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Measurement, Inhibition, and Killing Mechanisms of Cytotoxic Granule Serine Proteases

by

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ABSTRACT

Natural killer (NK) cells and cytotoxic T lymphocytes (CTL) are critical for the protection of organisms against pathogens and cancer. The process by which these cells eliminate infected or transformed cells are through two basic mechanisms, receptor-mediated interactions, or delivery of contents from intracellular cytotoxic granules. Granules are comprised of perforin and a family of serine proteases, called granzymes. Upon entry into target cells, these proteins work together to initiate cellular death pathways. Previous and extensive biochemical studies had already established that granzyme B (GrB) was a powerful inducer of apoptosis, but sensitive assays to confirm its release from cytotoxic cells were lacking. We hypothesized that GrB release, measured by ELISPOT, directly assessed the lytic potential of antigen-specific cytotoxic cells. Indeed, data provided in this thesis established a strong correlation between GrB release and target cell lysis. Our results imply that GrB could be a promising tool to assess cell-mediated immunity during vaccine development.

However, several other independent studies in *grB*$^{-/-}$ mice demonstrated that additional granzymes were capable of clearing viruses and tumorigenic cells. Granzyme H (GrH) is highly and constitutively expressed in human NK cells, and therefore, we hypothesized that it was also an effective cytotoxic molecule. Our experiments established that GrH-induced cell death by a mechanism distinct from those of GrB and Fas. We identified a GrH substrate, DFF45/ICAD, and showed that GrH induced mitochondrial damage through a Bid-independent mechanism. Furthermore, cell death was dependent on Bax and/or Bak, but

independent of caspase activation. Hence, we have elucidated an alternative cytotoxic pathway that could be employed to eliminate target cells with immune evasion strategies targeted to GrB or Fas.

Finally, control of serine proteases by endogenous inhibitors is important to numerous biological processes, including apoptosis. We hypothesized that as GrH displayed chymase activity, the serine protease inhibitor anti-chymotrypsin (ACT) would impair GrH function. Our data established that ACT effectively attenuated GrH cytotoxicity and prevented proteolysis of a GrH substrate. Collectively, this thesis describes a novel GrH inhibitor, provides a new tool to evaluate cell-mediated immunity, and provides evidence of an alternative mechanism of cytotoxicity.

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Abbreviations

ΔΨm	mitochondrial transmembrane potential
^{51}Cr-release	^{51}Chromium-release
7-AAD	7-amino-actinomycin D
Ac-IEPD-pNA	Acetyl-Ile-Glu-Pro-Asp-paranitroanilide
ACT	Antichymotrypsin
Ad5	adenovirus 5
Apaf-1	apoptotic protease activating factor-1
ATP	adenosine triphosphate
Bak	Bcl-2 antagonist killer 1
Bax	Bcl-2-associated X protein
Bcl-2	B cell CLL/ Lymphoma 2
Bcl-xL	Bcl-2- related gene, long isoform
BH3	Bcl-2 Homology Domain 3
Bid	Bcl-2- interacting domain death agonist
Bim	Bcl-2- interacting mediator of cell death
CARD	caspase recruitment domain
CrmA	Cytokine response modifier A
CTL	cytotoxic T lymphocytes
DED	death effector domain
DFF40/CAD	DNA Fragmentation Factor 40/ caspase-activated Dnase
DFF45/ICAD	DNA Fragmentation Factor 45/ Inhibitor of caspase-activated Dnase
DISC	death-inducing signal complex
DN	dominant negative
DTNB	5,5'-dithiobis (2-nitrobenzoic acid)
E/T ratio	effector/target ratio
EGTA	ethyleneglycoltetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunospot
gp	Glycoprotein
GrB	Granzyme B
GrH	Granzyme H
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
IAP	inhibitor of apoptosis protein
IL	Interleukin
IVTT	*in vitro* transcription/ translation
LAK	Lymphokine Activated Killer cells
LCMV	Lymphocytic choriomeningitis virus
Mcl-1	myeloid cell leukemia 1

MCMV	murine cytomegalovirus
MHC	major histocompatability complex
MOMP	mitochondrial outer membrane permeabilization
MPT	mitochondrial permeability transition
NK	natural killer
NP	Nucleoprotein
PARP	poly (ADP-ribose) polymerase
PBS	phosphate-buffered saline
PI	propidium iodide
PI9	protease inhibitor 9
PTP	permeability transition pore
Puma	p53-upregulated modulator of apoptosis
RCL	reactive centre loop
ROS	reactive oxygen species
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SMAC/ DIABLO	second mitochondria-derived activator of caspase/ Direct IAP-binding protein with low pI
SPI-6	serine protease inhibitor 6
Suc-FLF-Bszl	Succinyl-Phe-Leu-Phe-thiobenzyl ester
TMRE	tetramethyl-rhodamine, ethyl ester, percholate
TNF	tumor necrosis factor
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling
z-VAD-fmk	N-Benzyloxycarbonyl-Val-Ala-Asp Fluoromethyl Ketone

CHAPTER 1 – INTRODUCTION

THE CYTOTOXIC CELLS OF THE IMMUNE SYSTEM

Cell-mediated immunity is essential for protecting higher organisms against pathogens and tumorigenesis. Initial encounter between host and pathogens elicits the release of anti-viral and inflammatory molecules, limiting viral replication and promoting the recruitment of phagocytes and professional antigen-presenting cells (APCs) (Janeway, 2005). Natural killer (NK) cells are the first line of cytotoxic cells recruited to sites of infection and inflammation, and are characterized by a repertoire of activating and inhibitory receptors at their cell surface (Lanier, 2008). These receptors are designed to engage ligands on potential cellular targets and discriminate between healthy and altered/stressed cells. Virally-infected and tumorigenic cells display ligands that tip the balance toward activation, stimulating the NK cell to form synapses with these cells and attack them. Target cell killing is mediated by the engagement of death receptors or release intracellular cytotoxic contents (Orange, 2008).

Exposure of professional APCs to inflammatory stimuli and the acquisition of antigens result in their migration to secondary lymphoid tissues (Janeway, 2005). Here, they present antigen to cells of the adaptive immune compartment, including circulating $CD8^+$ T cells. T cells are characterized by a T cell receptor (TCR), a cell surface molecule that has exquisite specificity for antigen displayed by self antigen-presenting molecules, major histocompatibility complex (MHC) in mice, or Human Leukocyte Antigen (HLA) in humans. Recognition of T cell cognate antigen:MHC complexes in the presence of accessory signals induces a proliferation and differentiation program. In $CD8^+$ T

cells, the differentiation process results in the expression and storage of cytotoxic molecules, whereupon these cells become designated cytotoxic T lymphocytes (CTL) or effector $CD8^+$ T cells. Cytotoxic T cells are subsequently recruited to sites of viral infection or tumorigenesis through inflammatory chemotactic gradients (Campbell et al., 2003). Upon recognition of cellular targets displaying cognate antigen, CTLs deploy their cytotoxic arsenal to induce target cell death (Bossi and Griffiths, 2005).

However, there are many circumstances where if not carefully controlled, cytotoxicity may not be advantageous to the host organism. Indeed, autoimmune disease can be a consequence of dysregulated cytotoxic immunity (Russell and Ley, 2002; Trapani and Smyth, 2002). Cytotoxic cells are also implicated in transplant rejection and graft versus host disease (Choy, 2009; Trapani and Smyth, 2002).

The arsenal of cytotoxic cells is composed of molecules expressed at the cell surface, or a number of proteins stored within specialized secretory lysosomes (also known as cytotoxic granules) (Bossi and Griffiths, 2005; Lieberman, 2003). The constituents of secretory lysosomes involved in target cell death include perforin and a family of serine proteases, known as granzymes (Chowdhury and Lieberman, 2008; Cullen and Martin, 2008). Contact between effector and target cells results in the directional mobilization of secretory lysosomes towards contact sites. The release of granules from the cytotoxic cell requires sequential docking and fusion with the effector cell membrane, followed by the expulsion of contents

into the intercellular space, a process referred to as exocytosis/ degranulation (Bossi and Griffiths, 2005; Orange, 2008).

Once granule proteins gain entry to target cells, they initiate complex signaling cascades that instruct target cells to die (Barry and Bleackley, 2002; Chowdhury and Lieberman, 2008; Cullen and Martin, 2008).

Traditionally, CTL/NK- induced target cell death was monitored *in vitro*, using radioactive-based assays. Cell lysis was evaluated by measuring the leakage of a radioactive label, ^{51}Cr-sodium chromate, from target cells with compromised plasma membranes (Brunner et al., 1968). Later, studies of cytotoxic-mediated cell death led to the observations that target cells displayed oligonucleosomal degradation of DNA. This process could be measured by the release of ^{3}H-thymidine or ^{125}I-iododeoxyuridine radiolabeled DNA fragments from the nucleus of apoptotic cells (Matzinger, 1991). As the molecular processes involved in promoting cell death have been elucidated, many other assays have now been developed to monitor cell viability.

CLASSIFICATION OF CELL DEATH

Cell death is an essential process for multicellular organisms, with roles in homeostasis, development, and the control and/or removal of pathogens. The dysregulation of cell death processes has been implicated in the development of cancer, autoimmune disease, tissue injury, and ineffective immune responses (Duprez et al., 2009). Several pathways are now recognized: apoptosis, necrosis (necroptosis), autophagy, and pyroptosis (Duprez et al., 2009; Kroemer et al.,

2009). Classification of these death strategies is based on morphological and biochemical characteristics (Summarized in Table 1-1). One should note, however, that not all death stimuli induce one set of criteria, and may appear to have characteristics of more than one type of cell death program.

Apoptosis

Apoptosis is the classical mechanism of controlled cell death. It was first morphologically described by Kerr *et. al*., and now is associated with membrane blebbing, cell shrinkage, and chromatin condensation. Apoptotic cells are quickly recognized and cleared by phagocytes (Duprez et al., 2009; Kerr et al., 1972; Kroemer et al., 2009).

Biochemically, apoptosis is initiated by extrinsic or intrinsic signals that result in the activation of a family of intracellular cysteine proteases (caspases – discussed later), and/or a family of proteins that control the integrity of the mitochondria (Bcl-2 family – discussed later). The execution of apoptosis results in measureable changes in dying cells that include: the externalization of phosphatidylserine at the plasma membrane, activation of pro-apoptotic Bcl-2 family proteins that lead to mitochondrial outer membrane permeabilization (MOMP) and loss of mitochondrial transmembrane potential ($\Delta \Psi_m$), oligonucleosomal DNA fragmentation, and usually (but not always) the activation of cysteinyl aspartate-specific proteinases (caspases) (Kroemer et al., 2009). A major difference between apoptosis and necrosis (discussed below) is the maintenance of the plasma membrane integrity. Apoptotic cells remain

Cell Death	Morphological Features	Biochemical Featus
Apoptosis	• Cell shrinkage • Membrane blebbing • Chromatin condensation	• PS exposure • Caspase activation • Pro-apoptotic Bcl-2 proteins activated • MOMP • Dissipation of $\Delta\Psi_m$ • ROS generation • DNA laddering • PARP cleavage
Necrosis	• Cytoplasmic & organelle swelling • Loss of plasma membrane integrity • Release of intracellular content • Low – moderate chromatin condensation	• PS exposure • Permeable to vital dyes • Caspase-independent • Dissipation of $\Delta\Psi_m$ • ROS generation • ATP depletion • Elevated Ca_2^+ • Active calpains, cathepsins • Lysosomal membrane permeabilized • Alternative PARP cleavage
Pyroptosis	• Loss of plasma membrane integrity • No chromatin condensation	• Caspase-1 dependent • PS exposure • Permeable to vital dyes • $\Delta\Psi_m$ maintained
Autophagy	• Vacuolization of cytoplasm • No chromatin condensation	• Beclin-1 dissociation from Bcl-2/xL • LC3- I to LC3-II conversion

Table 1-1 Cell Death Classification

PS (phosphatidylserine)
MOMP (mitochondrial outer membrane permeablization)
$\Delta\Psi_m$ (mitochondrial transmembrane potential)
ROS (reactive oxygen species)
PARP (Poly (ADP-ribose) polymerase)
LC3-I (Light Chain-3 – I)
Bcl-2 (B cell CLL/ Lymphoma 2)

impermeable to vital dyes such as propidium iodide (PI) and 7-amino-actinomycin D (7-AAD). The non-inflammatory signature of apoptotic cell death arises from maintenance of the plasma membrane, preventing the release of intracellular contents, and rapid removal of apoptotic cells by phagocytes. However, in the absence of phagocytes, apoptotic cells eventually lose plasma membrane integrity, leading to characteristics that appear similar to necrotic cells, often termed secondary necrosis (Krysko et al., 2008).

Necrosis (Necroptosis)

Necrosis was initially presumed to be an uncontrolled cell death in response to extreme physiochemical stresses. However, recent findings have strongly implied that necrosis occurs in response to physiological stimuli and proceeds through a controlled process (Duprez et al., 2009; Kroemer et al., 2009; Krysko et al., 2008).

Morphologically, cells undergoing necrosis display rapid cytoplasmic and organelle swelling, with plasma membrane rupture and subsequent loss of intracellular contents. Chromatin condensation is very modest or not observed.

Biochemically, necrosis proceeds in the absence of caspase activation. Mitochondria are disturbed, as evidenced by extensive reactive oxygen species (ROS) generation, decreased levels of ATP, and loss of $\Delta \Psi_m$. MOMP is observed, but is not entirely controlled by Bcl-2 proteins. Elevated intracellular calcium levels, derived from damaged organelles such as the endoplasmic reticulum (ER), lead to the activation of cathepsins and calpains. Other effects

include lipid degradation mediated by phospholipases, lipoxygenases, and sphingomyelinases. Nuclear changes are induced by hyperactive poly (ADP-ribose) polymerase-1 (PARP-1), which eventually also leads to depletion of NAD^+ and a drop in ATP levels. In some forms of necrosis, there is a role for the kinase RIP1, a pathway termed necroptosis (Declercq et al., 2009).

However, it should be noted that some of these features also appear late in apoptosis, termed secondary necrosis, and so these features are not always *bona fide* necrotic characteristics. The most striking difference between apoptosis and necrosis is the elicitation of inflammation. As the plasma cell membrane becomes permeable, pro-inflammatory intracellular contents are released and induce responses from surrounding innate cells via recognition of damage-associated molecular patterns (DAMPs). Additionally, necrotic cells are cleared through macropinocytosis, a slower process than phagocytosis, leading to conditions more favorable to inflammation. The loss of plasma membrane integrity can be assessed by the accumulation of vital dyes, such as PI and 7-AAD, or the release of intracellular proteins, such as high mobility group box-1 protein (HMGB-1).

Pyroptosis

Pyroptosis has been recently recognized as a unique form of cellular death distinct from both apoptosis and necrosis (Bergsbaken et al., 2009; Duprez et al., 2009). It has been observed in response to microbial pathogens and exposure of cells to DAMPs. The process is entirely dependent on caspase-1, also known as interleukin-1β-converting enzyme (ICE), and does not involve apoptotic

executioner caspases (such as caspases-3 and -6). Caspase-1 promotes the proteolytic maturation of pro-inflammatory cytokines, pro-IL-1β and pro-IL-18, which are then released by cells into the extracellular milieu as part of the innate immune response. Certain stimuli induce very robust caspase-1 activation, leading to the formation of discrete pores in the plasma membrane, water influx, substantial cellular swelling, and eventually, cell lysis. Additional features of pyroptosis include the loss of $\Delta \Psi_m$, nuclear condensation, and DNA fragmentation. The integrity of the mitochondrial outer membrane is maintained, thereby preventing the release of cytochrome C and activation of apoptotic caspases. Essentially, pyroptotic death occurs with the release of pro-inflammatory cytokines and intracellular content, and thus, cell death by this pathway elicits a highly inflammatory response.

Autophagy

Autophagy is a catabolic pathway that can have multiple outcomes (He and Klionsky, 2009). Basal levels of autophagy promote the turnover of cellular components. Proteins and organelles are sequestered into specialized double-membrane vesicles. These "autophagosomes" subsequently fuse with lysosomes, where the constituents are degraded by hydrolases. Autophagy is elevated in response to stress, such as nutrient deprivation or pathogen invasion, and actually protects the cell from cell death by eliminating harmful organelles and protein aggregates. However, under severe conditions of stress, cell death occurs upon massive degradation of cytoplasmic constituents, a process designated

"macroautophagy" (Duprez et al., 2009; Kroemer et al., 2009). Under these conditions, autophagosomes accumulate and extensively fuse with lysosomes to generate autolysosomes, large cytoplasmic vacuoles that are very distinguishable from other cellular vesicles under transmission electron microscopy (Klionsky et al., 2008).

Macroautophagy is characterized by cell death that occurs independently of chromatin condensation, but is instead accompanied by extensive vacuolization of the cytoplasm. Biochemically, autophagy is regulated by a number of genes, the autophagy-related genes (Atg). One marker is the conversion of LC3-I (Atg8), in the cytoplasm, to LC3-II (through a lipidation process), a form associated with autophagosomic membranes (Klionsky et al., 2008).

MEDIATORS OF CELL DEATH

CASPASES

Caspases are a family of *c*ysteinyl *asp*artate-specific protein*ases*, characterized by a catalytic cysteine residue that induces nucleophilic attack at aspartate residues within target proteins (Chowdhury et al., 2008; Pop and Salvesen, 2009). Their targets comprise a wide variety of cellular proteins that affect the structural integrity and genetic material of the cell.

Caspases are produced as zymogens, requiring proteolytic processing to produce functional heterotetramers. The proteins are synthesized as 32-55 kD

polypeptides composed of three common domains; a 17-21 kD central large internal domain containing the large catalytic subunit (p20), a 10-13 kD small C-terminal domain that contains the small catalytic subunit (p10), and a 3-14 kD N-terminal domain called the Death Domain (DD).

Caspases can be classified according to their structure and function (Figure 1-1). Two groups of caspases contain large N-terminal prodomains, either caspase-recruitment domains (CARDs), or Death Effector Domains (DEDs). These domains are relevant for pro-caspase recruitment to macromolecular caspase activation platforms. Here, the low intrinsic activity of the zymogens can permit autoactivation of caspases in close proximity of each other, a process dubbed "proximity-induced activation" (Logue and Martin, 2008).

Class I caspases are characterized by N-terminal CARDs that interact with adapter proteins of "inflammasomes". In humans, this class includes caspases-1, -4, -5, -12, -13 and -14. Although traditionally viewed as inflammatory mediators, caspase-1 has recently received attention for its role in pyropoptosis (Bergsbaken et al., 2009; Duprez et al., 2009; Ting et al., 2008).

Class II caspases are "initiator" caspases which induce the activation of "executioner" caspases. These proteins are also recruited to macromolecular complexes for activation. Upon the trimerization of death receptors of the Tumor Necrosis Factor (TNF) family, a Death-Inducing Signaling Complex (DISC) is formed that promotes the activation of caspases-8 or -10 (Chowdhury et al., 2008; Duprez et al., 2009; Rupinder et al., 2007). These receptors contain cytosolic Death Domains (DDs) that recruit adapter proteins and procaspase-8,

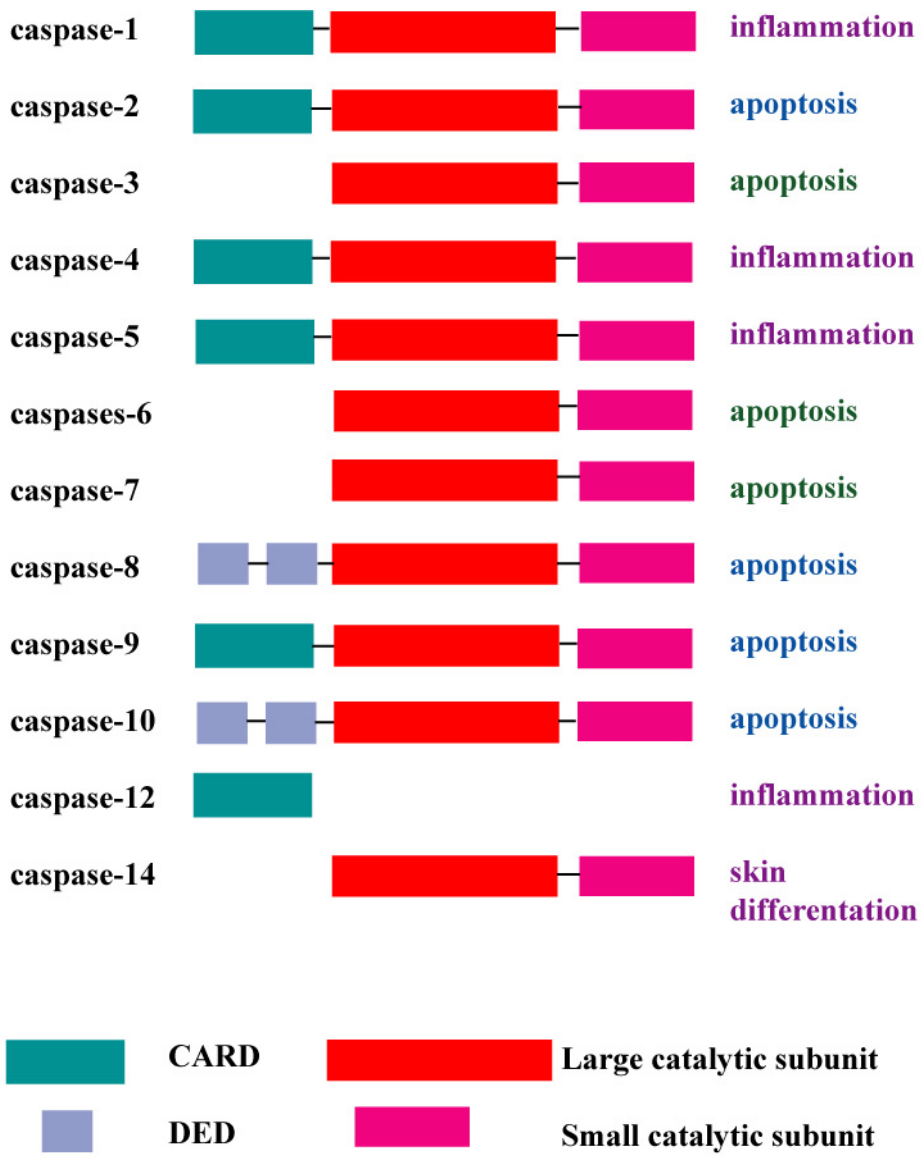


Figure 1-1 Caspase Structure and Function. Group I caspases have inflammatory functions, Group II caspases are initiators of apoptosis, Group III caspases are down-stream effector caspases of apoptosis.

and -10 via homotypic DED interactions. Once recruited to the complex, caspase-8 and -10 become activated through a proximity-induced mechanism.

The activation of Caspases-9 requires the formation of the "apoptosome" (Chowdhury et al., 2008; Pop and Salvesen, 2009). The apoptosome is comprised of cytosolic adapter apoptotic-protease activating factor-1 (Apaf-1), caspase-9, adenosine triphosphate (dATP), and the mitochondrial-derived cytotchrome C. Thus, caspase-9 activation is dependent on contributions from the mitochondrial death pathway (see below), and acts as a link between mitochondrial and caspase apoptotic pathways. Binding of cytochrome C with Apaf-1, in the presence of dATP, leads to Apaf-1 oligomerization. The recruitment of procaspase-9 is based on CARD-CARD protein interactions with Apaf-1, leading to proximity-induced activation of caspase-9.

Class III caspases, which include caspases 3,6, and 7, are executioner caspases and are characterized by short N-terminal prodomains. These caspases are responsible for the cleavage of proteins involved in cell cycle, DNA integrity, and structural proteins, and ultimately, drive the cell towards death. Executioner procaspases exist as dimers, needing proteolytic cleavage to initiate their activation process (Logue and Martin, 2008; Pop and Salvesen, 2009). Caspase-3 activation follows a two-step mechanism, initiated by caspase/GrB proteolysis at aspartate^{175}, a site that lies between the large and small catalytic units. This cleavage generates products of 20 kD and 12 kD (Martin et al., 1996; Nicholson et al., 1995). Full maturation requires an autocatalytic cleavage at the N-terminus, generating products of 17 kD and 12 kD. Production of active caspase-3 can be

impaired with chemical inhibitors or by inhibitor of apoptosis proteins (IAPs) (Chowdhury et al., 2008; Martin et al., 1996).

Once activated, caspases have a multitude of cellular substrates; see online database CASBAH http://bioinf.gen.tcd.ie/casbah/ (Luthi and Martin, 2007) . These include proteins such as Rho-associated kinase-1 (ROCK-1), vimentin, gelsolin, and Gas2, leading to plasma-membrane blebbing and cell contraction (Brancolini et al., 1995; Byun et al., 2001; Coleman et al., 2001; Kothakota et al., 1997; Sebbagh et al., 2001). Nuclear membrane degradation and chromatin condensation is mediated by caspase-dependent cleavage of lamins and acinus (Orth et al., 1996; Rao et al., 1996; Sahara et al., 1999). Cleavage of PARP and ICAD/DFF45 promote DNA degradation (Enari et al., 1998; Liu et al., 1997; Tang and Kidd, 1998).

MITOCHONDRIAL CELL DEATH EFFECTORS

The mitochondria are a major target for most cell death pathways, as their disruption promotes the release of pro-apoptotic molecules, the depletion of energy stores, and generation of reactive oxygen species (ROS) (Orrenius et al., 2007; Wang and Youle, 2009). Structurally, the mitochondria are composed of an inner membrane, which encloses the matrix, and an outer membrane, which encloses the intermembrane space. The inner membrane is highly convoluted, and contains proteins involved in the respiration-driven electron transport chain. Electron transport maintains an electrochemical gradient across the inner mitochondrial membrane (mitochondrial transmembrane potential; $\Delta \Psi_m$), and

ejects protons into the mitochondrial intermembrane space to drive the generation of ATP by oxidative phosphorylation.

The permeabilization of the outer mitochondrial membrane induces the release of pro-apoptotic proteins, such as cytochrome C and SMAC/DIABLO, from the intermembrane space into the cytoplasm. Mitochondrial permeability can be achieved by two basic mechanisms; the formation of oligomers by pro-apoptotic Bcl-2 proteins, or through Ca^{2+}-dependent mitochondrial permeability transition pores (PTP) that induce mitochondrial swelling and outer membrane rupture.

Bcl-2 Proteins

Members of the Bcl-2 (B cell CLL/ Lymphoma 2) family are characterized by the presence of one to four α-helical segments designated as Bcl-2 homology (BH) domains (Chipuk and Green, 2008; Youle and Strasser, 2008). There are three classes, based on structural and functional characteristics (Figure 1-2.A). The anti-apoptotic members are composed of four BH3 domains (BH1-4), and include Bcl-2, Bcl-x_L, Mcl-1, Bcl-w, and A1. These proteins are localized to the mitochondrial outer member and functionally serve to protect the integrity of the mitochondria.

There are two classes of pro-apoptotic Bcl-2 family members: proteins consisting of three BH domains (BH1-3), and the BH3-only proteins. The former family is composed of Bax, Bak, and Bok. These 'effector' molecules undergo

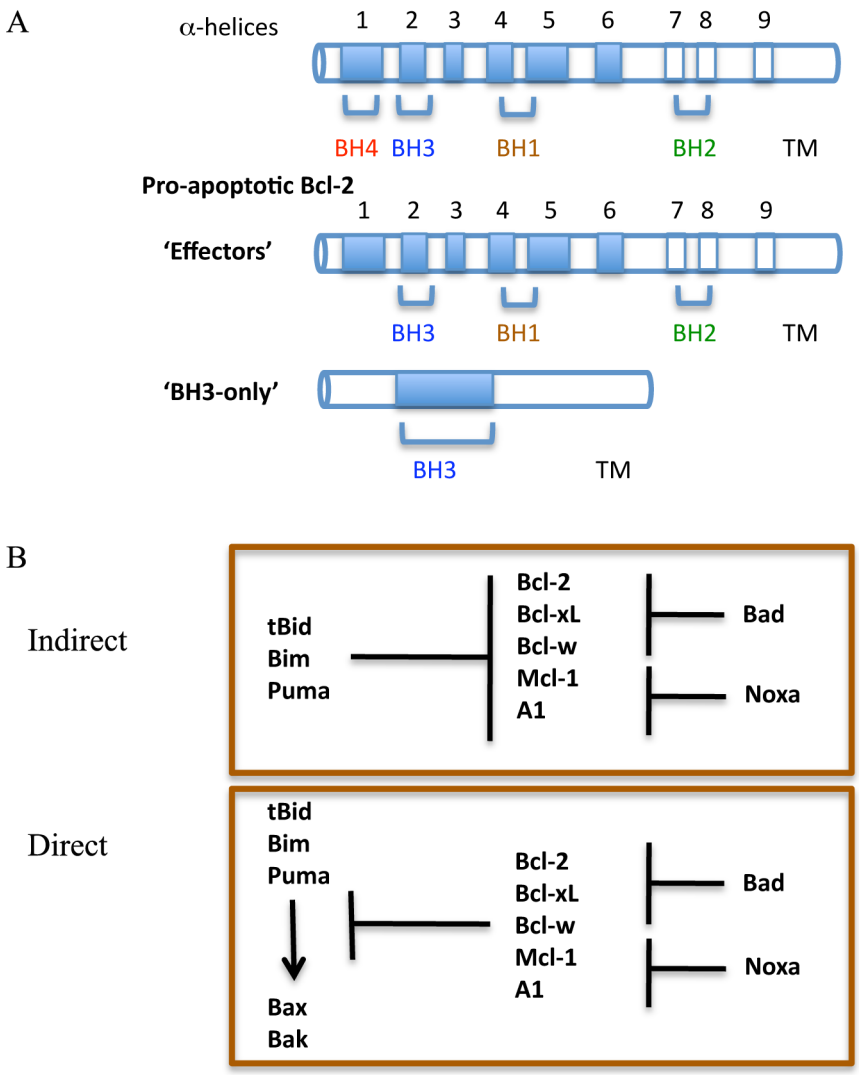


Figure 1-2 The Bcl-2 Family
A) Structural Organization of Bcl-2 proteins. Anti- and Pro-apoptotic proteins are composed of nine α-helices and contain BH1-4 and BH1-3 domains, respectively. BH3-only proteins are unstructured, with only on BH3 domain. A few also contain a transmembrane (TM) domain.

B) Bax / Bak Activation Models: Top- Indirect Activation Model states that BH3-only proteins neutralize anti-apoptotic Bcl-2 proteins to relieve the suppression of Bax/Bak.
Bottom- Direct Activation Model states that Bid, Bim and Puma directly activate Bax/Bak, while the remaining BH3-only proteins neutralize anti-apoptotic Bcl-2 proteins, sensitizing Bax/Bak for activation.

oligomerization at the mitochondrial outer membrane and promote MOMP. Major BH3-only proteins include Bid, Bim, Bad, Bik, Noxa, Puma, and Hrk, and these proteins act as sentinels of cell robustness and serve to modulate the functions or BH1-4 and BH1-3 molecules.

Cell fate decisions of survival or death are based on the integration of signals being transmitted to Bcl-2 proteins during exposure to various pathological and physiological stimuli (Chipuk and Green, 2008; Youle and Strasser, 2008). The canonical pathway proposes that BH3-only proteins are activated by transcriptional or post-transcriptional mechanisms that ultimately converge on Bax or Bak. These proteins then undergo localization and conformational changes that induce their oligomerization at the outer mitochondrial membrane. The importance of Bax/Bak in mitochondrial permeability has been demonstrated in *bax/bak* $^{-/-}$ double deficient cells, which are extremely resistant to multiple pro-apoptotic stimuli (Lindsten et al., 2000; Wei et al., 2001). In contrast, cells from single knockout mice remain responsive to pro-apoptotic stimuli.

Two models have been proposed to describe the mechanism of Bax/ Bak activation and oligomerization (Figure 1-2.B). The indirect theory proposes that anti-apoptotic Bcl-2 proteins interact with Bax and Bak and prevent their activation. BH3-only proteins act cooperatively with each other to relieve anti-apoptotic Bcl-2 protein repression of Bax/Bak (Willis et al., 2007). Bim, tBid, and Puma peptides can interact with all anti-apoptotic proteins, while other BH3-only proteins are selective. Bad only interacts with Bcl-2, Bcl-xL, and Bcl-w, and

Noxa only interacts with Mcl-1 and A1 (Chipuk and Green, 2008; Youle and Strasser, 2008). This implies that, in some situations, multiple BH3-only proteins would need to work together to effectively permit Bax/Bak activation.

The direct activation model proposes that BH3-only proteins Bid, Bim, and Puma directly interact with Bax and Bak proteins to facilitate activation and oligomerization (Gavathiotis et al., 2008; Kim et al., 2009; Kuwana et al., 2005). The remaining BH3-only proteins, such as Bad and Noxa, neutralize anti-apoptotic Bcl-2 proteins, preventing the inhibition of "activator" BH3-only proteins (Letai et al., 2002).

Activation of BH3-only proteins involves either transcriptional and/or post-translational controls. Bid is activated upon proteolytic cleavage by caspase-8, GrB, or calpains, promoting its translocation from the cytosol to the outer mitochondrial membrane, where it promotes MOMP (Barry et al., 2000b; Chen et al., 2001; Sutton et al., 2000). However, Bid cannot induce MOMP in the absence of Bax/Bak, and serves only to facilitate the initiation of Bax/ Bak oligomerization (Kim et al., 2009; Lovell et al., 2008).

Oxidative Stress and Calcium Overload

Mitochondria are a major source of Reactive Oxygen Species (ROS), produced as a consequence of mitochondrial respiration. Components of the electron transport chain, predominantly Complexes I and III, are likely the highest source of ROS, and the proteolytic inactivation of Complex I proteins by caspases or GrA has been reported to generate ROS during cell death (Martinvalet et al.,

2008; Ricci et al., 2004). Indeed, oxidative stress is implicated in both apoptotic and necrotic cell death, although the mechanisms are not fully understood (Orrenius et al., 2007; Ricci et al., 2003; Wang and Youle, 2009). The generation of ROS induces damage to DNA, impairs respiration, and induces lipid peroxidation.

Damage to DNA by ROS causes modification of purine and pyrimidine bases, DNA breaks (single or double-stranded), and can promote cross-linking with other molecules. The mitochondrial DNA (mtDNA) is a major target of ROS, and its damage has implications in the synthesis of proteins integral to electron transport and ATP generation. Consequently, cells exposed to oxidative stress become energy deprived, and the mitochondrial membrane potential is disrupted.

Reactive oxygen species production also promotes lipid peroxidation, a process that results in the oxidative degeneration of lipids, and ultimately damage to membranes. In the mitochondria, this leads to reduced mitochondrial respiration and oxidative phosphorylation, loss of $\Delta \Psi_m$, and impaired Ca^{2+} buffering capacity.

Mitochondria also play an integral role in intracellular Ca^{2+} homeostasis. Exposure to oxidative stress and excess Ca^{2+} can induce the mitochondrial membrane permeability transition (MPT), leading to loss of $\Delta \Psi_m$, mitochondrial swelling, and rupture of the mitochondrial outer membrane (Lemasters et al., 2009; Orrenius et al., 2007; Tsujimoto and Shimizu, 2007; Wang and Youle, 2009). The MTP is mediated by the PTP (permeability transition pore), which is

a protein complex putatively composed of voltage-dependent anion channel (VDAC), adenine nucleotide translocase (ANT), cyclophylin D (CyP-D), and a few other ill-defined proteins. It is localized at contact sites between the outer and inner mitochondrial membranes, where PTP blocking anti-apoptotic Bcl-2 proteins are also localized (Susnow et al., 2009). Researchers have observed that cyclosporine A, an inhibitor that interacts with cyclophilin D, can block MPT in some situations. However, PTP involvement in cell death pathways is still being debated. Currently, it seems to be more relevant during necrotic cell death, as the knockout of several components of the PTP show little resistance to a number of apoptotic stimuli (Wang and Youle, 2009).

