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Review

A quarter century of granzymes

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Granzymes (Grs) were discovered just over a quarter century ago. They are produced by cytotoxic T cells and natural killer cells and are released upon interaction with target cells. Intensive biochemical, genetic, and biological studies have been performed in order to study their roles in immunity and inflammation. This review summarizes research on the family of Grs.
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Facts

- Granzymes (Grs) are a family of closely related serine proteases expressed in cytotoxic T lymphocytes and natural killer cells.
- Grs are packaged in cytoplasmic granules that are exocytosed toward a bound target cell.
- Perforin facilitates the transfer of Grs into the target cell cytoplasm.
- GrB cleaves substrates at aspartic acid allowing it to activate caspase-3 and initiate apoptosis.
- GrA is a tryptase and acts by disruption of the ER-associated SET complex.
- Grs cleave a variety of intracellular and extracellular substrates.

Open Questions

- What are the implications of species-specific differences in Gr substrate specificities?
- Why do mice have so many Grs relative to humans?
- Why does Bcl-2 block GrB *in vitro* killing but not CTL-induced death?
- How do tumors inhibit CTL killing?
- What are the implications of cleavage of extracellular substrates to disease pathogenesis?
- Is GrA inflammatory or lethal or both?

Twenty-five years ago Jürg Tschopp's group[1] published a blockbuster paper in cell on the purification and characterization of granzymes (Grs). These are a family of serine proteases found in the cytolytic granules of cytotoxic T lymphocytes and natural killer cells. Jürg's group was able to purify eight members of the family, investigate their substrate specificities, and determine partial amino acid sequences (see Box 1). Two of them corresponded to proteins encoded by cytotoxic cell protease (CCP) genes cloned by the Bleackley laboratory.[2] Over the next few years, there was lively debate concerning the naming of these molecules. When Bleackley lab members agreed to call them Grs, it was clear who was the winner. At the time, the conventional wisdom was that another granule protein perforin (a.k.a. cytolysin) was the sole mediator of target cell destruction. The discovery of Grs set the stage for an exciting time in understanding cell-mediated cytotoxicity. Since then, over 3000 papers have been published on Grs. This review is dedicated to the memory of Jürg, not only a trail-blazer, but also a friend.

Box 1 Jürg Tschopp's contributions to our understanding of granzyme-mediated death

Identified and sequenced many of the granzyme cDNAs.

Coined the name 'granzymes' after he had isolated and characterized this novel family of serine proteases from the lytic granules of cytotoxic T lymphocytes.

Purified and characterized perforin, the molecule that facilitates the entry of granzymes into target cells.

Demonstrated that granzymes and perforin exist as a complex with proteoglycans in granules.

Knocked out the perforin gene. This lead to the discovery of a perforin/granzyme-independent pathway that acted via Fas/Fas ligand.

The Cytotoxic Granule Components

Cytotoxic lymphocytes contain specialized cellular compartments, the secretory lysosomes, which are membrane-bound organelles equipped with cytolytic proteins.[3] The primary cytolytic molecules, delivered to target cells upon contact with cytotoxic cells, are perforin and Grs. Currently, the mechanism of perforin-facilitated entry of Grs into target cells remains unresolved. Models proposed include the liberation of Grs from endosomal compartments of the target cells to the cytosol, or a Ca^{2+}-driven plasma membrane repair process initiated by perforin damage.[4]

