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Review

The biology of cytotoxic cell granule exocytosis pathway: granzymes have evolved to induce cell death and inflammation

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Abstract

The granule exocytosis pathway of cytotoxic lymphocytes (Tc and NK cells) is critical for control of tumor development and viral infections. Granule-associated perforin and granzymes are key components in Tc cell-mediated function(s). On the basis of studies that showed granzymes A, B, C, K and M, to induce apoptosis *in vitro*, all granzymes were thought to also induce cell death *in vivo*. This review summarizes our present understanding of the biological processes elicited by purified granzyme A and granzyme as well as the processes induced by the more physiologically relevant cytotoxic cells secreting these proteases. The combined evidence supports the concept that the granule secretion pathway is not mono-specific but rather poly-functional including induction of pro-inflammatory cytokines, besides their widely appreciated apoptotic properties.

Keywords: Granule exocytosis; Perforin; Granzymes; Cell death; Inflammation

1. Introduction

Cytotoxic T lymphocytes (Tc cells) and Natural Killer (NK) cells are key components of the host immune system that control infection by intracellular pathogens, primarily viruses and neoplasms. Both cytotoxic effector cells act through either the death receptor pathway (i.e. Fas/FasL) and/or the granule secretory pathway. The latter is a phenomenon unique to mammalian vertebrate biology where biologically active molecules are secreted by the effector cells to penetrate the cytosol of the responding target cell. After engaging a target cell, cytoplasmic granules of the cytotoxic cell migrate toward the contact site (i.e., immunological synapse) and fuse with the plasma membrane. The released granule contents then diffuse toward the target cell [1], where perforin (PFN) facilitates intracellular delivery of granzymes (gzms), encoded in 10 gzm genes in mice (*gzmA, gzmB–G, gzmK, M and N*) and five genes in humans (*gzmA, gzmB, gzmK, gzmH* and *gzmM*; see Table 1) [2].

Although the concerted action of PFN with gzmA and gzmB is critical in the control of viral infections [2–5] their

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Table 1
In vivo granzyme expression found in several cell types.

	Specie	Enzyme-like activity	*In vivo* expression[a]
Granzyme A	Human, mouse	Tryptase	Tc cell, NK, NKT, γδ
Granzyme B	Human, mouse	ASPase	Tc cell, NK, NKT, γδ, MC, CD4, Bas, Mac, DC, Ser
Granzyme C	Mouse	?	?
Granzyme D	Mouse	Chymase	?
Granzyme E	Mouse	Chymase	?
Granzyme F	Mouse	Chymase	?
Granzyme G	Mouse	Chymase	?
Granzyme H	Human	Chymase	NK
Granzyme K	Human, mouse	Tryptase	NK*, NKT, Tc cell, γδ
Granzyme M	Human, mouse	METase	NK, NKT, γδ
Granzyme N	Mouse	?	Sper

[a] Cell type in which the protein has been found *in vivo:* Tc cell: cytotoxic T lymphocyte. NK: Natural Killer cell. NK*: CD56 (bright) NK cell. NKT: Natural Killer T cell. MC: mast cell. CD4: T helper cell. Bas: basophil. Mac: macrophage. γδ: γδ T cell. DC: dendritic cell. Ser: sertoli cells. Sper: spermatocytes. Mem CD8: memory $CD8^+$T cell.

importance in the elimination of tumor cells is less clear [6–9]. Our understanding of the biological roles played by other gzms, termed orphan gzms (gzmC–G, K, L, N and M) is even more rudimentary. What then is the totality of the biological function of the granule secretion pathway and why have so many varied specificities of gzms evolved to become participants [10,11]?

2. Which granzymes are the real cytotoxic executioners?

The original finding that gzms are contained within cytoplasmic granules of Tc or NK cells and are released upon ligation of the Tc receptor led to the concept that gzms may act extracellularly and intracellularly, thereby facilitating diverse processes, such as extracellular matrix degeneration, extravasation, inflammation and cytolysis [12]. However, the important discovery that, in the presence of PRF, gzms, initially gzmA [13] and later on also gzmB [14], induce apoptosis in target cells, focused research on their biological role in cell death and lead to the prediction that all gzms participate in the cytolytic processes mediated by NK/Tc cells. Support for this view has been underpinned by numerous *in vitro* studies [15] in which either purified mouse (Mo) or human (Hu) gzms (A, B, C, H, K and M) were used in combination with PRF from different species (human, mouse, rat) or other delivery agents including adenoviral particles, streptolysin O (SLO) and occasionally listeriolysin [16].