Exposure to oxidative stress has also been implicated in the oxidation of an inner membrane lipid, cardiolipin (Orrenius et al., 2007). Cardiolipin keeps cytochrome C bound to the inner mitochondrial membrane through electrostatic interactions. Oxidation of cardiolipin disrupts these interactions and promotes the release of cytochrome C from the intermembrane space into the cytosol (discussed below).

Pro-apoptotic Mitochondrial Intermembrane Proteins

Once the mitochondrial outer membrane becomes permeable, a number of pro-apoptotic proteins are released; cytochrome C, second mitochondrial activator of caspases/ direct IAP-binding protein of low pI (SMAC/DIABLO), and apoptosis-inducing factor (AIF). The most extensively studied proteins released

from the mitochondrial intermembrane space are cytochrome C and SMAC/DIABLO.

Cytochrome C is localized in the intermembrane space, where it functions as an essential part of the respiratory chain, acting as an electron shuttle between complex III and complex IV. Upon MOMP, it is released to the cytosol, where it interacts with APAF-1 through lysine^{72} and becomes part of the apoptosome (Brenner and Mak, 2009; Garrido et al., 2006; Wang and Youle, 2009). To dissect the contribution of cytochrome C to apoptosis, independent of its critical role in oxidative phosphorylation, knock-in Cyto C K72A mice were generated. Cells expressing Cyto C K72A were unable to form a functional apoptosome, and caspase-9 and -3 activation was severely diminished in response to apoptotic stimulation in a number of cell types (Hao et al., 2005). However, in thymocytes, caspase-3 was still activated in response to various pro-apoptotic stimuli, despite the absence of an apoptosome. These results, and others, have indicated that there may be additional cytochrome C-independent mechanisms that activate caspases following MOMP (Marsden et al., 2004; Marsden et al., 2002). However, it remains unknown how these cytochrome C-independent mechanisms work.

SMAC/DIABLO is a 26 kDa protein that that is also localized at the intermembrane space of healthy mitochondria. However, upon its release from the mitochondria, it interacts with IAPs, proteins that interfere with caspase activation and target them for proteosomal degradation. The interactions of SMAC and IAPs are mediated through an N-terminal IAP-binding motif domain

of SMAC with the Baculovirus IAP Repeat (BIR) domains of IAPs (Brenner and Mak, 2009; Wang and Youle, 2009).

AIF is a flavin-adenosine dinucleotide binding oxidoreductase localized to the inner mitochondrial membrane. The N-terminus faces the matrix, while the C-terminal portion faces the intermembrane space. Proteolytic cleavage at leucine^{101} produces a liberated fragment which, upon subsequent translocation to the nucleus, interacts with DNA (Wang and Youle, 2009). This interaction leads to chromatin condensation and DNA fragmentation. It has been widely speculated that AIF contributes to caspase-independent cell death.

INDUCTION OF CELL DEATH BY CYTOTOXIC CELLS

Cytotoxic cells have two major mechanisms to induce target cell death; a surface FasL pathway, or via the perforin-dependent pathway that involves the directional secretion of cytoplasmic granules from cytotoxic cells to target cells (Kagi et al., 1994b; Lowin et al., 1994b).

FAS-MEDIATED CELL DEATH

Fas-mediated apoptosis is initiated upon the trimerization of Fas receptors, and the assembly of the DISC. The DISC consists of Fas, FADD (Fas-associated Death Domain), procaspase-8, and a modulator protein cFLIP (FLICE [a.k.a. caspase-8) inhibitory protein (Boldin et al., 1996; Boldin et al., 1995; Chinnaiyan et al., 1995; Muzio et al., 1996). Fas contain cytosolic DDs that promote the recruitment of FADD through homotypic protein interactions (Boldin et al., 1995;

Chinnaiyan et al., 1995). FADD is a bipartite adapter that also contains DEDs enabling it to interact with DEDs of procaspase-8, and -10 molecules (Boldin et al., 1996; Muzio et al., 1996). Caspase-8 and -10 become activated through proximal-induced mechanisms, and activated caspases are released to interact with cytosolic substrates. Fas-mediated apoptosis can proceed by two mechanisms, either directly through activation of caspase-3 (Type I), or through the mitochondrial pathway following the cleavage of Bid (Type II) (Fernandes-Alnemri et al., 1996; Li et al., 1998; Luo et al., 1998; Scaffidi et al., 1998). Fas-mediated cell death that follows the mitochondrial pathway induces MOMP, releases cytochrome C, promotes the formation of the apoptosome and results in caspase-3 activation (Figure 1-4.A). When anti-apoptotic Bcl-2 proteins are over-expressed, cytochrome C is not released and Fas-mediated apoptosis is attenuated (Adachi et al., 1997; Boise and Thompson, 1997; Vander Heiden et al., 1997). Expression of dominant-negative caspase-9 in Type II also impairs Fas-mediated killing by preventing caspase-3 activation (Li et al., 1997; Pan et al., 1998).

Once executioner caspases have been activated, either directly through caspase-8 or via the apoptosome, Fas-stimulated target cells die predominantly through apoptosis. The features of Fas-mediated apoptosis in Type II cells are summarized in Table 1-2.

GRANULE-MEDIATED CELL DEATH

The Cytotoxic Granule Components

Cytotoxic cells contain specialized cellular compartments, the secretory lysosomes, which are membrane-bound organelles equipped with cytolytic proteins (Bossi and Griffiths, 2005). The primary cytolytic molecules, delivered to target cells upon contact with cytotoxic cells, are perforin and a family of serine proteases called granzymes. Granule-mediated cytotoxicity is ablated in the absence of perforin, demonstrated by *in vivo* and *in vitro* studies using cytotoxic cells from *perforin* $^{-/-}$ mice, and in humans with perforin mutations, called familial hemophagocytic lymphohistiocytosis (Kagi et al., 1994a; Lowin et al., 1994a; Zur Stadt et al., 2006). Currently, the mechanism of perforin-facilitated entry of granzymes into target cells remains unresolved. Models proposed include the liberation of granzymes from endosomal compartments of the target cells to the cytosol, or a Ca^{2+}- driven plasma membrane repair process initiated by perforin damage (Cullen and Martin, 2008).

Granzymes are serine proteases, characterized by a His-Asp-Ser catalytic triad (Chowdhury and Lieberman, 2008; Kam et al., 2000). In humans, there are 5 granzymes (A, B, H, K, and M) while in mice there are 10 functional granzymes (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 (Chowdhury and Lieberman, 2008; Grossman et al., 2003) (Figure 1-3). The GrA cluster, at human chromosome 5 and murine chromosome 5, includes GrA and GrK. Granzymes A and K are tryptases, cleaving after basic amino acid residues

(designated the P1 residue) (Masson et al., 1986; Pasternack and Eisen, 1985; Wilharm et al., 1999).

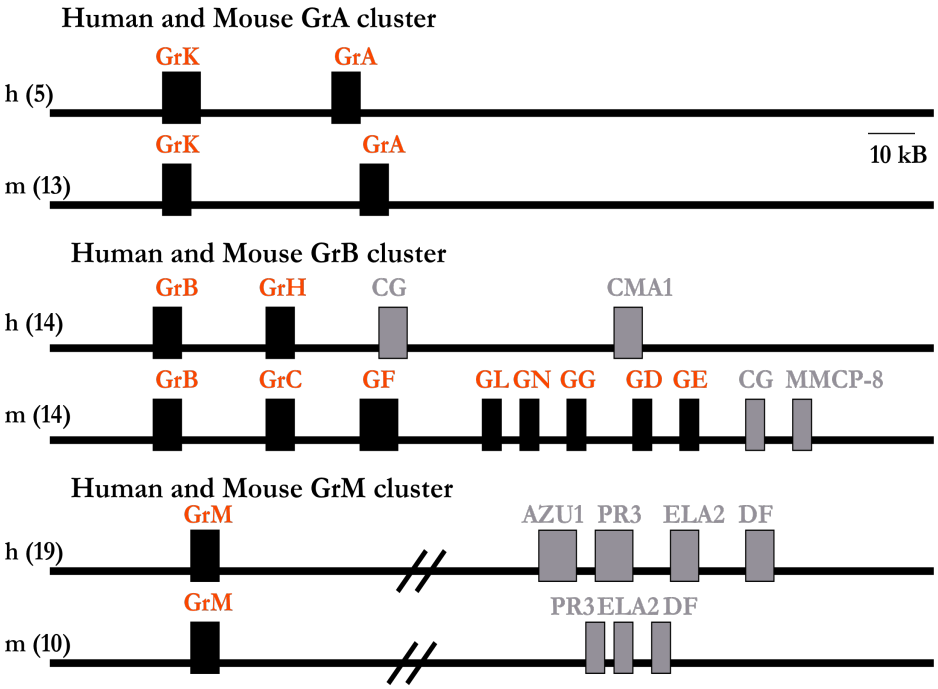
The GrB cluster is found at chromosome 14 of both human and mice but is composed of different enzymes. In humans, the cluster consists of GrB, GrH, cathepsin G, and mast cell chymase-1. In mice, the cluster contains GrB, C, F, N, G, D, E, cathepsin G, and mast cell chymase. Granzyme B is the most extensively studied granzyme, characterized by the unusual capacity to cleave substrates at aspartic acid residues (Odake et al., 1991; Poe et al., 1991).

Granzymes H and GrC -G, are believed to be chymases, established either through molecular modeling or cleavage of synthetic substrates at hydrophobic residues phenylalanine, leucine, tyrosine, and methionine (Edwards et al., 1999; Kaiserman et al., 2009; Kam et al., 2000). 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 (Smyth et al., 1993). Granzyme M is a metase, preferentially cleaving substrates at methionine, or leucine residues (Rukamp et al., 2004).

Granzyme Expression

The expression of granzymes within the lymphocyte population varies among cell types, and even among subsets. It should also be noted that many expression analyses have been based on RT-PCR, and there is emerging evidence



from Grossman et al. (2003) Curr. Opin. Immunol.

Figure 1-3 Genomic Organization of Serine Proteases.

Granzyme genes, found primarily in lymphoid-derived cytotoxic cells are indicated in **red**. (Mouse GrL is a pseudogene.)

Myeloid-derived serine proteases are indicated in **grey**.

Abbreviations: **Gr** = granzyme, **CG** = cathepsin G, **CMA1** = mast cell chymase, **MMCP-8** = mast cell protease-8, **AZU1** = azurocidin, **PR3** = proteinase 3, **ELA2** = human neutrophil elastase

that message and protein levels can differ, possibly indicative of post-transcriptional controls (Chowdhury and Lieberman, 2008; Fehniger et al., 2007). Most granzyme expression profiles in humans have now been determined by single-cell flow cytometry methods (the exception is GrH, where they have used Western blot analysis of enriched lymphocyte populations).

Human NK cells highly express GrA, H, and M, while GrB levels been reported to be low to intermediate in resting NK cells, but can be up-regulated upon stimulation (Fehniger et al., 2007; Fellows et al., 2007; Grossman et al., 2004b; Sayers et al., 2001; Sedelies et al., 2004). Granzyme K is only found in $CD56^{bright}$ cells, cells that have low cytotoxicity (Bade et al., 2005; Bratke et al., 2005).

Expression of granzymes within human T cell populations varies among subsets. Early differentiated memory $CD8^+$ T cells express GrA and GrK, usually in the absence/ low levels of perforin (Bratke et al., 2005). As $CD8^+$ T cells acquire more differentiated phenotypes, GrK becomes more reduced, while GrA, GrB, and GrM positive cells accumulate, along with the acquisition of perforin (Bade et al., 2005; Bratke et al., 2005; de Koning et al., 2009). Western blot analysis of human CTL showed that GrH levels were nearly undetectable (Fellows et al., 2007; Sedelies et al., 2004). However, we have detected GrH in our human $CD8^+$ T cell line by a number of techniques, including mass spectrometry (see Appendix Figure 2.D and Table -1). Human effector $CD4^+$ T cells can express GrA, B, H, and M, although usually at lower levels and frequencies compared to $CD8^+$ T cells (Appay et al., 2002; Bade et al., 2005; de

Koning et al., 2009; Fellows et al., 2007; Grossman et al., 2004b; Sedelies et al., 2004). Inducible human regulatory T cells have also been reported to express GrB (Grossman et al., 2004a; Grossman et al., 2004b).

In mice, expression patterns have been discerned primarily through molecular techniques, although there are now serological reagents for GrA, B, and C. Flow cytometry studies have demonstrated the expression of GrA in both resting and activated NK cells, while GrB levels are low in resting NK cells, but quickly increase upon their stimulation (Fehniger et al., 2007). A recently developed anti-GrC mAb has indicated that GrC expression can be induced in both $CD8^+$ T cells and NK cells upon *in vitro* activation (Cai et al., 2009). Granzyme C expression was also induced *in vivo* in NK cells of *Rag1* $^{-/-}$ mice infected with mouse cytomegalovirus (MCMV), the first demonstration of *in vivo* GrC protein expression (Cai et al., 2009). Effector $CD8^+$ T cells stimulated *in vivo* highly express GrB in response to various pathogens, while very little is detected in central memory cells (Byers et al., 2003; Laouar et al., 2008; Lawrence et al., 2005; Sarkar et al., 2008; Wherry et al., 2003c; Wolint et al., 2004b). Recently, effector $CD8^+$ T cells from mice infected with influenza virus A have demonstrated heterogeneity among antigen-specific populations with respect to GrA and GrB expression (Moffat et al., 2009). Two populations of antigen-specific $CD8^+$ T cells were observed; GrA^-/GrB^+, and GrA^+/GrB^+. Interestingly, GrA expression was higher among T cells specific for less immunodominant epitopes. The reason or significance of this finding is unknown. Granzyme B is also present in regulatory T cells (Cao et al., 2007).

The remaining murine granzymes have only been detected by molecular techniques in cells extensively manipulated *in vitro* (Grossman et al., 2003).

Mechanisms of Cytotoxicity and Physiological Roles of Granzymes

Our understanding of the function of the majority of granzymes is very limited. The exception is GrB, which has been established as a potent pro-apoptotic molecule (discussed below) as a result of extensive studies using biochemical techniques and purified or recombinant proteins. The cytotoxic mechanisms of remaining granzymes are still active areas of research. In the following section, the current understanding of granzyme-mediated cytotoxicity is reviewed. Table 1-2 summarizes the features displayed by target cells treated *in vitro* with purified/ recombinant granzymes, and the current models of cytotoxic-mediated cell death are shown in Figures 1-4A-D. Moreover, evidence of the physiological relevance of granzymes in cellular immunity will be presented.

GRANZYME B CLUSTER

Granzyme B

Several groups identified GrB in cytotoxic lymphocytes during the mid-1980s (Brunet et al., 1986; Lobe et al., 1986a; Lobe et al., 1986b; Masson and Tschopp, 1987; Young et al., 1986). Initial studies indicated that GrB induced *in vitro* cell lysis (^{51}Cr-release), rapid DNA fragmentation, and chromatin condensation (Nakajima et al., 1995; Shi et al., 1992a; Shi et al., 1992b). 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 impermeability to vital dyes at early timepoints (Froelich et al., 1996b; Heibein et al., 1999; MacDonald et al., 1999; Zamzami et al., 1995).

A pivotal piece of information that led to the discovery of the cytotoxic mechanism of GrB was its ability to cleave aspartic acid residues (Odake et al., 1991; Poe et al., 1991). Up to that point, only caspases were known to have this unusual specificity. Consequently, 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. -10) (Adrain et al., 2005; Andrade et al., 1998; Darmon et al., 1995; Martin et al., 1996; Medema et al., 1997; Van de Craen et al., 1997). These observations lead to a model wherein the activation of caspase-3 by GrB was largely responsible for many of the features induced by CTL killing (Adrain et al., 2005; Atkinson et al., 1998; Darmon et al., 1996; Darmon et al., 1995). Further studies revealed that GrB could also promote cell death via the mitochondrial pathway (Davis et al., 2000; Heibein et al., 1999; Pinkoski et al., 2001). 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 (Alimonti et al., 2001; Barry et al., 2000b; Heibein et al., 2000; Sutton et al., 2000). The release of a mitochondrial intermembrane molecule, SMAC/DIABLO, was later shown to relieve XIAP inhibition of autocatalytic caspase-3 maturation, allowing full

Granzymes

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

Table 1-2 Summary of Death Pathways Induced by Cytotoxic Cells

* Type II cells (such as Jurkat T cells)
? – have not been reported
N/ Y – reported results have been contrary

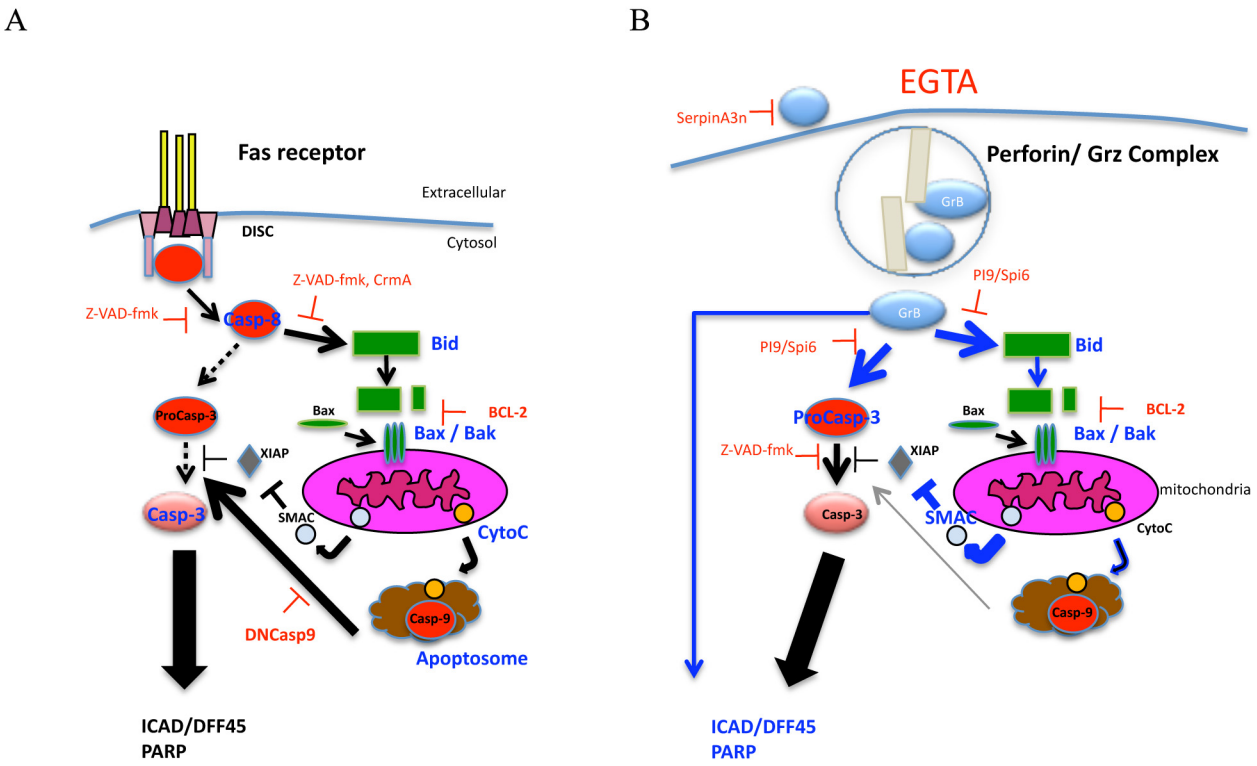


Figure 1-4 Cell Death Pathways. A) Fas can induce cell death through direct activation of caspase-3 (Type I) or through the mitochondrial pathway (Type II). The pathway is insensitive to EGTA. B) GrB initiates caspase andmitochondrial pathways as well as directly processing a number of other pro-apoptotic molecules. Granule-mediated cytotoxicity is ablated in the presence of EGTA.

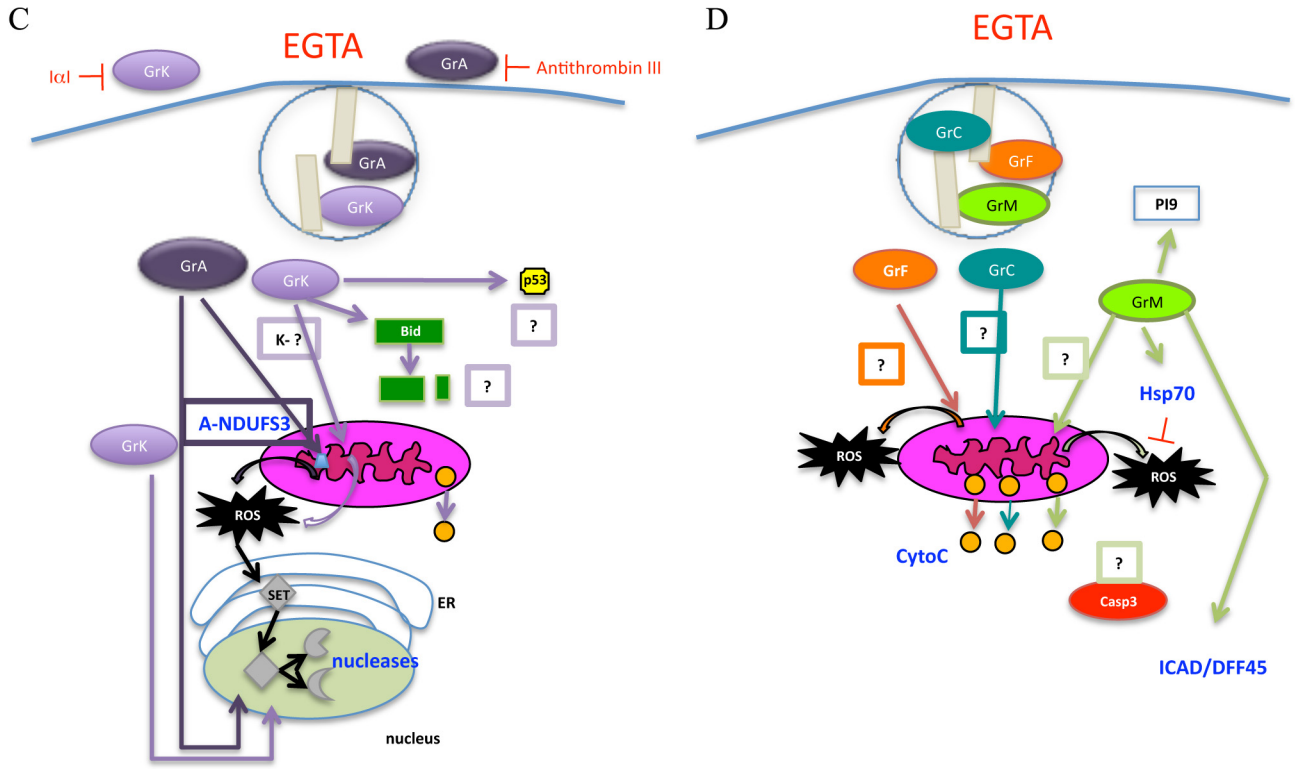


Figure 1-4 Cell Death Pathways continued C) Granzyme A cluster - GrA & GrK induce ROS production and the translocation of the SET complex from the ER to the nucleus. DNases from the complex initiate DNA damaage. GrK may also target Bid and p53. D) GrC, GrF, and GrM induce MOMP through unknown mechanisms. 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.

activation of caspase-3 (Goping et al., 2003; Sutton et al., 2003). Thus, in humans rapid GrB-mediated apoptosis is dependent on the integration of GrB proteolytic processing of caspase-3 and 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 (Adrain et al., 2005; Cullen et al., 2007).

In the absence of caspase activation, however, human GrB can still induce cell death through the mitochondria, although largely in the absence of DNA fragmentation (Heibein et al., 1999; MacDonald et al., 1999; Sarin et al., 1997; Trapani et al., 1998). 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 (Thomas et al., 2001). 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 (Andrade et al., 1998; Froelich et al., 1996a; Goping et al., 2006; Sebbagh et al., 2005; Sharif-Askari et al., 2001; Thomas et al., 2000; Zhang et al., 2001a).

Granzyme B deficiency in mouse models established that the *in vitro* role of GrB is rapid target cell killing and DNA fragmentation (Heusel et al., 1994; Shresta et al., 1995). *In vivo*, mice with targeted *grB*$^{-/-}$ knockout have shown enhanced susceptibility to MCMV and *Trypanosoma cruzi* (Fehniger et al., 2007; Muller et al., 2003). However, in various other models, GrB-deficient mice remain competent at viral and tumor clearance, which is likely a reflection of granzyme redundancy in these animals (Smyth et al., 2003).

Granzyme H

Granzyme H was identified through screening cDNA libraries derived from cytotoxic cells (Klein et al., 1990; Meier et al., 1990). It was observed that it contained a high level of homology to GrB, having arisen from gene duplication (Haddad et al., 1991). However, GrH did not demonstrate aspase activity and, instead, was shown to possess chymase activity (Edwards et al., 1999).

To date, two groups have assessed the cytotoxic potential and mechanism of action of purified recombinant GrH (Fellows et al., 2007; Hou et al., 2008). 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 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 (Hou *et al*., 2008). They proposed that GrH cytotoxicity was likely mediated by GrH-dependent proteolysis of Bid. However, the mechanism of Bid or DFF45 proteolysis was not explored. Monitoring of Bid and DFF45 cleavage in target cells was not assessed in the presence of caspase inhibitors, cleavage sites were not identified, and GrH-killing was not performed in Bid-deficient cells.

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 to untreated samples (Woodard et al., 1994).

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*., have reported GrH-proteolytic inactivation of a potent adenovirus-derived GrB inhibitor, LK-100K (Andrade et al., 2007). 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 towards an adenovirus DNA replication protein, and a Hepatitis-C translation factor, La, (Andrade et al., 2007; Romero et al., 2009).

Granzymes C-G

These enzymes are part of the mouse GrB gene cluster, and very little is known about their function. Most of these granzymes were identified through N-terminal sequencing of proteins acquired from purified granules, or screening cDNA libraries derived from Lymphokine-Activated Killer cells and CTLs (Bleackley et al., 1988; Jenne et al., 1988a; Jenne et al., 1988b; Jenne et al., 1989; Lobe et al., 1986a; Lobe et al., 1986b; Prendergast et al., 1991).

Granzyme C, 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 (Johnson et al., 2003). 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. Granzyme C 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 (Shi et al., 2009). 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 authors reported strong ROS production, and loss of ATP production, implying that GrF induced mitochondrial dysfunction. Interestingly, the release of cytochrome C, a sign of outer mitochondrial membrane permeability, was not prevented in *bid* $^{-/-}$ or *bax* $^{-/-}$/ *bak* $^{-/-}$ deficient mouse embryonic fibroblast targets. Other forms of mitochondrial apoptosis and outer membrane permeability are highly resistant to the death in the combined absence

of Bax and Bak (Wei et al., 2001). The authors could not establish any targeted proteolysis of members of the respiratory chain that could provide an explanation for the rapid effects of GrF-induced mitochondrial dysfunction. Further studies will be needed to ascertain the mechanism of this unusual form of cell death.

Attributing roles of cluster B orphan granzymes in granule-mediated cytotoxicity has come indirectly from studies of *grA*x*grB* $^{-/-}$ mice. The initial *grA*x*grB* $^{-/-}$ mouse strain contained severely reduced levels of downstream granzymes GrC, D, and F. More recent studies have evaluated GrC, D, and E by comparing them to a knockout mouse strain that has only knocked out the GrB gene. These comparative experiments have indicated a role of these enzymes in killing tumor cells *in vitro* and controlling *in vivo* responses to allogeneic tumors (Revell et al., 2005a). Assessment of *grB cluster* x *GrA*-deficient mice compared to *grA*x*grB* $^{-/-}$ mice also implied a role for GrC and F in the control of γ-herpesvirus latent infection, where viral reactivation was reduced in the presence of orphan granzymes (Loh et al., 2004).

GRANZYME A CLUSTER

Granzyme A

Granzyme A was the first serine protease identified in cytotoxic granules (Brunet et al., 1986; Kramer et al., 1986; Masson et al., 1986; Pasternack and Eisen, 1985; Young et al., 1986). Early studies using purified native GrA indicated a role for GrA in cell-mediated cytotoxicity, measured by ^{51}Cr-release and DNA fragmentation assays (Hayes et al., 1989; Shi et al., 1992a).

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 (Nakajima et al., 1995; Shiver et al., 1992).

Later studies, with the use of recombinant GrA and purified perforin, lead 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 (Beresford et al., 1999; Martinvalet et al., 2005). Cell death occurred in the absence of caspase activity, was insensitive to Bcl-2 over-expression, and damage to the mitochondria did not induce the loss of cytochrome C (Beresford et al., 1999; Martinvalet et al., 2005). These findings supported the hypothesis that GrA induced a cell death program distinct from another 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) (Chowdhury et al., 2006; Fan et al., 2003a; Fan et al., 2002; Fan et al., 2003b; Martinvalet et al., 2008; Zhang et al., 2001a; Zhang et al., 2001b; Zhu et al., 2009b; Zhu et al., 2006). The current molecular model of GrA-mediated cell death, proposed by Judy Lieberman's group, suggests that GrA enters the mitochondrial matrix, through an ill-defined mechanism, and proteolytically inactivates complex I protein NDUFS3. This leads to the generation of

mitochondrial superoxide, and loss of $\Delta \Psi_m$ (Martinvalet et al., 2008). Reactive oxygen species then somehow induce the translocation of the redox-sensitive ER-associated SET complex to the nucleus (Martinvalet et al., 2005). 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 (Chowdhury et al., 2006; Fan et al., 2003a; Fan et al., 2002; Fan et al., 2003b).

Studies using *grB* $^{-/-}$ CTLs have observed target cell death with a few characteristics of purified or recombinant GrA, which were attributed to GrA activity, although other granzymes cannot be discounted (Martinvalet et al., 2005; Pardo et al., 2002; Pardo et al., 2004; Shresta et al., 1999; Shresta et al., 1997). 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 (Ebnet et al., 1995). 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 (Mullbacher et al., 1996; Pereira et al., 2000). 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 (Metkar et al., 2008).

Granzyme K

Granzyme K was discovered and purified from LAK cells (Hameed et al., 1988). Initial studies using purified human or rat GrK revealed that GrK induced cell lysis, DNA damage, and affected the mitochondria of target cells (Hameed et al., 1996; MacDonald et al., 1999; Shi et al., 1992a). Treatment of target cells with recombinant human GrK via 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 over-expression (Zhao et al., 2007b). 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 (Zhao et al., 2007a). 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 authors did not ascertain the cleavage site in Bid, nor was GrK-mediated killing evaluated in the absence of Bid.

Recently, Zuzen Fan's group has also reported that GrK proteolytically processes p53 to pro-apoptotic forms (Hua et al., 2009). This would be a unique pro-apoptotic mechanism among granzymes, although caspase-3 has been reported to cleave and activate p53 at alternative residues (Sayan et al., 2006). The mechanism underlying GrK cleavage of p53 and its connection to cell death remains unknown.

Granzyme K-mediated killing by cytotoxic cells, in the absence of other granzymes, has not been assessed. Thus, its relevance in immunity still remains untested. Recently, a group has reported that *grA*x*B* $^{-/-}$ cytotoxic T cells were fully competent in the removal of influenza-infected target cells *in vivo* and proposed that GrK was responsible for this protection (Regner et al., 2009). However, these cells were also producing RNA to granzymes, C, F, K, and M. Furthermore, in human $CD8^+$ T cells, most GrK^+ cells do not contain perforin, so it is questionable whether GrK is intimately involved in cytotoxicity (Bade et al., 2005; Bratke et al., 2005).

GRANZYME M

Cytotoxic studies, using recombinant GrM have lead to conflicting results. Mark Symth'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 over-expression, and did not induce DNA fragmentation, cytochrome C release, ROS production, or loss of $\Delta \Psi_m$ (Kelly et al., 2004). 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 (Cullen et al., 2009). 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 (Lu et al., 2006). They reported direct proteolysis of ICAD at serine^{107} (not a previously reported P1 cleavage residue), and PARP at an unknown cleavage site. Mutagenesis studies were not performed, and cleavage of these substrates within target cells was not performed in the presence of caspase inhibitors. Of interest, these results have recently been refuted (Cullen et al., 2009). 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 (Hua et al., 2007). Granzyme M also disrupts the microtubule network of cells in the presence of zVAD-fmk, and specifically targets ezrin and α-tubulin at numerous cleavage sites (Bovenschen et al., 2008).

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 (Pao et al., 2005).

Granzyme M functions outside of direct killing have also been proposed, as GrM proteolytically inactives a GrB endogenous inhibitor, PI9 (Mahrus et al., 2004). The authors proposed a model wherein GrM could assist GrB-induced killing by relieving PI9 inhibition of GrB. However, it is still unknown whether GrM interaction with PI9 can to overcome the high $k_{association}$ rate of GrB and PI9.

ADDITIONAL ROLES OF GRANZYMES

Granzymes share homology with serine proteases derived from myeloid cells, such as neutrophils and mast cells. Serine proteases derived from these cells, such as mast cell chymases, cathepsin G, and neutrophil elastase, have long been implicated in extracellular functions, such as inflammation and tissue remodeling (Korkmaz et al., 2008; Pejler et al., 2007). 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.

Granzyme B

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 (Boivin et al., 2009). Granzyme B may also be released *in vivo* from activated cytotoxic cells in the absence of targets, both in active and zymogen forms (Prakash et al., 2009). Furthermore, it has become appreciated that GrB is expressed in the absence of perforin by a variety of non-lymphoid cells (Hirst et al., 2001; Pardo et al., 2007; Strik et al., 2007; Tschopp et al., 2006). 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 such as aggrecan, cartilage proteoglycans, vitronectin, fibronectin, fibrinogen, and laminin (Buzza et al., 2008; Buzza et al., 2005; Froelich et al., 1993). 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 (Choy et al., 2004). Alternatively, cleavage of these proteins may also influence cellular adhesion and migration processes. Other substrates have included proteins involved in blood clotting, and angiogenesis (Buzza et al., 2008; Mulligan-Kehoe et al., 2007). 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 (Gahring et al., 2001; Loeb et al., 2006).

Granzyme A

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 (Sower et al., 1996a; Sower et al., 1996b, c). 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 (Metkar et al., 2008). The process did not require perforin, but did require target cell internalization. However, Judy Lieberman's group has contested these findings, questioning the level of endotoxin contamination in the GrA preparations, which could provoke pro-inflammatory responses in monocyte-like cells (Zhu et al., 2009b). Interestingly, the caspase-1 inhibitor, WEHD-fmk, reduced the level of pro-inflammatory cytokines. It remains unresolved how GrA activates caspase-1. Murine GrA has also been

reported to directly proteolytically activate human proIL-1β *in vitro*, albeit with lower biological activity than caspase-1 processing (Irmler et al., 1995).

Other groups have reported GrA degradation of extracellular proteins, such as collagen type IV, fibronectin, and proteoglycans (Chowdhury and Lieberman, 2008). 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 (Tak et al., 1999) Additionally, a number of plasma inhibitors have been reported, further supporting a potential GrA extracellular function (described below). But, a direct causative effect of GrA and immunopathology has not been clearly established.