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Keywords: granzymes; apoptosis; cytotoxic T cells; NK cells
Abbreviations: Gr, granzyme; CG, cathepsin G; CMA1, mast cell chymase
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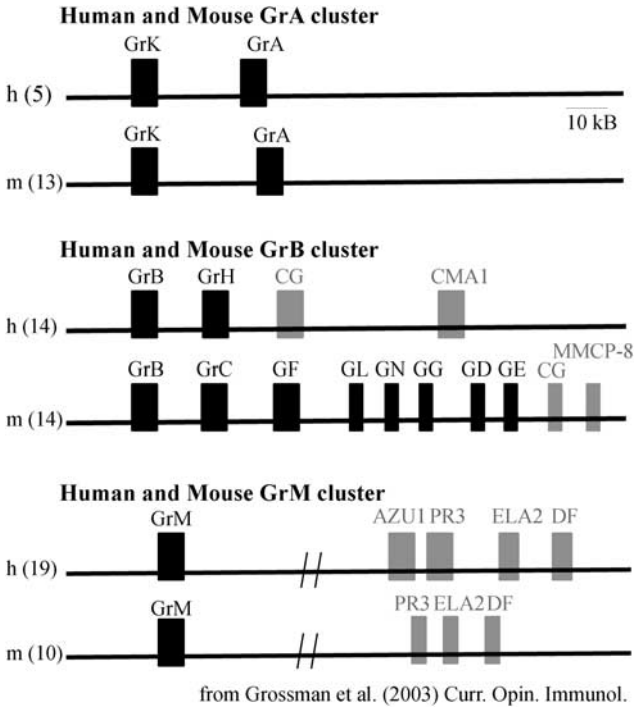

Figure 1 Genomic organization of serine proteases. Gr genes, found primarily in lymphoid-derived cytotoxic cells are indicated in black. (Mouse GrL is a pseudogene.) Myeloid-derived serine proteases are indicated in grey

Grs are serine proteases, characterized by a His-Asp-Ser catalytic triad.[5] In humans, there are 5 Grs (A, B, H, K, and M) while in mice there are 10 functional Grs (A, B, C, D, E, F, G, K, M and N). The genes are found in clusters among three different chromosomes, the GrA cluster, GrB cluster, and GrM cluster **(**Figure 1). The GrA cluster, at human chromosome 5 and murine chromosome 5, includes GrA and GrK. Grs A and K are tryptases, cleaving after basic amino acid residues (designated the P1 residue).[6,7]

The GrB cluster is found at chromosome 14 of both human and mice but is composed of different enzymes. In humans, the cluster consists of GrB, GrH, cathepsin G (CG), and mast cell chymase-1 (CMA1). In mice, the cluster contains GrB, C, F, N, G, D, E, CG, and CMA1. GrB is the most extensively studied Gr, characterized by the unusual capacity to cleave substrates at aspartic acid residues that is dependent on the presence of an arginine residue within the binding pocket (Figure 2).[8] Grs H and GrC–G, are believed to be chymases, and cleave synthetic substrates at hydrophobic residues.[5] These proteins are also highly similar at the amino acid level, from 57–61% identity using BLAST alignment analysis.

The GrM cluster is found on chromosome 19 in human and chromosome 10 in mice, and consists of GrM and neutrophil proteases.[9] GrM is a metase, preferentially cleaving substrates at methionine, or leucine residues.[10] However, human and mouse GrM display divergent substrate specificities.[11]

Mechanisms of Cytotoxicity and Physiological Roles of Grs

Our understanding of the function of the majority of Grs is very limited. The exception is GrB, which has been established as a potent pro-apoptotic molecule as a result of extensive studies using biochemical techniques and purified or recombinant proteins. The cytotoxic mechanisms of remaining Grs are still active areas of research. Table 1 describes the phenotype of Gr-deficient mice in their ability to induce target cell death and respond to immunological challenges. Table 2 summarizes the features displayed by target cells treated *in vitro* with purified/ recombinant Grs, and the current models of cytotoxic-mediated cell death are shown in Figures 3(a–d).

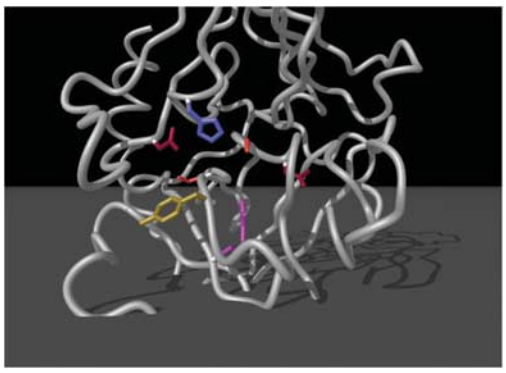

Figure 2 The binding pocket structure of GrB. Residues that line the binding pocket are shown in red (Asp), blue (His), orange (Ser), yellow (Tyr), and pink (Arg). The arginine residue at 226 is critical for cleavage of substrates at aspartic acid residues

