Beyond target cell death – granzyme serine proteases in health and disease

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Abstract

Granzymes are a family of small (~32kDa) serine proteases with a range of substrate specificities that are stored in, and released from, the cytoplasmic secretory vesicles ('granules') of cytotoxic T lymphocytes and natural killer cells. Granzymes are not digestive proteases but finely tuned processing enzymes that target their substrates in specific ways to activate various signalling pathways, or to inactivate viral proteins and other targets. Great emphasis has been placed on studying the pro-apoptotic functions of granzymes, which largely depend on their synergy with the pore-forming protein perforin, on which they rely for penetration into the target cell cytosol to access their substrates. While a critical role for granzyme B in target cell apoptosis is undisputed, both it and the remaining granzymes also influence a variety of other biological processes (including important immunoregulatory functions), which are discussed in this review. This includes the targeting of many extracellular as well as intracellular substrates, and can also lead to deleterious outcomes for the host if granzyme expression or function are dysregulated or abrogated. A final important consideration is that granzyme repertoire, biochemistry and function vary considerably across species, probably resulting from the pressures applied by viruses and other pathogens across evolutionary time. This has implications for the interpretation of granzyme function in preclinical models of disease.

1. Introduction

A key feature of the immune system is the capacity to selectively identify and eliminate cells that are either pathogen infected or malignantly transformed. The cellular effectors that mediate immune killing are collectively referred to as cytotoxic lymphocytes, and they comprise Cytotoxic T lymphocytes (CTL) and natural killer (NK) cells. When appropriately triggered by either their specific T cell receptor in the case of CTL, or via the appropriate cell surface receptors in the case of NK cells, cytotoxic lymphocytes form a synapse with their target cell and initiate target killing. Target cell death is typically triggered by two main pathways: ligation of tumour necrosis factor receptor (TNFR) family receptors (such as Fas) (reviewed in (Trapani, 1998)), and perforin/granzyme-dependent killing via granule exocytosis. Granule exocytosis-dependent killing in particular plays critical and non-redundant roles in target cell death in a range of contexts spanning from pathogen control to tumour cell killing.

In this review, we will examine the biology of granzymes, a family of cytotoxic serine proteases that mediate granule exocytosis-dependent killing. We will explore the myriad of cytotoxic and non-cytotoxic functions of granzymes, and examine how alterations in granzyme biology could contribute to human disease.

2. The granule exocytosis mechanism and molecular mediators of target cell death

A central feature of cytotoxic lymphocytes is that they possess specialised cytoplasmic secretory vesicles, commonly known as ‘granules’ because of their electron-dense core, whose exocytosis mediates target cell elimination following the formation of a stable immune synapse between the killer cell and its target. Granules first form during the differentiation of both T cells and NK cells into cytotoxic effector cells. While the pathways controlling expression of various granule contents have been extensively studied (Pipkin et al., 2010), the exact transcriptional pathways that lead to formation of the secretory granules themselves remain poorly characterised. These lysosome-like secretory vesicles combine both typical lysosomal degradative proteases (largely, members of the cysteine cathepsin family) and the safe storage of more specialised toxins that will ultimately be released *via* exocytosis into the immune synapse. The two key types of toxin present in granules are granzymes that possess a variety of substrate specificities, and a unique pore-forming protein, perforin. Once cognate

target cell recognition occurs, calcium signalling in the CTL/NK cell prompts granule mobilisation along the microtubular apparatus to the site of cell-cell contact ('polarisation') (Pores-Fernando and Zweifach, 2009). Following granule membrane fusion with the plasma membrane, the granule contents are released into the immune synapse; their subsequent diffusion across the intercellular space results in target cell apoptosis that typically occurs within minutes (Lopez et al., 2013; Sutton et al., 2000). Detachment of the killer cell from the target then enables the cytotoxic lymphocyte to 'recycle' to other target cells nearby, a process colloquially known as 'serial killing' (Jenkins et al., 2015).

Perforin and granzymes synergise in a unique way to bring about target cell death. Upon their release, monomers of perforin bind extracellular calcium at their membrane-proximal C2 domain, undergo a radical change in tertiary structure that endows the C2 domain with high affinity for plasma membrane lipids, and ultimately enables them to coalesce into ring-shaped transmembrane pores with 24-fold symmetry and an internal diameter of 18 nm. Though repaired within 60-90 seconds by the target cell, this transient breach of the membrane is sufficient for far smaller granzyme molecules (4 nm) to enter the target cell cytosol by passive diffusion (Law et al., 2010; Leung et al., 2017; Lopez et al., 2013). We strongly believe that simple passive diffusion accounts for granzyme passage across the target cell membrane, however an alternative possibility proposed by others is that perforin pores are internalised into the target cell in endosome-like structures formed during membrane repair, and granzyme escape from this compartment is due to perforin's disruptive actions on the endosomal membrane (Shi et al., 1997; Thiery et al., 2011). In any event, there is consensus that once in the cytosol, granzyme proteolysis activates various non-redundant apoptotic pathways that trigger the rapid death of the target cell.

3. Granzyme repertoire, substrate specificity and cross-species variation

While a large number of granzyme genes exist, the most important pro-apoptotic granzymes are granzyme B (GzmB) and, to a lesser extent, granzyme A (GzmA), whose cytotoxic activity is far weaker, particularly in humans (Kaiserman et al., 2006; Susanto et al., 2013). GzmB's potency is related to its unusual substrate specificity – it is the only mammalian serine protease that processes substrates specifically after a

P1 acidic residue, almost invariably Asp. This substrate preference closely mimics that of pro-apoptotic cysteine proteases (caspases), which are constitutively expressed as zymogens in virtually every cell type. As pro-caspases are activated via processing after selected Asp residues, pro-caspases are key substrates for GzmB, and much of the target cell damage caused by GzmB is mediated by effector caspases 3 and 7 (Sutton et al., 2000; Sutton et al., 2003).