INHIBITORS OF GRANZYMES – THE SERPINS

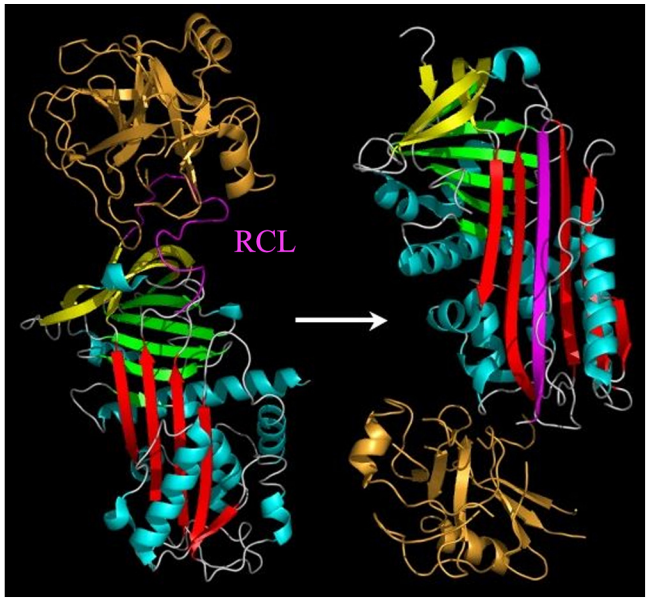
Serpin Structure and Mechanism of Inhibition

Serpins are a large, diverse family of proteins found within vertebrates, plants, fungi, bacteria, and viruses (Kaiserman and Bird, 2009; Schechter and Plotnick, 2004). They are classified into clades; in higher animals there are nine clades, A-I, viral serpins are divided into two clades, and plants, insects, nematodes, and horseshoe crabs each comprise a single clade (Kaiserman and Bird, 2009; van Gent et al., 2003). In vertebrates, most serpins control proteolytic cascades, but other functions include storage (like ovalbumin), hormone transport, and even tumor suppression. They play imperative roles in inflammation, blood

coagulation, and apoptosis, and their dysfunction has immunopathological manifestations (Gooptu and Lomas, 2009; Kaiserman and Bird, 2009; Travis and Salvesen, 1983; van Gent et al., 2003).

All serpins have similar architecture, comprised of 9 α-helices, 3 β-sheets, and variable region called the reactive centre loop (RCL) that dictates the specificity for target proteases (Schechter and Plotnick, 2004; van Gent et al., 2003). In its native state, the RCL is solvent exposed, protruding away from the body of the molecule, a state described as the "stressed" state (Figure 1-5.A). Upon recognition of the target RCL sequence by proteases, a serpin's conformation dramatically changes, with the RCL becoming inserted into β-strand A, a state described as the "relaxed state". The mechanism involves proteolytic nucleophilic attack of the peptide bond of the P1 residue of the serpin, the formation of an initial reversible Michaelis complex, and the enzyme and serpin substrate form an ester-bond (Kaiserman and Bird, 2009; Schechter and Plotnick, 2004). This covalent enzyme-substrate complex is called an acyl-enzyme intermediate (Figure 1-5B). In most protease reactions, the ester bond is further hydrolyzed (deacylation by water), releasing the remains of the substrate. However, during protease-serpin interaction, this acyl-enzyme intermediate is hydrolyzed extremely slowly, and acts as the signal for the conformational change of the serpin from a stressed to a relaxed transition state. The trapped protease remains bound to the serpin, in an inactive conformation. Serpin and protease reactions

A



B

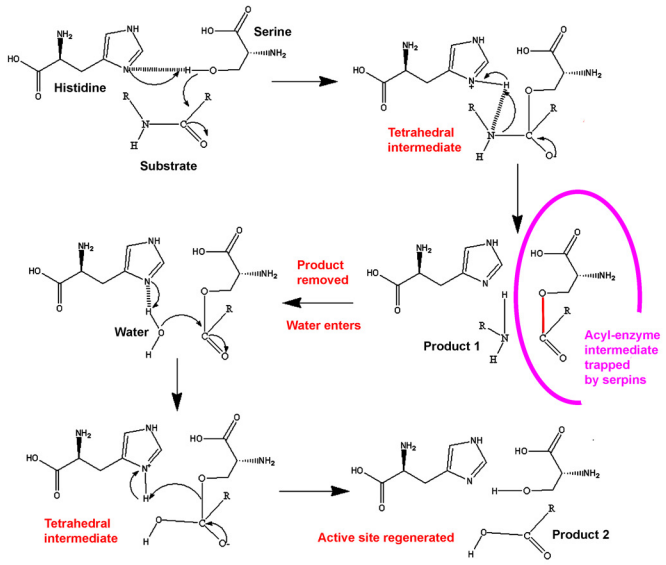


Figure 1-5 Serpin Inhibitory Mechanism. **A**) Left - The serine protease on top is depicted in gold, the serpin is on the bottom with its reactive centre loop (RCL) ,depicted in purple, protruding away from the body of the serpin. Right - Attack of the serpin suicide substrate by the protease induces a dramatic conformational change of the serpin and the protease is re-positioned and distorted in an inactive conformation.
B) Formation of the acyl-enzyme intermediate upon partial hydrolysis of the serpin substrate by the protease.
Both images are from Wikepedia.

are characterized by their association rates (K_{ass}), and the stoichiometry of inhibition (SI). K_{ass} describes the rate at which the Michaelis complex is converted to the cleaved intermediate, and SI describes the number of serpin molecules that are required to inhibit one protease molecule (Schechter and Plotnick, 2004). Most physiological interactions occur with a K_{ass} of $1x10^5$ M^{-1} s^{-1} to $1x10^7$ M^{-1} s^{-1}, and a SI close to 1. If the K_{ass} rate becomes too high, the deacylation step will proceed, leaving the serpin cleaved and inactive, and the active protease is released through a regular proteolytic reaction (Kaiserman and Bird, 2009). Thus, most serpins contain less favorable residues at the P1 amino acid residue, compared to more ideal protease substrates.

Anti - Chymotrypsin

Serpins that have been particularly well characterized are plasma resident clade A members, such as anti-trypsin (serpin A1 in humans) and anti-chymotrypsin (serpin A3 in humans). Anti-trypsin is highly abundant in plasma and controls an inflammatory mediator, human neutrophil elastase. Anti-chymotrypsin becomes highly elevated during inflammatory conditions, and controls neutrophil cathepsin G and mast cell chymase (Travis et al., 1978; Travis and Salvesen, 1983).

Anti-chymotrypsin is primarily produced in the liver, although it has also been reported in tumor cells of breast, stomach, lung, and prostate epithelial origins, and by astrocytes of Alzheimer patients, usually upon exposure to inflammatory cytokines (Abraham et al., 1988; Cichy et al., 1995a, b;

Higashiyama et al., 1995; Laursen and Lykkesfeldt, 1992; Tahara et al., 1984; Travis and Salvesen, 1983). The protein has also been observed in the nuclei of tumor cells (Takada et al., 1988b; Tsuda et al., 1986). Due to these observations ACT has received attention as a potential marker of cancer progression and metastasis (Montel et al., 2005; Yu et al., 2005).

Serpin – Granzyme Interactions

Granzyme-specific serpins have been identified for GrA, B, K, and M, while endogenous inhibitors of human GrH or murine GrC-G have not been identified. Human Granzyme A is inhibited by antithrombin III (serpin C1) (Masson and Tschopp, 1988). Antithrombin III is an abundant plasma inhibitor that has been established primarily as a mechanism to control proteinases involved in coagulation (Travis and Salvesen, 1983). Although the kinetics of the interaction is poor, the abundance of antithrombin III may still be sufficient to inhibit GrA. However, it has been reported that GrA complexed to proteoglycans (as it is during exocytosis) is resistant to antithrombin III inhibition (Spaeny-Dekking et al., 2000).

Granzyme B is inhibited by the intracellular serpins, human Protease Inhibitor-9 (PI9), and a functional murine orthologue SPI-6 (Bird et al., 1998; Sun et al., 1996; Sun et al., 1997). The expression of PI9 has been demonstrated in cytotoxic cells and various other cell types that can produce GrB (presumably to prevent cell death upon GrB leakage from granules), as well as cells in immune-privileged sites (Bladergroen et al., 2001; Medema et al., 2001). The interaction

between human GrB and PI9 is very strong, and there are reports suggesting that PI9 can protect a number of cell types from GrB-mediated cell death (reviewed by (Kaiserman and Bird, 2009)). An intracellular viral inhibitor of GrB has also been identified, the orthopoxvirus serpin Spi-2/CrmA (Quan et al., 1995). However, there is good evidence that CrmA is more effective at inhibiting caspases (Komiyama et al., 1994; Tewari et al., 1995). Cells infected with single CrmA/Spi-2 deletion mutant virus remained resistant to granule-mediated CTL killing, but after inactivation of both CrmA/Spi-2 and an additional serpin, Spi-1, target cells were effectively killed (Macen et al., 1996). This raises the question whether Spi-1 can impair additional cytotoxic proteases. Indeed, rabbit poxvirus Spi-1 has been reported to bind and inactivate cathepsin G, a protease with enzymatic activity similar to human GrH and mouse GrC-G (Moon et al., 1999).

Recently, murine serpin A3n, isolated from Sertoli cells, has been identified as an extracellular GrB inhibitor (Sipione et al., 2006). Sertoli cells are highly resistant to GrB-mediated apoptosis, and susceptible target cells ectopically expressing serpin A3n were rendered GrB-insensitive. The most similar human orthologue of serpin A3n, ACT, does not impair GrB activity.

Granzyme K activity has been inhibited *in vitro* by the inter-α -trypsin complex (IαI) (Wilharm et al., 1999). The serpin is a highly abundant plasma inhibitor, strongly implicated in regulating inflammatory reactions, such as LPS-induced sepsis (Kobayashi, 2006; Rucevic et al., 2007). However, the physiological relevance of the association between GrK and IαI is still unexplored.

Human Granzyme M has been reported to very weakly associate with ACT *in vitro* and it's unknown whether this is physiologically relevant (Mahrus et al., 2004). Mouse GrM has been proposed to interact with an intracellular clade B serpin, serpinb9b (SPI-CI) (Bots et al., 2005). This study revealed that tumor targets expressing both SPI-6 and SPI-CI were more resistant to cytotoxic attack. They also presented evidence that SPI-CI was expressed in cytotoxic cells, killing with purified rat GrM and perforin was abrogated in SPI-CI transfected targets, and SPI-CI and rat GrM formed a covalent complex. Currently, there are no biochemical data on the K_{ass} or SI between SPI-CI and mouse GrM, nor has the possibility that SPI-CI could interact with additional chymase-like granzymes been explored.

THESIS HYPOTHESES AND AIMS

The contribution of cytotoxic cells for the protection of host organisms from infection and tumorigenesis is well established. Cytotoxic molecules are also implicated in transplant rejection and numerous autoimmune diseases. As evidenced by extensive *in vitro* biochemical studies, GrB is a potent protease capable of inducing cell death. In order to firmly establish correlation between GrB expression and protection from immunological threats or its contribution to immunopathology, reliable measures were required. We hypothesized that GrB release by antigen-specific CTLs, detected by a novel ELISPOT assay, would correlate with cell death, traditionally assessed by the "gold standard" ^{51}Cr-release assay.

However, *in vivo* studies in mice have implied that the absence of GrB does not greatly impair cell-mediated cytotoxic responses to viral and tumorigenic challenges. These results indicated that other granule-derived molecules must have cytotoxic potential. The role of one such protease, GrH, was relatively unexplored. We hypothesized that GrH was also a cytotoxic protease, but it would induce cell death by a mechanism distinct from that of GrB and Fas.

Additionally, inhibition of serine proteases is highly relevant to the prevention of immunopathologies. We have identified a potential serine protease inhibitor for GrH, antichymotrypsin (ACT). We hypothesized that ACT could inhibit GrH proteolysis of death substrates and abrogate GrH – mediated cytotoxicity.

Therefore, the aims of this thesis are the following:

1) To develop a direct means of evaluating GrB release from antigen-specific cytotoxic cells and demonstrate its correlation to target cell death.
2) To evaluate the ability of GrH to contribute to granule-mediated cytotoxicity, and determine its mechanism of action.
3) To characterize the interaction between GrH and an abundant human protease inhibitor, antichymotrypsin.

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CHAPTER 2 – EVALUATING ANTIGEN-SPECIFIC CYTOTOXIC T LYMPHOCYTE RESPONSES BY A MOUSE GRANZYME B ELISPOT ASSAY[1]

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Introduction

The immune system is highly dependent on the $CD8^+$ T cell population for defense against infectious agents, both during a primary, acute exposure, and for long-term protection by antigen-specific memory T cells. Clearance or control of viruses is typically dependent on one or a combination of two mechanisms that cytotoxic cells employ to eliminate target cells: the Ca^{2+}-dependent delivery of cytotoxic granule contents to target cells and the ligation of death receptors on the target cell (eg. Fas/FasL) (Barry, 2002). The granule-mediated process requires the exocytosis of perforin, along with a number of serine proteases, known as granzymes, to initiate target cell apoptosis. A primary mediator of apoptosis is granzyme B (GrB), whose intracellular substrates include pro-apoptotic proteins Bid and caspase-3, and has been shown to directly induce target cell apoptosis (Barry et al., 2000a; Darmon, 1995).

Traditionally, the ability of cytotoxic cells to induce target cell apoptosis has been measured by the rupture of target cell membranes through the release of ^{51}Chromium (^{51}Cr-release). However, the process of evaluating the cytotoxic T lymphocyte (CTL) immune response has recently focused on single-cell based assays and has undergone significant changes, due to the development of sensitive serological reagents. Such reagents have been utilized in humans to assess intracellular cytotoxic mediator stores such as GrB, GrA, or perforin by flow cytometry (Hamann et al., 1997; Zhang et al., 2003), the externalization of CD107 antigen by flow cytometry (Betts et al., 2003), or the release of GrB by ELISA (Spaeny-Dekking et al., 1998) and ELISPOT (Rininsland et al., 2000; Shafer-

Weaver et al., 2003). However, specific and sensitive reagents have been lacking for similar analysis in rodent systems at the single cell level. Flow cytometric analysis has been described for intracellular staining of mouse GrB using an antibody (clone GB12) originally developed for human GrB, and CD107 staining has also been demonstrated in murine infectious models (Wherry et al., 2003b; Wolint et al., 2004a). However, the ability to directly assess the release of cytolytic proteins, including mouse GrB, has not yet been developed.

As the complexity of $CD8^+$ T cell subsets, defined by phenotypic markers and effector molecule expression during infection become more apparent, the ability to directly assess the production and release of effector molecules has become increasingly important for defining correlates of immunity. Chronic infection in humans with a number of viruses appears to result in distinct phenotypic subsets of $CD8^+$ T cells, with similarly distinct effector functions (Appay and Rowland-Jones, 2004; van Lier, 2003). Infections such as HIV can result in a lack of effector molecules, such as perforin, stored within memory and effector $CD8^+$ T cells, resulting in a dysfunctional cytotoxic response (Appay et al., 2000; Zhang et al., 2003). However, the ability of these HIV antigen-specific $CD8^+$ T cells to produce IFN-γ is unaffected. Similarly, recent studies in mouse models of chronic infection have also demonstrated that memory $CD8^+$ T cells may be functionally impaired (Wherry and Ahmed, 2004). Memory $CD8^+$ T cells progressively lost their ability to proliferate and to produce cytokines such as IL-2, IFN-γ, and TNF-α during chronic infection (Fuller et al., 2004; Wherry et al., 2003a). It remains controversial whether the cytotoxic capacity of $CD8^+$ T cells

exposed to persistent antigen is also similarly impaired in mice (Byers et al., 2003; Wherry et al., 2003a), or whether their functionality is dependent upon antigen or tissue distribution (Obar et al., 2004; Wherry et al., 2003a; Zhou et al., 2004). Therefore, it is important to be able to directly detect the production and/or release of effector molecules to ascertain the functional capabilities of antigen-specific $CD8^+$ T cell populations. Herein, we describe a novel assay for the single-cell detection of mouse GrB, and have demonstrated a significant correlation between degranulation of GrB by antigen-specific CTL and the induction of *in vitro* target cell lysis. Thus, the assay has significant utility for assessing antigen-specific CTL responses in mouse models of infection and, as a result, may accelerate the early development of human vaccines and immunotherapies.

Results

Sensitivity and specificity of mouse GrB ELISA and ELISPOT

The ability to detect and measure mouse GrB upon release from CTL following antigen stimulation would be useful in assessing CTL effector function in the mouse. We were able to detect mouse GrB by ELISA using anti-human GrB antibody GB12 and biotinylated anti-mouse GrB, as capture and detection reagents, respectively (Figure 2-1.A). Sensitivity of the mouse GrB ELISA was determined by analysis of sequential dilutions of either a commercially available recombinant mouse GrB (not shown), or a stock of native mouse GrB generated in our lab from the murine cytotoxic T cell line MTLA (see Materials and

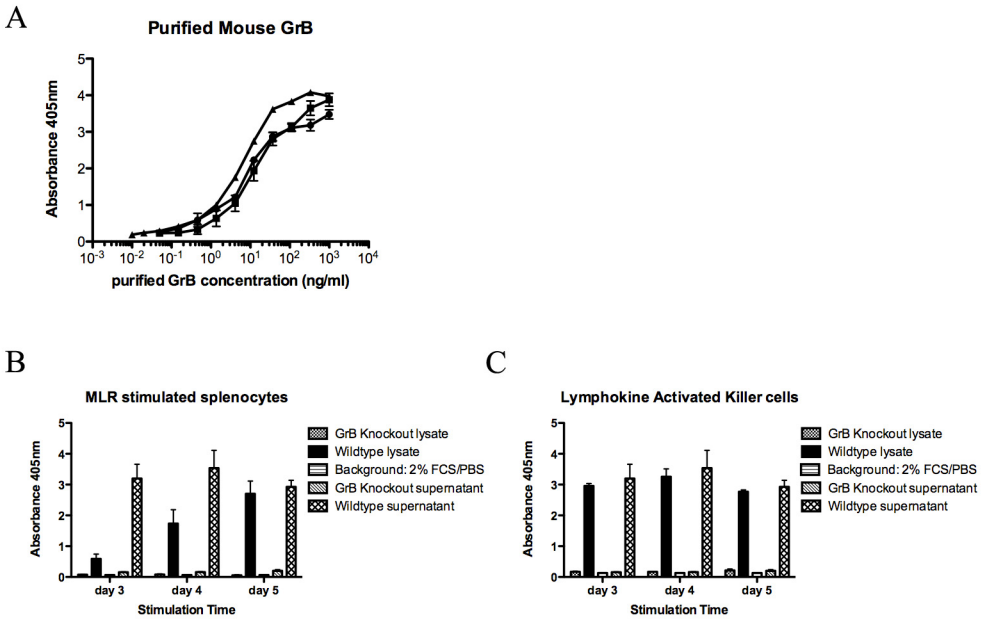

Figure 2-1 Sensitivity and specificity of mouse GrB ELISA. A) Native mouse GrB serially diluted to determine the sensitivity of GrB ELISA assay. Mean absorbance ± S.D.s. Samples from three independent experiments were assayed to determine inter-assay variability and the mean coefficient of variability. B) Cytotoxic lymphocytes derived from wildtype or GrB deficient mice were generated by MLR and assayed on day 3, 4, or 5 of culture. C) LAK cells were generated with 1000 U/ml rhIL2 and assayed for GrB after 5, 7, or 10 days of culture. Supernatants - Wildtype (hatched bars), GrB deficient (diagonal line bars); Lysates (equivalent of $1x10^5$ cells) - wildtype (black bars), GrB deficient (light stipled bars); Background (2% FCS/PBS) (horizontal bars). Mean absorbances ± S.D., of samples run in triplicate, with two mice per group.

Methods). The limit of detection was determined to be 50 pg/ml with inter-assay variability of 11%, calculated as the mean coefficient of variation (Figure 2-1.A). No cross-reactivity was observed with recombinant mouse granzyme A (data not shown).

Splenocytes derived from wild-type or GrB-deficient mice were stimulated in a mixed lymphocyte reaction and cell lysates and cell culture supernatants were collected at different days of culture and tested by ELISA. ELISA reactivity was found in lysates and supernatants of wild-type but not GrB-deficient cell cultures (Figure 2-1.B). As LAKs have been previously shown to express a number of different mouse granzyme species (Revell et al., 2005b), we also tested cell culture supernatants and cell lysates at different days of culture from LAK cells of wild-type and GrB-deficient mice. Again, ELISA reactivity was only found in lysates and supernatants of wild-type but not GrB-deficient LAK cell cultures (Figure 2-1.C). These results indicate that the ELISA is specific for mouse GrB.

Comparative analyses of GrB ELISPOT and ^{51}Cr-release assays of CTLs entirely dependent on granule-mediated cytotoxicity

To examine the relationship between target cell killing and release of GrB by single cell analysis, we developed an ELISPOT assay for mouse GrB, using GB12 as the capture reagent and biotin-conjugated anti-mouse GrB antibody as the detection antibody. The top row of Figure 2-2.A showed that CTL-10 cells incubated with EL4 targets bound with an irrelevant peptide, $NP_{366-374}$, produced

very few spots. In contrast, EL4 target cells bound with the HY peptide antigen and exposed to CTL-10 cells (specific for D^b-HY peptide) induced strong antigen-specific release of GrB, indicated by the numerous spots seen in the bottom row of Figure 2-2.A. The number of GrB spots induced was substantially higher with HY-peptide pulsed EL4 targets than EL4 alone or EL4 pulsed with the non-antigenic influenza NP peptide at all cell concentrations tested (Figure 2-2.B). These results indicated that we were able to detect antigen-specific release of mouse GrB by ELISPOT. Release of GrB by CTL-10 was reduced to basal levels in the presence of EGTA (Figure 2-2.B), indicating that the secretion of GrB was occurring through the Ca^{2+}-dependent granule cytotoxic pathway.

In parallel with GrB ELISPOT assays, antigen-specific target cell lysis, detemined by ^{51}Cr-release, was observed (Figure 2-2.C). Addition of EGTA also abrogated target cell lysis, consistent with killing of targets cells by CTL-10 via a Ca^{2+}-dependent granule-mediated mechanism (Figure 2-2.C). Analyses of results showed significant correlation over a range of E/T ratios between the number of GrB-releasing CTL-10 cells in response to peptide antigen and the percentage of antigen-specific target cell lysis (R^2= 0.9541; $p<0.0001$) (Figure 2-2.D).

Comparative analyses of GrB ELISPOT and ^{51}Cr-release assay by a granule- and FasL-dependent CTL

We further applied the mouse GrB ELISPOT assay to measure GrB

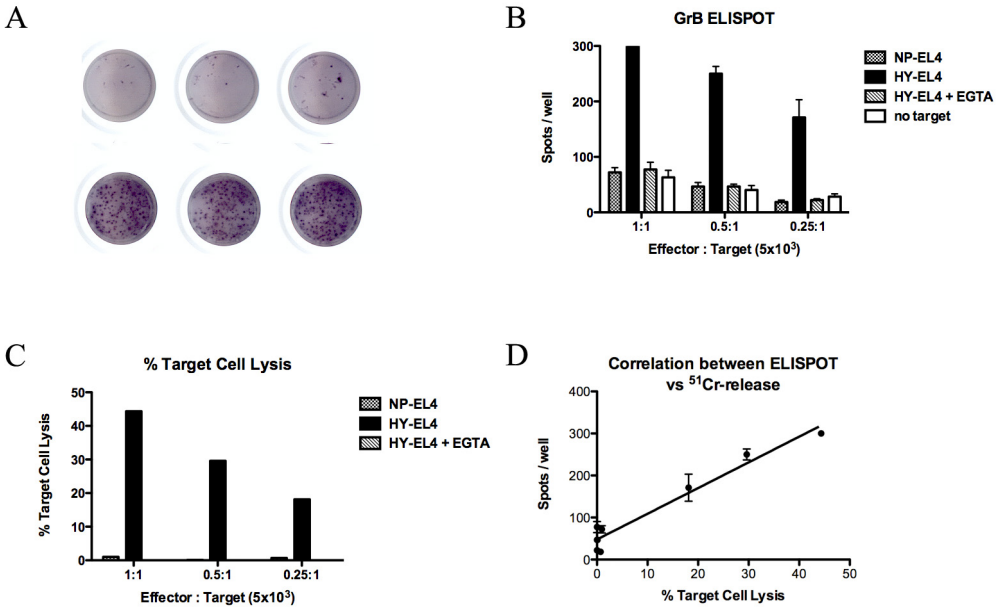

Figure 2-2 Mouse GrB ELISPOT directly correlates with antigen-specific target cell lysis. A HY-specific CTL clone was incubated with syngeneic EL4 target cells pulsed with Influenza virus $NP_{366-374}$ peptide (control) or HY peptide pulsed EL4 target cells. A) ELISPOT generated with control peptide (A; top row) and HY peptide (A; bottom row). B) Graphical results of ELISPOT assay and C) ^{51}Cr-release assays. Further control groups included CTL-10 only, and HY peptide pulsed EL4 cells in the presence of 4mM EGTA. Target cells, $5x10^3$ cells/ well, were incubated with varying numbers of CTL-10 effector cells, in triplicate. Results are means ± S.D. of two independent experiments.
D) Correlative analysis was performed on ELISPOT and target cell lysis results by Spearman's test for correlation (R^2=0.9541; $p<0.0001$).

release by an alloantigen-specific CTL, clone AB.1, which has been demonstrated to use degranulation and FasL-dependent lytic mechanisms (Ostergaard et al., 1987). When H-2^d AB.1 effector cells were incubated with H-$2D^d$/ H-$2K^d$ syngeneic targets L1210, very few spots were detected in a GrB ELISPOT (Figure 2-3.A). However, when AB.1 effectors were challenged with L1210 cells transfected to express a chimeric class I MHC molecule composed of the α1 and α2 domains of the K^b alloantigen fused to the α3 domain of D^d, the frequency of spots detected by ELISPOT increased substantially over control targets. The frequency of spots also varied in an effector cell dose-dependent manner. Similar responses were found when target cell lysis was measured by ^{51}Cr-release (Figure 2-3.B), where L1210 targets were not lysed, but chimeric MHC class I alloantigen-expressing L1210 targets were killed in a cell concentration and antigen-specific manner. Interestingly, the addition of EGTA abrogated GrB release detected in ELISPOT (Figure 2-3.A) but only partially reduced target cytotoxicity of the AB.1 effectors (Figure 2-3.B), indicating that a residual Ca^{2+}-independent lytic mechanism was also being employed. Numerous examples where Fas interactions may potentiate granule-mediated killing have been reported, but the ability of investigators to clearly demonstrate its presence is still very difficult, particularly in physiological conditions. Therefore, the combination of using GrB ELISPOT and ^{51}Cr-release assays has the potential of revealing whether specific effector populations have the capability of utilizing Fas-FasL to induce target cell killing. Poor release of GrB, directly measured by the GrB ELISPOT, in the continued presence of cytotoxicity may strongly

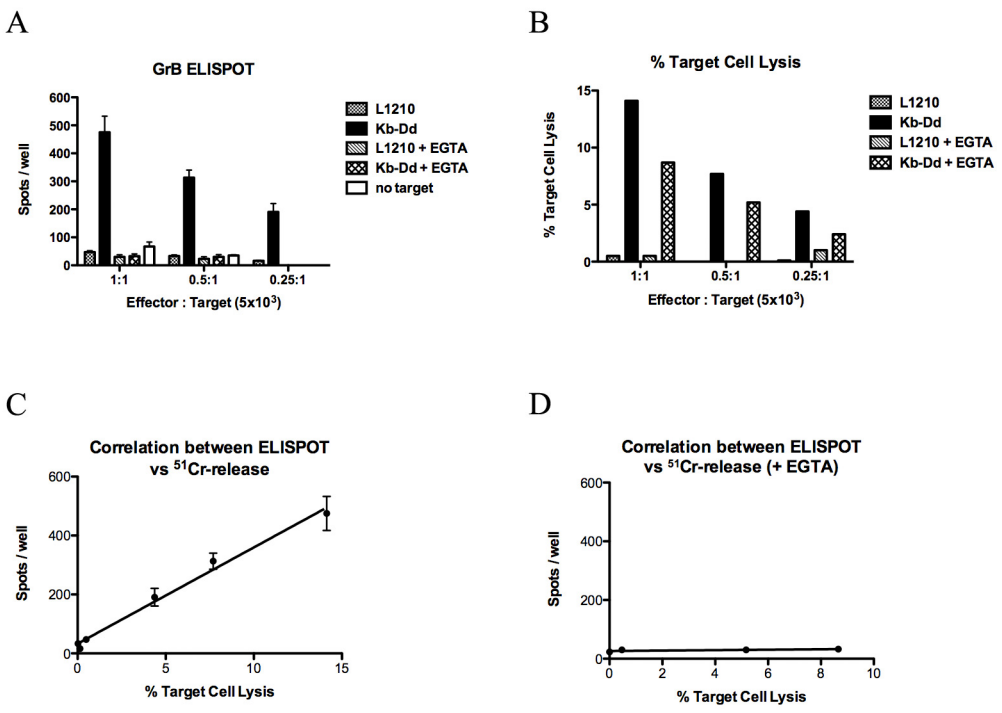

Figure 2-3 Mouse GrB ELISPOT strongly correlates with Ca^{2+}-dependent antigen-specific target cell lysis. The H-2K^b allo-antigen -specific CTL clone, AB.1 (H-2^d), was incubated with syngeneic H-2^d L1210 target cells, or L1210 target cells transfected to express a K^b-D^d fusion MHC class I protein. A) Mouse GrB ELISPOT and B) ^{51}Cr-release assay. Varying numbers of AB.1 cells were stimulated with 5x10^3 target cells, either in control media, or in the presence of 4mM EGTA. An additional ELISPOT control included AB.1 cells only. Samples were run in triplicate, and are shown as means ± S.D. of two independent experiments. Correlative analysis, performed on ELISPOT and target cell lysis results was derived by Spearman's test for correlation (R^2=0.9889; p<0.0001) C) in the presence of Ca^{2+} D) or in the absence of Ca^{2+} (R^2= 0.5313; p=0.2711).

indicate the activity of FasL. Indeed, our data corroborates previous reports where Fas-mediated cytotoxicity was demonstrated by AB.1 cells, as significant correlation between the GrB ELISPOT and ^{51}Cr-release could be established when assays were performed in the presence of Ca^{2+} (R^2=0.9889; p<0.0001). However, this correlation is clearly lost in conditions of Ca^{2+} depletion (i.e. with EGTA), where target cell lysis still occurs in the absence of GrB release (R^2=0.5313; p=0.2711) (Figure 2-3.D). Thus, the GrB ELISPOT analysis is capable of indirectly indicating the contribution of Fas-mediated killing, but most importantly, these results further establish the correlation between GrB release and Ca^{2+}-dependent target cell killing.

Detection of LCMV gp33-specific effector CTL responses

Mice were infected with LCMV by intraperitoneal injection for the assessment of *in vivo*-generated cytotoxic T cell responses by ELISPOT and ^{51}Cr-release. Splenocytes, isolated from mice on day 7 post-infection mice and directly tested in the mouse GrB ELISPOT assay (Figure 2-4.A), demonstrated significant GrB release towards LCMV gp33 pulsed EL4 targets compared to control targets (p<0.001) at both E/T ratios of 10:1 and 1:1. Similar to the ELISPOT data, the ^{51}Cr-release assay demonstrated strong and significant (p<0.001) cytolytic activity only against gp33 peptide-pulsed EL4 targets, at both 10:1 and 1:1 E/T ratios (Figure 2-4.B). Both assays demonstrated only low levels of CTL activation against control target cells, where $1x10^5$ splenocytes generated 50 spots/ well in the ELISPOT assay, and 5% or less target cell lysis by ^{51}Cr-

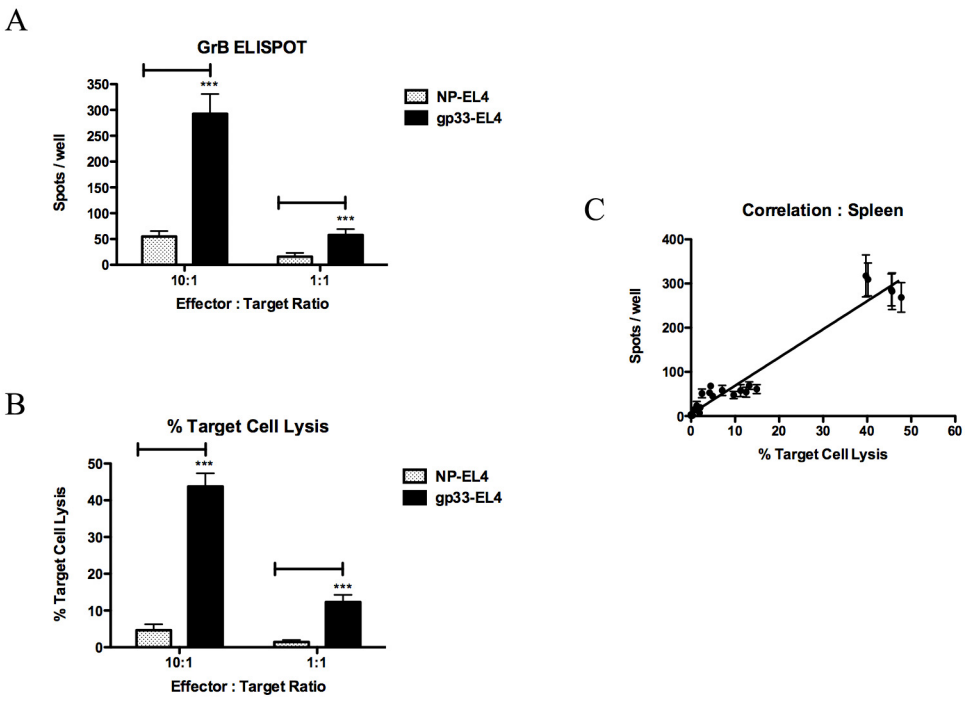

Figure 2-4. ***Ex vivo*** **effector responses demonstrate a strong correlation between GrB ELISPOT and target cell lysis**. Splenocytes were isolated from mice on day 7 post-infection with LCMV (N=5) and directly assessed, *ex vivo,* for gp33 peptide-specific cytotoxic T cell responses. Effector cell populations were incubated with 1x 10^4 control NP peptide or LCMV gp33 peptide- pulsed EL4 target cells, in triplicate, at two E/T ratios. A) Mouse GrB ELISPOT (B) or ^{51}Cr-release assay. C) Correlative analysis, performed on ELISPOT and target cell lysis results, was tested by Spearman's test for correlation (R^2=0.9524; p<0.0001). (***p<0.001)

release. This finding is likely due to a constitutive leakage of granular contents from a minor population of activated splenocytes. Notably, when ^{51}Cr-release and GrB ELISPOT responses were directly compared, a significant correlation (R^2=0.9524; $p<0.0001$) was demonstrated between the assays, which included responses to both control peptide and gp33 pulsed target cells (Figure 2-4.C). Thus, the GrB ELISPOT assay is able to effectively and sensitively detect the lytic function of *in vivo* generated antigen-specific cytotoxic T cells.