Table 1 Mouse models of granzymes

Mouse	Phenotype and apoptotic response	References
$grB^{-/-}$(cluster)	Rapid DNA fragmentation and caspase activation affected	42–44
	Moderate susceptibility to MCMV and ectromelia virus	45,46
	Reduced MHC class I-dependent GVHD	47
$grB^{-/-}$ (targeted)	Reduced clearance of allogeneic tumors	48
	Control of gammaherpervirus latent infection	104
$grA^{-/-}$	Apoptotic response not affected	71
	Moderate susceptibility to ectromelia virus	72
	Control of herpes virus spread to peripheral nervous system	73
$grA^{-/-}$ x $grB^{-/-}$	Highly susceptible to ectromelia virus	46
	Defective clearance of *Trypanosoma cruzi*	105
	Control of tumor growth	106
$grM^{-/-}$	Apoptotic response not affected	85
	Mild susceptibility to MCMV	

Granzyme B. Several groups identified GrB in cytotoxic lymphocytes during the mid-1980s.[1,2,12–14] It was also known variously as CCP1 and cytotoxic T lymphocyte associated-1 (CTLA1) before GrB was widely adopted. Initial studies indicated that GrB-induced *in vitro* cell lysis (^{51}Cr-release), rapid DNA fragmentation, and chromatin condensation.[15–17] Indeed, identification of the DNA fragmenting activity (fragmentin) of GrB was an important piece of the puzzle in understanding the mechanism of CTL-induced death.

Table 2 Summary of granzyme death pathways

	Granzymes						
	A	**B**	**H**	**K**	**M**	**C**	**F**
Cell lysis	Yes	Yes	Yes	Yes	Yes	Yes	Yes
z-VAD-fmk sensitive	No	Yes	Y/N	No	Y/N	No	No
Bcl-2 inhibition	No	Yes	?	No	No	?	?
MOMP	No	Yes	Y/N	Yes	Y/N	Yes	Yes
$\Delta\psi_m$	Yes	Yes	Yes	Yes	Y/N	Yes	?
ROS	Yes	Yes	Transient	Yes	Y/N	?	Yes
ATP Depletion	Yes	Later	?	?	?	?	Rapid
PS exposure	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Loss plasma membrane	Rapid	Late	Rapid?	Rapid	Rapid Late	Rapid	Rapid
Chromatin condensation	Yes	Yes	Yes	Yes	Yes	Yes	Yes
DNA fragmentation	Yes SS	Yes DS	Yes DS?	Yes SS	Y/N	Yes SS	?
PARP	Yes	Yes	?	?	Y/N	?	?
ICAD/DFF45	No	Yes	Y/N	?	Y/N	No	?
Bid	No	Yes	Y/N	Yes	?	No	No

Abbreviations: DS, double stranded; MOMP, mitochondrial outer membrane permeability; SS, single stranded; Y/N, reported results have been contradictory; ?, have not been reported.

Later experiments revealed that purified GrB and perforin or adenovirus treatment induced many classical features of apoptosis, such as membrane blebbing, phosphatidylserine exposure, release of cytochrome C, dissipation of $\Delta\psi_m$, generation of ROS, and plasma membrane permeability to vital dyes at early timepoints.[18,19]