Three analogous genetic loci encode granzyme genes in both humans and mice, but granzyme repertoire varies with species (Smyth et al., 1996) (Table 1). The *GzmB* gene is linked in both species to granzyme genes that encode chymotrypsin-like ('chymase') substrate preference. Mice have 5 such genes (Gzms C-G, all tightly linked), but only a single human orthologue exists, GzmH. Both species have two tightly linked genes for granzymes with trypsin-like specificity ('tryptase'; cleavage after basic residues Lys or Arg), GzmA and GzmK. A third locus encodes GzmM, (Met-ase) which is also common to both species and cleaves after long-chained unbranched amino acids at the P1 position (Met, Leu). In both humans and mice, Cathepsin G, a serine protease expressed in myeloid cells but not in T or NK cells, is encoded within a locus that houses multiple granzyme genes, indicating that serine protease genes expressed in various types of immune cell have co-evolved over hundreds of millions of years. In humans, the gene encoding CatG is located between those for GzmB and its structurally close homologue, GzmH, which arose from fusion of the 5' end of the CatG gene and the final three 3' exons of the GzmB gene (Haddad et al., 1991).

Immunologists and other researchers often do not appreciate that the granzyme repertoire pertinent to a given species needs to be considered when designing experiments on viral or tumour immunity. Even closely related species such as mouse and rat have different numbers of *Gzm* genes, and some granzymes have multiple alloforms. Only granzymes B, A, K and M are common to mice, rats and humans, suggesting they serve important roles. It is likely that evolutionary pressures imposed by viruses specific for each species have shaped granzyme repertoire, as viruses and their host typically engage in a 'tit for tat' battle for survival over eons of evolutionary time. This theme will be taken up in some detail below. Evidence for this hypothesis also comes from the fact that multiple alloforms exist for some granzymes, especially

mouse GzmB. We previously identified 13 distinct *Gzmb* alleles in outbred *M. musculus musculus* (Thia and Trapani, 2007), several of which encoded amino acid changes that alter protease specificity sufficiently to alter the peptidome of cells infected with murine cytomegalovirus (MCMV), and drastically affect survival after *in vivo* infection with a pathogenic MCMV strain (Andoniou et al., 2014) . A 'founder effect' is probably responsible for the fact that all inbred mouse strains are homozygous for a single GzmB allele (Thia and Trapani, 2007). Several human GzmB alleles have also been identified, but they do not impinge on the substrate-binding pocket, and so do not affect proteolytic or cytotoxic activity (Sun et al., 2004).

As a related observation, human and mouse GzmB vary somewhat in their preference for amino acid residues flanking the P1 Asp, so that human GzmB is relatively less efficient at direct pro-caspase processing than mouse GzmB, but 100-fold more efficient at processing pro-apoptotic Bid to truncated (t) Bid, which then activates Bax/Bak and death signalling via the mitochondrial pathway (Cullen et al., 2007; Kaiserman et al., 2006). Consistent with this finding, human GzmB-mediated cell death is blocked by Bcl-2 overexpression in the target cell, while that induced by its mouse counterpart is not (Sutton et al., 1997). Physiological serine protease inhibitors (serpins) important in preventing inadvertent cell death due to GzmB therefore also have species-specific pseudo-substrate cleavage sites in their reactive site loops, and interestingly this commonly results in a P1 Glu instead of Asp, which confers inhibitory activity against GzmB but not caspases (Bird et al., 1998).

Species differences have also been noted for the cytotoxicity of granzyme A, as careful quantitative and kinetic analyses indicate that while the mouse ortholog clearly has some cytotoxic activity, that of its human counterpart is far weaker (Kaiserman et al., 2006). Two distinct signalling pathways have been proposed for granzyme A-induced cell death, both of which do not require caspase activation. One operates through proteolytic processing of members of the nuclear SET complex that triggers DNA nicking (Martinvalet et al., 2005), and the other through cytoskeletal disruption that triggers a form of cell death known as athetosis (Susanto et al., 2013).

More recently, granzyme-mediated cleavage of a pore-forming family of proteins called gasdermins has been implicated in granzyme-dependent induction of a non-

apoptotic and inflammatory form of cell death called pyroptosis. GzmB has been specifically implicated in pyroptosis induction via cleavage of gasdermin E (Liu et al., 2020; Zhou et al., 2020), while GzmA can trigger pyroptosis by gasdermin B cleavage (Zhou et al., 2020). Interestingly, gasdermin cleavage specificity appears to be conserved between mouse and human Gzms A and B (Liu et al., 2020; Zhang et al., 2020; Zhou et al., 2020). Gasdermins are not universally expressed, with evidence of tissue-specific expression and/or induction upon certain stimuli (eg. IFNγ can induce gasdermin B expression (Zhou et al., 2020)). Thus, this form of granzyme-mediated cytotoxicity is restricted to cells that express their specific gasdermin substrates. Nevertheless, as many tumours harbour inactivating mutations in gasdermin E (Zhang et al., 2020), this pathway may be important in the context of tumour immune surveillance.

4. Transcriptional control of granzyme expression

4.1. Cell-specific expression of granzymes

Granzymes are expressed predominantly by CTL and NK cells, although other cells such as mast cells (Rönnberg et al., 2014), testicular Sertoli cells (Hirst et al., 2001) and skin keratinocytes have also been reported to express them, usually at the mRNA level (Berthou et al., 1997; Hernandez-Pigeon et al., 2006). Granzyme N is also expressed only in the testis (Takano et al., 2004). In these latter cases, the functional relevance of granzyme expression is not always clear. Typically, granzyme expression needs to be induced, usually in response to viral infection, however granzyme A levels are constitutively high in some mouse and human NK cells (Sedelies et al., 2004). In all CTLs and most NK cell subtypes, granzyme expression and secretory vesicle synthesis are switched on only upon cell activation (Liu et al., 1989; Trapani et al., 1988). These processes typically take 24-72 hours, depending on the nature of the stimulus and which granzyme is being considered; GzmB is usually switched on a day or so earlier than GzmA. Indeed, using a fluorescent chimeric protein in mice, OT-I T cells showed high GzmB expression as early as 40 hours after primary, and 14 hours after secondary responses to *Listeria*-OVA infection *in vivo* (Mouchacca et al., 2015). Similarly, GzmB expression was evident within 60 hours of activation in a vaccination model (Parish et al., 2009), and within the first 2 days of both influenza and acute