Detection of memory LCMV gp33-specific CTL responses

To establish whether the mouse GrB ELISPOT could be utilized to assess memory cell responses, splenocytes from mice on day 32 post-infection with LCMV were stimulated *in vitro* for 5 days with 0.2 μM LCMV gp33 peptide (Figures 2-5.A-C). Following stimulation, cells were seeded at 10:1 and 1:1 E/T ratios for both the GrB ELISPOT and ^{51}Cr-release assays. A significant response to LCMV gp33 pulsed target cells was demonstrated compared to control target cells ($p<0.001$ and $p<0.05$ for 10:1 and 1:1 E/T; respectively), as measured by the mouse GrB ELISPOT assay (Figure 2-5.A). Similar to the ELISPOT results, target cell lysis was only detected against LCMV gp33 pulsed target cells ($p<0.001$) (Figure 2-5.B). Antigen-specific GrB release and target cell lysis by memory T cells demonstrated significant correlation (R^2=0.7983; $p<0.0001$). Cells were also isolated from mesenteric lymph nodes or were isolated and pooled from axillary and inguinal lymph nodes from mice on day 35 post-infection with LCMV (Figure 2-5.D). These cells were then stimulated *in vitro* with gp33

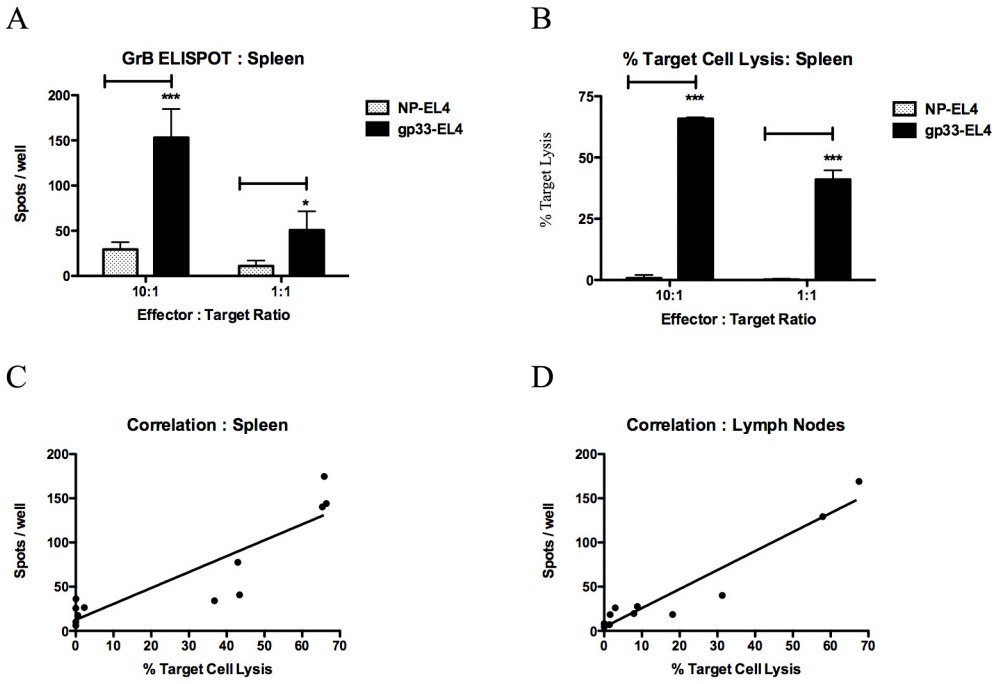

Figure 2-5. **Memory responses demonstrate a significant correlation between GrB ELISPOT and target cell lysis.** Cells derived from the spleens (N=3) (A-C) and mesenteric or pooled axillary and inguinal lymph nodes (N=2) (D) of mice 32 days following infection with LCMV mice were stimulated *in vitro* for 5 days with 0.2 μM LCMV gp33 peptide. Two concentrations of effector populations were incubated with $1x10^4$ irrelevant NP_{366} peptide pulsed EL4 or LCMV gp33 peptide pulsed EL4 target cells. A) Mouse GrB ELISPOT (B) ^{51}Cr-release assay. Correlative analysis showed significant correlation between the assays for both C) spleen (R^2=0.7983; $p<0.0001$) D) and lymph node (R^2=0.9225; $p<0.0001$). (***$p<0.001$; *$p<0.05$)

peptide for 5 days before incubation with target cells in the GrB ELISPOT and ^{51}Cr-release assays. Antigen-specific memory T cell responses again indicated significant correlation between the GrB ELISPOT and ^{51}Cr-release in the lymph node populations (R^2=0.9225; $p<0.0001$) (Figure 2-5.D). Taken together, these results establish the mouse GrB ELISPOT assay as a reliable alternative to the ^{51}Cr-release assay for the measurement of mouse antigen-specific memory cytotoxic T cell responses.

Discussion

The ability to detect antigen-specific responses of cytotoxic T lymphocytes is an integral component of studies directed toward the improvement of vaccines designed to include or enhance cell-mediated cytotoxicity. The ^{51}Cr-release assay has long remained the 'gold standard' for the measurement of an effective cell-mediated cytotoxic response. However, this technique, while quite sensitive, requires the employment of radioactive reagents and relatively large numbers of effector cells. Moreover, the assay is limited to target cells that can take up the radioactive label effectively, and the output only measures the bulk cytotoxicity of any effector cell population.

Recently, a number of assays have been employed to address the deficiencies of the ^{51}Cr-release assay. The externalization of granule resident proteins, Lysosomal-Associated Membrane Proteins (LAMP-1/2) (CD107a/CD107b), has garnered attention as a reliable flow cytometry-based technique, as it was found to detect the degranulation of antigen-specific $CD8^+$ T

cells (Betts et al., 2003; Betts et al., 2004; Rubio et al., 2003). While the assay may demonstrate antigen-specific responses and is compatible with tetramer and intracellular cytokine staining, evidence exists to suggest that $CD107^+$ externalization can take place in the absence of effective stores of cytotoxic proteins, and that such T cell memory populations are not immediately cytotoxic (Wolint et al., 2004a). Thus, CD107 staining should also be performed with the detection of cytotoxic mediators, such as GrB, in order to correctly assess functional CTL responses to stimuli. A drawback, however, of using a flow cytometric approach is that degranulation results in a loss of these proteins at short time points of target challenge, thus the assessment of functional cytotoxic responses is indirect and, potentially, underestimated.

The ELISPOT approach allows for the direct assessment of the release of cytotoxic mediators, such as GrB. The feasibility of this approach has been demonstrated using an ELISPOT for the detection of human GrB, where antigen-specific $CD8^+$ T cell responses to specific viral peptides were detected in *ex vivo* PBMC populations (Rininsland et al., 2000). Comparisons of the human GrB ELISPOT to CD107 staining, and ^{51}Cr-release responses in effector cytotoxic cell populations have shown a strong correlation (Rininsland et al., 2000; Shafer-Weaver et al., 2004; Shafer-Weaver et al., 2003), similar to our results with the mouse GrB ELISPOT system. Additionally, the GrB ELISPOT may be employed in a dual-based ELISPOT system, such as for the secretion of GrB and cytokines, as there has been evidence of responses to specific antigens where cytotoxic responses and IFN-γ production and are disparate at the single $CD8^+$ T cell level

(Kleen, 2004; Snyder et al., 2003). The sensitivity of the assay is also amenable to detecting memory and/or effector responses in both lymphoid and nonlymphoid tissues, as it requires few cells, potentially aiding our understanding of cell-mediated cytotoxicity responses in various tissues, at sites of infection, as well as following changes in the localization of cytotoxic effector cells during an immune response. Collectively, this information may be of significance to the characterization of specific $CD8^+$ T cell subsets, and the establishment of correlates of immunity.

A caveat to the ELISPOT approach is that the assay doesn't directly assess whether target cells are killed. However, the presence of GrB and the direct measurement of its release has been consistent with target cell lysis by ^{51}Cr-release, as demonstrated by studies using ELISPOT, or by the presence of GrB aspase activity in cell culture supernatants (Ewen et al., 2003a).

The mouse GrB ELISPOT provides a reliable and sensitive means in which to detect cytotoxic $CD8^+$ T cell responses at the single cell level by directly measuring the release of a potent, established cytotoxic mediator. The assay has potential to be utilized in various mouse models of infection, potentially accelerating the development of vaccine and immunotherapies relevant to humans.

Materials and methods

Mice and injections

Female mice of strains C57BL/6 and BALB/c mice were obtained from Charles River Laboratories (Kingston, ON, Canada) and handled according to the guidelines of the Canadian Council on Animal Care and the University of Alberta Health Animal Policy and Welfare Committee. Mice of C57BL/6 wild-type and GrB-deficient backgrounds were maintained in the breeding colony at the University of Alberta. To generate primary *in vivo* antigen-specific immune responses, mice were injected intraperitoneally with 200 μl of 10^5 p.f.u Armstrong strain LCMV (gift from Pamela Ohashi, University of Toronto, Canada). At indicated time points following injection, mice were sacrificed and the spleens or lymph nodes were removed, and then teased through a nylon mesh.

Cells

Splenocytes were treated with ammonium chloride to lyse erythrocytes (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.2), then diluted in complete media, RPMI 1640 (Life Technologies, Burlington, ON, Canada) supplemented with 10% (v/v) FBS (Hyclone, Logan, UT), 10 mM HEPES, 50 U/ml penicillin, 50 μg/ml streptomycin, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids (all from Life Technologies), and 100 μM 2-ME (Sigma, St. Louis, MO). Primary LCMV *ex vivo* effector responses were tested directly in GrB ELISPOT or ^{51}Cr-release assays. For secondary *in vitro* LCMV-specific stimulation, splenocytes or cells derived from mesenteric lymph nodes or pooled

axillary and inguinal lymph nodes were diluted to $5x10^5$/ 200 μl, seeded into 96-well Falcon tissue culture plates (Becton Dickinson Labware, Franklin Lakes, NJ) and incubated for 5 days with 0.2 μM LCMV gp33 peptide (CANVAC core facilities, Montreal, QC, Canada) in complete media supplemented with 10 U/ml recombinant human IL-2, produced in our laboratory.

Primary *in vitro* activation of splenocytes by mixed lymphocyte reaction was established using $2x10^6$ splenocytes from wild type or GrB deficient responders and $2x10^6$ irradiated (2500 rads) BALB/c stimulators in 12-well tissue culture plates (Nalge-Nunc International, Copenhagan, Denmark) with complete media supplemented with 80U/ml recombinant human IL-2. At the indicated time points, supernatants from a brief spin at 1500 rpm (400 x g) were collected, and pelleted cells were lysed in a buffer composed of 150 mM NaCl, 20 mM Tris pH 7.2 and 1% Triton-X at $4x10^6$ cells/ml. For the generation of lymphokine-activated killer cells (LAK), splenocytes were diluted to $1x10^6$ cells in complete media supplemented with 1000 U/ml rhIL-2, seeded to 24-well tissue culture plates (Corning Incorporated, Corning, NY) and after various days of culture, supernatants and lysates were generated as described above.

Cytotoxic T lymphocyte clones CTL-10 (H-$2D^b$ HY male antigen-specific) and AB.1 (H-$2K^b$ alloantigen-specific) were maintained as previously described (Greenfield et al., 1996; Kane et al., 1989). Target cell lines, EL4 and L1210 were obtained from American Type Culture Collection (ATCC) (Manassas, VA), while preparation of L1210 transfectants expressing a chimeric class I MHC molecule composed of the α1 and α2 domains of the K^b class I

molecule fused to the α3 domain and cytoplasmic tail of D^d have been described previously (Durairaj et al., 2003).

GrB ELISA

ImmunoMaxisorp (Nalge-Nunc International) plates were coated with anti-human GrB antibody clone GB12 (Sanquin, Amsterdam, The Netherlands) at 5 μg/ml in PBS at 4°C overnight. Plates were washed twice with PBS, then blocked with RPMI 1640 media supplemented with 10% FBS for 1 hour at room temperature. Samples were added, in 2% FCS/ PBS, at indicated dilutions. The mouse GrB standards utilized were either *P. pastoris*-derived recombinant mouse GrB (Sigma) or a purified, native mouse GrB stock prepared in our lab from a IL-2 independent variant (designated MTLA) of the murine cytotoxic line MTL2.8 (Bleackley et al., 1982), following a procedure previously described for the purification of human GrB (Caputo et al., 1999b). Following an incubation of 1-4 hours, wells were washed four times with PBS-Tween 20 (0.05% v/v). Biotinylated anti-mouse GrB (R&D Systems Inc., Minneapolis, MN) was diluted to 0.5 μg/ml in 1% BSA (Sigma)/PBS and added to wells for incubation at room temperature for 90 minutes. Wells were washed with PBS-T four times, then streptavidin-alkaline phosphatase (Jackson Immunoresearch Laboratories, West Grove, PA) was diluted 1/1000 in 1% BSA/PBS and incubated in the plate wells at room temperature for 1 hour. Wells were washed 4 times with PBS-T and twice with PBS before the addition of the substrate Di (Tris) *p*-nitrophenyl phosphate (pNPP) (Sigma). Absorbances at 405 nm were recorded by a

Multiskan Ascent spectrophotometer (Thermo Lab-systems, Helsinki, Finland) at regular intervals. Statistical analyses were analyzed by Instat™ statistical software.

GrB ELISPOT

Ninety-six well Multi Screen Immobilon-IP plates (0.45 μm) (Millipore, Bedford, MA) were coated as indicated in the ELISA procedure with 5 μg/ml GB12 antibody. Wells were washed twice with PBS and blocked with 10% FBS/ RPMI for 1 hour at room temperature. Cells were diluted in 2% FCS-RPMI media for CTL-10 and AB.1 cells, or complete media for primary effector cells, to the indicated concentrations, using L1210, H-2K^b-transfected L1210, or EL4 cells as targets at either 1x10^4 or 5x10^3 cells per well. Target cells were pulsed with 40 μM H-2D^b restricted HY male minor histocompatibility peptide Uty$_{246-254}$ (WMHHNMDLI) (Multiple Peptide Systems, San Diego, CA), Influenza virus nucleoprotein (NP)$_{366-374}$ peptide (ASNENMETM)(CANVAC Core Facilities), or LCMV gp33 peptide (KAVYNFATM) (CANVAC Core Facilities) for one hour at 37°C, then washed before addition to ELISPOT plates. Effector cells, CTL-10, AB.1, or spleen or lymph node cells of LCMV-infected or naïve mice were seeded into wells at various concentrations, then targets were added to give a final volume of 150 μl per well. To inhibit degranulation, 4 mM of EGTA and 8 mM $MgCl_2$ was added to target cells and incubated for 10 minutes prior to the addition to effector cells. Cells were incubated for 4 hours at 5% CO_2 and 37°C, then were rapidly and vigourously washed twice with ice-cold distilled water, four times

with ice cold PBS-T and twice with ice-cold PBS. Biotin-conjugated anti-mouse GrB antibody was diluted to 1 μg/ml in 1% BSA/PBS and incubated for 1 hour at room temperature. Wells were washed 4 times with PBS-T, then streptavidin-alkaline phosphatase was added for 1 hour, diluted 1/1000 in 1% BSA/PBS. Wells were washed six times with PBS-T and twice with PBS prior to the addition of 5-brom-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) substrate (Sigma). Wells were washed in distilled water upon substrate development and then air-dried before enumeration under a dissecting microscope. Images were produced by a DuoScan f40 Scanner (Agfa-Gavaert N.V., Mortsel, Belgium) and Adobe® Photoshop 7.0.1 (San Jose, CA).

^{51}Cr-release assay

Target cell lysis was measured by ^{51}Cr-release assays which were performed as previously described (Ewen et al., 2003a). Briefly, $1x10^6$ target cells were labeled with 100 μCi $Na_2{}^{51}CrO_4$ (Perkin-Elmer Life Sciences, St. Laurent, Quebec) following a pulse with 40 μM HY peptide, H-2D^b Influenza Virus peptide NP_{366}, or H-2D^b LCMV peptide gp33. Target cells were incubated at $3x10^3$ or $1x10^4$ per sample with varying numbers of effector cells. Supernatants were collected at 4 hours, analyzed by a Wallac 1470 gamma counter (Wallac Oy, Turku, Finland) and percent target cell lysis was calculated using the formula percent target lysis = experiment cpm – spontaneous cpm / total cpm - spontaneous cpm x 100.

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CHAPTER 3 - MECHANISM OF GRANZYME H - MEDIATED CELL DEATH

Introduction

The cytotoxic cell-mediated response, mediated primarily by natural killer (NK) and cytotoxic T cells (CTL), is central for ability of the immune system to eliminate virally-infected and transformed cells (Barry and Bleackley, 2002; Chowdhury and Lieberman, 2008). These cells employ two basic mechanisms; granule-mediated, or receptor-mediated cytotoxicity (Kagi et al., 1994; Lowin et al., 1994). Target cell death is characterized by the activation of caspases, condensation of chromatin and oligonucleosomal damage to DNA, and dysfunction of organelles including mitochondria, endoplasmic reticulum and lysosomes (Kroemer et al., 2009).

Receptor-mediated responses, such as Fas, are initiated by caspase 8/10, following their recruitment and activation at the Death Inducing Signal Complex (DISC) (Boldin et al., 1996; Muzio et al., 1996). Fas signalling can occur through two pathways (Scaffidi et al., 1998). In Type I cells, the activation of caspase-8 sufficiently and directly activates caspase-3 to promote cell death (Fernandes-Alnemri et al., 1996). In type II cells, such as Jurkat, cleavage and activation of Bid (BH3-interacting domain death agonist) via caspase-8 facilitates the activation and oligomerization of Bax (Bcl-2 associated X protein) and/or Bak (Bcl-2 antagonist killer 1) (Li et al., 1998; Luo et al., 1998). This results in the permeabilization of the outer mitochondrial membrane and release of pro-apoptotic molecules such as cytochrome C, and the loss of the mitochondrial transmembrane potential ($\Delta\Psi_m$). Apoptosome formation, composed of caspase-9, cytochrome C, dATP, and Apaf-1, induces cell death by activating caspase-3.

Perforin and granzymes are the primary effector molecules of granule-mediated cytotoxicity. The best characterized granzymes are GrB (granzyme B) and GrA (Chowdhury and Lieberman, 2008). Granzyme B can directly activate caspase-3 to induce cell death (Darmon et al., 1996; Darmon et al., 1995). Alternatively, the protein can also initiate the mitochondrial pathway through direct Bid cleavage. This promotes Bid translocation from the cytosol to the mitochondria, where it induces Bax/Bak oligomerization (Barry et al., 2000; Heibein et al., 2000; Sutton et al., 2000; Waterhouse et al., 2005). Full caspase-3 autocatalytic maturation can be attenuated by inhibitor of apoptosis proteins (IAPs), but these proteins can be neutralized by the mitochondrial intermembrane protein SMAC/DIABLO (Second Mitochondria-derived Activator of Caspases). Thus, in GrB-mediated cell death, full caspase-3 activation is promoted by direct caspase-3 interaction with GrB and SMAC/DIABLO release from permeabilized mitochondrial outer membranes (Goping et al., 2003; Sutton et al., 2003). Additionally, GrB can directly promote DNA fragmentation via proteolysis of ICAD (inhibitor of caspase-activated DNAse)/DFF45, and the translocation of CAD to the nucleus (Sharif-Askari et al., 2001; Thomas et al., 2000).

Granzyme A induces cell death by a caspase-independent mechanism, targeting the mitochondrial respiratory chain protein NDUFS3. This results in the generation of reactive oxygen species, the translocation of the SET complex from the ER to the nucleus, and the generation of single-stranded DNA breaks (Chowdhury et al., 2006; Fan et al., 2003a; Fan et al., 2003b; Martinvalet et al., 2008; Zhu et al., 2009; Zhu et al., 2006).

Human NK cells produce additional granzymes H, K, and M, but relatively little is understood about their cytotoxic potential (Bratke et al., 2005; Sayers et al., 2001; Sedelies et al., 2004). Granzyme K induces cell death through cleavage of components of the SET complex, and perhaps through direct Bid processing (Guo et al., 2008; Zhao et al., 2007a; Zhao et al., 2007b). Granzyme M has also been reported to induce cell death and substrates identified, thus far, include ICAD/DFF45 and alpha tubulin (Bovenschen et al., 2008; Kelly et al., 2004; Lu et al., 2006). Additionally, GrM may enhance cytotoxicity of killer cells by inactivating an endogenous inhibitor of GrB, proteinase inhibitor-9 (PI9) (Mahrus et al., 2004). Similarly to GrM, GrH has been shown to enhance NK cytotoxicity towards adenovirus-infected cells via cleavage of the adenovirus-derived GrB inhibitor, LK-100K (Andrade et al., 2007)

There is also evidence that GrH directly induces cell death, but the mechanism is controversial (Fellows et al., 2007; Hou et al., 2008). Fellows *et al.* reported GrH cell death was mediated via a caspase-independent mechanism, in the absence of direct GrH-mediated proteolysis of Bid or ICAD/DFF45, and without the release of cytochrome C. In contrast, Hou *et al*. have demonstrated that GrH exposure to target cells resulted in the direct cleavage of Bid and ICAD/DFF45, the release of cytochrome C, and the activation of caspase-3 by an unknown mechanism.

The focus of our studies was to resolve the disparities between these studies, and further refine the model of GrH-mediated cell death. We also sought to compare GrH effects to well characterized GrB and Fas death pathways and

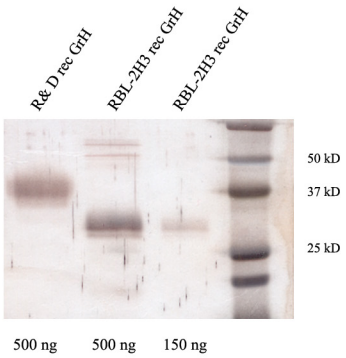
reveal differences among these pathways under similar conditions. We have evidence to show that GrH directly cleaves ICAD/DFF45, but does not efficiently directly cleave Bid. Death is reduced by the over-expression of Bcl-2 or the double deficiency of Bax and/or Bak, and induces the release of cytochrome C and SMAC/DIABLO. However, unlike GrB and Fas, GrH does not strongly promote the activation of caspase-3, and the contribution of caspase-3 to GrH-mediated cell death varies among target cell lines.

Results

Production of GrH

Granzyme H is a serine protease, initially produced within cytotoxic cells in a zymogen form and requires the activity of granule-resident cathepsin C to become active (Pham and Ley, 1999). Moreover, the functional uptake of granzymes has been shown to be affected by eukaryotic post-translational modifications (Motyka et al., 2000; Veugelers et al., 2006). To produce functional, physiologically relevant GrH, we inserted human cDNA derived from Lymphokine Activated Killer (LAK) cells into a pcDNA3.1 expression vector (Meier et al., 1990). The rat mast cell tumour line, RBL-2H3 was stably transfected with pcDNA3.1-GrH, as these cells have previously been reported to produce functional granzymes (Nakajima et al., 1995). By isolating GrH from granules of transfected RBL-2H3 cells, we produced pure, enzymatically active stocks of GrH (Figure 3-1.A and B). We also generated mutant (S202A) inactive GrH from stably transfected RBL-2H3 cells (Figure 3-1.B). Aspase and tryptase

A



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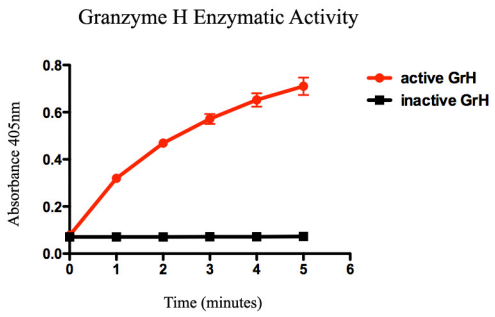

Figure 3-1 Production of RBL-2H3-derived recombinant human Granzyme H. A) Silver stain of 12% SDS-polyacrylamide gel loaded with 500 ng of commercially available zymogen recombinant GrH (R & D Systems; lane 1), or 500 ng (lane 2) or 150 ng (lane 3) active recombinant GrH purified from RBL-2H3 cells. B) Enzymatic activity of 10 µg/ml active or inactive S202A mutant recombinant GrH from three independent protein preparations was assessed by proteolytic hydrolysis of a peptide substrate Suc-Phe-Leu-Phe-Thiobenzyl ester. (N=3; mean ± SD).

activity was absent in purified GrH stocks.

Titration of GrH activity

To determine the cytotoxic potential of GrH, various target cells were treated for 4 hours with a series of concentrations of GrH, along with replication-deficient Adenovirus 5 (Ad5), which acts as a perforin substitute that promotes granzyme trafficking (Froelich et al., 1996). Cell death in target cells K562, T cell lymphoma Jurkat, and EBV-transformed B cell line RPMI-8866 was measured by multi-parameter flow cytometry for poly-caspase activation (FITC-VAD-fmk), and plasma membrane damage (Annexin V and 7-AAD). Cells were analyzed for apoptotic ($7\text{-}AAD^{-}$ / $AnnexinV^{+}$) and necrotic features ($7\text{-}AAD^{+}$ / $AnnexinV^{+}$) and demonstrated that concentrations at and above 20 μg/ml of GrH were capable of inducing cell death by all these parameters (Figure 3-2.A-D). Necrosis was more pronounced at higher concentrations of GrH in all cells, and was more prominent in K562 and RPMI-8866 cells (Figure 3-2.B and D). Jurkat cells had the highest proportion of apoptotic cells at all concentrations of GrH tested (Figure 3-2.C). Inactive mutant GrH only induced cell death at very high concentration (80 μg/ml), perhaps a reflection of very minimal chymase activity in these active-site mutants (Johnson et al., 2003; Wu et al., 2009).

Caspase activation, detected by staining with poly-caspase detector FITC-VAD-fmk, was observed in all cell lines in a similar concentration-dependent manner as membrane damage (Figure 3-2.E-H). Based on these data, concentrations from 20 – 50 μg/ml were utilized in subsequent experiments.

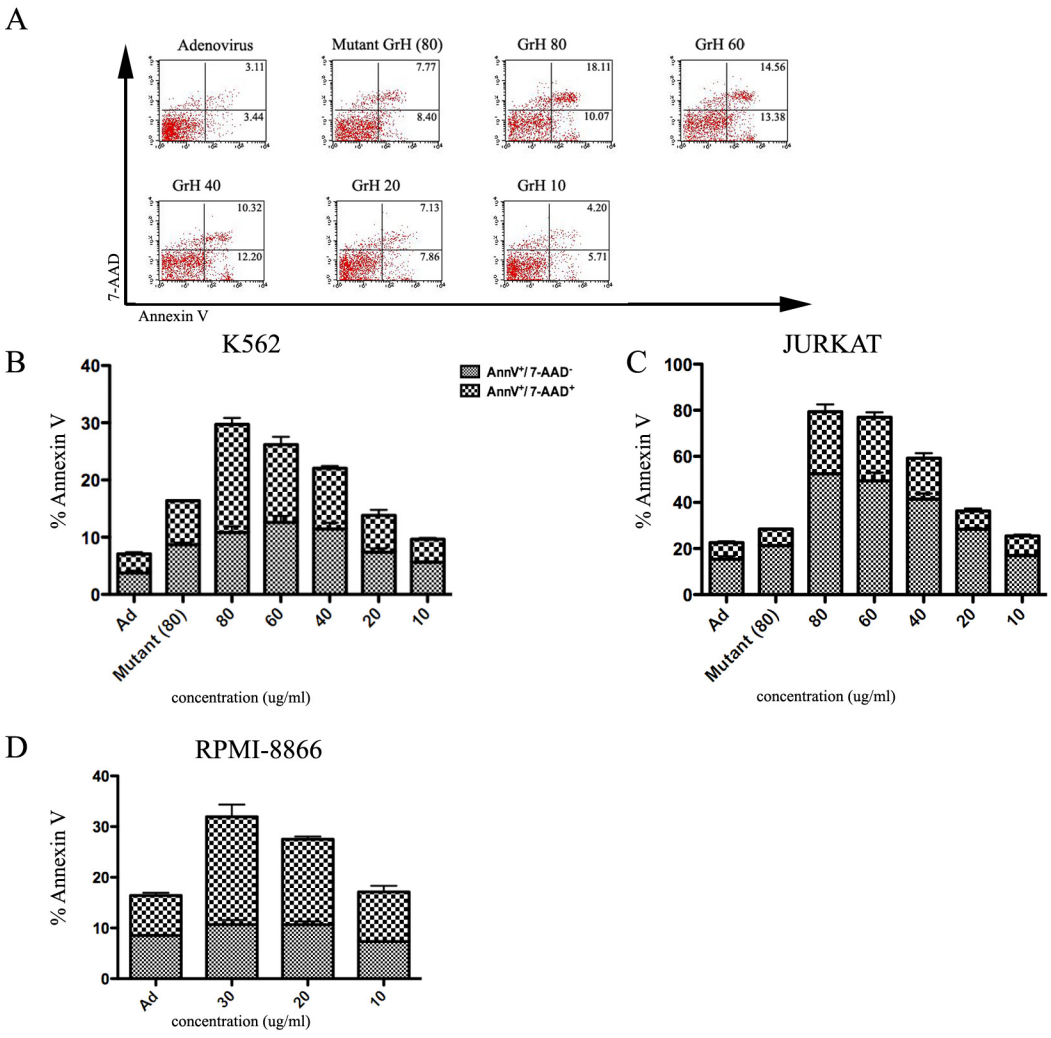

Figure 3-2 Titration of recombinant GrH Killing Activity. Cell death was monitored after 4 hours of exposure to 200 p.f.u./cell Ad5 and numerous concentrations of GrH or mutant S202A GrH (μg/ml). A) Dot plots of K562 cells stained with Annexin V and 7-AAD. B-D) Graphical results of cell death - either by Annexin V^+/7-AAD^- (light speckle) or by Annexin V^+/ 7^-AAD (dark speckle) staining. B) K562, C) Jurkat, D) and RPMI-8866 target cells.

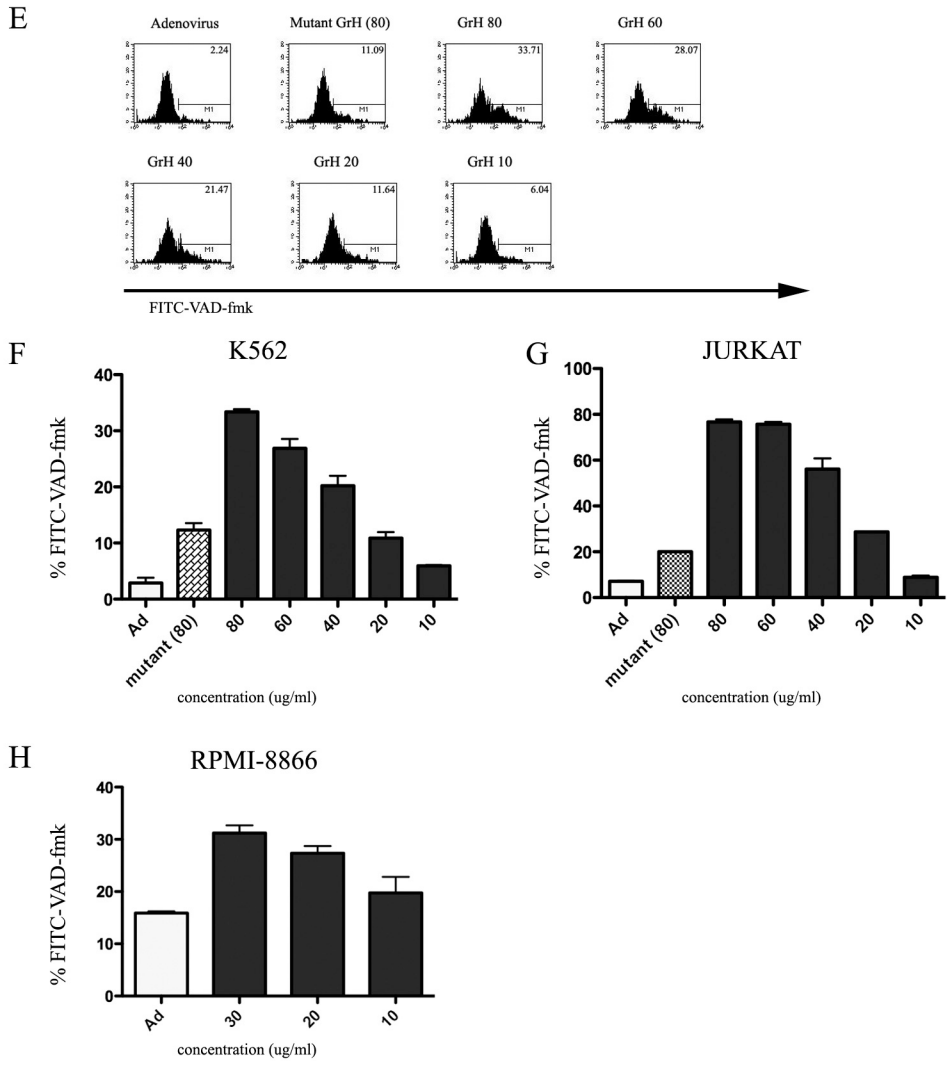

Figure 3-2 continued. E) Histograms of polycaspase activation in K562 cells. Graphical results of caspase activation in F) K562, G) Jurkat, H) and RPMI-8866 target cells. Data is representative of ≥ 3 independent experiments. (Mean ± SD).

GrH-mediated cell death is insensitive to Protease Inhibitor-9

Plasma membrane damage was also assessed following treatment with Ad5 and either GrH or GrB in K562, Jurkat, and RPMI-8866 cells (Figure 3-3). Granzyme H induced similar levels of cell death in all target cell lines, while GrB showed significant variability among cell lines (Figure 3-3.A and B). As indicated in Figure 3-3.C, this strongly correlated with the expression of the GrB-specific serpin, PI9. Thus, while GrB was susceptible to PI9 inhibition, GrH was insensitive to levels of PI9 and implied that GrH could have a role in the elimination of cells when GrB activity is impaired.

Time-course of GrH Induced Cell Death

The kinetics of numerous features of cell death were measured in Jurkat and K562 cells over 8 hours following the addition GrH (Figure 3-4). Multi-parameter flow cytometry was used to assess poly-caspase activity (FITC-VAD-fmk), loss of $\Delta\Psi_m$ (TMRE), and phosphatidyserine externalization (Annexin V) in one combination, while DNA breaks (TUNEL) and active caspase 3 was assessed in another set of cells treated in parallel. Most features of cell death following GrH treatment were not readily apparent until 4 hours, and then steadily increased until nearly all cells showed signs of cell death at 8 hours.