A pivotal piece of information that led to the discovery of the cytotoxic mechanism of GrB was its ability to cleave aspartic acid residues.[8] This cleavage specificity is unique among eukaryotic serine proteases. Up to that point, only caspases were known to have this unusual specificity. Subsequently, GrB was found to proteolytically activate a number of caspases *in vitro* (caspases-2, -3, -6, -7, -8, and -9) and *in vivo* (caspases-3, -7, -8, and -10).[20–25] These observations led to a model wherein the activation of caspase-3 by GrB was largely responsible for many of the features induced by CTL killing.[20,22,26,27] Further studies revealed that GrB could also promote cell death via the mitochondrial pathway.[18,28] These experiments demonstrated that human GrB proteolytically activated Bid at aspartate[75], resulting in its translocation to the mitochondrial outer membrane, and the facilitation of oligomerization of Bax/Bak molecules.[29–31] It was initially thought that this resulted in the release of cytochrome C and apoptosome formation. This proved not to be the case, but rather involved inhibitor of apoptosis proteins (IAPs) that are potent antagonists of caspase activity. The release of a mitochondrial intermembrane molecule, SMAC/DIABLO, was later shown to relieve XIAP inhibition of autocatalytic caspase-3 maturation, allowing full activation of caspase-3.[32,33] Thus, in humans rapid GrB-mediated apoptosis is dependent on the integration of GrB proteolytic processing of caspase-3 and mitochondrial outer membrane permeabilization (MOMP). In mice, however, GrB may not efficiently process Bid, and it is speculated that GrB-mediated cell death is due primarily through direct caspase activation.[20,34] Thus, in humans GrB can activate caspases and disrupt mitochondria through Bid, whereas in mice both cell death pathways act via caspases. This would result in different sensitivities to caspase inhibitors between species.

However, in the absence of caspase activation human GrB can still induce cell death through the mitochondria, although largely in the absence of DNA fragmentation.[18,19] Treatment with GrB results in damage to mitochondria detected by the dissipation of $\Delta\psi_m$, even in the absence of Bid, Bax, or Bak.[35] This implies that unidentified GrB substrates may also be present at the mitochondria. Additional cellular GrB substrates are: ICAD/DFF45, PARP, DNA-PK, α-tubulin, ROCK-1, lamin B, and NUMA.[21,36–41] The physiological significance of these other GrB substrates in normal killing is unclear. If caspases are blocked, the major short-term consequences of GrB-mediated apoptosis are blocked. This suggests that GrB cleavage of caspases is the pivotal event. However, in certain cases when the caspase pathway is blocked, in for example, virus infection of overexpression of IAPs, then the cleavage of these other proteins could become very important in mediating cell death.

GrB deficiency in mouse models confirmed that the *in vitro* role of GrB is rapid target cell killing, DNA fragmentation, and caspase activation.[42–44] *In vivo*, *grB*$^{-/-}$ knockout mice have shown enhanced susceptibility to mouse cytomegalovirus (MCMV), ectromelia virus, reduced graft *versus* host disease, and a reduced ability to clear allogeneic tumors.[45–48] However, in various other models, GrB-deficient mice remain competent at viral and tumor clearance, which is likely a reflection of Gr redundancy in these animals.[49]

Granzyme H. GrH was identified through screening cDNA libraries derived from cytotoxic cells.[50,51] To date, two groups have assessed the cytotoxic potential and mechanism of action of purified recombinant GrH.[52,53] Interestingly, some of their findings were highly disparate. Both groups observed that GrH cytotoxicity resulted in phosphatidylserine exposure, some loss of plasma membrane integrity, chromatin condensation, DNA damage (although they observed different patterns), and mitochondrial damage. However, Fellows *et al.* proposed a novel pathway that was independent of Bid or ICAD/DFF45 cleavage, caspase activity, did not induce cytochrome C release or DNA laddering, and transiently induced ROS production. In contrast, Zuzen Fan's group