LCMV infection (Bannard et al., 2009; Kakaradov et al., 2017). However, consistent with the idea that GzmA induction is more delayed, for influenza A (IAV) infection in mice, it was shown that GzmB is expressed earlier at the mRNA level than GzmA (Jenkins et al., 2008), with heterogeneous GzmA and B expression seen at the protein level. GzmA+ B+ CTLs predominate early after IAV infection (day 6), whereas at peak (day 8-9) and later during infection (day 14-17) GzmA- B+ CTLs become the major population (Moffat et al., 2009). Varying GzmA expression levels were further found for CTLs with different IAV-specificity (Moffat et al., 2009). In contrast to productive activation, one of the hallmarks of $CD8^+$ T cells that undergo tolerance induction is impaired upregulation, or even active downregulation, of GzmA and GzmB expression (Parish et al., 2009).

Within effector and memory T cell populations that form and persist later during the immune response, additional variability in granzyme expression has been observed. As a general rule, GzmB (and to a lesser extent GzmA) expression are most elevated within more differentiated effector and memory cell subsets. This expression pattern is evident as early as the first division, where GzmB expression is elevated in more effector-like cells, and persists into memory, where GzmA and GzmB expression are typically elevated within effector memory and resident memory cells, but largely absent within the less differentiated central memory cells (Arsenio et al., 2014; Kakaradov et al., 2017; Kurd Nadia et al., 2020). Nevertheless, lineage tracing studies have shown that at least a portion of central memory cells express GzmB during the effector phase prior to losing expression during memory differentiation (Bannard et al., 2009; Jacob and Baltimore, 1999), although there may still remain stem-like memory subsets that emerge early during the immune response and never express GzmB (Johnnidis Jonathan et al., 2021; Pais Ferreira et al., 2020). Similarly, within exhausted T cells, less differentiated progenitor cells typically lack GzmB and GzmA expression while more terminally exhausted cells express both granzymes (Im et al., 2016; Leong et al., 2016; Utzschneider et al., 2016; Zheng et al., 2021).

Expression of granzymes during differentiation beyond Gzms A and B is poorly studied, in part due to the lack of good antibodies to study protein expression. GzmK was recently identified as a biomarker of exhausted $CD8^+$ T cells both within chronic viral infection and within the exhausted T cells that accumulate with age (Mogilenko et

al., 2021; Sandu et al., 2020). Similar to Gzms A and B, GzmK is elevated within more terminally exhausted T cells (Chen et al., 2021; Kanev et al., 2019). GzmK is less well studied in the context of acute infection, but the ImmGen expression database (www.immgen.org) reveals that during acute infection GzmK largely parallels GzmB expression within $CD8^+$ T cells, although expression is better retained within central memory T cells (in line with (Jenkins et al., 2007)) and less highly expressed within NK cells than GzmB. There are limited studies examining GzmK expression in tissue resident memory cells, but there is evidence of GzmK expression within gut resident cells (Kurd Nadia et al., 2020). Despite often being considered an NK-restricted granzyme, GzmM is also expressed in $CD8^+$ T cells, although it has a reciprocal expression pattern to most of the other granzymes as it is depleted from resident memory and exhausted cells, but enriched within central and effector memory cells (Beura et al., 2018; Hayward et al., 2020; Pan et al., 2017; Pritykin et al., 2021). In fact, unlike other granzymes, GzmM expression increases as cells differentiate into circulating memory cells (www.immgen.org). Beyond these granzymes, only GzmC is detected as largely lowly expressed in a pattern similar to GzmB, with Gzms D, E, F, G and N not detected in mouse T cells and NK cells (www.immgen.org) (Jenkins et al., 2007). Further work is needed to confirm to what degree these expression patterns are conserved in humans, although GzmK expression also appears to be a conserved marker of exhaustion in humans (Zheng et al., 2021), while the human specific granzyme, GzmH, has expression patterns that are interestingly discordant with GzmB in both NK and T cells despite close gene linkage (Sedelies et al., 2004).

4.2. Transcriptional control of granzyme expression

Beyond GzmB, surprisingly little is known regarding the transcriptional control of granzyme expression. A range of key transcription factors linked to $CD8^+$ T cell and NK cell differentiation are known to directly control expression of the *Gzmb* gene. The T-box transcription factors (TFs) T-bet (encoded by *Tbx21*) and Eomesodermin (encoded by *Eomes*), which promote effector cell differentiation, are known to directly and cooperatively induce *Gzmb* expression (Intlekofer et al., 2005; Pearce et al., 2003; Townsend et al., 2004) as does the TF Runx3 (Cruz-Guilloty et al., 2009). All three TFs have been broadly linked to induction of cytolytic genes including perforin (Cruz-Guilloty et al., 2009; Intlekofer et al., 2005; Pearce et al., 2003; Townsend et al., 2004). The TF Blimp1, which also induces effector differentiation, promotes sustained *Gzmb*

expression (Kallies et al., 2009; Rutishauser et al., 2009), but this is likely an indirect effect downstream of Bcl6 repression, as Bcl6 is known to directly repress *Gzmb* (Yoshida et al., 2006). Direct *Gzmb* repression by Bcl6 likely contributes to its limited expression in central memory cells, where elevated Bcl6 expression promotes a memory phenotype (Ichii et al., 2002). Similarly, the TF TCF1, which also is elevated in central memory cells and programs the memory state, directly represses *Gzmb* (Jeevan-Raj et al., 2017). In addition to these TFs, within human NK and T cells, NF-κB TFs have been implicated in *Gzmb* induction (Huang et al., 2006). Despite low expression of *Gzmb* during memory, elevated histone acetylation is maintained at the *Gzmb* locus and this allows more rapid re-expression upon re-stimulation in memory versus naïve cells (Araki et al., 2008).