In Jurkat cells, GrH treatment was also compared to the kinetics of GrB or Fas-induced cell death at concentrations previously reported to induce cell death (Figures 3-4.A-E, and Figures 3-4.K-M) (Barry et al., 2000; Heibein et al., 1999; Motyka et al., 2000). Granzyme H kinetics were rather similar to activating Fas

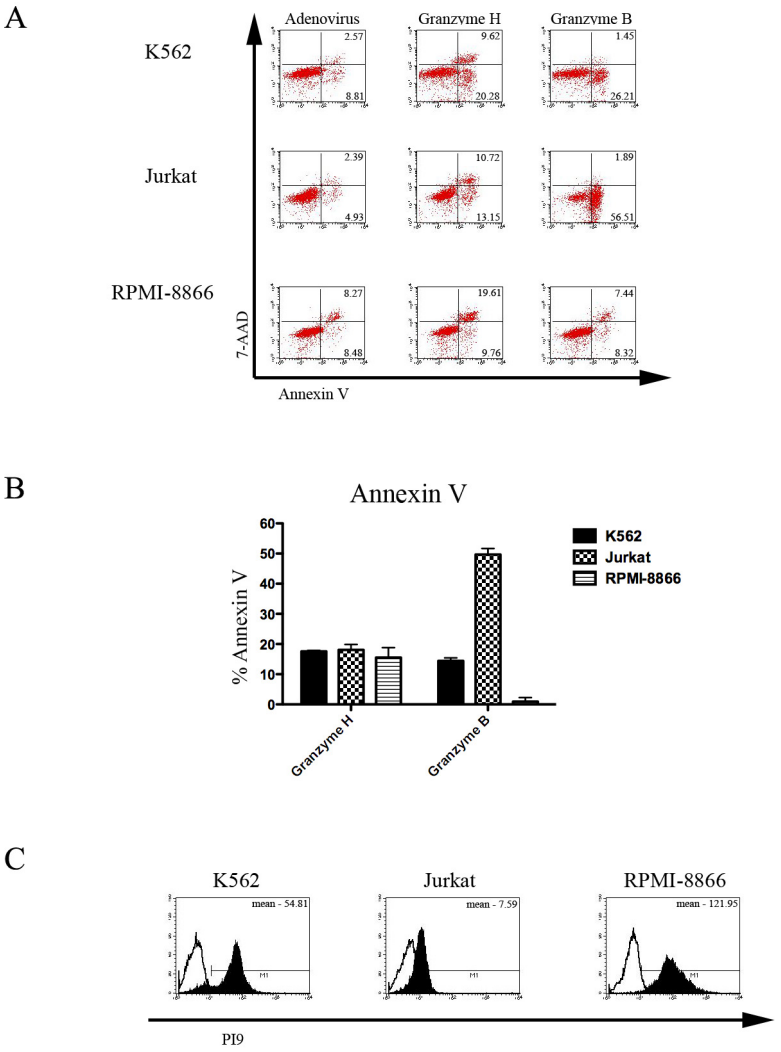

Figure 3-3 Granzyme H kills in the presence of Proteinase Inhibitor-9. Cell death was monitored by Annexin V and 7-AAD staining following 4 hours of exposure to 200 p.f.u./cell Ad5 and 30 µg/ml GrH or 1.2 µg/ml GrB. A and B) K562, B) Jurkat, and RPMI-8866 cells. Background Ad5 levels were substracted from all groups. C) Levels of PI9 were measured by intracellular staining with anti-PI9 antibody and flow cytometry. Data is representative of ≥ 3 independent experiments. (Mean ± SD).

antibody treatment, while GrB kinetics were considerably more rapid. The earliest (≤ 2 hours) features of GrH-mediated cell death in Jurkat cells included a very modest activation of caspases (FITC-VAD-fmk; Figures 3-4.B and L), loss of $\Delta\Psi_m$ (% TMRE Loss; Figures 3-4.C and L), and Annexin V positivity (Figures 3-4.A and K). The activation of caspase-3 (Figures 3-4.D and M), and the appearance of TUNEL-positive cells (Figures 3-4.E and M) were later features (≥ 4 hours). This differs strikingly from GrB-mediated cell death in Jurkat, where the vast majority of cells displayed apoptotic features within 2 hours of treatment. At one hour following GrB exposure cells displayed substantial reduction of $\Delta\Psi_m$, strong poly-caspase activation, and rapid caspase-3 activation. The externalization of phosphatidlyserine and appearance of TUNEL-positive cells occurred slightly later.

Cell death induced by GrH and GrB was also compared in K562 cells. Granzyme H treatment of K562 cells showed similar kinetics to Jurkat cells with early modest phosphatidylserine externalization (Figures 3-4.F and N), poly-caspase activation (Figures 3-4.G and O), and loss of $\Delta\Psi_m$ (Figures 3-4.H and O). However, TUNEL staining did not become apparent until very late time-points (Figures 3-4.J and P) and caspase-3 activation was very low, even at late time-points (Figures 3-4.I and P). Granzyme B treatment of K562 cells was less efficient than GrH in inducing most features of cell damage, with the exception of caspase-3 activation. Caspase-3 activation occurred earlier with GrB treatment, although levels never reached those observed in Jurkat cells. Lower levels and slower kinetics of cell death in GrB-treated K562 cells were likely due to PI9

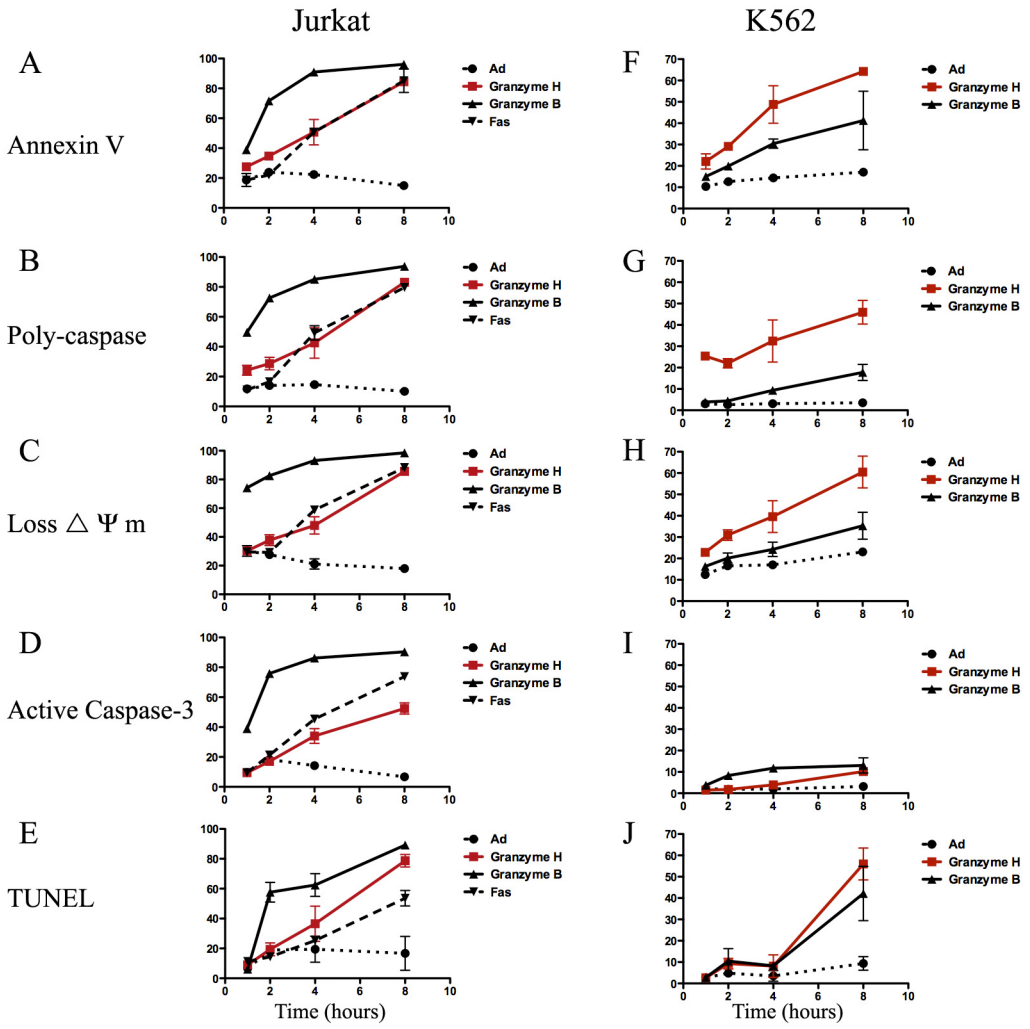

Figure 3-4 Kinetics of cell death following treatment with GrH. A-E) Jurkat cells were treated with 200 p.f.u./cell Ad5 (dotted line) plus either 20 µg/ml GrH (red line), 500 ng/ml GrB (solid line), or with 1 µg/ml anti-Fas antibody (dashed line) for indicated times (hours). F-J) K562 cells were treated with Ad5 (dotted line), 40 µg/ml GrH (red line), or 1 µg/ml GrB (solid line) for indicated times (hours). Graphical representations of Annexin V (A and F), FITC-VAD-fmk (B and G), TMRE (C and H), active caspase-3 (D and I), and TUNEL (E and J) Data are representative of 2 independent experiments. (Mean ± SD).

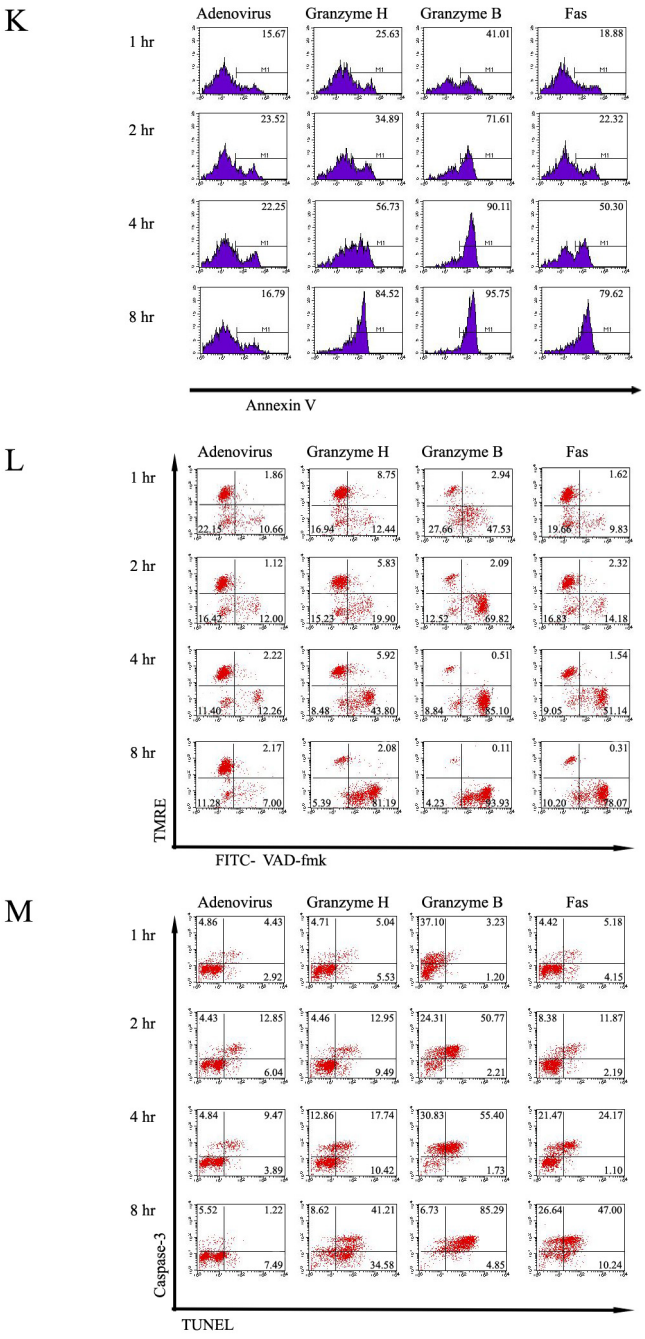

Figure 3-4 Kinetics continued. Individual representative flow cytometry plots for Jurkat cells. K) Annexin V; L) TMRE vs FITC-VAD-fmk; and M) Active caspase-3 vs TUNEL.

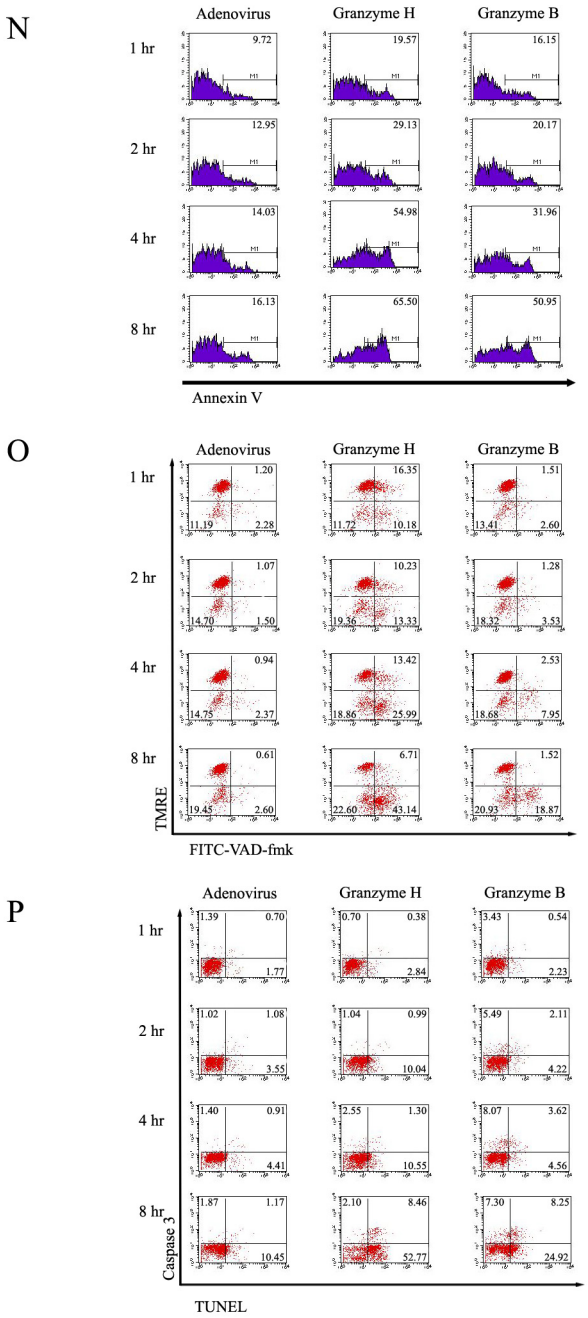

Figure 3-4 Kinetics continued. Individual representative flow cytometry plots for K562 cells. N) Annexin V; O) TMRE vs FITC-VAD-fmk; and P) Active Caspase-3 vs TUNEL.

activity (Figure 3-3).

Collectively, time-course results demonstrated an early, modest effect on plasma membrane, $\Delta\Psi_m$, and poly-caspase activity following GrH treatment. Most indicators of cell death required at least 4 hours to become substantial, and TUNEL and active caspase-3 generation were noticeably delayed. Indeed, caspase-3 activity remained rather moderate in all GrH treated cells, an observation distinct from that of GrB and Fas treatments.

Effects of GrH on $\Delta\Psi_m$ and the Plasma Membrane

To ascertain the effect of GrH-induced caspase activation on $\Delta\Psi_m$ or the externalization of phosphatidylserine during GrH-mediated cell death, experiments were performed in the presence of a poly-caspase inhibitor (z-VAD-fmk). Treatment of either Jurkat or K562 cells with z-VAD-fmk did not prevent GrH-induced loss of $\Delta\Psi_m$ (Figures 3-5.A and B, or Figures 3-5.D and E; respectively). Inhibition of caspases did not prevent GrB-mediated $\Delta\Psi_m$ loss either, as was expected (Heibein et al., 1999). In contrast, phosphatidylserine exposure was partially reduced in GrH-treated Jurkat and K562 cells (Figures 3-5.A and C, and Figures 3-5.D and F; respectively), and considerably reduced in GrB-treated cells. These observations indicated that the externalization of phosphatidylserine is less caspase-dependent during GrH-mediated cell death than during GrB-induced apoptosis.

To determine whether Bcl-2 family members could affect GrH-induced $\Delta\Psi_m$ loss or plasma membrane changes, cell death was measured in Jurkat cells

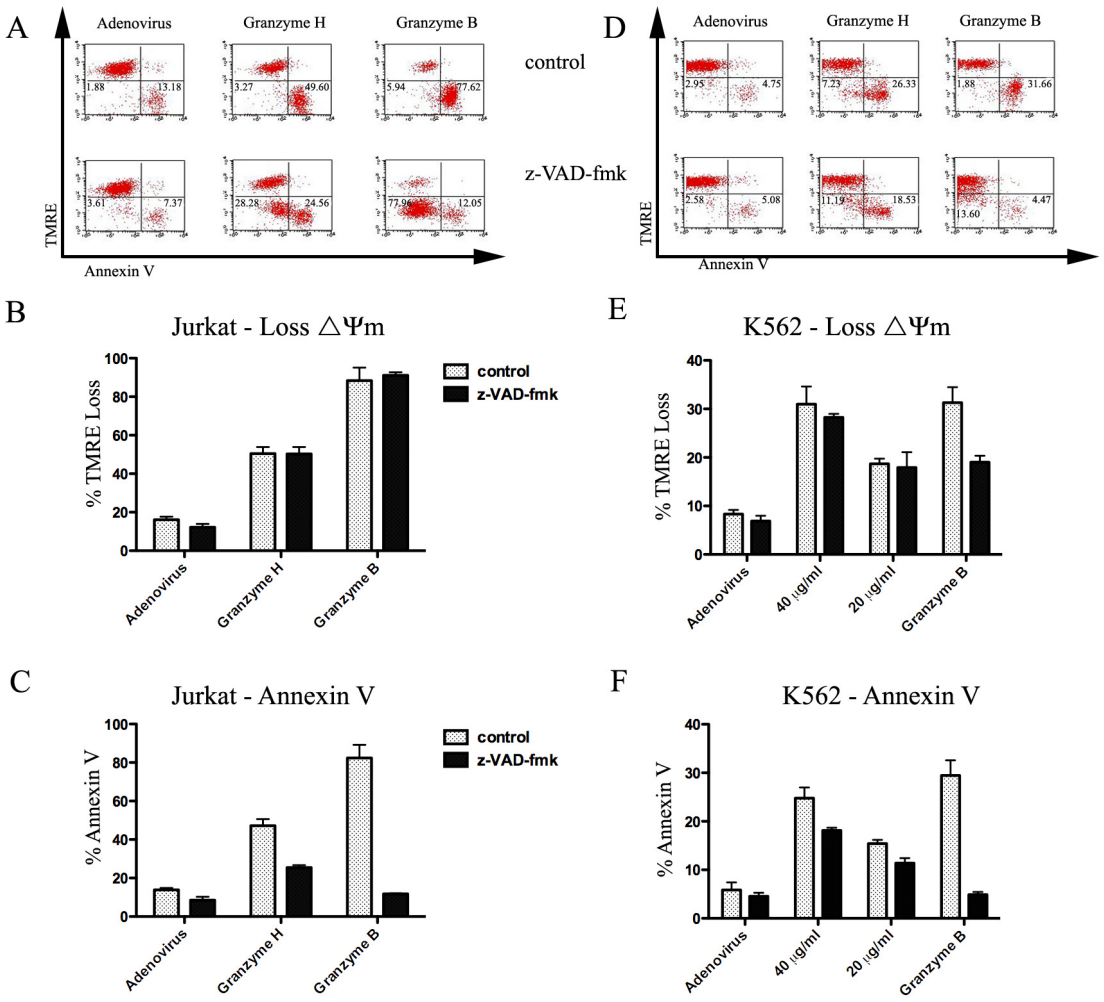

Figure 3-5 Mitochondrial Transmembrane Potential Loss induced by GrH is independent of caspases. A-C) Jurkat cells were treated with Ad5 and 20 µg/ml GrH or 1.2 µg/ml GrB in the presence of DMSO control (light bars) or 50 µM z-VAD-fmk (dark bars) for 4 hours before being stained with TMRE and Annexin V. D-F) K562 cells were similarly treated and stained with TMRE and Annexin V. Data is representative of 2 independent experiments. (Mean ± SD).

either over-expressing Bcl-2 (J-Bcl2) or doubly deficient in Bax and Bak (JR cells) (Figure 3-6) (Han et al., 2004). The results, in Figures 3-6.A and B, indicated that Bcl-2 strongly impaired GrH-induced $\Delta\Psi_m$ loss (protection of 78%). Bcl-2 over-expression similarly prevented GrB-mediated $\Delta\Psi_m$ loss (protection of 88%), as has been previously reported (Davis et al., 2000; Pinkoski et al., 2001). Results also indicated that when $\Delta\Psi_m$ loss was impaired by Bcl-2 during GrH- or GrB-mediated cell death, phosphatidylserine exposure was also prevented (Figure 3-6.A). Thus, for both GrH and GrB, phosphatidylserine exposure was likely downstream of mitochondrial damage.

Bax/Bak double-deficient cells (JR) were almost completely protected from GrH-induced $\Delta\Psi_m$ loss, while only partially protected from GrB (protection of 92% and 68%; respectively). Granzyme B has been reported to induce $\Delta\Psi_m$ dissipation in the absence of Bid, Bax, and Bak, (Thomas et al., 2001). In contrast, our findings suggest that GrH-induced $\Delta\Psi_m$ loss is more sensitive to the absence of Bax and/or Bak than GrB. Additionally, annexin V and TMRE staining demonstrated that cells with $\Delta\Psi_m$ did not expose phosphatidylserine, and that this was likely a downstream event of mitochondrial damage (Figure 3-6.A).

GrH and Bid Activation

Bid is a highly pro-apoptotic protein, with central roles in both GrB- and Fas-mediated cell death (Barry et al., 2000; Heibein et al., 2000; Li et al., 1998; Luo et al., 1998). However, the role of Bid in GrH-mediated cell death has been controversial; one group demonstrated that Bid was not a direct substrate of GrH,

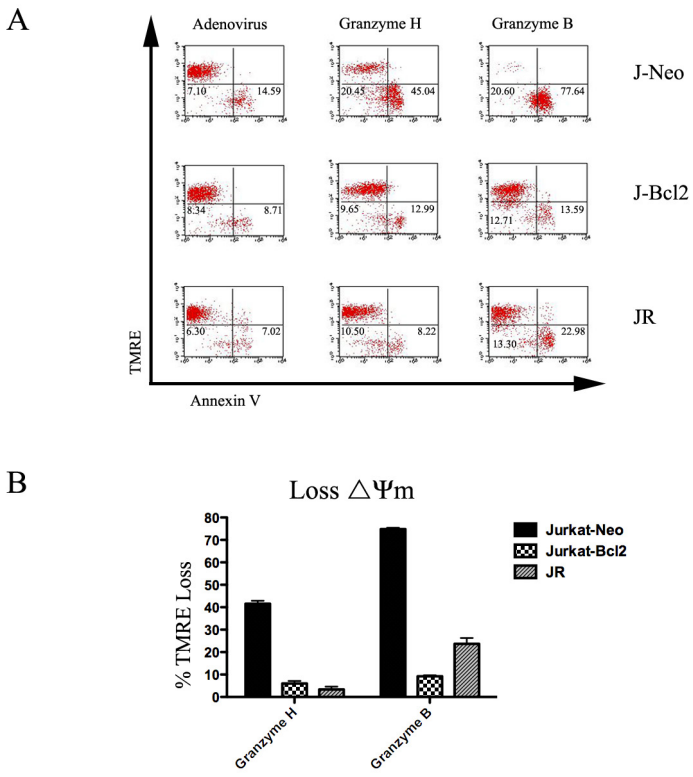

Figure 3-6 Mitochondrial Transmembrane Potential Loss induced by GrH is modulated by Bcl-2 family proteins. Jurkat-Neo (dark bar), Jurkat-Bcl-2 (thick hatched bar), and JR (light speckled bar) cells were treated with 40 µg/ml GrH or 1 µg/ml GrB in the presence of 200 p.f.u./cell Ad5 for 4 hours, then stained with TMRE and Annexin V. Ad5 levels were subtracted from all treatment groups. Data is representative of 3 independent experiments. (Mean ± SD).

while another group reported that direct cleavage of Bid by GrH was a central mechanism of GrH-induced cell death (Andrade et al., 2007; Chowdhury and Lieberman, 2008; Fellows et al., 2007; Hou et al., 2008). To resolve this conflict, various experiments were performed to evaluate the role of Bid during GrH-mediated cell death. Firstly, Bid processing was assessed in Jurkat – Neo and Jurkat- Bcl2 cells following treatment with GrH, GrB, or Fas. Jurkat-Neo cells were treated with DMSO or z-VAD-fmk to determine whether Bid cleavage was affected by caspases. As shown in Figures 3-7.A and B, Bid cleavage in GrH-treated Jurkat-Neo cells was only observed in control cells, and was not evident in the presence of z-VAD-fmk. In control samples, Bid processing was ablated by z-VAD-fmk in Fas-stimulated cells, while GrB cleavage was only slightly reduced, as was expected. Next, Bid processing was evaluated in Jurkat-Bcl2 cells following GrH, GrB, and Fas treatment (Figure 3-7.C). Bid cleavage could not be detected in GrH treated cells, while both GrB and Fas produced detectable levels of processed Bid. These results indicated that detectable levels of processed Bid was not produced directly by GrH, but was likely due to caspases activated by GrH treatment, and was downstream of mitochondrial damage.

To rule out the possibility that Bid antibody reagents were not sensitive enough to detect *in vivo* GrH-processed Bid, cleavage was assessed following granzyme treatment of either cell lysates *in vitro*, or with *in vitro* transcription/ translation (IVTT)-derived Bid. In Figure 3-7.D, GrH or GrB was added to Jurkat or MCF-7 cell lysates and Bid processing was assessed after 4 hours of treatment.

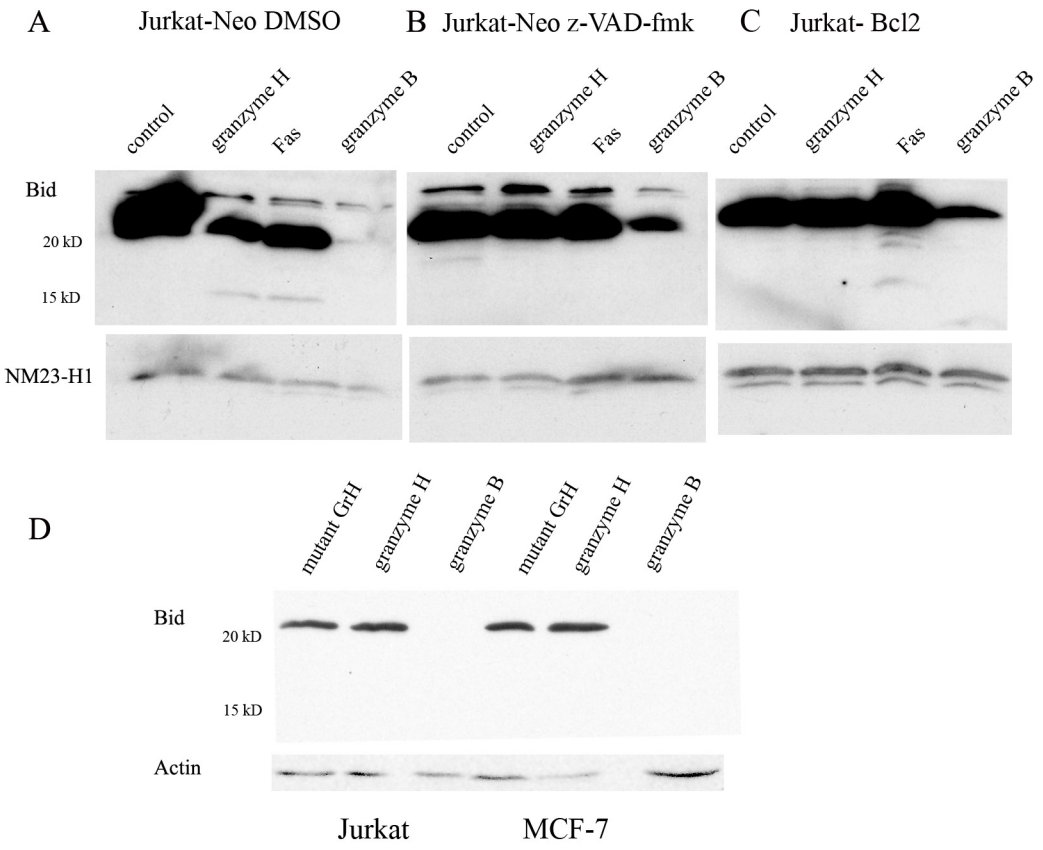

Figure 3-7 Granzyme H does not efficiently process Bid. A-C) Immunoblot of Bid in A) Jurkat-Neo, and C) Jurkat-Bcl2 lysates from cells treated with Ad5 (control), Fas, 50 µg/ml GrH and Ad5, or 1.5 µg/ml GrB and Ad5 for 4 hours. B) Jurkat-Neo cells were treated with GrH, GrB, and Fas in the presence of 50 µM z-VAD-fmk. D) Cell lysates from the equivalent of $2x10^5$ Jurkat or MCF-7 cells were treated with 30 µg/ml inactive S202A mutant GrH (control) or active GrH, or 1 µg/ml GrB for four hours, then immoblotted for Bid.

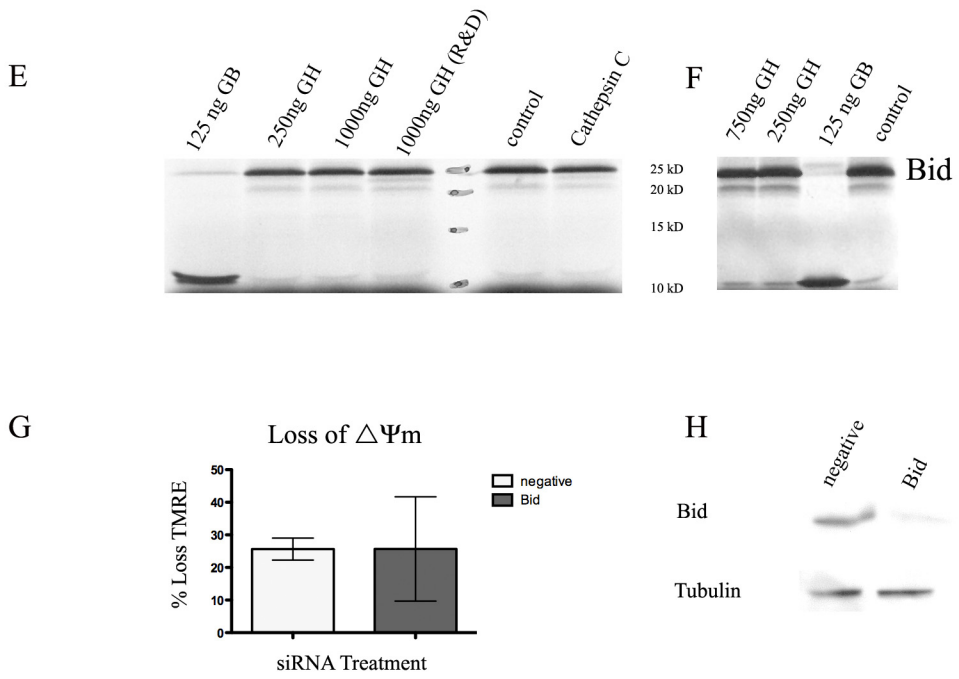

Figure 3-7 continued. E and F) ^{35}S-Met labelled and *in vitro* transcription/translated-Bid was treated with GrH from RBL-2H3 cells, or commercially available GrH (R&D) activated *in vitro* with Cathepsin C, or with GrB for E) 2 hours or F) 11 hours then visualized on a 15% SDS-polyacrylamide gel by audioradiography. G) Hela cells were treated were control (negative) (light bars) or Bid (dark bars) siRNA 48 hours prior to exposure to GrH. Graph represents the average loss of ΔΨm in GrH-treated Hela cells over 3 independent experiments (Mean ± SD). Ad5 background levels were substrated from both treatment groups. H) Immunoblot for Bid (top panel) and loading control tubulin (bottom panel) in Hela cells treated with negative or Bid siRNA.

Granzyme B completely degraded Bid while GrH failed to induce any changes in Bid levels, or produce detectable fragments. *In vitro* transcription/translation-derived Bid was completely processed by GrB within 2 hours of exposure (Figure 3-7.E). In contrast, GrH had no effect on Bid after 2 hours of incubation, but began to show very faint bands at 16 kD after 11 hours of incubation (Figure 3-7.F). These findings demonstrated that GrH does not efficiently process Bid. Finally, in experiments performed in Hela cells that were pretreated with siRNA against Bid, indicated that the reduction of Bid did not affect GrH-mediated cell death (Figure 3-7.G and H).

Release of Pro-apoptotic Proteins from Mitochondria

Cytochrome C and SMAC/DIABLO are mitochondria-resident proteins that are released into the cytosol upon exposure to apoptotic stimuli and strongly promote cell demise (Brenner and Mak, 2009). Previous reports have shown disparate results in the ability of GrH to induce cytochrome C loss from the mitochondrial intermembrane space (Fellows et al., 2007; Hou et al., 2008). Therefore, to resolve this issue, numerous cell lines were treated with GrH, and cytochrome C release was assessed by either cellular fractionation (Figure 3-8.A) or flow cytometry (Figures 3-8.B and C). After 4 hours of GrH treatment, cytosolic cytochrome C could be detected using cell fractionation and immunoblotting in Jurkat and K562. Granzyme B was used as a positive control. Additionally, cytochrome C levels were reduced in membrane fractions of both GrH- and GrB- treated cells.

Cytochrome C loss from the mitochondria was also evaluated in Jurkat, RPMI-8866, and Hela using flow cytometry (Figures 3-8.B and C). Cells were gently treated with digitonin before fixation and intracellular anti-cytochrome C antibody staining. Digitonin treatment gently permeabilized the cell membrane, and permitted the leakage of cytosolic cytochrome C from cells, while mitochondria bound cytochrome C was retained in cells (eg. control cells). Following GrH treatment, Jurkat, RPMI-8866, and Hela cells displayed reduced intensity for cytochrome C staining compared to control cells, indicative of translocation of cytochrome C from the mitochondria to cytosolic compartment (Figure 3-8.B). Granzyme B treatment reduced cytochrome C staining in Jurkat and Hela cells, but not GrB –resistant RPMI-8866 cells (Figure 3-3).

SMAC/DIABLO has been shown to relieve inhibition of caspase activity upon translocation to the cytosol by interacting with IAPs (Du et al., 2000). SMAC/DIABLO release following granzyme treatment was evaluated in Jurkat and RPMI-8866 cells by cell fractionation (Figure 3-8.D) and flow cytometry (Figures 3-8.E and F). Cytosolic SMAC/DIABLO was detected in both GrH and GrB treated Jurkat cells by immunoblotting, or by staining digitonin treated cells with anti-SMAC/DIABLO using the same procedure as was done for cytochrome C. SMAC/DIABLO translocation was only observed in RPMI-8866 cells exposed to GrH, as GrB activity is strongly impaired in these cells.