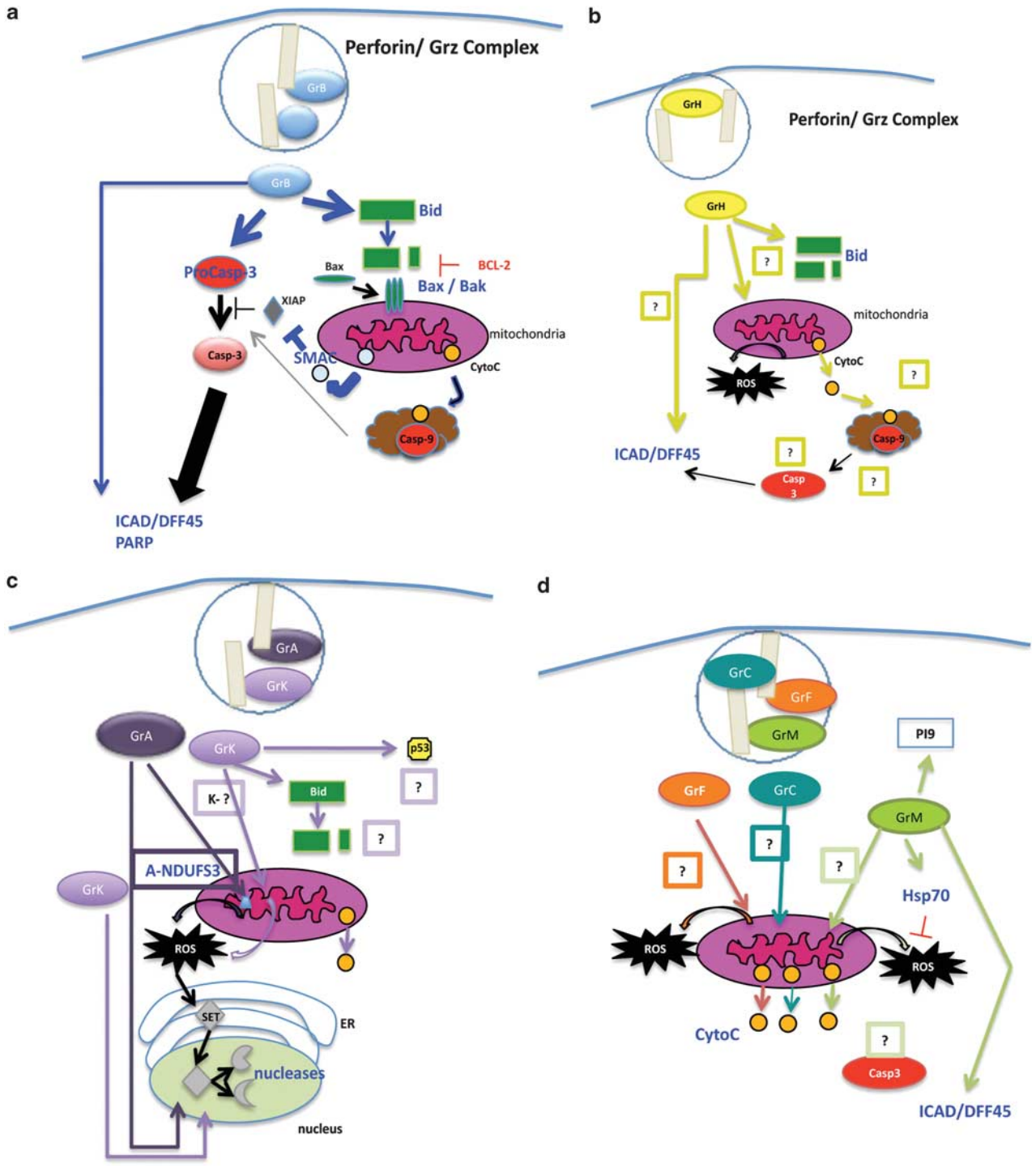

Figure 3 (**a**) GrB initiates caspase and mitochondrial cell death pathways, as well as directly processing a number of other pro-apoptotic molecules. (**b**) GrH cell death induces cell death by targeting the mitochondria and causes DNA through mechanisms that have not been clearly resolved. (**c**) GrA Cluster – GrA and GrK induce ROS production and the translocation of the SET complex from the ER to the nucleus. DNases from the complex initiate DNA damage. GrK may also target Bid and p53. (**d**) Grs C, F, and M – GrC, GrF, and GrM induce MOMP through unknown mechanisms. GrM also targets Hsp70 to promote ROS generation and may directly process ICAD/DFF45. GrM has been shown to proteolytically inactivate the GrB inhibitor PI9

observed direct GrH proteolysis of ICAD/DFF45, some sensitivity to zVAD-fmk and the activation of caspase-3, DNA laddering, cytochrome C release, and mitochondrial swelling. They proposed that GrH cytotoxicity was likely mediated by GrH-dependent proteolysis of Bid.