In contrast to GzmB, very little is known about the direct transcriptional regulators of other granzyme genes. The glucocorticoid receptor has been shown to directly repress *Gzma* expression, albeit in a human lymphoma line (U et al., 2004), but otherwise the factors directly controlling expression of *Gzma* and other granzymes are unreported. It is possible that the various granzyme gene clusters are controlled by shared enhancer elements, but this remains to be proven.

5. Are granzymes required for immune cell cytotoxicity?

Given the long-standing dogma that granzymes eliminate infected or mutated host cells via apoptosis, it is perhaps surprising that mouse gene knock out models produce only mild phenotypes during most infections and tumours (Davis et al., 2001; Smyth et al., 2003). This may be because, unlike perforin, individual granzymes are often dispensable for cytotoxicity, probably reflecting a level of functional redundancy (Joeckel and Bird, 2014). For example, while perforin deficiency fully blocks *in vitro* cytotoxicity of CTL and NK cells (Kägi et al., 1994), no equivalent granzyme single or compound knock-out model has been identified that phenocopies perforin loss *in vitro* (Voskoboinik et al., 2015). GzmB loss does significantly impair the nuclear manifestations of apoptosis without fully eliminating cytotoxicity (Heusel et al., 1994), but additional deletion of other granzymes does not appreciably further limit killing capacity (Waterhouse et al., 2006). This is mirrored by *in vivo* findings from infection models. For example, while perforin deficient mice fail to clear the mouse pathogen

LCMV (Walsh et al., 1994), all granzyme knockout mice thus far tested have displayed little or no defect in viral control (Balkow et al., 2001; Joeckel et al., 2017; Zajac et al., 2003). Similarly, Sendai virus is cleared normally in granzyme B deficient mice (Salti et al., 2011). In contrast, granzyme deficient mice have severely impaired MCMV control, although unlike perforin deficient mice, granzyme deficient mice survive infection again highlighting differences in perforin versus granzyme function (van Dommelen et al., 2006). In the case of MCMV, direct cleavage of MCMV proteins by granzymes may be more important for viral control than granzyme-dependent cytotoxicity (Andoniou et al., 2014; Sutton et al., 2020). Ectromelia is one of the few reported viral models where granzyme deficiency can phenocopy perforin loss in terms of mortality during infection (Müllbacher et al., 1999a; Müllbacher et al., 1999b). Nevertheless, unlike perforin knock-out mice, *in vivo* CTL killing capacity is intact in Ectromelia infected mice lacking Gzms A and B (Regner et al., 2009), meaning it again remains possible that direct granzyme-dependent cleavage of viral proteins contributes to control given the widely reported role of granzymes in cleaving viral proteins across diverse infections (de Jong et al., 2021). Consistent with the idea that granzyme deficiency does not phenotype perforin loss, while patients with severe deficiencies of perforin function often develop significant (sometimes fatal) pathology, no pathology has ever been reported in granzyme deficient patients (see discussion below). The reasons for this discrepancy in phenotype remain unclear. It could be that the presence of the other remaining granzymes compensates for the loss of the key cytolytic granzymes (ie. Gzms A, B, K). Alternatively, perhaps other lysosomal proteases can substitute for granzymes in their absence.

6. Direct and indirect immunoregulatory roles of granzymes

While granzymes undoubtedly contribute to target cell death in synergy with perforin, there is considerable evidence that the perforin/granzyme pathway can also dampen T cell responses and thus serve as an immune checkpoint (Figure 1). Notably, granzymes and/or perforin have been proposed to play important roles in the activity of regulatory cell types, including regulatory T cells (Tregs), and regulatory B cells. For example, cytotoxic Tregs were shown to kill dendritic cells (DCs) in an antigen-dependent fashion during T cell priming in a murine tumour setting, reducing both DC numbers and T cell priming in the draining lymph node (Boissonnas et al., 2010). GzmB expressing Tregs were similarly postulated to restrain T cells responses to

Sendai virus infection (Salti et al., 2011). However, in both settings direct evidence that Treg-restricted GzmB was functionally required for the phenotype using conditional knock-out models was not provided. Similarly, GzmB-expressing regulatory B cells have been proposed to kill target cells, including T cells, and thereby regulate immunity (Lindner et al., 2013). Again, functional knock-out evidence proving a direct role for GzmB was lacking, and while the absence of perforin in B cells would imply a perforin-independent mechanism, the molecular mechanism was not resolved. Finally, NK cell cytotoxicity has also been demonstrated to limit $CD8^+$ T cell immunity through killing of helper $CD4^+$ T cells in chronic viral infection (Lang et al., 2012; Waggoner et al., 2011), and direct killing of antiviral $CD8^+$ T cells in acute infection in situations where interferon receptor signalling is defective (Crouse et al., 2014; Xu et al., 2014). While perforin has been implicated in these processes, the role of granzymes is again unclear.

Unless appropriately regulated, granzymes have the capacity to directly kill the cells that express them due to cytoplasmic leakage from granules. In the mouse, Serpinb9 (formally called Spi6) is expressed in the cytosol of CTL, and CTL lacking Serpinb9 have a significant survival defect (Zhang et al., 2006). Survival is recovered in cells that lack both GzmB and Serpinb9, implying that death is GzmB-dependent and that Serpinb9 operates by neutralising GzmB that leaks into the cytoplasm. However, the pseudo-substrate loop of Spi6 is devoid of acidic residues (Ser and Asn are in the likely P1 position). As shown by Zhang et al., highly stable complexes comprising Serpinb9 and mouse granzyme B are still able to form, indicating that the protease must be able to recognise Ser or Asn as the P1 residue. In the human context, the potent granzyme B-specific SERPINB9 is expressed at high levels in the cytosol of CTL/NK cells, as well as in antigen-presenting DC and B cells, suggesting a role in protecting these cells in diverse bystander settings (Hagn et al., 2014; Watanabe et al., 2021). A similar protective mechanism is also proposed to operate in human mast cells, which are also able to express and store granzyme B under certain conditions (Bladergroen et al., 2005).