Collectively, Figure 3-8, demonstrated that GrH had the capacity to promote the release of various pro-apoptotic mitochondria resident proteins that

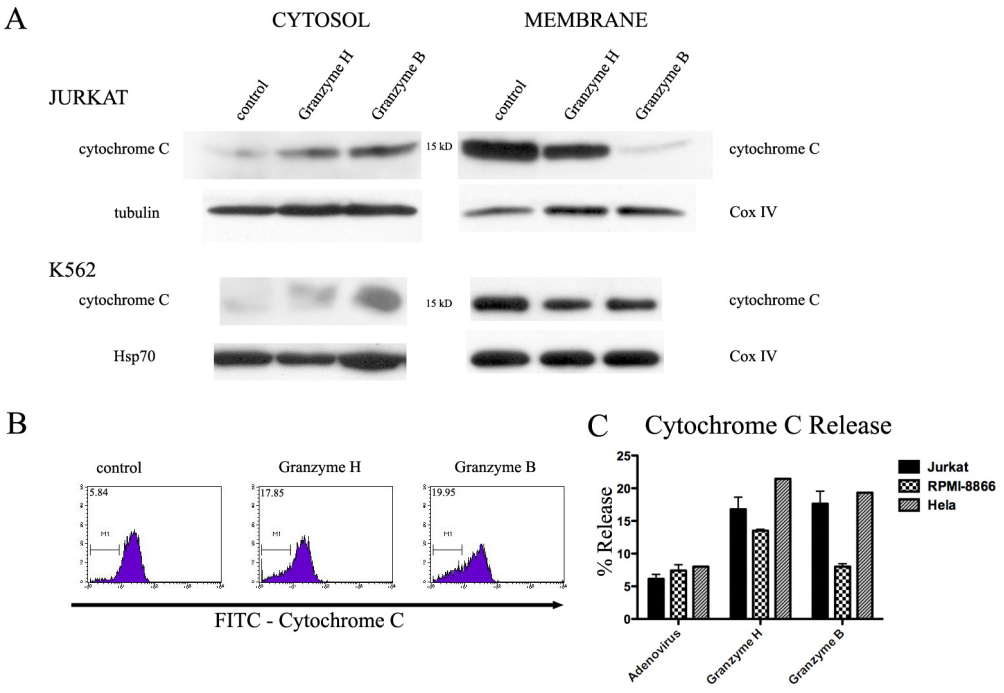

Figure 3-8 Granzyme H induces the release of apoptogenic proteins from the mitochondrial intermembrane space. A) Jurkat cells (top panel) and K562 cells (bottom panel) were treated with Ad5 (control) and 50 µg/ml GrH and Ad5, or 1.5 µg/ml GrB and Ad5 for 4 hours prior to cell fractionation. Cytosolic (left panel) and membrane (right panel) fractions were generated and immunoblotted for cytochrome C, or stripped and re-probed for loading controls Cox IV (membrane fraction) and Hsp70 or alpha-tubulin (cytosolic fraction). B) Cytosolic flow cytometry staining of cytochrome C in Jurkat cells treated with Ad5 (control) or with 30 µg/ml GrH or 1 µg/ml GrB. C) Graphical representation of translocated cytochrome C detected by flow cytometry in various cell lines. (N=3; mean ± SD).

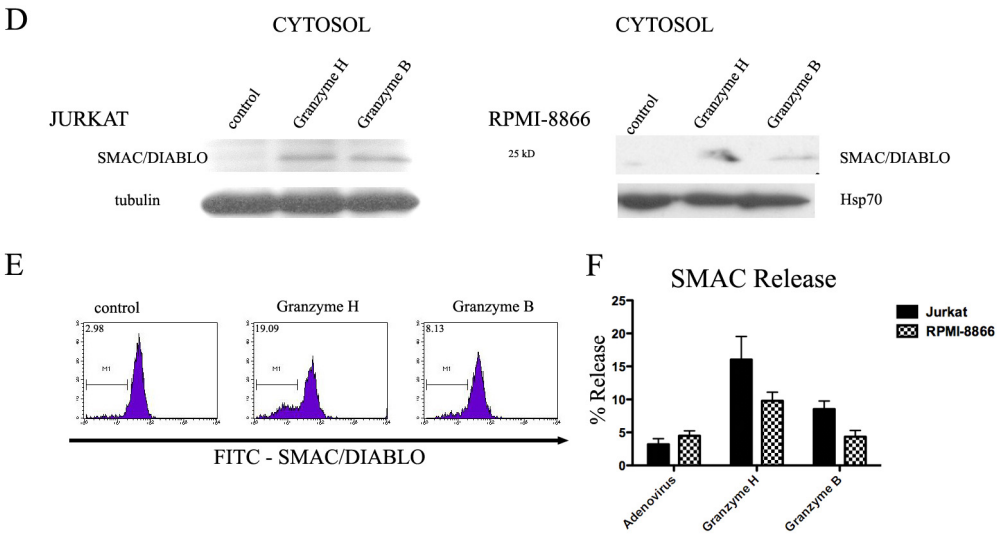

Figure 3-8 continued. D) Immunoblot of SMAC/DIABLO in cytosolic fractions of Jurkat cells (left panel) and RPMI-8866 cells (right panel) treated with Ad5 (control) and either 50 µg/ml GrH or 1.5 µg/ml GrB. Blots were stripped and re-probed with loading controls alpha-tubulin or Hsp70. E) Cytosolic detection of SMAC/DIABLO by flow cytometry staining of Jurkat cells treated with Ad5 (control) and 30 µg/ml GrH or 1 µg/ml GrB. F) Graphical representation of translocated SMAC/DIABLO detected by flow cytometry in Jurkat and RPMI-8866 cells. (N=3; Mean ± SD).

could potentially generate the formation of the apoptosome, relieve endogenous caspase inhibitors, and activate executioner caspases.

Activation of caspases

As demonstrated in Figures 3-2.E - H, caspases were activated in Jurkat, K562, and RPMI-8866 cells following GrH treatment. Subsequently, poly-caspase activation was measured in Jurkat- Neo, Jurkat- Bcl-2, Jurkat- Bax and Bak deficient (JR cells), and Jurkat - dominant negative caspase 9 (Jurk-DNCasp9) cells. These experiments were performed to determine whether Bcl-2 family proteins could affect caspase activity, and whether the apoptosome was central to the activation of caspases (Figure 3-9.A). Cells were treated with GrH, GrB, or anti-Fas antibody for 4 hours prior to staining with FITC-VAD-fmk. Jurkat over-expressing Bcl-2 resulted in a drop from 40% to 10% of poly-caspase activity in GrH-treated cells (75% reduction), compared to an 80% reduction for GrB, and nearly a 100% reduction for Fas-stimulated cells. JR cells were even more strongly impaired, with nearly a complete ablation of caspase activity in GrH treated cells, similar to Fas-activated cells. Granzyme B was slightly less affected at a 75% reduction.

Dominant negative caspase-9 partially impaired caspase activation following GrH exposure, a 45% reduction, while it did not impair GrB treatment, as caspases were activated independently of the apoptosome (Andrade et al., 1998; Atkinson et al., 1998). These experiments implied that caspase activity was

strongly modulated by Bcl-2, and Bax/Bak proteins. Also, our results demonstrated that the apoptosome was not soley responsible for the activation of caspases during GrH-mediated.

Caspase-3 is a central executioner of apoptosis, and so, intracellular antibody staining for active caspase-3 was performed to study its mechanism of activation in Jurkat-Neo, Jurkat- Bcl2, JR, and Jurk- DNCasp9 cells. As demonstrated in Figure 3-9.B, caspase-3 activation was absent at 4 hours in GrH-treated Jurkat-Bcl-2 and JR cells. Bcl-2 over-expression and Bax/Bak double-deficiency also completely impaired caspase-3 activation in Fas-activated cells, while expected low levels of caspase-3 could still be detected in GrB-treated cells (Goping et al., 2003). Dominant negative caspase-9 partially impaired caspase-3 activation in GrH-treated cells (65% reduction), completely impaired caspase-3 in Fas-stimulated cells, and had little effect on GrB-induced caspase-3 activation. These observations indicated that the Bcl-2 and Bax/Bak proteins are central to the control of GrH-induced caspase-3 activation in Jurkat cells, similar to Fas and GrB. However, unlike Fas, other mechanisms must be present in addition to the apoptosome to induce GrH-mediated caspase-3 activation.

Granzyme B is capable of direct cleavage of the zymogen form of caspase-3 to a p20 form that, in the absence of IAPs, can autocatalyze to a fully active p17 and p10 form (Goping et al., 2003; Sutton et al., 2003). To determine whether GrH could similarly directly interact with the zymogen form of caspase-3, or could indirectly initiate partial processing in a Bcl-2 or caspase-9 insensitive manner, caspase-3 cleavage was assessed by immunoblotting in Jurkat- Neo,

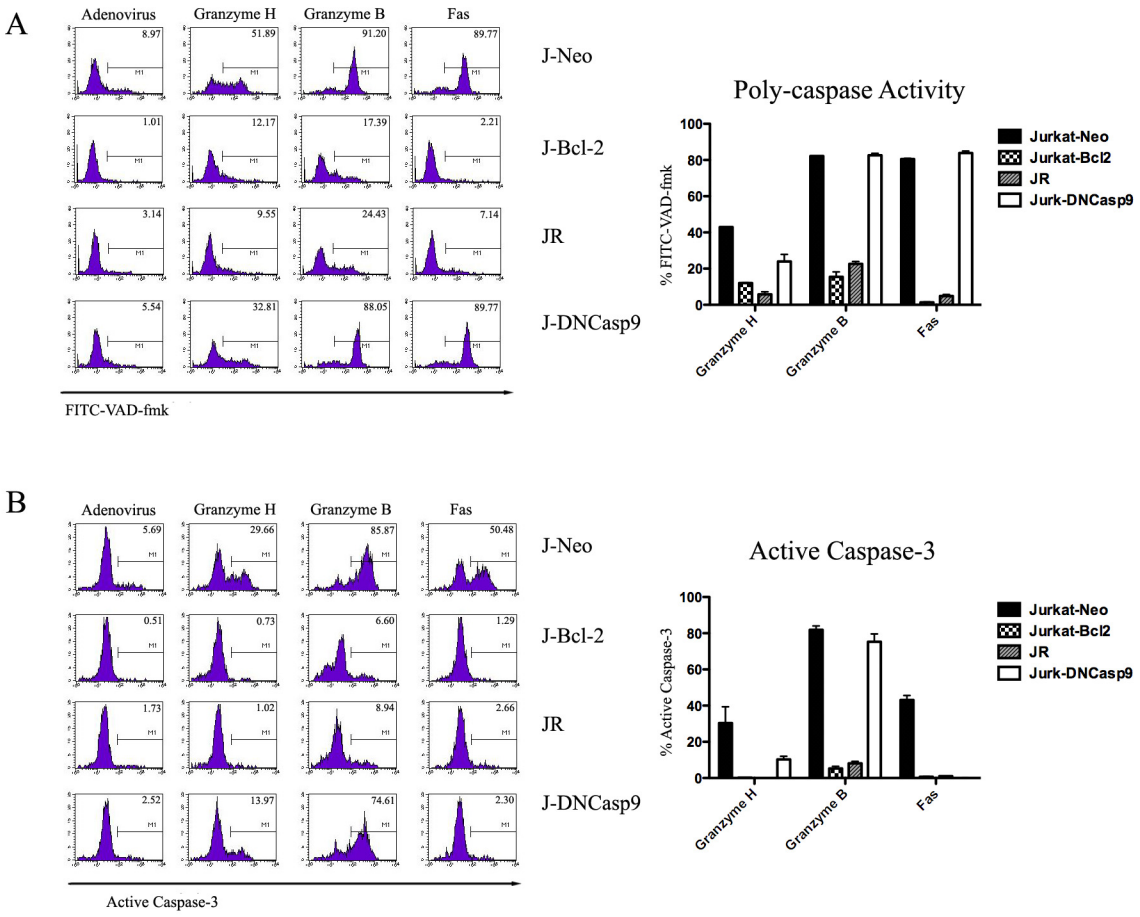

Figure 3-9 Caspase activation by GrH is modulated by Bcl-2, Bax and/or Bak, and caspase-9. A&B) Jurkat cell lines Jurk-Neo (dark bars), Jurk-Bcl2 (thick hatched bars), JR (light hatched bars) and Jurk-DNCasp9 (clear bars) were treated with Ad5 and 40 µg/ml GrH or 1 µg/ml GrB for 4 hours prior to the measurement of A) polycaspase activation, or B) active caspase-3. Ad5 background levels were subtracted each group. Data is representative of 3 independent experiments (mean ± SD).

C

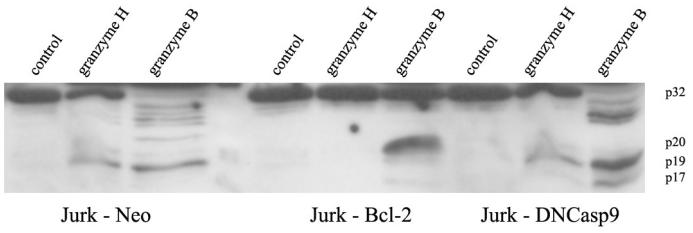

Figure 3-9 continued C) Immunoblot of Caspase-3. Cleavage of caspase-3 was assessed in Jurk-Neo (lanes 1-3), Jurk-Bcl2 (lanes 4-6), and Jurk-DNCasp9 (lanes 7-9) following 4 hours of exposure to Ad5 plus 20 µg/ml GrH, or control fractions (identical column – purified fractions collected from parental RBL-2H3 cells – see Methods and Materials), or 1.2 µg/ml GrB.

Jurkat- Bcl2, Jurk- DNCasp9 cells (Figure 3-9.C) Following 4 hours of GrH treatment, fully processed caspase-3 could be detected in GrH treated Jurkat-Neo and Jurk-DNCasp9, although at lower levels compared to GrB. The over-expression of Bcl-2 resulted in partial processing of caspase-3 by GrB, while GrH did not produce any caspase-3 cleavage products. These findings indicated that GrH does not have any capacity to directly process caspase-3, and further substantiated results in Figure 3-9.B that the apoptosome was redundant in the activation of caspase-3 during GrH mediated cell death.

DNA Damage

Previous reports have established that GrH treatment induces nuclear condensation and DNA damage. However, the mechanism underlying DNA damage is controversial. To address whether GrH treatment induces the fragmentation of DNA, TUNEL assays were performed on Jurkat and K562 cells (Figure 3-10).

As previously observed in Figure 3.4, TUNEL-positive cells were detected in Jurkat cells. These cells were also co-stained with anti-active caspase-3 antibody and this revealed that many cells treated with GrH were TUNEL-positive in the absence of active caspase-3 (Figure 3-4.M). In contrast, most Jurkat cells treated with GrB or anti-Fas were double positive (summarized in Figure 3-10.A). These data pointed to a possible difference of caspase contribution between GrH and GrB or Fas – mediated DNA damage.

K562 cells were also evaluated with TUNEL and anti-caspase-3. Similarly to Jurkat cells, a large proportion of K562 cells treated with either GrH or GrB were TUNEL-positive in the absence of caspase-3 (Figures 3-4.P and summarized in Figure 3-10.B). This implied that GrH could potentially mediate DNA damage in the absence of caspase-3.

Caspases have several mechanisms that promote DNA fragmentation (Enari et al., 1998; Lazebnik et al., 1994; Tang and Kidd, 1998). Experiments were performed in the presence of caspase inhibitors in Jurkat-Neo following 4 hours of GrH treatment to ascertain the level of caspase contribution to GrH induced DNA damage (Figure 3-10.C and D). As expected, z-VAD-fmk and the caspase 3/7 inhibitor, z-DEVD-fmk, nearly completely ablated TUNEL staining in GrB treated and Fas-stimulated Jurkat cells. In contrast, TUNEL staining was clearly reduced (80% for z-VAD, 67% for z-DEVD) in GrH treated cells, but a small proportion of TUNEL positive cells persisted. In K562 cells, z-VAD-fmk treatment did not prevent DNA damage, detected by TUNEL staining (Figure 3-10.D). These data suggested that GrH was able to initiate DNA damage in the absence of caspases.

Granzyme H and ICAD/DFF45

Many proteins that promote oligonucleosomal DNA breaks, such as DFF40/ CAD have been identified. Indeed, GrB and GrM have been reported to roteolytically inactivate its inhibitor DFF45/ ICAD to promote cell death

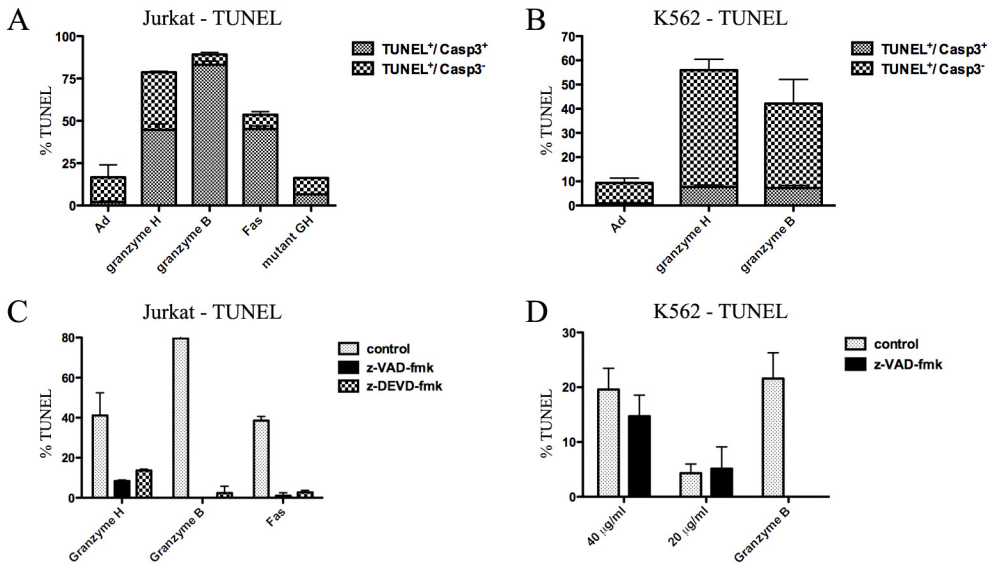

Figure 3-10 DNA Fragmentation induced by GrH is largely caspase-independent A) Jurkat cells were treated with Ad5 alone (control) or with 20 µg/ml GrH, 500 ng/ml GrB, or with 1µg/ml anti-Fas for 8 hours, and stained for active caspase-3 and TUNEL. B) K562 cells were treated with Ad5 and 40 µg/ml GrH or 1 µg/ml GrB for 8 hours, and stained for active caspase-3 and TUNEL. Cells single-positive for TUNEL (thick hatched bars) and doubly-positive for TUNEL and active caspase-3 (light hatched bars) were observed. C&D) TUNEL staining was assessed in C) Jurkat and D) K562 cells treated with DMSO (control; light bars), or 50 µM z-VAD-fmk (dark bars), or z-DEVD-fmk (thick hatched bars) following 4 hours of exposure to 40 µg/ml GrH, 1µg/ml GrB, or 1 µg/ml anti-Fas. Ad5 background levels were subtracted from treatments. All experiments are representative of ≥ 2 independent experiments (mean ± SD).

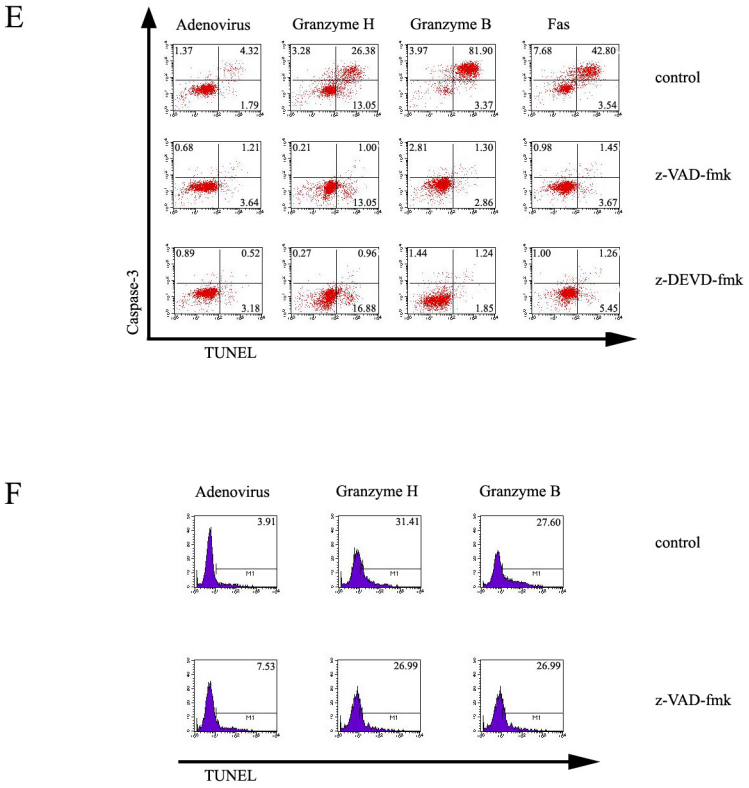

Figure 3-10 continued. E) Individual representative dot plots of TUNEL and caspase-3 staining in Jurkat cells, treated as indicated above. F) Individual histograms of TUNEL staining in K562 cells, treated as indicated above.

(Lu et al., 2006; Thomas et al., 2000). Caspase-3 also directly cleaves DFF45 (Enari et al., 1998; Sakahira et al., 1998; Wolf et al., 1999). However, GrH-mediated cleavage of DFF45 in previous reports has been controversial. To assess whether GrH could directly cleave DFF45, Jurkat-Neo and Jurkat-Bcl-2 (where GrH treatment could not produce active caspase-3 – see Figure 3-10.B), cells were treated with GrH for 4 hours, and DFF45 levels were assessed by immunoblotting (Figure 3-11.A). Granzyme H treatment clearly induced the proteolysis of DFF45/ICAD in Jurkat-Neo, likely through caspase-3 (a 12kD fragment). However, a modest cleavage product of 30 kD could be observed in Jurkat-Bcl2, possibly a result of direct GrH activity.

To better assess whether GrH could directly process DFF45, cell lysates were treated with GrH for 2 hours. Granzyme H treatment resulted in a fragment of 30 kD in both Jurkat and MCF-7 (which does not contain active caspase-3) lysates (Figure 3-11.B). Next, ^{35}S-Met labeled in vitro transcription/translation derived DFF45 was treated with a titration of GrH concentrations for 2 hours, and GrH directly processed DFF45/ICAD to cleavage products of 30 kD and 15 kD (Figure 3-11.C). In Figure 3-11.D, the proteolysis by GrH and GrB were compared and indicated that the cleavage site of GrH was distinct from that of GrB (and caspase-3).

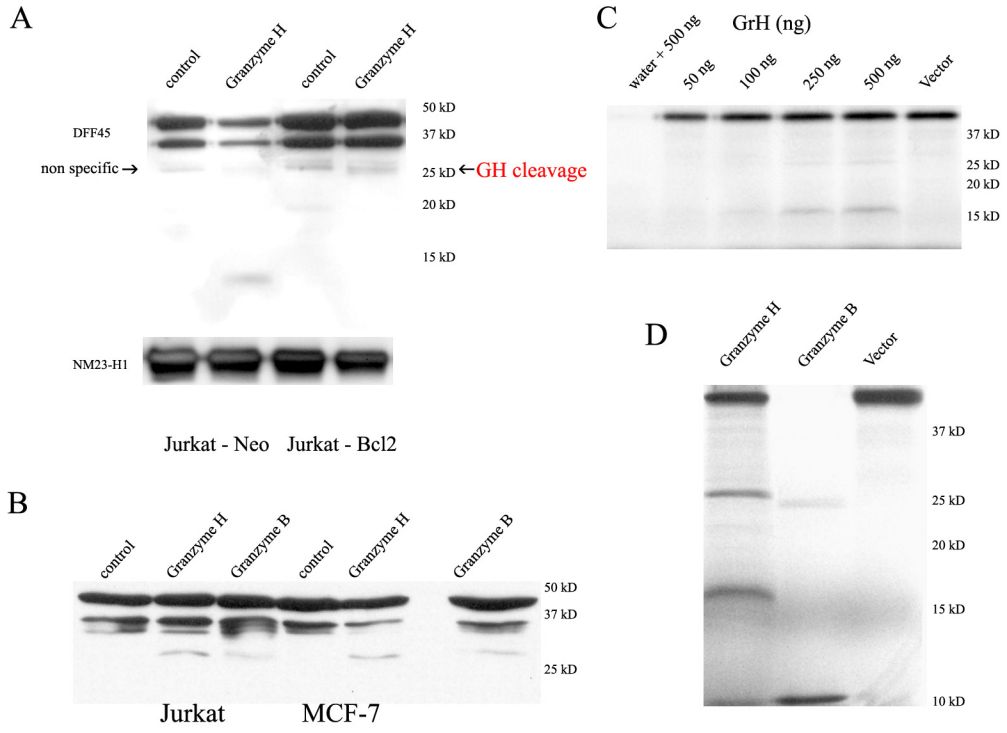

Figure 3-11 DFF45/ICAD is a substrate of GrH A) Jurk-Neo (lanes 1 &2) or Jurk-Bcl2 (lanes 3 & 4) were treated with Ad5 and 20 µg/ml GrH, or fractions isolated from parental RBL-2H3 cells (control) for 4 hours prior to cell lysis and immunoblotting for DFF45/ICAD (top panel). Loading control NM23-H1 (bottom panel). B) Cell lysates, the equivalent of $2x10^5$ Jurkat (lanes 1-3) or MCF-7 (lanes 4-6) cells, were treated with mutant GrH (control) or 25 µg/ml active GrH, or 1 µg/ml GrB, then DFF45/ICAD cleavage was assessed by immunoblot. C) IVTT-produced DFF45/ICAD was treated with increasing concentrations of GrH for 2 hours, and then visualized by autoradiography. D) IVTT-produced DFF45/ICAD was treated with 250 ng GrH or 125 ng GrB for 2 hours, and then visualized by autoradiography.

Discussion

Our studies have revealed that: GrH could directly proteolytically process DFF45/ICAD, but not Bid; GrH death was heavily modulated by Bcl-2, Bax and/or Bak; GrH induced the release of pro-apoptotic proteins from the mitochondria; and while caspase activation was observed, was not required for GrH-mediated cell death.

Previous studies have reported disparate results on the direct cleavage of Bid by GrH. Our studies indicated that GrH weakly processed Bid at high concentrations and with lengthy exposure to Bid. Likewise, Hou *et al.* only detected Bid processing using very high GrH concentrations added directly to either cell lysates, or using high concentrations of recombinant Bid. They also demonstrated *in vivo* Bid cleavage, but despite findings that caspases were activated during GrH-mediated cell death, caspase inhibitors were not utilized in these experiments. Furthermore, the authors did not assess the pro-apoptotic activity of GrH- truncated Bid generated during GrH-mediated cell death, and experiments were not performed in the absence of Bid or with mutants to assess whether GrH-mediated cell death was affected. Fellows *et al*. performed many of their experiments in K562 cells, where we detected limited caspase activity compared to Jurkat cells, and so this may explain why they did not observe Bid cleavage. Our findings indicated that GrH treatment only induced caspase-dependent Bid cleavage, generating a truncated Bid similar in size following Fas stimulation. Thus, Bid could enhance cell death at later time-points following caspase activation, but is not central to the initiation of GrH-mediated cell death.

As JR cells were highly resistant to GrH, our studies strongly infer that Bax and/or Bak are central mediators of GrH-induced cell death. However, it remains uncertain how these pro-apoptotic proteins are activated. It is possible that other BH3-only proteins, such as Bim, PUMA, Bad, NOXA, or Bik, could modulate their activity (Brenner and Mak, 2009; Lomonosova and Chinnadurai, 2008).

Our findings also demonstrated that the over-expression of Bcl-2 strongly inhibited GrH-mediated cell death. This is similar to many other mitochondrial-dependent death pathways, where Bcl-2 protects cells from cell death (Brenner and Mak, 2009; Chipuk and Green, 2008). These observations strongly imply that mitochondria are central to GrH-induced cell death.

We also found that GrH treatment promoted the release of cytochrome C and SMAC/DIABLO in various cell lines. This is in agreement with Hou *et al.*, where cell fractionation and immunofluorescent-based experiments demonstrated release of cytochrome C in Hela cells (Hou et al., 2008). In contrast, Fellows *et al.* did not observe cytochrome C release using cell fractionation on GrH-treated K562 cells (Fellows et al., 2007). Potentially, this may be a reflection of differential enzymatic activity, or the delivery method used. Interestingly, although cytochrome C release was observed, it is unlikely that it is critical for GrH-mediated cell death or caspase-3 activation. Dominant negative caspase-9 cells still displayed most signs of apoptosis, albeit, at slightly lower levels than control counterparts. However, other factors released from the mitochondria may contribute to GrH cytotoxicity, such as apoptosis inducing factor (AIF). Its

release would also be inhibited by Bcl-2 over-expression and double Bax/Bak deficiency, and remain insensitive to z-VAD-fmk treatment (Brenner and Mak, 2009). Fellows *et al.* also reported the generation of reactive oxygen species during GrH-mediated cell death, but we did not address this finding in our studies.

Both groups reported effects on the nuclear compartment following exposure to GrH. Our findings indicated that GrH-treatment leads to DNA damage, detected by TUNEL staining. DNA damage was reduced in GrH-treated Jurkat cells in the presence of caspase inhibitors, but was not completely impaired. We also observed many TUNEL-positive Jurkat cells in the absence of active caspase-3. Additionally, K562 cells displayed no change in TUNEL positive cells in the presence of z-VAD-fmk and the vast majority of these cells did not show signs of active caspase-3. Therefore, it was likely that a caspase-independent mechanism was contributing to GrH-mediated DNA damage.

In agreement with Hou *et al,* we clearly demonstrated that GrH directly cleaves DFF45 to a product of 30 kD and 15 kD (Hou et al., 2008). Fellows *et al.* did not conclude that GrH processed DFF45 cleavage, but did not attempt to assess direct GrH cleavage *in vitro* with recombinant DFF45.

Finally, our findings suggested that caspase activity was present in all cell lines treated with GrH, but at variable levels. However, the contribution of caspases, particularly caspase-3 was dependent on the cell line. In Jurkat cells, where active caspase-3 was generated, partial sensitivity to caspase inhibitors was observed. Conversely, K562 generated very little active caspase-3, and displayed a stronger caspase-independent mechanism of cell death. Therefore, we

concluded that GrH-mediated killing in the presence of caspases was enhanced, but not dependent on caspases to induce cell death.

In summary, our data established that DFF45 is a substrate of GrH. However, as Bcl-2 over-expression and loss of Bax/Bak significantly affected target cell death, GrH-mediated cytotoxicity is greatly amplified through a mitochondrial pathway (Figure 3-12). The initiation of this pathway is independent of Bid activation, as GrH cannot directly process Bid. Further studies will be required to elucidate the mechanism of GrH-induced mitochondrial damage.

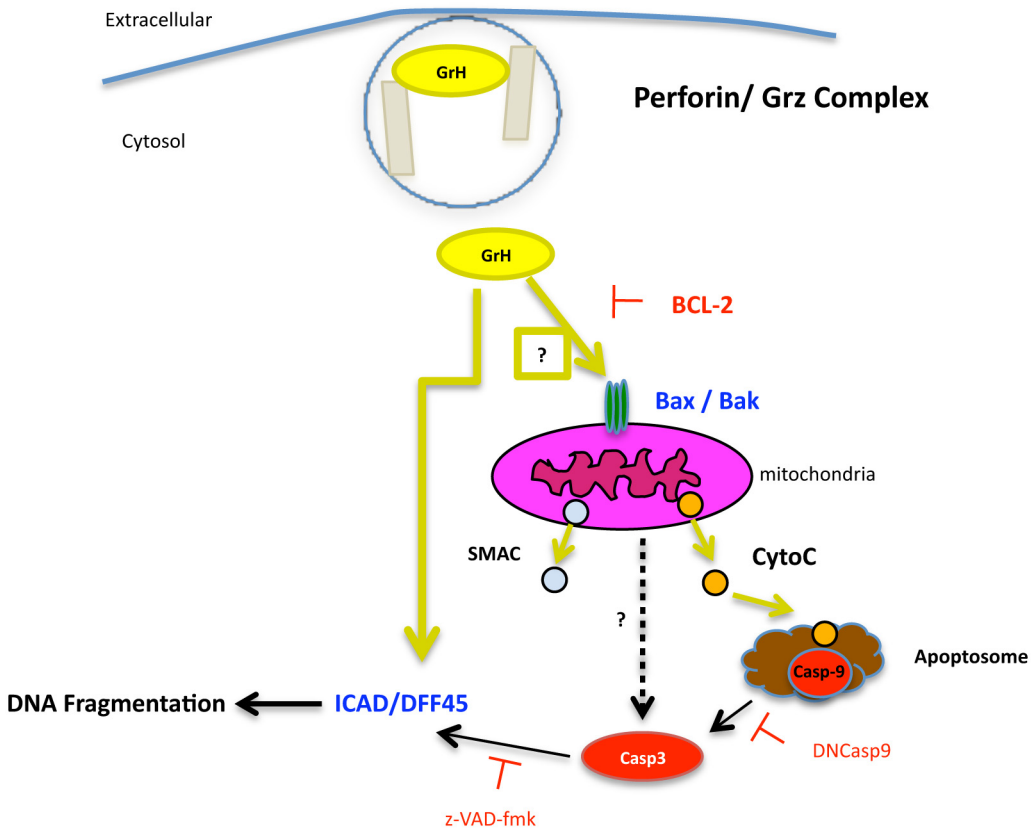

Figure 3-12 Mechanism of GrH-mediated Cell Death. Granzyme H induces Bax/Bak activation through an unknown, Bcl-2 sensitive mechanism. Mitochondrial intermembrane proteins are released into the cytosol and may induce modest levels of caspase-3 activation. Caspase-3, if activated, contributes to cell death. However, GrH can induce DNA damage, independently of caspases, through direct proteolysis of DFF45/ICAD.

Materials and Methods

Cell Lines and Chemicals

All chemicals were from Sigma (Sigma, St. Louis, MO), unless otherwise stated. Cell lines Jurkat (American Type Tissue Collection (ATCC), Manassas, VA), K562 (ATCC), RPMI-8866 (Sigma), and JR (a gift from Hanna Rabinowich) were maintained in RHF media (RPMI 1640 medium (Life Technologies, Burlington, ON, Canada), supplemented with 10% Fetal Calf Serum (v/v) (FCS ; Sigma), 25 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), and 100 U/ml penicillin/ streptomycin (both from Life Technologies)). Jurkat-Neo, Jurkat-Bcl-2 (Heibein et al., 2000), Jurkat-Dominant-Negative caspase-9 (Goping et al., 2003) were maintained in RHF supplemented with 0.8 mg/ml geneticin (Life Technologies). Hela and MCF-7 cells (ATCC) were maintained in DMEM (Life Technologies) supplemented with 10% FCS, 100 U/ml penicillin/streptomycin, and 2 mM L-glutamine (Life Technologies). RBL-2H3 cells (ATCC) were maintained in MEM (Life Technologies) supplemented with 10% FCS, 100 U/ml penicillin/ streptomycin, non-essential amino acids, and sodium pyruvate (Life Technologies). Inactivated human recombinant GrH and active recombinant cathepsin C were from R & D Systems (R&D Systems, Minneapolis, MN).

Production of human GrH and inactive GrH Mutant

Generation of mammalian expression vector

The full sequence of human GrH cDNA was derived from human

Lymphokine Activated Killer (LAK) cells, as previously described (Meier et al., 1990). Subcloning the GrH sequence into pcDNA3.1 was accomplished by a PCR reaction using an Advantage® –HF- PCR Kit (BD Biosciences, Palo Alto, CA) and 5'(CGTACTCGAGACCATGCAGCCATTCCTTCCTTCCTGTTG) primer and (GGACCAGATCTTCAGTGGTGGTGGTGGTGGTGGAGGCGC TTCATTGTTCTCTTTATC) 3' primer incorporating a C-terminal hexahistidine tail both (from Integrated DNA Technologies, Inc., Coralville, IA). The GrH was firstly cloned into a TA cloning vector pCR®II (Invitrogen, Carlsbad, CA), followed by a restriction digest and ligation into Hind III and Xba I sites of pcDNA3.1 (Invitrogen). Inactive mutant GrH was generated by a single mutation at Serine^{202} to Alanine by site-specific mutagenesis and a QuickChange® II Site-Directed Mutagenesis Kit (Stratagene; Agilent Technologies Company, La Jolla, CA).