However, the apparent cytotoxicity observed for any purified gzm under *in vitro* conditions should be interpreted with caution since any serine protease is potentially toxic after intracellular delivery [17]. Many variables may influence the outcome of experiments designed to understand the function of individual gzms and are subject to a variety of interpretations. For example, conditions need to be clearly established to mirror physiological conditions and that gzms are effectively yet safely delivered into the targets before death parameters and other biochemical measurements can be meaningfully interpreted [18]. Also of concern are results obtained by using synergizing components from different species in protein- or cell-based cytotoxicity assays. For example, gzm orthologs may have distinct substrate specificities [19–21], which could result in *in vitro* artifacts.

Any putative cytotoxic function identified for a given purified gzm using such experimental systems, needs to be verified at the whole cell level using effector cells designed to selectively secrete the individual protease of interest.

This goal is achieved, at least in part, by using *ex vivo*-derived Tc or NK cells from wild type (wt) and KO mice, which express almost exclusively gzmA and/or gzmB. One possible contaminant being gzmK, which is expressed in both, *in vivo*-derived and *in vitro*-propagated Tc cells [22]. Depending on the *in vitro* conditions, propagated Tc cell lines may also express gzmC–G [7,22–25]. Studies with *ex vivo*-derived Tc cells from gzm KO mice, clearly demonstrated that cell death delivery is markedly impaired in the absence of gzmB [26–28] and even more so when both gzmA and gzmB are absent [27–29]. The observation that all *ex vivo* Tc cells similarly express gzmK, but in the absence of gzmB cytolytic activity is greatly diminished, indicates that gzmB is the main cytotoxic effector molecule whereas gzmA and gzmK are less cytotoxic, or not at all.

Finally, when using *in vitro* cytolytic assays it is very important that multiple parameters such as membrane damage, PS externalization, mitochondrial depolarization, ROS induction and DNA breakage are analyzed by flow cytometry. In addition the proliferation potential of affected target cells (clonogenic assay, thymidine incorporation) needs to be evaluated. Only after cytotoxic capacity of a gzm using isolated protease assays are assessed with relevant cytotoxic cell systems like *ex vivo*-derived virus-immune Tc cells and results are compatible can one assign biological function to individual gzms.

3. Cell death induced by purified gzmA and by Tc cells containing gzmA ($gzmA^+$Tc)

The first report that native mouse gzmA (nMogzmA) is a cytotoxic serine protease was published by the Henkart lab showing that treatment of EL-4 target cells with isolated nMogzmA and native mouse PFN (nMoPFN) caused significant target DNA breakdown [13]. Shortly thereafter, Greenberg and Shi isolated native rat gzmA (nRagzmA) observing that the protease could induce apoptosis (DNA fragmentation) in the presence of native rat PFN (nRaPRF) [14]. At the same time, Shiver et al. reported that transfection of rat basophilic leukemia (RBL) cells with both MogzmA and MoPFN genes endowed the line with the potential to induce target cell DNA breakdown [30]. Studies of the mechanism by which gzmA kills cells remained unknown and awaited the *in vitro* expression and isolation of gzmA.

Lieberman's group, using recombinant HugzmA (rHugzmA) and nRaPFN, described biochemical schemes for

gzmA-mediated cytotoxicity (Fig. 1) [15]. After release into the cytosol of target cells, gzmA enters the mitochondrial matrix as well as the nucleus. Within mitochondria, gzmA processes a complex I mitochondrial protein, NDUFS3, stimulating the generation of reactive oxygen species (ROS). The increased level of ROS then activates cellular detoxification mechanisms including the ER-associated SET complex [15]. The activated SET complex translocates to the nucleus facilitating the repair of the ROS-induced oxidative DNA damage. However, gzmA cleaves several components of the SET complex, leading to the release of the endonuclease, NM23H1, which induces single-stranded DNA damage (nicking), resulting in cell death.

Recent studies however have cast some doubt as to whether isolated Hu- or MogzmA is able to induce cell death under normal physiological conditions. Indications are that when Hu- and MogzmA are used at concentrations previously reported to be cytotoxic (micromolar), the protease induces, in concert with HuPFN or SLO rapid membranolysis [31]. However, if either HugzmA or MogzmA is used under conditions employed to demonstrate the cytolytic potential of gzmB (nanomolar concentrations), cell death was not observed. This was established during multiple short term readouts as well as in long term proliferation assays [31]. Thus, the described biochemical effects attributed to gzmA may have occurred in necrotic cells, which became permeable to the gzm.