Extracellular GzmK has been proposed to contribute to inflammation and aging independent of cytotoxic effects. Treatment of fibroblasts with GzmK in combination with IFNγ can promote secretion of inflammatory factors, such as IL-6 and various

chemokines, and this is proposed to contribute to senescence during aging (Mogilenko et al., 2021). Secreted GzmK can also trigger IL-1β production by primary mouse macrophages, which was proposed to contribute to LCMV control in mice that lack Gzms A and B (Joeckel et al., 2011). While this remains to be functionally tested, mice that lack GzmK alone at least control and respond to LCMV normally (Joeckel et al., 2017).

Finally, cell intrinsic granzyme expression can aid T cell migration independent of cytotoxicity. For example, extracellular secreted GzmB contributes to CTL diapedesis by cleaving components of the basement membrane (Prakash et al., 2014). Additionally, $CD4^+$ T cells perform transendothelial diapedesis using stored GzmK for immune surveillance in the CNS (Herich et al., 2019). Lymphocyte migration may also be facilitated by granzyme B-mediated cleavage of several extracellular matrix proteins (Buzza et al., 2005).

7. Congenital diseases related to loss of function mutations in perforin and granzymes.

A number of disease states result from inherited defects in the granule exocytosis mechanism of cell death. By far the most serious relate to defects of perforin function, and intriguingly, this was first appreciated in perforin (pfp) gene-*null* mice, which have severe defects in CTL/NK cell-mediated cytotoxicity, and are thus highly susceptible to poxvirus infection, particularly ectromelia, the ortholog of smallpox in humans (Müllbacher et al., 1999a). These mice survive and breed happily in pathogen-free lodgings, but ~70% spontaneously develop transplantable, aggressive B cell lymphomas as they age (12-18 months), reflecting a fatal lapse in cancer immune surveillance (Smyth et al., 2000). Humans who inherit complete loss of function *PFP1* gene mutations comprise 1/60,000 live births in most societies and die within months of birth from the severe autosomal recessive immunoregulatory disorder Type 2 Familial Haemophagocytic Lymphohistiocytosis (FHL2), marked by fatal cytokine hypersecretion and myeloid cell hyperactivation (Stepp et al., 1999). Hypomorphic PFP1 gene mutations are far more common, and may be well tolerated unless inherited with a null allele (‘compound heterozygosity’). However, in some instances, atypical, partial and/or delayed manifestations of FHL can result, and these cryptic

clinical presentations can be difficult to diagnose (House et al., 2015). Mutations that disrupt several other genes (UNC13D, STX11, STXBP2) can also cause FHL, due to failure to deliver or release otherwise functional perforin into the immune synapse (Lopez et al., 2018). As a result, we recently suggested the term 'perforinopathy' to describe the functional consequences of all congenital causes of perforin deficiency in humans (Voskoboinik and Trapani, 2013).

Interestingly, there are no known human disease states related to granzyme dysfunction. Unlike perforin, which is encoded by a single-copy gene, the multiplicity of *Gzm* genes is probably sufficient to ensure survival under most circumstances, and some redundancy of pro-apoptotic function clearly exists – although GzmB is undoubtedly the most potent cytotoxic granzyme, cytotoxicity has been attributed to virtually all of the others, but only in idealised *in vitro* cell death models that utilise purified recombinant granzyme proteins (Susanto et al., 2012). As described above, mice deficient in any one granzyme have no obvious phenotype, but pathologies emerge in the response to some pathogenic viruses if the loss of function extends to multiple granzymes. As an interesting corollary, congenital loss of Cathepsin C (CatC), which is co-located with granzymes in cytotoxic granules and is the major convertase for processing granzymes from their zymogenic to their active forms, results in a florid disease known as Papillon-Lefevre Syndrome, but the phenotype is restricted to myeloid cells (particularly neutrophils) and not CTL or NK cells. The syndrome presents in children aged 3-6, almost invariably with severe suppurative gum infections severe enough to cause chronic gingivitis, severe halitosis and tooth loss extending to adulthood (Toomes et al., 1999). Despite this florid myeloid-related pathology, patients with Papillon-Lefevre Syndrome have no reported defects of T cell function. Mice engineered to have total loss of CatC function likewise have no defect of T/NK cell effector function in viral or cancer models (Andoniou et al., 2011; Regner et al., 2009; Sutton et al., 2007), despite the cytotoxicity of these cells being reduced (but not abolished) *in vitro* (Pham and Ley, 1999).

Why is granzyme processing required for protease activity? Granzymes are synthesised and processed through the secretory pathway, commencing with cleavage of the signal sequence in the ER; however, acquisition of protease activity requires further processing and occurs only when the granzyme molecules are

transported into the acidic lysosome-like secretory vesicles in the CTL/NK cytoplasm. The final, crucial step in activating pro-granzymes is mediated by lysosomal CatC, a dipeptidylpeptidase that is most active at the acidic lysosomal pH (pH4.5 - 5.0). Removal of an 'activation dipeptide' from the amino-terminus allows the mature, fully processed amino-terminus to fold snugly into a niche close to the catalytic site of the granzyme, activates the electron transfer chain and thus switches on its proteolytic activity. Fully activated granzymes are thus confined to and sequestered in CTL/NK secretory vesicles until their release into the immune synapse following target cell binding (Pham and Ley, 1999). While neutrophil proteases rely totally on CatC for activation, mice with gene-engineered inactivating mutations of both CatC alleles have only partly diminished CTL/NK cell cytotoxicity, but lose little protection against most pathogenic viruses studied to date. These mice totally lack granzyme A activity, but retain some active granzyme B; thus, it appears that processing of mouse granzyme A to its active form relies totally on CatC, but the same is not so for granzyme B (Sutton et al., 2007). The fact that CatC+/+ mice that are *null* for expression of granzymes A and B are highly susceptible to poxvirus infection, whereas CatC-deficient mice are not, is further evidence that CatC is not the only lysosomal protease capable of processing pro-granzymes to their active state. Indeed, we showed some time ago that in the absence of CatC, mouse pro-GzmB can also be processed to full activity by Cathepsin H (CatH), and by a third protease, although this protease was not identified (D'Angelo et al., 2010). There is to the present time no similar report of pro-granzyme processing in humans, however, the fact that individuals with Papillon-Lefevre syndrome have no clinical defect in CTL/NK cell function suggests that redundancy in pro-granzyme processing must also be present in humans. Recently, a small molecule CatC inhibitor with nanomolar potency was described and is now being trialled in patients with recurrent suppurative lung infections secondary to bronchiectasis (Doyle et al., 2016). This highly selective inhibitor provides a new pharmacological tool that may be useful in identifying granzyme convertases other than CatC.

