to initiate granzyme B independent death signals are the other granzymes expressed by CL. Humans and rodents share four structurally homologous granzyme subgroups (A, B, K and M); granzyme H is specific for human, and a further five granzymes are specifically expressed by rodents (C, D, F, G and L). The relative importance of these enzymes in CL-mediated killing is not known, but granzymes A, C, K and M have been shown to act with perforin in vitro to induce cell death.

Granzyme A is a 65 kDa homodimer with trypsin-like activity, cleaving after basic amino acids [44]. The manner of cell death induced by granzyme A treatment is reminiscent of apoptosis, but, strikingly, it is caspase independent and cannot be regulated by Bcl-2 family members [45]. The process involves rapid loss of cell membrane integrity, single strand DNA nicks and loss of mitochondrial transmembrane potential. Several cellular substrates for granzyme A have been identified including the nucleosome assembly protein SET, the DNA bending protein

HMG-2, the base excision repair enzyme Ape-1, histone H1, nuclear lamins and IL-1β, but surprisingly not caspases [46]. One study has suggested that cleavage of SET by granzyme A releases the tumour suppressor NM23-H1 and this leads to cell death, but the other elements of this pathway have not been established [46,47]. A number of intriguing questions still surround granzyme A-induced cell death: Are there further granzyme A substrates? Which substrates are cleaved by granzyme A in the context of CL-induced cell death and what is their relative importance to the process? How and why does the death program avoid caspase activation? Finally, what is the importance of granzyme A-induced cell death to immune responses? Regardless of the results of such studies, there must be yet other death-inducing proteins within CL granules as mice that are doubly deficient for granzyme A and B still possess functional CLs.

Granzyme C is a 32 kDa serine protease whose substrate specificity is yet to be determined. Similar to granzyme A, granzyme C-induces cell death by a caspase independent pathway. Granzyme C-induced cell death is characterized by loss of plasma membrane integrity, mitochondrial swelling, nuclear condensation and single strand DNA nicking, but little DNA fragmentation. To date, no granzyme C substrates have been reported, however caspases, Bid and ICAD have all been excluded [48]. The ability of antisense to granzyme C to modulate the cytotoxic activity of a T cell clone indicated that it might be a critical effector of CL-induced death. However, granzyme A/B doubly deficient mice also lack granzyme C and as CLs from these mice remain competent killers, it appears that, like the other granzymes, there is a degree of redundancy to its function. Granzyme K has been shown to induce cell death by a mechanism that involves the generation of reactive oxygen species, however this does not appear to involve MOMP or nuclear changes indicative of apoptosis [49]. Most recently, granzyme M has been shown to induce rapid cell death [50]. The exact parameters of granzyme M-induced cell death are unclear, however the death induced is known to be caspase independent, and is not regulated by Bcl-2. Granzyme M is expressed exclusively in natural killer cells suggesting that it is likely to have a more specialized role than other granzymes [51].

Aside from granzymes A, B, C, K and M little is known about the ability of other granule components to synergise with perforin to induce cell death. Even Granzyme H, which is highly homologous to granzyme B (71%) but has a chymotrypsin-like activity, has no known death-inducing function. The best-characterized non-granzyme granule protein that is reported to have cytolytic ability is granulysin, a CL specific member of the saposin-like family of proteins with anti-microbial properties [52]. Granulysin is also reported to kill tumour cells and its crystal structure suggests that it acts by embedding within membranes then breaking them open by a scissor-like action [52]. This model points to a direct lytic mechanism of cell death, but like granzyme B, granulysin-mediated killing of tumour cell targets involves cytochrome c release, is blocked by Bcl-2 and is linked to caspase activation [53]. These observations are characteristic of an apoptotic cell death, but the molecular mechanisms that trigger granulysin-induced apoptosis are unclear. The importance of granulysin in CL killing of mammalian cells remains unclear, as perforin-deficient CLs should remain capable of releasing granulysin yet are unable to kill targets.

6. Epilogue

The mechanism of action of the granule components, their relative contribution to CL-mediated cell death and their importance to immune function are of critical interest. Although several important mediators of cell death and their mechanisms of action have been determined, there is much more to be learned about CL-mediated killing. CL induce apoptosis in target cells and the only granule protein known to induce death in this manner is granzyme B, suggesting that this enzyme must be a key player in CL-induced death. This raises an important question: if granzyme B alone is capable of synergising with perforin to induce apoptosis, then why are there so many different granule proteins that can all induce cell death?

We propose that, in vivo, the other granzymes probably all contribute to deliver an overwhelming death signal, of which the granzyme B pathway is only one part. The effects of other granzymes may only become apparent when the potent granzyme B-mediated signal is removed. It is also possible that different granzymes are expressed in discrete subsets of CL. In this case, removing a granzyme would reduce target cell death simply because the population of CLs expressing this protein would no longer be capable of delivering a death signal. Finally, it is possible that redundancy exists among granzymes to ensure that CLs can still kill target cells that have acquired resistance, either by mutations or expression of viral inhibitors, to a particular death stimulus. Thus, if granzyme B were unsuccessful in eliciting its apoptotic program, the target cell would still be subjected to the effects of other granzymes and death triggered by a different molecular mechanism. It is unlikely that a cell could simultaneously acquire resistance to the diverse range of death pathways that can be induced by the contents of cytolytic granules. There is not yet any proof of this theory, but it is known is that certain pathogens are particularly sensitive to specific granzymes. For example, loss of granzyme A renders animals more sensitive to infection by the pox virus ectromelia [54] and the adenoviral protein L4-100K has been reported to be an efficient inhibitor of granzyme B [55]. Therefore it is possible that the diverse specificity of the granzymes may reflect specific virus activity. To fully understand the cumulative and/or redundant nature of CL-mediated killing a more comprehensive study the

activity of CLs from gene targeted mice must be performed.

In conclusion, delving into CL-mediated killing may be likened to delving into a gangland massacre days after the event. Doubtless death has occurred, but what happened may be difficult to deduce. We are now aware of the chief perpetrators, the weapons of choice have been somewhat defined, but there appear to be more weapons deployed than needed. Was granzyme B the primary bullet, or did the other granzymes play a role? If so, were the other granzymes essential? Would perforin have been sufficient? To date we may have isolated some key players and have even re-enacted some of the murder scene, but until we can identify all the accomplices and define their individual roles it will be difficult to put the case to bed.

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