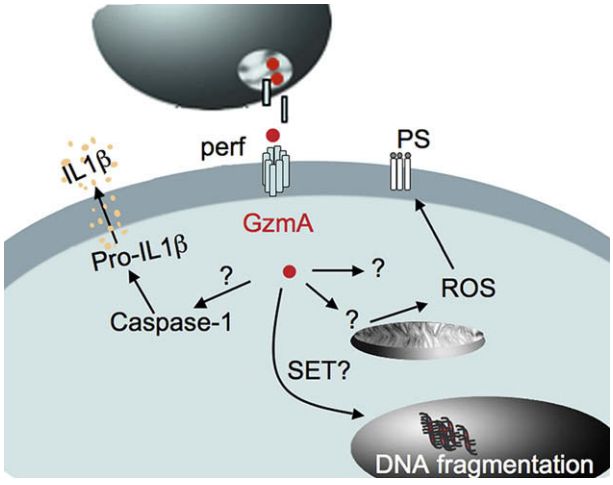

Fig. 1. **The role and mechanism of *ex vivo* Tc cells delivered gzmA on target cells**. Tc cells recognize and bind to target cells (virus infected or transformed cells) and secrete perforin (perf) and granzymes (gzm). Perf facilitates gzm entry into the cytosol of the target cell where it can induce several processes. GzmA can induce two different processes. Left: in macrophages GzmA is able to promote cleavage of Pro-IL1β to generate mature IL1β, which is secreted in the extracellular media. This process depends on caspase-1 activity. By this mean GzmA is able to activate pro-inflammatory signals. In fact absence of GzmA improves mouse survival after LPS challenge. Right: GzmA may induce cell death in some but not all target cells. However, this process may depend on additional molecules present in the Tc cell or the target cell. The mechanism activated by GzmA in susceptible target cells is not clear at present. ROS contribute to some extent to PS translocation, but the role of the SET complex (Ape-1, SET and the endonuclease NM23H1) is still pending of validation by using Tc cells.

Since Hu/MogzmA is generally non-toxic *in vitro*, except under extreme conditions, the protease is unlikely to induce cell death at concentrations mirroring those after secretion by a cytotoxic effector cell. Thus data derived from experiments that examine the cytotoxicity of *ex vivo* immune Tc and NK cells secreting the gzm will be more informative. Analysis of data derived from experiments using *ex vivo* mouse gzmA$^+$Tc cells revealed their potential to induce many of the characteristic signs of cell death, however only in some (EL-4 thymoma or A1.1 and TA3 lymphoma cells) but not other (mouse embryo fibroblasts (MEF), L1210 leukemia cells) target cells, a phenomenon at present not understood [27,28,32] (and J. Pardo and M.M. Simon, unpublished observations).

Importantly however, compared to *ex vivo* mouse gzmB$^+$ and wt Tc cells, the potency of cytotoxicity is low [32] and contrasts with data obtained using purified MogzmA [33]. Furthermore, when *ex vivo* virus-immune Tc cells were used [27,28,32] the pattern of DNA fragmentation observed was oligonucleosomal and not single-stranded DNA nicking, as shown for HugzmA [33]. In addition, cleavage of putative gzmA substrates such as Ape-1, HMGB2, Ku70, histones, and NDUFS3 is not apparent under conditions where gzmA-secreting Tc cells are employed [34–39].

Since purified MogzmA is not cytotoxic, mouse gzmA$^+$Tc-induced killing of specific target cells must depend on either additional molecules present in cytoplasmic granules of the cytotoxic cells (e.g., other gzms and/or cathepsins), or a different quality of Tc-delivered gzmA or on an unusual susceptibility of the target cell to the effector gzmA$^+$Tc cells [28]. The inconsistencies observed between the cytotoxic activity of purified MogzmA and those mediated via gzmA$^+$Tc necessitate re-evaluation of published data with experimental designs in which expression of all gzms beside gzmA is inhibited by siRNA or by gene inactivation.