Apart from proteolytic processing at their N terminus, the other significant post-translational modification of granzymes involves their glycosylation. As with many other proteins trafficked through the secretory pathway, simple (high-mannose) glycosylation in the proximal endoplasmic reticulum is followed by phosphorylation on

the 6 position to form mannose-6-phosphate (M6P) moieties. The mannose phosphate receptor pathway is important for trafficking of granzymes to the secretory vesicles (Griffiths and Isaaz, 1993). I-cell disease results from the congenital loss of activity of the enzyme UDP-N-acetylglucoseamine-1-phosphotransferase, necessary for mannose 6-phosphorylation. Among the many developmental defects seen in I-cell disease, patient T and NK cells have markedly reduced levels of granzyme stored in their cytotoxic vesicles. However, ~30% of the proteases still reach the vesicles, indicating that additional pathways exist for granzyme trafficking (Griffiths and Isaaz, 1993). The MPR pathway is also important for granzyme re-uptake and recycling through the endosomal pathway; as with most lysosomal proteins, granzymes are also subject to constitutive secretion and re-uptake in addition to the quantal secretion that occurs in the direction of the immune synapse upon conjugation of a target cell (Trapani et al., 2003).

8. Non apoptotic, extracellular roles of granzymes in human pathology

As indicated above, gene knock-out studies in mice have shown that Gzms A and B are indispensable in the defence against natural viral pathogens such as ectromelia and MCMV. In the following section, we consider roles potentially played by granzymes in other disease states, particularly autoimmune diseases and diseases of the cardiovascular system, which are at least partly mediated by immunomodulatory granzyme functions that may not include the death of target cells.

8.1. Skin pathologies linked to non-apoptotic functions of granzymes

Granzymes are typically not detectable in healthy tissue, but elevated levels are frequently observed in extracellular fluid and serum in various pathologies, including autoimmune diseases and inflammatory responses, suggesting they may play some role in disease pathology. Granzymes may accumulate and become detectable in the extracellular environment through various mechanisms. As with most secreted proteins, GzmB is constitutively released and undergoes re-uptake through the mannose-6-phosphate receptor pathway (Trapani et al., 2003). It has also been suggested that around one third of the released GzmB leaks out from the immunological synapse during a CTL-target cell interaction (Isaaz et al., 1995). Other suggested pathways of granzyme release include pathogen-, chemokine- or cytokine-induced degranulation, or secretion after integrin-ECM interaction (Bouwman et al.,

2021; van Daalen et al., 2020). As no cognate extracellular inhibitor of GzmB exists in humans (Kaiserman and Bird, 2010), secreted GzmB maintains its proteolytic activity and accumulates in the extracellular space. Perforin-independent roles of GzmB have therefore been postulated in wound healing, pathologic skin blistering (such as pemphigus) and other autoimmune conditions including lupus and vitiligo.

Cytotoxic (granzyme high) $CD8^+$ T cells are a frequent feature of many post-infectious and auto-immune skin pathologies, but not of normal skin. A common autoimmune skin disease whose severity correlates with GzmB levels is vitiligo, in which melanocytes are attacked by skin-resident memory $CD8^+$ T cells (Boniface et al., 2018), typically leading to patches of depigmented skin. The common GzmB alloform 55R-94A 247H (RAH) was found to be more frequent among a European vitiligo cohort (Ferrara et al., 2013) and was associated in non-segmental vitiligo susceptibility in a Korean cohort (Jeong et al., 2021). However, the RAH and less common QPY variants have equivalent pro-apoptotic function (Sun et al., 2004), so the basis of granzyme involvement in disease is unknown. Other studies have provided evidence suggesting a role for GzmB in autoimmune blistering diseases such as epidermolysis bullosa, bullous pemphigoid and dermatitis herpetiforma, in which proteolytic degradation at the dermo-epidermal junction (DEJ) results in a loss of connectivity of the two anatomical skin zones (Hiroyasu et al., 2021; Russo et al., 2018). Accumulation of granzyme B secreted into the DEJ as a result of T cell infiltration, and its subsequent cleavage of α6/β4 integrin, collagen VII, and collagen XVII, is likely to contribute significantly to disease pathology. Granzyme B is also implicated in the causation of at least some forms of atopic dermatitis, as pruritis and disease severity are correlated with local granzyme B proteolytic activity (Turner et al., 2021). *In vivo*, mice that lacked granzyme B expression were relatively protected against dermatitis induced by the chemical agent oxazolone (Turner et al., 2021).