Studies aimed at finding the physiological relevance of GrH have been challenging. As there is no clear GrH homologue expressed in mice, knockout studies cannot be performed. However, cytotoxic studies using a chymase inhibitor with rat NK granules (which express a similar chymase to GrH)

diminished YAC-1 cell death compared with untreated samples.[54]

Alternatively, GrH may have other anti-viral functions, either targeting viral proteins or as an accessory role to other cytotoxic molecules such as GrB. Andrade *et al.*,[55] have reported GrH-proteolytic inactivation of a potent adenovirus-derived GrB inhibitor, LK-100K. In this context, GrH relieved the inhibition of GrB by LK-100K in adenovirus-infected cells, allowing GrB to regain the ability to induce target cell apoptosis. Andrade's group has also reported anti-viral functions of GrH toward an adenovirus DNA replication protein, and a hepatitis-C translation factor La.[55,56]

Granzymes C–G. These enzymes are part of the mouse GrB gene cluster, and very little is known about their function. Most of these Grs were identified through N-terminal sequencing of proteins acquired from purified granules, or screening cDNA libraries derived from lymphokine-activated killer cells and CTLs.[2,13,57,58]

GrC, located just downstream of GrB, is similar to human GrH and has been reported to induce cell death upon its delivery with perforin to target cells.[59] YAC-1 exposure to GrC resulted in phosphatidylserine exposure, plasma cell membrane permeability, single-stranded DNA breaks, chromatin condensation, mitochondrial swelling, loss of $\Delta\psi_m$, and the release of cytochrome C. GrC did not activate caspases, nor cause direct proteolytic cleavage of ICAD or Bid. Mitochondrial swelling induced by GrC was not sensitive to the classical PTP inhibitor cyclosporine A. Therefore, mechanisms responsible for biochemical changes leading to GrC-induced cell death remain unknown.

Recently, GrF has been reported to induce a novel mechanism of cell death.[60] Treatment of YAC-1 target cells with GrF and adenovirus, perforin, or streptolysin O resulted in a form of cell death that was characterized by phosphatidylserine exposure, plasma membrane permeability, mitochondrial swelling and damage, and partial chromatin condensation. Cell death was unimpeded in the presence of caspase inhibitors, and the cells did not show signs of caspase activation, despite the release of cytochrome C from the mitochondria. The investigators reported strong ROS production, and loss of ATP production, implying that GrF-induced mitochondrial dysfunction.

Granzyme A. GrA was first identified as a CTL-specific cDNA clone that was predicted to encode a protease named Hanukah factor.[61] Early studies using purified native GrA indicated a role for GrA in cell-mediated cytotoxicity, measured by ^{51}Cr-release, and DNA fragmentation assays.[16] Additionally, GrA-stably transfected rat mast cells (RBL-2H3) provided evidence that GrA could induce DNA fragmentation in target cells, although the greatest effects were observed when the cells were co-transfected with GrB.[15]

Later studies, with the use of recombinant GrA and purified perforin, led to observations that GrA-mediated cell death was characterized by phosphatidylserine exposure, chromatin condensation, single-stranded DNA nicking, the dissipation of $\Delta\psi_m$, and ROS production.[62] Cell death occurred in the absence of caspase activity, was insensitive to Bcl-2 overexpression, and damage to the mitochondria did not induce the loss of cytochrome C.[62] These findings supported the hypothesis that GrA induced a cell death program distinct from other cell-mediated pathways, particularly that of GrB. Substrates of GrA have been identified; lamins A–C, core histones, Ku70 (a double-strand DNA repair enzyme), PARP-1 (DNA repair enzyme), a component of the mitochondrial respiratory complex I, and components the ER-associated oxidative stress response SET complex (composed of SET, pp32, Ape1, HMG2, NM23-H1, and TREX-1).[41,63–70] The current molecular model of GrA-mediated cell death, proposed by Judy Lieberman's group,[67] suggests that GrA enters the mitochondrial matrix and proteolytically inactivates complex I protein NDUFS3. This leads to the generation of mitochondrial superoxide, and loss of $\Delta\psi_m$. Reactive oxygen species then induces the translocation of the redox-sensitive ER-associated SET complex to the nucleus.[62] There, GrA proteolytically degrades lamins to gain entry to the nucleus, and inactivates SET (an inhibitor of the exonuclease NM23-H1), Ape1 (a base excision repair endonuclease) and HMGB2 (a DNA-binding protein that recognizes distorted DNA), and liberates two nucleases NM23-H1 and TREX-1 to induce DNA damage.[63–66]