4. Cell death induced by purified gzmB or by selectively gzmB expressing Tc cells (gzmB$^+$Tc)

The observation that mouse, gzmB deficient, Tc cells failed to induce rapid DNA fragmentation was the first evidence indicating that gzmB is crucial for cell-mediated induction of apoptosis [26]. Since then, studies on the molecular mechanisms of gzmB-mediated apoptosis have employed native and more recently recombinant proteins delivered by PFN derived from various species (human, mouse, rat) or other delivery systems [18,40–42]. However, as mentioned above, the indiscriminate employment of gzmB, PFN and target cells' sources from different species does complicate data analysis.

The present consensus view on Hu/MogzmB-initiated apoptosis is a two pathway mechanism (Fig. 2): gzmB either directly activates the executioner caspases (-3 and -7) [43–45]; or caspase activation is initiated indirectly through mitochondrial permeabilization promoted by gzmB-mediated cleavage of Bid [46–50]. In the latter case, activated tBid induces oligomerization of Bak/Bax at the outer mitochondrial membrane leading to cytochrome c release and the assembly of the apoptosome, with subsequent activation of caspase 9 followed by

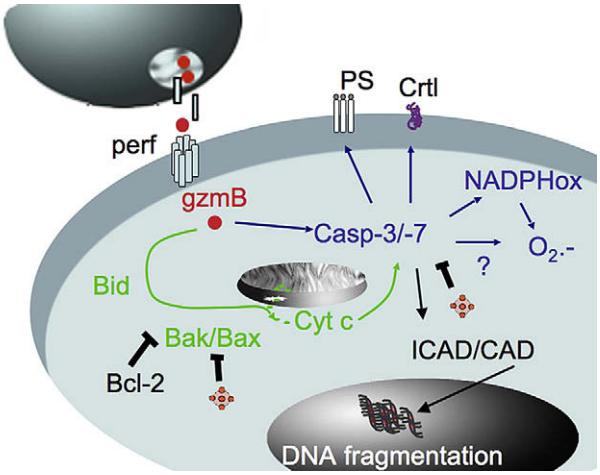

Fig. 2. **The role and mechanism of *ex vivo* Tc cells delivered-gzmB on target cells**. GzmB induces apoptotic cell death by two different pathways. Direct caspase-3 or caspase-7 activation is critical to induce PS and calreticulin (Crtl) translocation, ROS production and contributes to mitochondrial depolarization. Bid cleavage generates the active tBid form that induces Bak and/or Bax activation and promotes cytochrome c (cyt c) release from the mitochondria and subsequently caspase activation (by apoptosome formation). In addition, gzmB is able to induce ROS production outside of the mitochondria by a caspase dependent activation of NADPH oxidase(s) (Aguiló et al., unpublished observations). By blocking simultaneously caspase-3/-7 and Bak/Bax all apoptotic features are inhibited but gzmB is still able to kill the cells by an unknown mechanism. The pleiotropy of gzmB-induced cell death is likely due to viral pressure during evolution, since viruses are able to interfere at different levels of the apoptotic machinery.

caspases 3 and 7. Interestingly, purified HugzmB and MogzmB were reported to have a strikingly different substrate preference for Bid and caspase-3 respectively [19–21]. Therefore in mice gzmB is suggested to initiate death mainly through caspase-3 (s.a. below for gzmB$^+$Tc cell) while HugzmB gains the capacity to cleave Bid if counter-regulatory mechanisms dampen direct caspase-3 activation.

To evaluate if the death pathway(s) induced by purified gzms are the same or similar to those induced by *ex vivo*-derived mouse gzmB$^+$Tc cells (i.e., gzmA$^{-/-}$ Tc cells) experiments were performed using target cells (MEFs) with defects in either the effector caspases (MEF.casp3 × 7$^{-/-}$) or mitochondrial proteins (MEF.Bid−/−, MEF.Bak/Bax−/−) and cell death was monitored by flow based annexin reactivity, mitochondrial depolarization, ROS generation and proliferation potential [22]. GzmB$^+$Tc cells triggered Bid/Bak/Bax deficient targets to undergo cell death, associated with caspase-3/-7 dependent PS translocation, $\Delta\Psi_m$ loss and ROS production. On the other hand, in targets lacking caspase-3 and caspase-7, gzmB$^+$Tc-mediated cell death was accompanied by processing of Bid, leading to $\Delta\Psi_m$ loss and cytochrome c release, consistent with results obtained with HugzmB [51]. Blockade of both death pathways prevented PS translocation mitochondrial depolarization and ROS production, but not cell death [22].