Generation of stable expression clones for GrH

RBL-2H3 cells were transfected with 2 μg of pcDNA3.1-GrH or pcDNA3.1-inactive mutant GrH by Nucleofector™ I (Amaxa Biosystems, Germany) and selected with 0.8 mg/ml geneticin. Positive clones were identified using cell lysates using by either Western blotting with anti-GrB/GrH mAb (clone Gr7; AbD Serotec, Oxford, UK) or using a GrH-specific capture ELISA. Briefly, Maxisorp™ plates (Thermo Fisher Scientific, Roskilde, Denmark) were coated with 5 μg/ml capture GrH antibody (clone 185813; R & D Systems), blocked with 1% (w/v) BSA-PBS, treated with samples, then GrH was detected using 1 μg/ml biotin-conjugated anti-GrH (goat polyclonal Ig; R&D Systems) and streptavidin-

alkaline phosphatase (Jackson Immunoresearch, West Grove, PA). Reactions were monitored following development with p-Nitrophenyl Phosphate (pNPP; Sigma) on a Multiskan Ascent Spectrophotometer (Thermo Lab Systems, Helsinki, Finland).

Isolation of human GrH from stable transfectants

RBL-2H3 cells were grown to $2x10^9$ cells, washed in PBS, then freeze/thawed to generate crude homogenate, or were disrupted via nitrogen cavitation at 450 p.s.i. for 20 minutes (Model 4639, Parr Instrument Co., Moline, IL) in Heparin Column Binding Buffer (20 mM HEPES, 500 mM NaCl, pH 6.1) Cell debris and nuclei were removed by two centrifugation spins at 2500 rpm in a Beckman benchtop centrifuge for 10 minutes, followed by a 15-minute centrifugation at 13 000 rpm in a Sorvall SS34 rotor. Supernatants or granules were collected and passed through a 16-gauge needle and 50 ml syringe at least 6 times, then were filtered through a 0.22 μM filter (Millipore Corporation, Billerica, MA). Samples were applied to a 5 ml HiTrap™ Heparin column (GE Healthcare Biosciences, Uppsala, Sweden) and ÄKTA FPLC System (Amersham Pharmacia Biotechnologies, Uppsala, Sweden), and eluted via a linear salt gradient (500 to 1400 mM NaCl). Fractions were screened for GrH, then filtered through a 15 ml 10 000 MWCO Amicon filter (Millipore). Retentate was diluted 10-15 fold with HisTrap Binding Buffer (20 mM Sodium Phosphate, 250 mM NaCl, and 10 mM Imidazole, pH 7.4) and applied to a 5 ml HisTrap™ Column (GE Healthcare). Fractions were eluted with a linear imidazole gradient using 20

mM sodium phosphate, 500 mM NaCl and 500 mM Imidazole (gradient 10 – 500 mM imidazole). Fractions were screened for GrH, pooled, washed of salts and concentrated using 10 000 MWCO Amicon® Ultra filters, and finally diluted in PBS. The concentration of GrH was calculated by protein concentration using a BCA™ Protein assay (Pierce Scientific, Rockford, IL) and a GrH ELISA, using recombinant GrH (R&D Systems) as a standard. Protein purity was assessed by silver staining on a 12% SDS-polyacrylamide gel (BioRad Silver Stain Kit, BioRad Laboratories, Hercules, CA). Activity was confirmed by a colorimetric assay, using 200 μM Suc-FLF-SBzl (Succinyl-Phe-Leu-Phe-thiobenzylester) (MP Biomedicals, LLC, Solon, OH) and 200 μM DTNB (5,5'-dithiobis(2-nitrobenzoic acid)) as the substrate in a buffer containing 100 mM HEPES, and 500 mM NaCl, pH 7.5 (Edwards et al., 1999).

Production of GrB

Granzyme B was produced from YT Indy cells, as previously described, with some modifications (Caputo et al., 1999). Crude homogenates (in 20 mM HEPES, 500 mM NaCl, pH 6.1) were eluted from a 5 ml HiTrap Heparin Column using a linear gradient of 500 mM to 1.4 M NaCl and fractions were screened for GrH chymase activity using substrate Suc-FLF-Bszl. Granzyme B aspase activity was assessed using 200 μM Suc-IEPD-pNA (Kamiya Biomedical Co., Seattle, WA) in buffer containing 100 mM HEPES, 20% (w/v) sucrose, 0.2% (w/v) CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate), and 5 mM DTT (dithiothreitol) . Granzyme B fractions, free of GrH, were then further

fractionated on a 5 ml HiTrap™ SP cation exhange column (GE Healthcare). Granzyme B concentrations were determined using a quantitative activity assay, as previously described (Ewen et al., 2003).

Flow cytometry Apoptosis assays

Target cells ($5x10^4$ to $1x10^5$) in 25 μl RH-BSA were treated with granzymes in the presence of Adenovirus type 5 d170-3 (Ad5); purified in our lab according to (Bett et al., 1994), or anti-Fas mAb (clone CH11; Upstate Technologies, NY) for the indicated times. When indicated, caspase inhibitors z-VAD-fmk or z-DEVD-fmk (Kamiya Biomedical Company) were added one hour prior to the addition of granzymes/ anti-Fas, and remained through-out the entire incubation. Poly-caspase activation was assessed by staining with 10 μM CaspACE reagent (FITC-VAD-fmk) (Promega Corporation, Madison, WI) or 100 nM TMRE (tetramethylrhodamine, ethyl ester, perchlorate; Invitrogen) for 30 minutes at 37^{o}C, washed, and then cells werer stained with 0.8 μM 7-AAD (Invitrogen) and 1/400 Alexa-647 conjugated Annexin V (Invitrogen). For the detection of cytosolic-localized cytochrome C or SMAC/DIABLO, cells were treated with 5 μg/ml digitonin (Sigma) in 100 mM KCl/ PBS for 5 minutes, then fixed with 4% PBS-buffered paraformaldehyde for at least 30 minutes (Waterhouse and Trapani, 2003). Cells were treated with 0.05% (w/v) saponin /PBS/3% (w/v) BSA, then stained with 1/50 FITC-conjugated mouse anti-cytochrome C (clone 6H2.B4; eBioscience) or FITC-conjugated anti-SMAC/DIABLO (CytoLight, Assay Designs, Ann Arbor, MI). Active caspase-3,

and TUNEL-based DNA fragmentation were detected following fixation with 4% paraformaldehyde; cells were then and permeabilized with 0.1% saponin/ PBS/ 2% FCS, and stained with FITC-conjugated TUNEL label (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling) (Gavrieli et al., 1992)(Roche Diagnostics Canada, Laval, QC), or 1/20 biotin-conjugated anti-active caspase 3 (BD Biosciences, San Jose, CA). The biotin probe was detected with 1/20 streptavidin- APC (BD Biosciences). All samples were analyzed on a BD FACS Calibur flow cytometer, and CellQuest software.

***In vivo* cleavage**

A total of 5 x10^5 Jurkat-Neo, or Jurkat-Bcl2 cells were pre-treated with DMSO or 50 μM z-VAD-fmk for 45 minutes, then incubated either with granzyme and Ad5, or anti-Fas in RH-0.2% BSA for 4 hours. Cells were washed with PBS, then lysed in 20 μl buffer (50 mM HEPES pH 7.4, 1 mM EDTA, 1% NP-40, 165 mM NaCl, 100 μM z-VAD-fmk; Kamiya Biomedical Company and protease inhibitor cocktail). Lysates were loaded on to 15% gels, resolved by SDS-PAGE, wet-transferred to nitrocellulose membranes (GE Healthcare), blocked with 5% (w/v) skim milk in 0.1% (v/v) Tween-20 in PBS, and probed with 1/500 rabbit anti-human Bid (Cell Signaling Technology Inc., Danvers, MA), 1/1000 mouse anti-human DFF45 (BD Biosciences) or 1/10 000 rabbit anti-caspase-3 (a gift from Dr. D. Nicholson). Detection HRP-conjugated antibodies were goat anti-mouse IgG or goat anti-rabbit IgG, both diluted 1/3000 (BioRad Laboratories). Membranes were developed via the Enhanced Chemiluminescent

System (GE Healthcare) and Biomax films (Kodak; Carestream Health Inc, Rochester, NY). The loading control employed was 1/1000 rabbit anti-human anti-NM23-H1 (Santa Cruz Biotechnology, Santa Cruz, CA).

In vitro cell lysate cleavage of Bid and DFF45

Jurkat or MCF-7 (deficient in active caspase-3) cells were lysed at 1.6 x 10^7/ ml with lysis buffer (50 mM Tris-HCl pH 7.5, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.5% NP-40 (v/v); (Hou et al., 2008)). The equivalent of $2x10^5$ cells were incubated with granzymes in the presence of 100 μM of pan-caspase inhibitor z-VAD-fmk for 4 hours at 37°C. Upon the addition of SDS-loading buffer, heat-denatured samples were loaded to 15% gels, wet-transferred to nitrocellulose membranes, and probed with anti-DFF45 or anti-Bid antibodies. Loading control was 1/200 anti-actin (Santa Cruz).

Cell Fractionation for Cytochrome C and SMAC/DIABLO Translocation

Jurkat, K562, or RPMI-8866 cells, at $5x10^5$ cells/ sample, were incubated for 4 hours with granzymes and Ad5. Cells were washed with PBS, then incubated with 50 μl of CE1 buffer from a cell fractionation kit (QProteome Cell Compartment Fractionation Kit, Qiagen Inc., Mississisauga, ON, Canada). Following a 10 minute incubation, samples were spun at 1000 x g for 10 minutes, and the cytosolic supernatants were collected. Membrane pellets were collected following 30 minutes in 50 μl of CE2 buffer, and 10 minutes centrifugation at 6000 x g. Protein concentrations were measured using a BCA™ protein assay,

then samples were treated with 6x SDS loading buffer and heated to 100^{o}C for 5 minutes. Samples were loaded to 15% gels, resolved, and transferred to nitrocellulose membranes. Cytochrome C was detected using 1/2000 mouse-anti-cytochrome C (BD Biosciences), and SMAC/DIABLO using 1/1000 mouse-anti-SMAC (BD Biosciences). Loading controls were 1/ 2000 anti-Cox IV (Invitrogen), 1/10 000 anti-alpha tubulin (Sigma), or 1/200 anti-Hsp70 (Santa Cruz Biotechnology) antibodies.

Cleavage of IVTT DFF45 and Bid

For *in vitro transcription/ translation*, 1 μg of vectors pcDNA3.1C-DFF45 (a gift from Dr. Pierre Rafick-Sekaly) and pEST15-Bid (a gift from Dr. X. Wang) were added to 20 μl of IVTT master mix (TnT® T7 Quick Coupled Transcription/ Translation Systems Kit; Promega) and 1 μCi of ^{35}S-Methionine (Easy Tag; Perkin Elmer Life and Analytical Sciences, Boston, MA) and incubated at 30^{o}C for 90 minutes. For cleavage experiments, 2 μl of IVTT material was incubated with granzymes for at least 2 hours at 37^{o}C. Reaction mixtures were denatured for 5 minutes at 100^{o}C with 6x SDS loading buffer and loaded onto 15% gels. Gels were dried and then exposed to BioMax MR film (Kodak).

siRNA (short interfering RNA) knockdown of Bid

Hela cells were seeded at 5x10^{4} cells in a 12 well plate (Nunclon™ Roskilde, Denmark) overnight before being treated with 10 nM siRNA oligos,

either with AllStars negative siRNA (#1027280), or Bid siRNA (Hs_BID_7 HP Validated siRNA #SI02661911) and HiPerfect transfection reagent (all from Qiagen). At 48 hours, Hela cells were treated with 500 - 1000 p.f.u. /cell Ad5 and granzymes, and monitored for cell death by staining with TMRE.

PI9 Intracellular Staining

Intracellular PI9 staining was performed on $2x10^5$ cells, fixed with 4% paraformaldehyde, permeabilized with 0.1% saponin/2% FCS/ PBS, and treated with either 1 μg/ml mouse IgG1 isotype control or anti-PI9 antibody (clone 7D8; Medical and Biological Laboratories Co., Naka-ku Nagoya, Japan) for 30 minutes. Cells were washed, and stained with 1/500 Cy5.5 conjugated goat-anti mouse IgG (Jackson Immunoresearch) for 20 minutes. Washed cells were assessed by flow cytometry.

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CHAPTER 4 - INHIBITION OF GRANZYME H BY ANTICHYMOTRYPSIN

Introduction

Serpins are an enormous family of protease inhibitors, implicated in the control of numerous biological functions including inflammation, extracellular matrix degradation, coagulation, and apoptosis (Kaiserman and Bird, 2009; van Gent et al., 2003). Their secondary structure is highly conserved and is composed of 9 α-helices, 3 β-sheets, and a variable region called the Reactive Centre Loop (RCL). The RCL contains a sequence of amino acids that mimics a protease substrate site, dictating the specificity of interactions between proteases and serpins. However, rather than being fully hydrolyzed, proteolytic attack of the serpin RCL sequence results in a proteolytic intermediate and the inhibition of the protease (Kaiserman and Bird, 2009; Schechter and Plotnick, 2004). Mechanistically, initial hydrolysis of the peptide bond by the protease induces the formation of a covalent acyl-enzyme intermediate between the serpin and protease. This triggers a dramatic conformational change in the serpin resulting in the distortion and inactivation of the protease, and impairs the full hydrolysis of the peptide bond. The serpin itself also becomes inactive, remaining bound to the protease.

Granzymes are a family of serine proteases found mostly within lymphoid-derived cytotoxic cells and share similarity to myeloid cell serine proteases, such as mast cell proteases, and cathepsin G (Jenne and Tschopp, 1988; Salvesen et al., 1987). In humans, Granzymes A, B, H, K, and M have been identified (Chowdhury and Lieberman, 2008). Following recognition of infected or transformed target cells by cytotoxic cells, granzymes and perforin are

directionally released by cytotoxic cells and transferred to target cells (Chowdhury and Lieberman, 2008). Upon entry into the target cells, these granzymes, along with the perforin, initiate the process of target cell apoptosis (Chowdhury and Lieberman, 2008; Cullen and Martin, 2008). .

Serpins of human granzymes A, B, K, and M have been identified (Kaiserman and Bird, 2009). Granzyme B, an aspase, is a potent pro-apoptotic enzyme that targets intracellular substrates caspase-3 and Bid to induce cell death (Chowdhury and Lieberman, 2008; Cullen and Martin, 2008). Cytotoxic cells express a potent and specific intracellular GrB serpin, Protease Inhibitor-9 (PI9) in humans or Serpin Protease Inhibitor-6 (SPI-6) in mice, in order to protect themselves from their own GrB reserves (Bird et al., 1998; Sun et al., 1996; Sun et al., 1997). However, expression of PI9 or SPI-6 in other cell types has been observed, and can protect cells from GrB-mediated cytotoxicity (Kaiserman and Bird, 2009). Poxviruses have also evolved to evade GrB-induced apoptosis by expressing their own serpin, Cytokine response modifier A (CrmA) (Quan et al., 1995). However, CrmA interaction with caspases is stronger than with GrB, and the primary function of CrmA is likely the inhibition of caspases (Komiyama et al., 1994; Quan et al., 1995; Tewari et al., 1995).

Similar to myeloid-derived cell serine proteases, granzymes also have extracellular functions, such as extracellular matrix remodelling and inflammatory cytokine production (Boivin et al., 2009; Korkmaz et al., 2008; Metkar et al., 2008; Pejler et al., 2007). Thus, there is speculation that secreted serpins may exist to counteract extracellular granzyme functions. Recently a novel, secreted

GrB-specific serpin, SerpinA 3n, was identified in mice (Sipione et al., 2006). Cells over-expressing SerpinA 3n were resistant to killing by human GrB, suggesting the potential that the serpin also could modulate GrB proteolysis of substrates such as cartilage proteoglycans, aggrecan, vitronection, fibronectin, fibrinogen, and laminin (Boivin et al., 2009). Interestingly, the human orthologue of serpinA 3n, alpha-anti-chymotrypsin (ACT), did not similarly inhibit human GrB activity. Currently, there is no known extracellular GrB-specific serpin in humans.

Granzyme A is a tryptase which can be inhibited by the blood-derived serpin antithrombin III (serpin C1) (Masson and Tschopp, 1988). Extracellular substrates of GrA include anti-thrombin receptor, collagen type IV, fibronectin, and proteoglycans (Boivin et al., 2009) However, GrA is not inactivated by anti-thrombin while complexed to proteoglycans (as GrA is upon release from secretory lysosomes), so the physiological relevance of this interaction is still unclear (Spaeny-Dekking et al., 2000).

Granzyme K is also a tryptase, and has shown cytotoxic potential *in vitro* (Guo et al., 2008; Hua et al., 2009; Zhao et al., 2007a; Zhao et al., 2007b). However, its expression in human lymphocytes occurs predominantly in memory $CD8^+$ T cells that express low levels of perforin and are not directly cytotoxic (Bade et al., 2005; Bratke et al., 2005). Elevated levels of GrK have been reported in plasma and bronchoalveolar lavage fluid in patients undergoing septic shock, acute allergic asthma, and bronchopneumonia (Bratke et al., 2008; Rucevic et al., 2007). Coincidently, in these same patients a plasma-derived inhibitor that

has been observed to inhibit GrK activity *in vitro*, was detected at highly reduced levels (Rucevic et al., 2007; Wilharm et al., 1999).

Granzyme M, a metase, has demonstrated *in vitro* cytotoxic effects upon entry into target cells (Kelly et al., 2004; Lu et al., 2006). A murine serpin, serpinb9b, has been proposed to be an intracellular GrM inhibitor, but this interaction has not been well-characterized (Bots et al., 2005). No extracellular functions of GrM have been identified, but the *in vitro* enzymatic activity can be weakly inhibited by a plasma-derived serpin, antichymotrypsin (ACT) (Mahrus et al., 2004).

Granzyme H is a chymase, and also has been reported to be cytotoxic *in vitro* (Edwards et al., 1999; Fellows et al., 2007; Hou et al., 2008). Presently, no inhibitors of GrH have been reported. However, GrH shares homology with a myeloid-derived chymase, cathepsin G (Klein et al., 1990; Salvesen et al., 1987). This protease is released by neutrophils to induce inflammation and matrix remodelling, and its activity is potently inhibited by a plasma-derived acute phase protein, ACT (Kalsheker, 1996; Korkmaz et al., 2008; Travis et al., 1978).

Thus, although an extracelluar function of GrH has not yet been reported, given its similarity cathepsin G, we sought to examine whether GrH activity could be impaired by a serpin known to interact with cathepsin G. Here, we report the ability of ACT to interact with GrH and abrogate its enzymatic activity and its ability to induce cell death.

Results

Effect of ACT on GrH Activity

The *in vitro* enzymatic specificity of GrH has been demonstrated by Edwards *et. al.* using synthetic substrates, and showed a preference for cleavage following phenylalanine, methionine, tyrosine, and leucine residues (Edwards et al., 1999). Antichymotrypsin contains a leucine residue at the P1 residue, the site of proteolytic attack, which enables it to abrogate cathepsin G activity (Duranton et al., 1998; Lomas et al., 1995). To determine the ability of ACT to inhibit GrH activity, various concentrations of ACT were incubated with GrH for 1 hour at 37^{o}C and GrH enzymatic activity was assessed using a peptide thiobenzylester substrate (Edwards et al., 1999). These experiments demonstrated that increasing concentrations of ACT resulted in decreases in GrH enzymatic activity (Figure 4-1.A). Further analysis indicated that ACT inhibited GrH at a stoichiometry of inhibition of 1.6, a ratio consistent with other serine protease and serpin interactions (Figure 4-1.B) (Schechter and Plotnick, 2004).

Binding of GrH and ACT

The interaction between a serine protease and an inhibitory serpin is characterized by the formation of an SDS-stable complex (Schechter and Plotnick, 2004). To examine the ability of ACT to form covalent complexes with GrH, we incubated purified GrH with purified plasma ACT for an hour and resolved proteins by SDS-polyacrylamide gel electrophoresis. Coincubation of purified recombinant GrH and plasma-purified ACT resulted in the formation of a

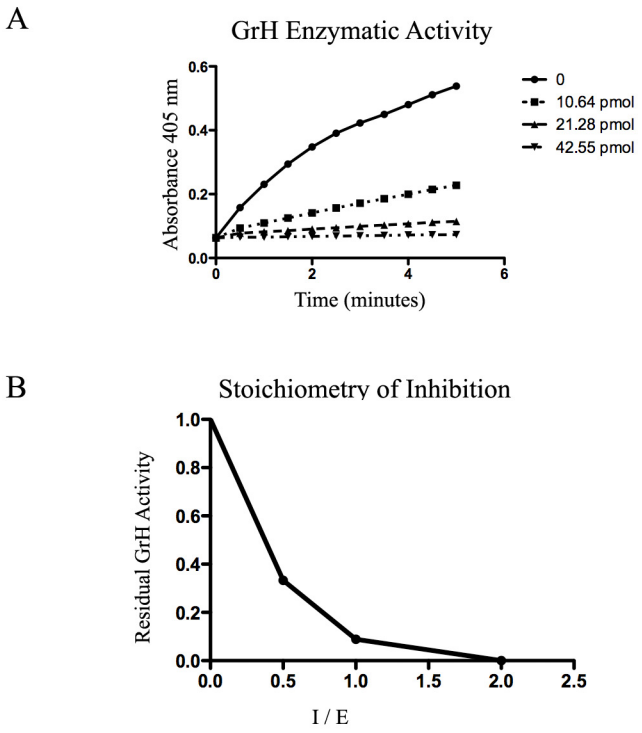

Figure 4-1 ACT inhibits GrH Enzymatic Activity. A) GrH (20 pmol) was pre-incubated with numerous concentrations of ACT (pmol) for 1 hour prior to addition to substrate Suc-FLF-thiobenzyl ester. Reaction was monitored over time (minutes).

B) Stoichiometry of Inhibition – GrH (20 pmol) hydrolysis of substrate monitored at five minutes reaction time following pre-treatment with numerous concentrations of ACT. Data is representative of at least 3 independent experiments.

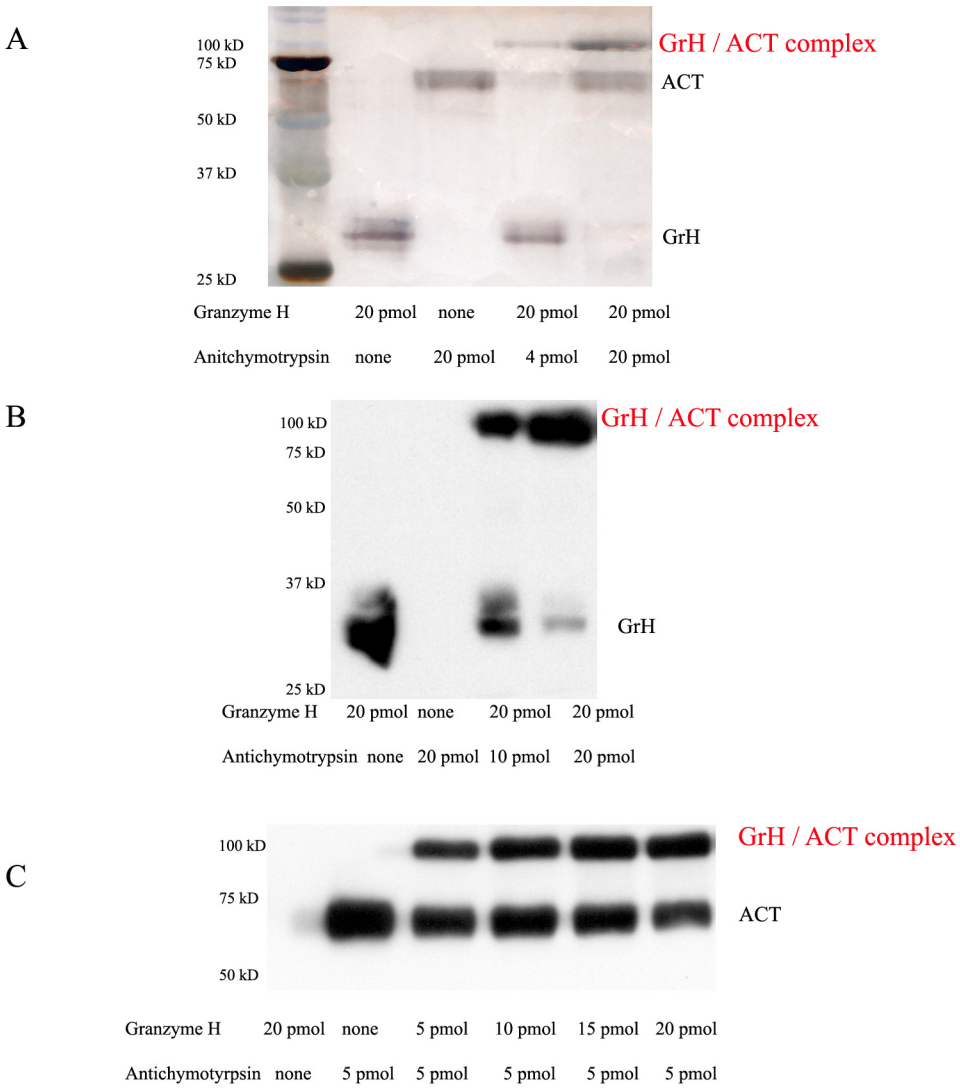

Figure 4-2 Granzyme H forms covalent complex with ACT. A) Silver Stain of Granzyme H pre-treated with ACT for 1 hour prior to separation on a 10% SDS-PAGE gel. B) Immunoblot of GrH. ACT was incubated with increasing concentrations of GrH for 1 hour. C) Immunoblot of ACT. GrH was incubated with increasing concentrations of ACT for 1 hour. Data is representative of 2 independent experiments.

95 kD GrH-ACT complex, visualized by silver-staining a 12 % SDS-polyacrylamide gel (Figure 4-2.A). A constant concentration of GrH incubated with increasing concentrations of ACT resulted in the generation of higher levels of GrH-ACT complexes. To confirm the identity of proteins within the complexes, immunoblots for GrH (Figure 4-2.B) or ACT (Figure 4-2.C) were also performed. Both immunoblots established that GrH-ACT complexes were produced in a dose-dependent manner, further establishing the specificity of the protease-serpin interaction.

ACT Inhibition of GrH-Mediated cleavage of DFF45

Granzyme H has been demonstrated to cleave DFF45/ICAD *in vitro* (Hou et al., 2008)(and see chapter 3). To ascertain whether ACT could prevent the proteolysis of a natural GrH substrate, ACT-treated GrH was added to cell lysates, and the ability of GrH to cleave DFF45/ICAD was assessed by immunoblot. As shown in Figure 4-3, pre-treatment of GrH with ACT prevented the proteolysis of DFF45/ICAD, demonstrated by the reduction of a 30 kD DFF45 cleavage product.

ACT Inhibits GrH-Mediated Cell Death

Granzyme H has been shown to induce cell death in tumour lines, demonstrated by the loss of mitochondria inner membrane potential (ΔY_m), and phosphatidylserine externalization (Fellows et al., 2007; Hou et al., 2008). To determine whether ACT could prevent GrH-mediated cytotoxicity, ACT-treated

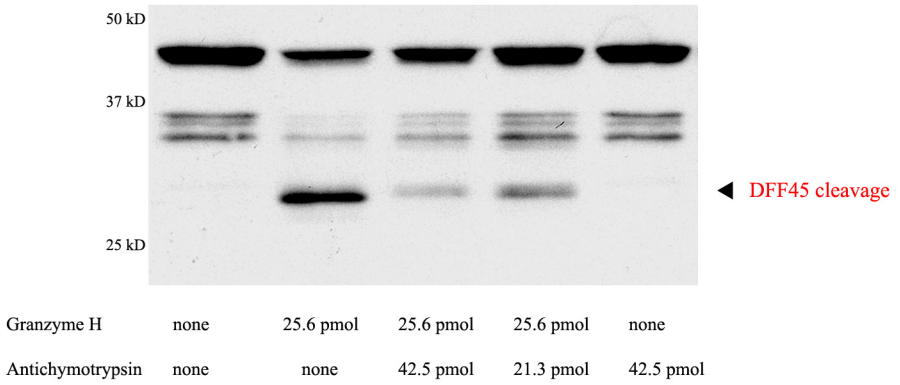

Figure 4-3 Antichymotrypsin impairs GrH cleavage of DFF45/ICAD.

Immunoblot of DFF45/ICAD. GrH (25.6 pmol) was incubated with ACT for 1 hour prior to its addition to lysate from an equivalent of $2x10^5$ Jurkat cells. Lane 1 – control; Lane 2 – GrH only; Lane 3- GrH + 42.5 pmol ACT; Lane 4- GrH + 21.3 pmol ACT; Lane 5- ACT only. Data is representative of 2 independent experiments.

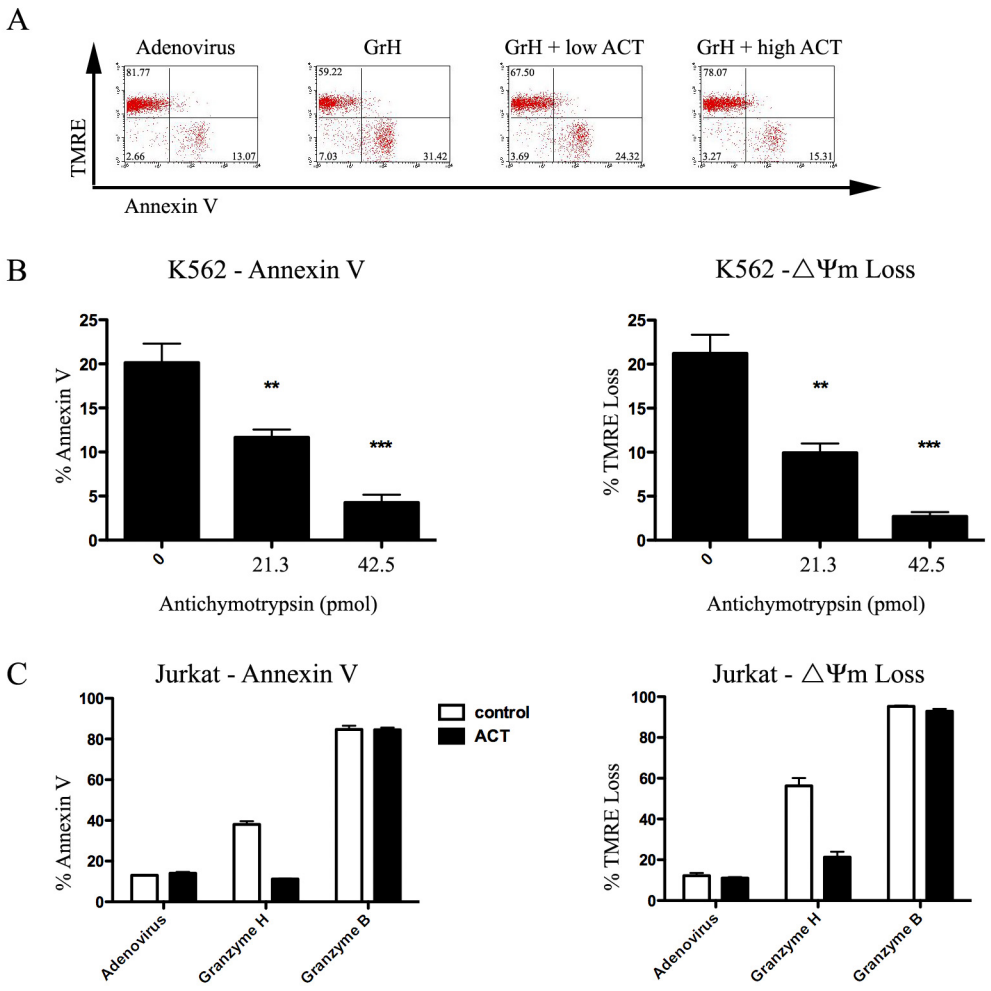

Figure 4-4 Antichymotrypsin impairs GrH-mediated cell death. A) Individual representative dot plots of K562 cells treated with 200 p.f.u./cell Ad5 (control) plus ACT pre-treated GrH (25.6 pmol) for 4 hours. Cells were stained with TMRE and Annexin V. B) Graphical representations of K562 cells treated with ACT pre-treated GrH (25.6 pmol) and stained with Annexin V (left) and TMRE (right). C) Graphical representation of Jurkat cells treated with ACT pretreated (42.5 pmol) GrH (25.6 pmol) or GrB (1 pmol) and stained with Annexin V (left) and TMRE (right). Data is representative of 2 independent experiments (N=3).

GrH was added with adenovirus to target cells. Cell death was monitored with the dye TMRE, as a measure of ΔY_m, and with Annexin V, as a measure of phosphatidylserine exposure. Both K562 and Jurkat cells were protected from GrH-mediated cell death in the presence of ACT (Fig. 4-4). The addition of increasing amounts of ACT provided significant protection against GrH cytotoxicity in K562 cells (**$p < 0.01$) (Figure 4-4.B). In contrast, ACT did not prevent GrB-mediated cell death in target Jurkat cells (Figure 4-4.C).

Discussion

Our studies have revealed a novel role for ACT. The interaction of ACT resulted in the inhibition of GrH proteolytic activity and impaired its ability to induce cell death. The stoichiometry of the interaction between GrH and ACT is within a physiologically relevant range, as described for other serpin-protease interactions (Schechter and Plotnick, 2004; Travis and Salvesen, 1983). Further studies will be required to calculate k_{ass} rates, and determine whether ACT can inhibit proteoglycan complexed GrH. Other factors that could effect k_{ass} rates, such as heparin, will also need to be addressed.

Previous reports have indicated that NK activity could be impaired by chymase synthetic inhibitors or plasma-derived serpins, including ACT (Hudig et al., 1984; Woodard et al., 1994). However, these studies did not evaluate the effect of these inhibitors on granzymes such as GrH, and there was no apparent mechanism proposed to explain their observation. Granzyme H is highly and constitutively expressed by human NK cells, possibly at levels higher that of GrB

(Fellows et al., 2007; Sedelies et al., 2004). Potentially, ACT produced by target cells could impair the contribution of GrH to NK-mediated cytotoxicity. Of interest, several studies have observed elevated ACT expression in numerous tumor lines during inflammatory conditions or in cells undergoing metastasis (Cichy et al., 1995a, b; Higashiyama et al., 1995; Laursen and Lykkesfeldt, 1992; Mbeunkui et al., 2007; Montel et al., 2005; Tahara et al., 1984; Yu et al., 2005). Moreover, ACT has been detected in the nuclei of tumor cells and GrH entry into target cells results in its localization to the nucleus (Hou et al., 2008; Takada et al., 1988a; Takada et al., 1988b; Tsuda et al., 1986). The interaction between intracellular ACT and GrH could be a novel strategy utilized by transformed cells to evade NK cytotoxicity.

Of additional interest, ACT and orthopoxvirus serpins Spi-1 are approximately 50 % similar by BLAST alignment analysis. It has been observed that cathepsin G forms covalent serpin-protease complexes with the Rabbitpox virus Spi-1, (Moon et al., 1999). The interaction between Cathepsin G and Spi-1 was dependent upon a phenylalanine residue at P1 within the RCL, and thus, this is a potentially attractive site for GrH as well. The function of Spi-1 is still unclear and its inhibition of a cytotoxic mediator would be novel viral immune evasion strategy employed by orthopoxviruses such as variola and smallpox.