An initial understanding of how granzymes can influence wound healing was gained with the finding that GzmB cleaves van Willebrand factor (VWF) in the platelet interacting regions necessary for cell aggregation and blood coagulation (Buzza et al., 2008), an early prelude to the subsequent stages of wound healing. In fact, in various infectious diseases causing skin ulceration, such as cutaneous leishmaniasis, elevated levels of $CD8^+$ T cells and granzymes in the skin are associated with

immunopathology, potentially contributing to the disease pathology (Bousoffara et al., 2004). Cleavage of substrates in the extracellular matrix such as decorin, fibronectin, vitronectin and laminin (Boivin et al., 2012; Buzza et al., 2005) is likely to contribute to these disorders by altering ECM and skin structure. If a pathogenic role for granzymes is confirmed in immune-mediated skin diseases, they would offer an attractive target for topical treatment strategies. Indeed, treatment with a small molecule GzmB inhibitor demonstrated efficacy in preclinical models of both dermatitis (Turner et al., 2021) and autoimmune blistering (Hiroyasu et al., 2021). As an alternative approach, a recent study that applied an IL-15 signaling inhibitor in cutaneous leishmaniasis found that topical treatment reduced GzmB expression in $CD8^+$ T cells with concurrent protection from severe skin lesions in mice (Novais et al., 2021).

8.2. Systemic autoimmune conditions and inflammatory responses

Studies showing the up-regulation of granzymes in human autoimmune diseases have mostly been correlative, and a causal role in immunopathology has not been demonstrated. For example, soluble GzmB, but not K and M, is elevated in SLE patients and correlates with disease severity (Kok et al., 2017; Shah et al., 2011). It was previously shown that cleavage of autoantigens by GzmB is involved in the generation of SLE-specific autoantigens (Graham and Utz, 2005). Conversely, in one study, the frequency of IL-21-induced GzmB-producing regulatory B cells was reduced in lupus nephritis (Rabani et al., 2018). Given the known role of regulatory B cells in restraining autoimmunity, and the proposed roles of GzmB in regulatory B cell-mediated immunosuppression, this could contribute to accumulation of the autoreactive B cells that drive lupus pathogenesis (Oleinika et al., 2019).

High extracellular granzyme levels have been proposed to result in vascular leakage due to increased local VEGF brought about by its release from extracellular matrix proteins, such as fibronectin, by granzyme B (Hendel et al., 2014; Wijelath et al., 2006). Increased vascular permeability then follows via increased VEGFR2 phosphorylation (Hendel et al., 2014). Another potential mechanism for granzymes contributing to generalised inflammation is either through the stimulation of cytokine release by monocytes, or by cleavage of certain cytokines into their active form, resulting in further recruitment of lymphocytes to the site of infection (Afonina et al., 2011). A direct facilitatory effect of granzyme B on T cell migration has also been

proposed, by enabling the diapedesis of T cells via basement membrane remodelling (Prakash et al., 2014).

In human subjects, granzymes have also been implicated in the pathology of severe asthma. In a study on 37 cases of fatal asthma, GzmA and B expression was elevated in the wall of large and medium bronchi, bronchioles, and in the peribronchial septa. However, the study authors were unable to conclude whether granzymes were involved in asthma pathogenesis, or reflected the presence of activated T cells recruited during agonal infections with virus or other pathogens (Annoni et al., 2015). Alternatively, there are several reports that mast cells are able to express granzymes (Burgener et al., 2021; Rönnberg et al., 2014), potentially pointing to a more direct role for granzymes in the pathogenesis of earlier less severe asthma. More compelling data has demonstrated that NK cell-derived GzmB is required for asthma induction through direct or indirect actions on lung epithelial cell PAR2 specifically in a maternal diesel particle exposure triggered asthma model (Qian et al., 2020).

8.3. Granzymes and cardiovascular disease.

High granzyme B levels have been identified in the serum of patients with a variety of cardiovascular conditions including atherosclerosis (Chamberlain et al., 2010). The loss of extracellular matrix integrity is a common feature of these diseases, and proteins such as fibrillin and decorin, which have been identified as GzmB substrates, have been found to be degraded. In combination with chronic inflammation, a consequence of this damage can result in atherosclerotic plaque rupture. In a mouse model of abdominal aortic aneurysm (AAA), it was reported that GzmB deficient mice had reduced levels of rupture and enhanced survival compared to wild type controls (Ang et al., 2011; Chamberlain et al., 2010), which had significant GzmB staining in the region of the aneurysms, often colocalised with infiltrating mast cells and macrophages. It was proposed that fibrillin cleavage by GzmB results in weakening of the aortic wall, leading to rupture. However, the source of the GzmB in this condition has not been identified and direct evidence for GzmB mediated cleavage of fibrillin and other extracellular substrates has been difficult to demonstrate. Although no extracellular serpin capable of inhibiting human GzmB exists (Kaiserman and Bird, 2010), a mouse extracellular serpin, Serpina3n has been reported to have some inhibitory activity against mouse GzmB (Sipione et al., 2006). Treatment with the

recombinant serpin gave some protection from aortic rupture and death in this model, and maintenance of decorin was observed (Ang et al., 2011). Granzyme-mediated cleavage of proteins in the extracellular matrix can also retard wound healing, as demonstrated in a variety of experimental mouse models, including those inflicted by thermal burns (Shen et al., 2018), or resulting from insulin-dependent diabetes (Hsu et al., 2014) or congenital apolipoprotein E deficiency (Hiebert et al., 2013). Once again, the fact that either small molecule (Shen et al., 2018) or serpin-mediated (Hsu et al., 2014) inhibition of granzyme B resulted in more rapid wound closure suggested the role of granzyme B to be causal in these pathologies.

8.4. Granzymes in severe sepsis and septic shock

Septic shock is a serious and potentially fatal syndrome of severe hypotension, vascular leakage, hypercoagulation, multiple organ failure and systemic hyperinflammation arising from disseminated bacterial infection, frequently a gram-negative organism arising in the gut (Minasyan, 2017). The pathogenesis of septic shock is complex and incompletely understood, involving many pro-inflammatory pathways and immune cell types, and typically the hypersecretion of cytokines by myeloid cells, particularly IL-1, IL-6 and TNFα (Minasyan, 2017). While in no way accounting for the totality of septic shock syndrome, a number of studies have suggested that granzymes may play a role in the generation of excessive circulating inflammatory cytokines, or in the response to bacterial endotoxins such as lipopolysaccharide (LPS). Clinically, a recent study reported that patients with severe sepsis have poorer outcomes if they also have high circulating levels of GzmA and GzmB (Napoli et al., 2012). In fact, a role for GzmA in eliciting the secretion of IL-6 and IL-8 from monocytes was postulated many years ago potentially through direct proteolytic activation of the pro-forms of these cytokines (Sower et al., 1996). A role for GzmA in regulating coagulation was also postulated many years ago, via direct cleavage of thrombin receptor (Suidan et al., 1994). A more recent study found that mice null for the expression of GzmA had lower mortality than wild type litter mates when challenged with septicaemia resulting from caecal ligation and puncture (Garzon-Tituana et al., 2021). The same study reported that treating GzmA-sufficient mice with GzmA inhibitors as well as antibiotics improved their survival in comparison with a group that received antibiotics alone, and this was associated with reduced