The finding that mouse *ex vivo* LCMV-immune [22,45] as well as *in vitro*-propagated alloreactive Tc [22,45] are able to induce, via gzmB, both pathways suggests that the differential proteolytic activity observed with purified MogzmB [19–21] does not reflect its actual potential, when delivered by intact Tc/NK cells. The fact that neither inhibition of Bcl-2 nor of caspases alone prevented cell death elicited by human NK cell-derived gzmB [52], extend this interpretation to the human system and suggests that both, human and mouse Tc/NK cells are able to independently activate both, the mitochondrial and the caspase death pathways.

Taking all the data into consideration [22,28,32,52–54] the following scheme can be proposed (Fig. 2): Depending on the concentration of gzmB delivered intracellularly, the quantities of substrates and counter-regulatory processes which may suppress apoptosis (Smac/Diablo), gzmB does initiate cell death by engaging different facets of the death program: one depends on mitochondrial Bid/Bak/Bax regulated cytochrome c release followed by caspase activation and the other involves direct activation of the executioner caspases. Finally, if this bi-functional death pathway is completely blocked, gzmB retains the capacity to kill the target cell by a yet undefined additional pathway [22].

This plasticity of mouse and most probably also human gzmB$^+$Tc to induce target cell apoptosis is most likely due to evolutionary pressures exerted by pathogens attempting to evade immune elimination. One example being the orthopoxviruses, which encode serine protease inhibitors that severely curtail FasL/Fas-mediated cell death. Thus mice deficient in PFN and/or gzmA × B are unable to control mouse pox, ectromelia (ECTV) [55]. Recent observations by us, that the ECTV is unable to inhibit apoptosis induced by gzmB$^+$Tc in competent cells, support this interpretation (Pardo et al., unpublished results). However, ECTV does block gzmB$^+$Tc-mediated apoptosis in caspase 3 × 7 or Bak × Bax deficient cells pointing to the importance of having multiple proapoptotic pathways activated by gzmB to combat viral infections and poxviruses in particular.

5. Cell death induced by gzms other than A and B

Although purified gzmC, gzmK, gzmH and gzmM were reported to induce cell death when delivered by a variety of agents, such as streptolysin O, perforin or liposomes (ProJect) their contribution to Tc/NK cell-mediated protective and/or inflammatory processes is not known (Fig. 3).

5.1. GzmC

Purified mouse gzmC was reported to induce cell death, accompanied by some (PS translocation, chromatin condensation and $\Delta\Psi_m$ loss) but not other apoptotic features (oligonucleosomal DNA fragmentation, caspase activation, Bid cleavage or cytochrome c release) [56].

The expression of gzmC protein has not been demonstrated *in vivo*. However the finding that a) *ex vivo* Tc cells from intravenously but not intraperitoneally LCMV infected mice express gzmC transcripts [22,57], b) *in vitro* generated Tc cells from CatC−/− mice (mice deficient in the granzyme processing enzyme, dipeptidyl peptidase I) express gzmC after

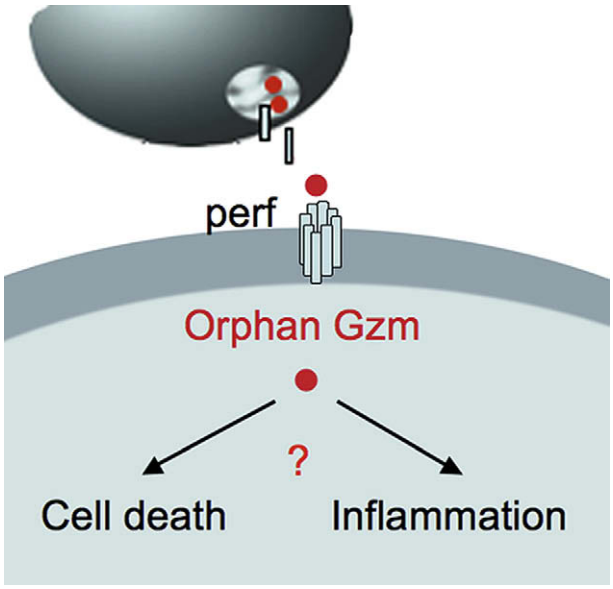

Fig. 3. **The role and mechanism of *ex vivo* Tc cells delivered-orphan gzms on target cells**. Nothing is known about the mechanism and consequences of *ex vivo* Tc cell-derived orphan gzms with *in vitro* cytotoxic potential (gzmC, gzmK, gzmH and gzmM). They may promote cell death, pro-inflammatory signals or other processes, but neither of them has been shown yet by using Tc or NK cells.

secondary activation [25], the blocking of which via RNAi partially rescued cell death elicited by these Tc cells, and c) mice deficient in gzmB, gzmC and gzmF are more resistant to tumor challenge than those deficient in gzmB alone [7], suggests that gzmC may contribute to Tc/NK cell-mediated proapoptotic processes *in vivo*. However, since the protein has not been found *in vivo*, it still remains speculative if it plays a role during cell death induced by killer cells.