Studies using *grB*$^{-/-}$ CTLs have observed target cell death with a few characteristics of purified or recombinant GrA, which were attributed to GrA.[47,62] However, there is strong debate as to whether GrA is truly a cytotoxic protease. Studies of granule-mediated responses in *grA*$^{-/-}$ knockouts have not shown any defects in response to numerous pathogens.[71] The exceptions are the response to poxvirus ectromelia, where mice displayed a delayed clearance of the virus, and during herpes simplex infection, where virus spreads more readily throughout the peripheral nervous system.[72,73] Work recently published by Chris Froelich's group has suggested that GrA can only induce cell death at 'non-physiological levels', and that the primary function of GrA is actually the elicitation of inflammation.[74]

Granzyme K. GrK was discovered and purified from LAK cells.[75] Initial studies using purified human or rat GrK revealed that GrK-induced cell lysis, DNA damage, and affected the mitochondria of target cells.[16,19] Treatment of target cells with recombinant human GrK and either a cationic liposomal delivery vehicle or adenovirus induced phosphatidylserine exposure, ssDNA nicks, loss of membrane integrity, and chromatin condensation in a manner insensitive to z-VAD-fmk and Bcl-xL overexpression.[76] Similarly to GrA, GrK directly cleaved SET, Ape1, HMG2, and induced the translocation of the nuclease NM23-H1 from the ER to the nucleus. Mitochondrial damage was also observed, demonstrated by loss of $\Delta\psi_m$, and the generation of ROS.[77] However, unlike GrA, GrK was reported to cause MOMP, measured by the release of cytochrome C (although caspase-3 was not activated). The mechanism of MOMP was proposed to be via direct GrK proteolytic activation of Bid. However, the investigators did not ascertain the cleavage site in Bid, nor was GrK-mediated killing evaluated in the absence of Bid.

Recently, Zuzen Fan's group[78] has also reported that GrK proteolytically processes p53 to pro-apoptotic forms.

This would be a unique pro-apoptotic mechanism among Grs, although caspase-3 has been reported to cleave and activate p53 at alternative residues.[79] The mechanism underlying GrK cleavage of p53 and its connection to cell death remains unknown.

Granzyme M. Cytotoxic studies, using recombinant GrM have led to conflicting results. Mark Smyth's group reported that GrM and perforin-induced cell death characterized by rapid lysis (^{51}Cr-release), phosphatidylserine exposure, permeability to PI, chromatin condensation, and cytoplasmic vacuolization. Killing lacked sensitivity to both zVAD-fmk and Bcl-2 overexpression, and did not induce DNA fragmentation, cytochrome C release, ROS production, or loss of $\Delta\psi_m$.[80] In support of this finding, another group also reported GrM-killing induced cell swelling and permeability to PI, was insensitive to zVAD-fmk, and did not activate caspase-3.[81] In contrast, Zuzen Fan's group reported recombinant GrM delivered by a cationic liposome reagent-induced rapid cell lysis (^{51}Cr-release), phosphatidylserine exposure (but remained largely impermeable to PI), and DNA fragmentation. Cell death readouts were also sensitive to zVAD-fmk, as caspase-3 was activated by an undetermined mechanism.[82] They reported direct proteolysis of ICAD at serine[107] (not a previously reported P1 cleavage residue), and PARP at an unknown cleavage site. Of interest, these results have recently been refuted.[81] Fan's group has also reported the release of cytochrome C, ROS production, $\Delta\psi_m$ dissipation (through a cyclosporine-A-sensitive mechanism), and GrM-proteolysis of heat shock protein 70, a protein that protects cells from ROS damage.[83] GrM also disrupts the microtubule network of cells in the presence of zVAD-fmk, and specifically targets ezrin and α-tubulin at numerous cleavage sites.[84]