circulating cytokine levels. It is important to note though that serpins such as anti-thrombin III that are abundant in human plasma cause GzmA to be rapidly inactivated (Masson and Tschopp, 1988), meaning that further work is needed to establish the importance of these GzmA-driven pathways in humans. Other studies have also found elevated levels of various granzymes in mouse models, although interpreting the functional significance of the results was problematic as there was much variation in which granzyme was elevated, depending on the pathogen studied (Garzon-Tituana et al., 2020). NK cells activated early in the innate immune response to pathogen have been postulated as a source of granzyme secretion, and evidence in support of this notion came from the demonstration that congenital deficiency of GzmM, which is expressed predominantly by NK cells, protected mice against shock induced by LPS, whereas GzmB deficiency had no effect (Anthony et al., 2010). Widespread granzyme secretion from NK cells early in the course of serious bacterial infection also raises the possibility that granzymes might play an adaptive role in suppressing bacterial numbers. Evidence to this effect came to light recently in a study showing that GzmB elaborated by NK cells was able to directly kill extracellular *Klebsiella pneumoniae* through a perforin-independent mechanism (Feehan et al., 2022).

9. Conclusions

The role of granzymes in human pathology remains an area of ongoing research with many open questions. At a fundamental level, a deeper understanding of the basic biology of granzymes is needed to better interpret how granzymes may be functionally contributing to human disease. In particular, it remains unclear to what degree granzymes are required for the cytotoxicity of immune cells. The potential redundancy of the many granzyme genes has made this difficult to functionally address, but perhaps future CRISPR approaches will enable complex compound knockout models to comprehensively address these questions. Furthermore, additional work is needed to functionally dissect how non-canonical and/or extracellular granzyme activity may contribute to normal immune function, as well as immune dysregulation in the context of disease.

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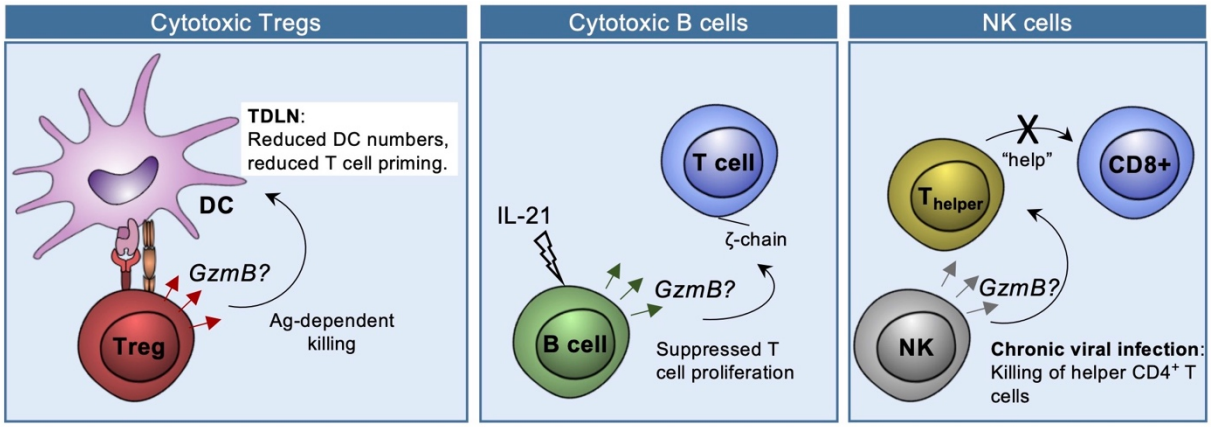

Figure 1. Potential immunoregulatory mechanisms of GzmB. GzmB has been implicated in immunoregulation by Tregs via killing of DCs (left), and regulatory B cell immunoregulation by killing of T cells (middle). NK cell-mediated killing of $CD4^+$ T helper cells also limits $CD8^+$ T cell immunity in chronic viral infection (right). To date, there is no functional proof for a specific role of GzmB in these situations, meaning further work with conditional knockout models is required.

Table 1 Cross-species variation in granzymes and their substrates

Human and Mouse granzymes

Family	Chymase	Tryptase	Metase
Locus -human	14q11-12	5q11-12	19p13
Locus- mouse	14D	13D	10q21
Family members – human	B,H	A,K	M
Family members – mouse	B,C,D,E,F	A,K	M
Cleavage specificity[1]	B cleaves after Asp, the rest after hydrophobic residues	Lys, Arg	unbranched, long or aromatic
Intracellular targets[2]	B cleaves Bid; pro-caspases 3,6,7,8; PARP, lamins, filamin[3], gasdermin E. H cleaves adenovirus DBP and L-100 capsid protein	SET complex gasdermin B	alpha-tubulins survivin
Extracellular targets[2]	α6/β4 integrin, collagen VII, collagen XVII, VWF, decorin, fibronectin, fibrillin, vitronectin, laminin, IL-1α	thrombin receptor, PAR-1 and PAR-2[4]	

[1]all granzymes cleave on the carboxyl side of a preferred P1 amino acid, but cleavage efficiency is also greatly influenced by up to 4 residues either side of P1 (P1-P4, P'1-P'4)

[2]partial list, see text for details. Unless indicated, relevant references are in the text.

[3] (Browne et al., 2000)

[4] (Kaiserman et al., 2022)