5.2. GzmH

Contradictory results have been reported for the proapoptotic potential of gzmH. Cell death induced by purified human gzmH was shown in one study to be caspase independent and accompanied by PS translocation, $\Delta\Psi_m$ loss, ROS generation, DNA degradation, and chromatin condensation, but not Bid or ICAD cleavage nor cytochrome c release [58]. The other report showed Bid and ICAD cleavage, cytochrome c release and caspase dependency of apoptosis [59].

Although gzmH is strongly expressed by human NK cells, its involvement in cytotoxic processes is questionable. Independent studies have shown that inhibition of gzmB in gzmH positive human NK cells leads to nearly total abolition of their cytotoxic potential [52,60,61].

5.3. GzmK

Contradictory reports also exist for the cytotoxic potential of HugzmK [62,63]. One study suggests that HugzmK induces processes, similar to HugzmA, including SET and Ape-1 cleavage and NM23H1 nuclear translocation but no caspase-3 activation [63]. The same group presented more recent data, indicating that gzmK induces Bid cleavage and cytochrome c release [62]. It is possible that caspase-3 was not activated even when cytochrome c was released from mitochondria due to the presence of inhibiting IAPs in the target cell [64,65]. However, caspase-3 was readily activated by gzmB under similar conditions [62,63].

Although gzmK is expressed by *ex vivo* LCMV-immune Tc cells, independent of their gzmA and/or gzmB expression [22,66,67], its biological function remains elusive.

5.4. GzmM

Purified human gzmM was reported to induce cell death in Jurkat cells with ROS production, caspase activation, ICAD cleavage and DNA fragmentation [68,69] or in the absence of any apoptotic feature [70,71]. Further more, purified human gzmM was found to cleave alpha-tubulin, thereby interfering with the integrity of the cytoskeleton [72]. As with gzmH, gzmM seems to be dispensable for NK cell induced cell death processes. However, the finding that gzmM deficient mice are slightly more susceptible to MCMV infection, but not ECTV infections, than wt mice suggests a possible role of this protease in the control of some viruses but not others [73].

6. GzmA and gzmA$^+$Tc/NK cells induce pro-inflammatory cytokines

As outlined above gzmA *per se* does not appear to induce cytotoxic effects after intracellular delivery into target cells thus leaving the question as to its biological function during an immune response open. Some clues as to that function has emerged recently from evidence which suggests that Hu/MogzmA may function as an inducer of pro-inflammatory cytokines [31]. Purified HugzmA as well as human NK cells selectively secreting gzmA induced human monocytes to secrete IL-1 and TNF in a caspase-1 dependent manner. Equally important, MogzmA and mouse gzmA$^+$Tc cells induce IL1β release from primary mouse macrophages and gzmA KO mice were found to resist LPS-induced toxicity [31]. Thus the granule secretory pathway does appear to play an already previous suggested role in inflammation with gzmA acting as an endogenous modulator [12,74–76]

7. Conclusions and future perspectives

Overall data gathered from studies using *ex vivo*-derived Tc cells, rather than from experiments using purified protease preparations, indicate that apoptosis induced by the granule exocytosis pathway is critically dependent on PFN and gzmB. Although purified orphan gzms have been shown to induce cell death *in vitro*, the biological significance of these findings has still to be elucidated. Furthermore, it is now becoming clear that the granule secretion pathway is not uniquely focused on the elimination of target cells but instead, gzmA and perhaps some or all of the orphan gzms may contribute to

host defense through the induction of Tc/NK-mediated pro-inflammatory cytokines. Further studies are required to elucidate the molecular basis and biological relevance of these phenomena. Finally, it is becoming more evident that other, non-cytotoxic functions, of gzms [83] have evolved in order to combat viruses [77,78] or regulate extracellular matrix dependent processes like atherosclerosis, anoikis or extravasation [79–82].

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