Mice recently generated with targeted GrM ablation display normal homeostasis, normal NK cytotoxicity, and an effective response against ectromelia virus. The only alteration in cell-mediated immunity was a partially impaired response to MCMV.[85]

GrM functions outside of direct killing have also been proposed, as GrM proteolytically inactives a GrB endogenous inhibitor, PI9.[86] The investigators proposed a model wherein GrM could assist GrB-induced killing by relieving PI9 inhibition of GrB.

Additional Roles of Grs

Grs share homology with serine proteases derived from myeloid cells, such as neutrophils and mast cells. Serine proteases derived from these cells, such as CMA1s, CG, and neutrophil elastase, have long been implicated in extracellular functions, such as inflammation and tissue remodeling.[87,88] Consequently, in addition to well-established roles in cytotoxicity, it is possible that lymphocyte-derived serine proteases modulate some of these extracellular processes as well.

Elevated levels of soluble GrB and GrB-expressing cells in plasma, synovial fluid, cerebrospinal fluid, and bronchoalveolar lavage are observed in patients suffering from a variety of inflammatory conditions.[89] GrB may also be released *in vivo* from activated cytotoxic cells in the absence of targets, both in active and zymogen forms.[90] Furthermore, it has become appreciated that GrB is expressed in the absence of perforin by a variety of non-lymphoid cells.[91–93] Thus, there is great speculation that GrB may have extracellular functions that promote extracellular matrix remodeling, cell death, and inflammation. In support of this theory, reports have demonstrated GrB-mediated proteolysis of extracellular matrix proteins.[94–96] A consequence of this may be perforin-independent cell death of adherent cells, via anoikis, where adherent cells die following detachment from the extracellular matrix. Alternatively, cleavage of these proteins may also influence cellular adhesion and migration processes. Other substrates have included proteins involved in blood clotting, and angiogenesis.[94,97] Additionally, GrB cleaves a number of cell receptors, which may influence their ability to respond to growth and survival signals, as well as promote the production of autoantigens.[98,99]

Several studies have suggested an inflammatory role for GrA. Treatment with native human GrA induced the production of inflammatory cytokines from epithelial and fibroblast cells, as well as human monocytes.[100] Recently, both purified native and recombinant forms of human and mouse GrA have been observed to induce the release of pro-inflammatory cytokines such IL-1β, TNF- α, and IL-6 from freshly isolated human adherent PBMCs.[74] The process did not require perforin, but did require target cell internalization. However, Judy Lieberman's *et al*.[69] has contested these findings, questioning the level of endotoxin contamination in the GrA preparations, which could provoke pro-inflammatory responses in monocyte-like cells. Of note, mouse GrK has also recently been shown to have pro-inflammatory potential.[101]

Other groups have reported GrA degradation of extracellular proteins, such as collagen type IV, fibronectin, and proteoglycans[102] The thrombin receptor is also susceptible to GrA proteolysis, affecting clotting reactions. Physiological evidence of GrA extracellular proteolytic activities originates from the observation that GrA levels are elevated in synovial fluid of rheumatoid arthritis patients.[103] Additionally, a number of plasma inhibitors of GrA have been reported, further supporting a potential GrA extracellular function. But, a direct causative effect of GrA and immunopathology has not been clearly established.

Clearly Gr research is still very active a quarter of a century on, and will continue to grow in the years to come. This is just one of the outstanding legacies that Jurg has left to the scientific world.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements. Many investigators have contributed to granzyme research. We apologize to those whose work was not cited because of a limitation in the number of references allowed. Work in the authors' laboratories is supported by the Canadian Institutes of Health Research and the Canadian Cancer Society. RCB is a Medical Scientist of Alberta Innovates Health Solutions.

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