Finally, ACT is found abundantly in plasma during acute phase responses (Travis and Salvesen, 1983). Traditionally, ACT has proposed been to control inflammatory and tissue remodelling processes. Therefore its ability to impair GrH activity could also be a reflection of unknown extracellular GrH functions.

Currently, several extracellular substrates for GrA and B have been identified, and a number of reports have observed a pro-inflammatory role for GrA (Boivin et al., 2009; Metkar et al., 2008; Sower et al., 1996a; Sower et al., 1996b). Additional studies will need to be performed to address whether GrH has extracellular functions, and whether ACT could counteract these activities.

Methods and Materials

Chemicals and Cell Lines

All chemicals were from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. Cell lines K562 and Jurkat were maintained in RPMI 1640 (Life Technologies, Burlington, ON, Canada), supplemented with 10 % (v/v) FCS (Sigma), and 100 u/ml penicillin/streptomycin (Life Technologies), and were obtained from American Tissue Cell Culture (ATCC; Manassas, VA). Antichymotrypsin was purchased from Sigma.

Production of Recombinant Purified Granzyme H

Granzyme H was purified from stable RBL-2H3 transfectants (as described in chapter 3). Purity was assessed by silver stain analysis and concentrations were calculated by BCA protein assays and by GrH ELISA (as described in chapter 3).

Formation of SDS-stable GrH and ACT complexes

Two concentrations of GrH were incubated with 500 ng of plasma-purified ACT in PBS for one hour at 37^0C. Reactions were stopped by boiling at 100^oC for 5 minutes in sample buffer, then loaded onto 8 – 12 % SDS-polyacrylamide gels. Silver staining was performed, as described in Chapter 3. Immunoblots for GrH and ACT were performed following wet-transfer to nitrocellulose membranes, blocked with 5% milk/ PBST, and incubation with 1 µg/ml anti-human GrH (clone 185813; R & D Systems, Minneapolis, MN) or 1 µg/ml anti-human serpin A3 (clone 213907; R & D Systems). Detection of proteins followed addition of 1/3000 HRP-conjugated goat anti-mouse IgG (BioRad Laboratories) and blot development with Enhanced Chemiluminescent Solution (GE Healthcare Biosciences, Uppsala, Sweden).

Calculation of Stoichiometry of Inhibition

Recombinant GrH (20 pmol) was titrated with ACT for one hour at 37^0C in PBS. The GrH-ACT mixture was added to GrH Enzyme Reaction Buffer (100 mM HEPES, 500 mM NaCl, pH 7.5) and hydrolysis of 200 mM Succinyl-Phe-Leu-Phe-thiobenzylester substrate (MP Biomedicals, LLC, Solon, OH) was measured in the presence of 200 mM DTNB with a Multiskan Ascent Spectrophotometer (Thermo Lab Systems, Helsinki, Finland) at an absorbance of 405nm.

Cleavage of DFF45/ICAD

Jurkat cells were lysed at $1.6x10^7$ cells/ml with lysis buffer (50 mM Tris-HCl pH 7.5, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.5% NP-40 (v/v)) in the presence of 100 mM pan-caspase inhibitor z-VAD-fmk. Recombinant GrH was pretreated with ACT for one hour at 37^0C in PBS, then added to the equivalent of $2x10^5$ cells for two hours at 37^0C. Heat-denatured samples were loaded on a 12% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose membranes and probed with 1/1000 mouse-anti human DFF45 (BD Biosciences, San Jose, CA).

Measurement of Cell Death

Recombinant GrH was pre-incubated with ACT for one hour, then added to Jurkat or K562 cells in 25 ml RPMI 1640 supplemented with 0.1% bovine serum albumin (w/v) and in the presence of 200 p.f.u./ cell Adenovirus 5. Cell death was monitored at four hours by assessing mitochondrial membrane potential (ΔY_m) using TMRE stain (Invitrogen, Carlsbad, CA) and phosphatidylserine exposure using Annexin V staining (BD Biosciences).

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CHAPTER 5 - GENERAL DISCUSSION AND FUTURE DIRECTIONS

The granule-mediated immune response has been implicated in the protection of organisms against numerous pathogens and transformed cells. It also promotes graft rejection and participates in autoimmune disease. Therefore, defining the functions of individual components stored within cytotoxic granules has implications in vaccine strategies and developing effective immunotherapies.

The most well-characterized enzyme released by cytotoxic cells is GrB. The mechanism by which it promotes target cell apoptosis is relatively well-defined, as reviewed in Chapter 1. What remained less clear was whether its expression was correlative to target cell death or with immunological protection against disease. Traditionally, effective cell-mediated cytotoxicity was assessed *in vitro* by cumbersome ^{51}Cr-release assays that could not clearly distinguish between Fas- or granule-mediated cytotoxicity. The mouse GrB ELISA/ELISPOT is a direct assessment of granule-mediated cytotoxicity and strongly correlates to target cell lysis. Thus, GrB-based assays can now be applied to vaccine models to establish whether GrB is a correlate of immunity, compared to various other traditional analyses. Indeed, human studies with our collaborator have indicated that GrB expression and release by CTLs are better correlates of protection against seasonal influenza virus than serum titres (McElhaney et al., 2009; McElhaney et al., 2006).

On the other hand, the absence of GrB release from cytotoxic cells may be reflective of the acquisition of inhibitory signaling molecules that interfere with degranulation, or defects in the machinery involved in the degranulation process. Recently, it has been proposed that chronic viral exposure leads to CTL non-

responsiveness through co-inhibitory interactions (reviews by (Ha et al., 2008; Kaufmann and Walker, 2009)). Granzyme B ELISPOT/ ELISA assays could be utilized to evaluate therapies targeted at restoring CTL granule-mediated cytotoxicity. Conversely, the assay could also be applied to finding strategies that attenuate CTL-mediated graft rejection.

The cellular machinery involved in mobilizing, docking, and fusion of secretory lysosomes to effector cell membranes has not been well characterized. A small number of genetic mutations and some recent targeted deletion studies have identified a few regulatory proteins involved in exocytosis (Andzelm et al., 2007; Haddad et al., 2001; Krzewski et al., 2008; Menager et al., 2007; Menasche et al., 2008; Stinchcombe et al., 2001; Zur Stadt et al., 2006). A proteomic approach that identifies the full constellation of secretory lysosome proteins is a promising means by which additional factors can be identified. A comprehensive assessment of secretory lysosomal proteins, isolated by biochemical techniques, from CTL or NK cells can be viewed in the Appendix (Appendix Figures 4,6 and Table 1). Genetic knockdown or siRNA strategies will be complemented by GrB release assays to identify essential components of the degranulation machinery. Ultimately, these studies could lead to therapeutic strategies against genetically or pathogen-driven defective cytotoxic responses.

Essentially, GrB is a well-established and powerful mediator in cell-mediated immunity. The ability of GrB to strongly and quickly target cells to cell death is mediated primarily through its ability to activate caspases (see Introduction). Thus, in the absence of direct GrB inhibitors or means to prevent

caspase activation, cells exposed to GrB rapidly die via apoptosis. However, GrB can more slowly kill targets in the absence of caspases through a more necrosis-like mechanism (Heibein et al., 1999). Fas receptor-mediated cytotoxicity also primarily targets caspase activation in order to eliminate target cells (see introduction). However, numerous viral and endogenous molecules interfere with GrB, Fas, and caspase pathways (Clem et al., 1991; Clem and Miller, 1994; Komiyama et al., 1994; Sun et al., 1996; Tewari et al., 1995). Hence, it would be logical that alternative mechanisms would exist that would provide compensatory means of killing transformed or infected cells. Studies in mice deficient in GrB and GrA have suggested that alternative granzymes may provide protection against immunological threats (Davis et al., 2001; Jenkins et al., 2008; Revell et al., 2005a). *In vitro* studies of these "orphan" granzymes have begun to illuminate the existence of additional cell death pathways that are largely caspase-independent, and induce forms of cell death that have features of apoptosis, necrosis, and perhaps even autophagy or pyroptosis (see introduction).

Thus, the understanding of additional granzymes and the implications of these alternative granule-mediated strategies are of great interest. We have shown evidence that GrH can contribute to granule-mediated cytotoxicity, and does so through a mechanism distinct from that of GrB and Fas. However, several important questions remain:

What is the mechanism that promotes GrH-mediated mitochondrial dysfunction?

Bid cleavage during GrH-mediated cell death is z-VAD-fmk sensitive and impaired by Bcl-2, suggesting that Bid cleavage occurs through caspases activated downstream of MOMP. Thus, Bid is likely a part of a positive amplification loop of GrH-mediated cell death, rather than the instigator. However, the killing mechanism utilized by GrH may involve other Bcl-2 proteins that could promote Bax/Bak oligomerization. Further experiments should directly assess cellular levels of numerous Bcl-2 proteins in cells, and determine whether any are elevated or reduced following exposure to GrH. Subsequent experiments using siRNA should provide further insights of the contributions of these proteins, as knockdown of pro-apoptotic BH3-only proteins may abrogate GrH damage to mitochondria and cell death (such as Bim, Bad, etc).

Moreover, given the delay of GrH-mediated cell death compared to GrB, *de novo* synthesis of BH3-only proteins, such as Puma or Bim_s is also a possibility. This could initially be assessed by killing experiments that include reagents such as cyclohexamide.

Another scenario that could lead to mitochondrial dysfunction observed upon GrH treatment is that of Ca^{2+} overload and ROS production. Effluxes of calcium from the ER lead to calpain activation and damage to mitochondria that lead to a loss of $\Delta\Psi_m$, possibly through a PTP-dependent mechanism (see introduction). The absence of Bax and Bak, or the over-expression of Bcl-2 has been shown to protect cells from calcium-induced cell death. The over-

expression of Bcl-2 has also been reported to protect against ROS damage. Various granzymes induce ROS production (see introduction), and Fellows *et. al.* detected transient elevated ROS levels in cells undergoing GrH-mediated cell death (Fellows et al., 2007). Our results demonstrated that mitochondria were protected from GrH in the presence of Bcl-2 over-expression and in the absence of both Bax and Bak. Subsequent experiments demonstrated that cells exposed to GrH activated calpains, but additional preliminary experiments using a calpain inhibitor did not affect multiple readouts of GrH-mediated cell death. Other experiments demonstrated that GrH-induced cell death was also insensitive to cyclosporine A, an inhibitor of the PTP. Therefore, most evidence supports the theory that GrH disrupts mitochondrial function through Bax/Bak MOMP.

What is the mechanism of GrH-induced DNA Damage?

Our results have shown that GrH directly cleaves DFF45/ICAD, an inhibitor that protects DNA from DFF40/CAD. However, the cleavage site has not been identified. A putative cleavage site $Ile^{138}Ile^{139}Leu^{140}Leu^{141}Ser^{142}$ was tested using targeted mutagenesis, as proteolysis at this site would produce fragments of approximately 30 kD and 15 kD. More enticingly, it showed similarity to the sequence present within the RCL of ACT (Ile^{P4}-Thr^{P3}-Leu^{P2}-Leu^{P1} – $Ser^{P1'}$). However, single and double leucine mutations of DFF45 at this site did not prevent GrH proteolysis (data not shown). Further studies with deletion mutants will be required to reveal whether the cleavage site is the N-terminal or C-terminal portion of the protein. Alternatively, N-terminal

sequencing of proteolytic fragments may also be employed to identify the cleavage site.

Damage to DNA may also proceed following PARP proteolysis by GrB and caspases. However, our experiments in cell lysates revealed that GrH did not directly process PARP, and treatment of Jurkat cells with GrH only induced caspase-dependent PARP cleavage (data not shown).

Why is caspase-3 poorly activated by GrH treatment?

Evidence presented in Chapter 3 showed that GrH does not directly process procaspase-3. However, the release of cytochrome C and SMAC/DIABLO following GrH exposure was observed. Despite the release of apoptosome constituents from the mitochondria, levels of active caspase-3 detected by intracellular staining remained rather low. Studies using recombinant mouse GrF and GrC have made similar observations, where MOMP did not result in the generation of active caspases (Johnson et al., 2003; Shi et al., 2009). The formation of an active apoptosome requires the assembly of cytosolic Apaf-1 monomers and caspase-9, as well as mitochondrial cytochrome C and ATP. During necrotic cell death ATP levels can be quickly depleted either through direct damage to the respiratory chain, or by hyperactive PARP that leads to a depletion of NAD^+. Our experiments have indicated that cells treated with GrH produce PARP fragments consistent with caspase-3 cleavage, not those produced during necrosis. A component of the respiratory chain, NDUFS3, has been reported to be a substrate of GrA (Martinvalet et al., 2008). It is also possible that GrH could target proteins involved in ATP generation. Further studies will

involve the measurement of ATP levels in cells treated with GrH to assess whether this could be a mechanism that disrupts caspase-3 activation.

In summary, our experiments have supported the hypothesis that GrH can induce cell death through a mechanism distinct from that of GrB and Fas. So then, what is the overall contribution of GrH to cell-mediated cytotoxicity? Many of the biochemical effects on cells by GrH are similar to those observed using other "orphan" murine GrB cluster enzymes (i.e. GrC and GrF). Human GrH and members of the orphan GrB cluster are highly homologous (60 – 70% similar by BLAST analysis) and all are proposed to be chymases (Kaiserman et al., 2009; Kam et al., 2000). Furthermore, these granzymes induce death pathways that target MOMP independently of Bid, and induce the release of cytochrome C, but kill cells independently of caspases. Also like GrH, the morphological features induced by GrC and GrF exposure include mitochondrial swelling and early plasma membrane permeability (Figure 3.2) (Fellows et al., 2007; Johnson et al., 2003; Shi et al., 2009). Hence, it appears that GrH, GrC, and GrF induce features of necrosis, and to a lesser extent, those of apoptosis. Indeed, with the exception of GrB, the remaining granzymes tested *in vitro* (including the GrA cluster, and GrM) promoted cell death programs that were largely caspase-independent and displayed features of necrosis (see introduction). The release of intracellular contents from cells with compromised plasma cell membranes is highly inflammatory, and the recent finding that GrA may also directly promote pro-inflammatory responses further questions whether other granzymes supply alternative killing pathways, modulate inflammatory responses, or do both.

In humans, GrA, H, and M are highly and constitutively expressed in NKs, while GrB expression is strongly elevated by exposure to cytokines. Therefore, the overall objective of the "orphan" granzymes, including GrH, may be to prime the early immunological environment for an effective adaptive response. Natural killer cells are initial cytotoxic responders, detecting cells displaying signs of infection or stress. The release of granzymes from NK cells could directly elicit production of pro-inflammatory cytokines upon their interaction with phagocytes, NKs, or even target cells. Alternatively, these granzymes may indirectly induce pro-inflammatory responses by promoting the release of intracellular contents from necrotic target cells, leading to phagocyte activation and the recruitment and activation of professional APCs.

The elevation of GrB expression in NK or CTLs redirects target cells to more a dominant apoptotic cell death pathway, and the concerted action of GrB/ caspases may actively counteract necrotic and inflammatory pathways (Akita et al., 1997; Declercq et al., 2009). Ultimately, this would then provide the final and efficient elimination of infected or transformed cells while preventing extensive exposure to inflammatory mediators and damage to tissues.

Additionally, in order to counteract inflammation or tissue degradation by degranulated granzymes, as well as neutrophil or mast cell-derived serine proteases, endogenous plasma inhibitors become elevated to avoid damage. One such inhibitor induced during the acute phase of the immune response is ACT. Historically, ACT has been implicated in the inhibition of the neutrophil inflammatory mediator cathepsin G (Travis et al., 1978). However, we have

demonstrated that ACT also interacts and inhibits GrH through a classical serpin mechanism, and does so at a stoichoimetry of inhibition close to 1 (Chapter 4). Preliminary kinetic studies have placed the K_{ass} rate at approximately $5x10^4$ $M^{-1}s^{-1}$, but this awaits further analysis. Of greater significance, treatment of GrH with ACT strongly impaired its ability to induce target cell death. The implications of this finding are that tumor cells over-expressing ACT may be more resistant to GrH-mediated killing, and provide a mechanism for NK immune evasion. However, what remains questionable is why the predominantly plasma mediator ACT would target an intracellular granzyme? One possibility is that GrH has extracellular functions. As was discussed in the introduction chapter, GrA and GrB, have demonstrated extracellular functions that include tissue remodeling, cell adhesion, and the induction of pro-inflammatory cytokines. Future studies will be aimed at determining whether GrH substrates include extracellular matrix constituents. Of note, RBL-2H3 cells stably transfected with GrH, as well as adherent cells treated with GrH, required lengthier exposures to trypsin to detach cells from cell culture dishes. The reason for this phenomenon is unclear, but may involve the modulation of adhesion molecules.

Lastly, exposure of freshly isolated NK cells to endogenous GrH elicited the production of IFN-γ and TNF-α, supporting a hypothesis that GrH may directly induce pro-inflammatory responses (Appendix Figure 1). Further experiments are planned to repeat this observation, and the ability of ACT to circumvent GrH-induced cytokine production will be evaluated. The mechanism by which GrH could evoke pro-inflammatory cytokine production may include

interactions with extracellular NK molecules, in a manner similar to Protease Activated Receptors (reviewed by (Shpacovitch et al., 2007)). Or, like GrA, GrH may induce pro-inflammatory cytokine maturation through direct proteolytic processing (Irmler et al., 1995). Human mast cell chymase has demonstrated an *in vitro* capacity to activate IL-1 and IL-18 (Mizutani et al., 1991; Omoto et al., 2006). Thus, GrH may potentially process these cytokines at similar sites.

In summary, data in this thesis establishes the correlation between GrB release and target cell death, probes the mechanism of an additional granzyme, GrH, to induce cytotoxicity, and reveals the modulation of GrH activity by an endogenous serine protease inhibitor. These findings have implications towards a better understanding of the control and effector mechanisms of granule-mediated cytotoxicity. Ultimately, this will be relevant to the production of effective vaccines, cancer treatments, and alleviating immunopathology.

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APPENDIX

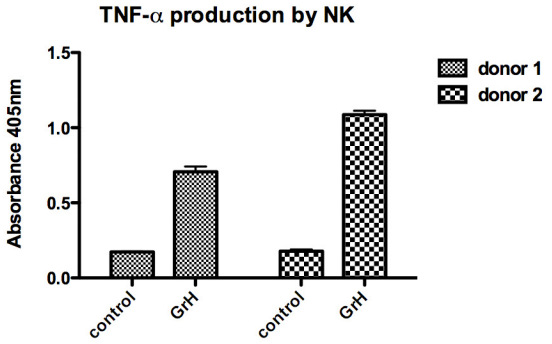
A) Granzyme H Treatment of *Ex Vivo* human NK Cells

Materials and Methods

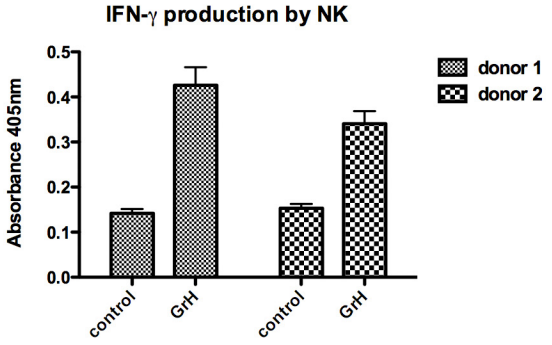
Lymphocytes were isolated from peripheral blood of healthy volunteers on Ficoll-Hypaque density (GE Healthcare Biosciences, Uppsala, Sweeden). Natural Killer cells were purified using a Negative Selection NK isolation cocktail (Miltenyi Biotech Inc, Auburn, CA). Following isolation, NK cells were diluted to $3x10^6$ cells/ ml in RPMI 1640 media supplemented with 10 mM HEPES, and $3x10^5$ cells were aliquoted to 96-well U-bottom tissue culture plates. Control samples were treated with media only, while other cells were treated with 20 ug/ml recombinant GrH (purified as described in Chapter 3) for 1 hour at 37^{o}C. Media containing 20% FBS was added to give a final concentration of 10% FBS, and cells were further incubated for 15 hours. Cells were spun and the supernatants were collected for cytokine secretion. Maxisorp™ plates (Thermo Fisher Scientific, Roskilde, Denmark) were coated with 2 mg/ml capture anti-human TNF-α or anti-human IFN-γ mAbs (both from BD Biosciences, San Jose, CA), in PBS overnight. Plates were washed with PBS and blocked using 1% BSA (w/v) / PBS. Samples were added to plates for 2 hours, washed using PBS-0.1% Tween-20 (v/v) before application of 2 ug/ml of biotinylated anti-human TNF-a or IFN-g detection antibodies (BD Biosciences). Wells were washed, incubated with 1/5000 of streptavidin-alkaline phosphatase (Jackson Immunoresearch Laboratories, West Grove, PA), and cytokines were detected by addition of the colorimetric substrate Di (Tris) *p*-nitrophenyl phosphate (pNPP)

(Sigma) and measurement at absorbance 405nm by a Multiskan Ascent spectrophotometer (Thermo Lab-systems, Helsinki, Finland).

A



B



Appendix Figure 1 : Production of Pro-inflammatory cytokines by NK cells treated with recombinant GrH. Granzyme H (20 ug/ml) was applied to $3x10^5$ purified NK cells from 2 healthy donors for 1 hour in the absence of serum. Media with 20 % FBS was added to cultures for another 15 hours of incubation. Supernatants were collected and analyzed by ELISA for TNF-α (Appendix 1-A) or IFN-γ (Appendix 1-B).

B) Proteomic Analysis of Secretory Lysosomes

Materials and Methods

Cell lines and Chemicals

All chemical were from Sigma (Sigma, St. Louis, MO) unless otherwise stated. YT Indy, a human NK leukemia cell line, (ATCC) was maintained in RPMI 1640 (Life Technologies, Burlington, ON, Canada) supplemented with 10% (v/v) FBS (Sigma), 10 mM HEPES, 50 U/ml penicillin, 50 μg/ml streptomycin, and 0.1 mM nonessential amino acids (all from Life Technologies).

The human $CD8^+$ T cell line was generated by treatment of peripheral blood from a healthy donor with RosetteSep™ Human CD8+ T Cell Enrichment Cocktail (StemCell Technologies, Vancouver, Canada) and Ficoll-Hypaque density (Veugelers et al., 2006). Cells were then stimulated by an irradiated, allogeneic EBV-transformed B cell line, RPMI-8866 (Sigma), for 3 days in RPMI 1640 media, supplemented with 10% FCS, HEPES, penicillin/ streptomycin, and 20 unit/ml of recombinant IL-2. Dead cells were removed upon centrifugation in Ficoll and further cultured for three days before undergoing subcellular fractionation.

U937 and Jurkat cell lines (both from American Type Culture Collection (ATCC) (Manassas, VA) were maintained in RRMI 1640 media, supplemented with 10% FCS and 10 mM HEPES.

Subcellular Fractionation

Cells were grown to at least $2x10^9$ cells before being harvested for subcellular fractionation. Cells were washed PBS, then in ice cold relaxation buffer [1x Borregaard Buffer (100mM KCl, 3 mM NaCl, 3.5mM $MgCl_2$, 1mM ATP, 1.25 mM EGTA, 10 mM piperazine-N,N'-bis (2-ethanesulfonice acid), pH 6.8)] (Borregaard et al., 1983). Cells were resuspended at $2x10^8$ cell/ml in 1x Borregaard Buffer for disruption in a nitrogen cavitation bomb (Model 4639, Parr Instrument Co., Moline IL) at 450 p.s.i. for 20 minutes. Nuclei and intact cells were removed via two spins at 2500 rpm in a Beckman labtop centrifuge. Five millilitres of supernatant were layered upon 25 ml of 40% continuous Percoll/ RediGrad gradient (density 1,130 g/ml, GE Healthcare, Uppsala, Sweden) in Beckman ultra-clear centrifuge tubes (25 x 89mm, Beckman Instruments, Palo Alto, CA) and spun in a Beckman Optima L-80 XP ultracentrifuge (Beckman Coulter, Mississagua, ON) and Type-70 Ti rotor at 20 900 rpm for 20 minutes (Davis et al., 2003). Three millilitre fractions were collected, beginning at the most dense fractions, to generate 10 fractions for every tube. Percoll was removed from fractions by centrifugation for 3 hours at 38 100 rpm. Material above the Percoll pellet was collected for subsequent analyses.

Assessment of Lytic Activity of Subcellular Fractions

Jurkat cells were labelled with 100 μCi $Na_2{}^{51}CrO_4$ (Perkin-Elmer Life Sciences, St. Laurent, Quebec) and diluted at $5x10^4$ cells/ 100ul in HBSS (Invitrogen), supplemented with 2 mM $CaCl_2$ and 0.1% (w/v) BSA. Fractions

were diluted in 100 μl HE buffer (10 mM HEPES, 150 mM NaCl), then added to labelled target cells. Supernatants were collected at 4 hours, analyzed by a Wallac 1470 gamma counter (Wallac Oy, Turku, Finland) and percent target cell lysis was calculated using the formula percent target lysis = experiment cpm – spontaneous cpm / total cpm - spontaneous cpm x 100.

Characterization of protein content of subcellular fractions

Protein concentration of fractions was determined by BCA™ assay (Pierce, Rockland, IL) using BSA standards and linear regression analysis following measurement at 590nm.

Assessment of enzymatic activity of subcellular fractions

Fractions were assessed for GrB activity using hydrolysis of 200 mM Ac-IEPD-pNA substrate (Kamiya Biomedical Company, Seattle, WA) (Ewen et al., 2003b). Absorbance at 405nm was measured by a Multiskan Ascent spectrophotometer.

ELISA for Perforin, FasL, and Granzyme H

Perforin was assessed using a human perforin ELISA kit (Abcam Inc. Cambridge, MA), FasL by a FasL ELISA kit (R&D Systems, Minneapolis, MN). Granzyme H was detected as described in Chapter 3. Briefly, Maxisorp™ plates (Thermo Fisher Scientific) were coated with 5 μg/ml of capture anti-perforin or 2 mg/ml anti-FasL in PBS at 4^{o}C overnight, washed with PBS, and then blocked

with 1% BSA (w/v) in PBS assay diluent for an hour. Samples were diluted in assay diluent and incubated for 2 hours at room temperature. Wells were washed with PBS-0.1% Tween-20 (v/v) before application of 1 μg/ml of biotinylated anti-perforin detection antibody or 50 ng/ml biotinylated anti-FasL detection antibody. Wells were washed, incubated with 1/5000 of streptavidin-alkaline phosphatase (Jackson Immunoresearch Laboratories) and perforin, GrH, and FasL were detected upon the addition of pNPP.

Immunoblots

Antibodies for immunoblot analyses: anti-cytochrome C, anti-GM130, anti-calnexin, and anti-EEA1were from BD Biosciences (San Jose, CA); anti-Cox IV was from Invitrogen (Carlsbad, CA); anti-histone H1 (clone AE-4) and anti-granzyme B (clone 2C5) were from Santa Cruz Technologies (Santa Cruz, CA). Goat anti-mouse and anti-rabbit HRP-conjugated antibodies were from BioRad Laboratories (Hercules, CA). Fifteen to 30 ml of fractions in 2x SDS Loading Buffer were loaded to 8% - 15% SDS-PAGE gels, and wet-transferred to PVDF or nitrocellulose membranes (both from GE Healthcare). Membranes were blocked with 5% milk in PBS-Tween 0.1% (v/v) for at least one hour. Primary antibodies were incubated overnight at 4^{o}C, washed with PBST, and incubated with HRP-conjugated secondary antibodies for 1 hour. Bands were visualized with ECL Plus Western Blot Detection Agent (GE Healthcare).

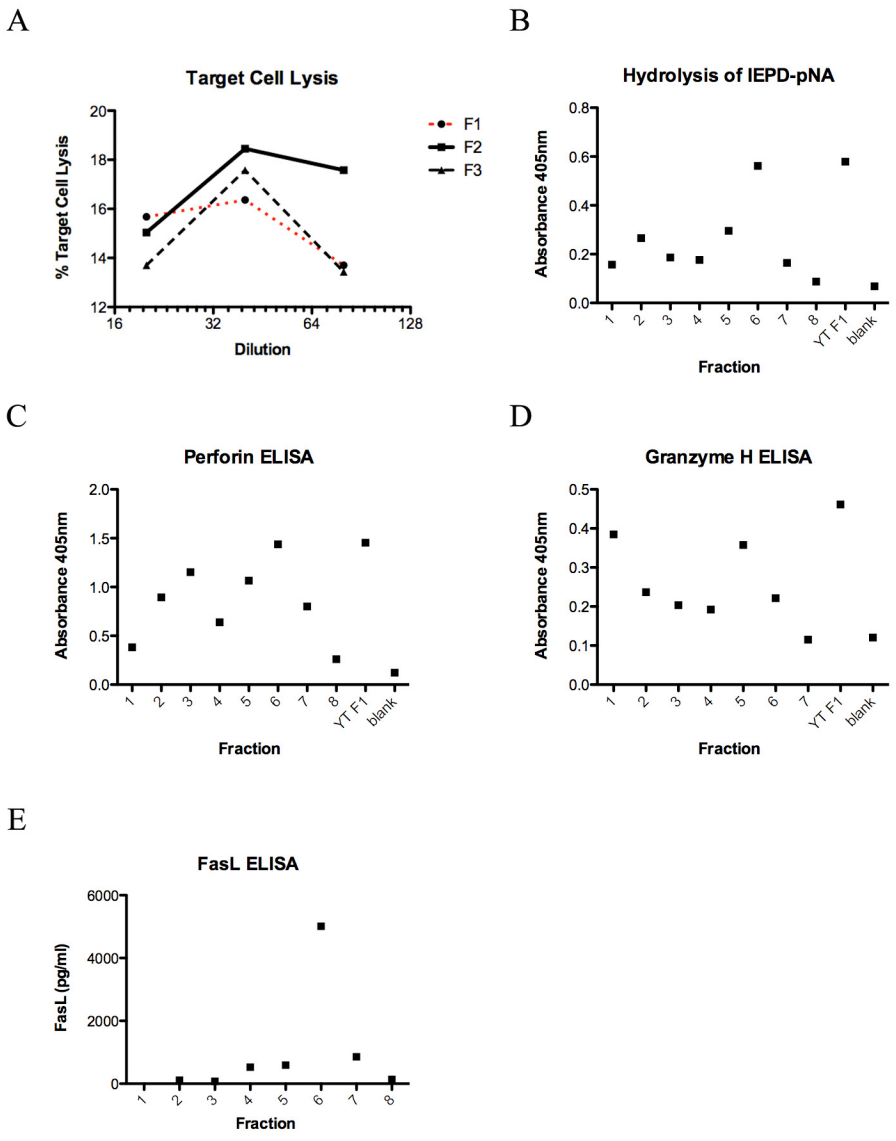
Mass Spectrometry

Fractions were sent to our collaborator, Dr. John Wilkins, at the Manitoba Centre for Proteomics & Systems Biology for mass spectrometry analysis.

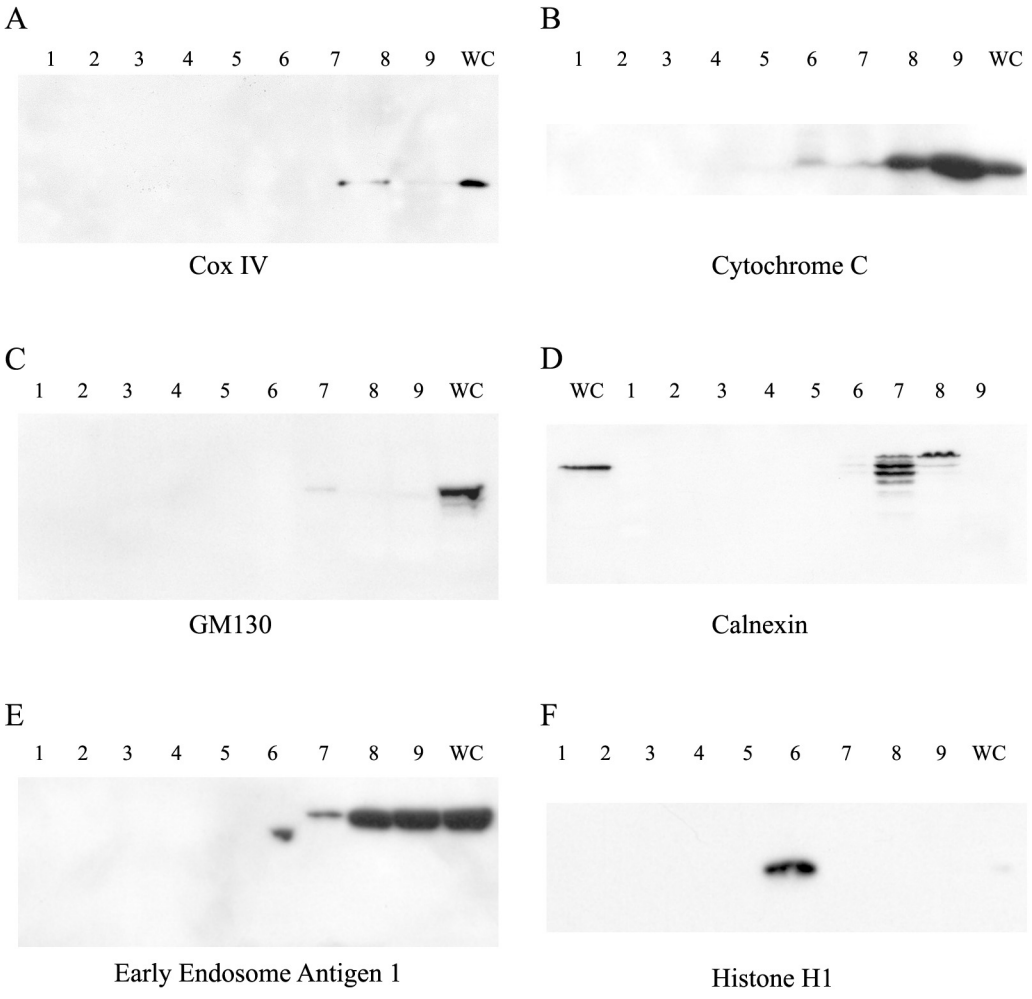
Results of Subcellular Fractionation and Proteomic Analysis

Human $CD8^+$ T cells

Fractions isolated from human $CD8^+$ T cells indicated that Fraction 2 possessed the highest lytic activity against Jurkat target cells (Appendix Figure 2-A). Levels of GrB activity, and perforin, and GrH protein were also high in fraction 2 (Appendix Figure 2B – D). However, FasL was not strongly detected in high density cellular fractions, indicating it was likely stored in compartments outside of secretory lysosomes (Appendix Figure 2E). This finding is in contrast to findings reported by Gillian Griffith's group (Bossi and Griffiths, 1999), but in agreement with findings from Hanne Ostergaard's lab (He and Ostergaard, 2007). Markers for mitochondria included cytochrome C and Cox IV (Appendix Figure 3-A &B), for Gogli apparatus, the cis-golgi marker GM130 (Appendix Figure 3-C), the endoplasmic reticulum marker was Calnexin (Appendix Figure 3-D), Early Endosomal Antigen was a marker for endosomal proteins (Appendix Figure 3-E), and Histone H1 was used as a marker for nuclear contamination (Appendix Figure 3-F). Whole cells (WC) lysates were used as positive controls. These results demonstrated that Fraction 2 contained lytic activity, cytotoxic granule proteins, and were relatively free of contaminants from the mitochondria, ER, gogli, and nucleus. A total of 153 proteins below log (e) -10 were reported by



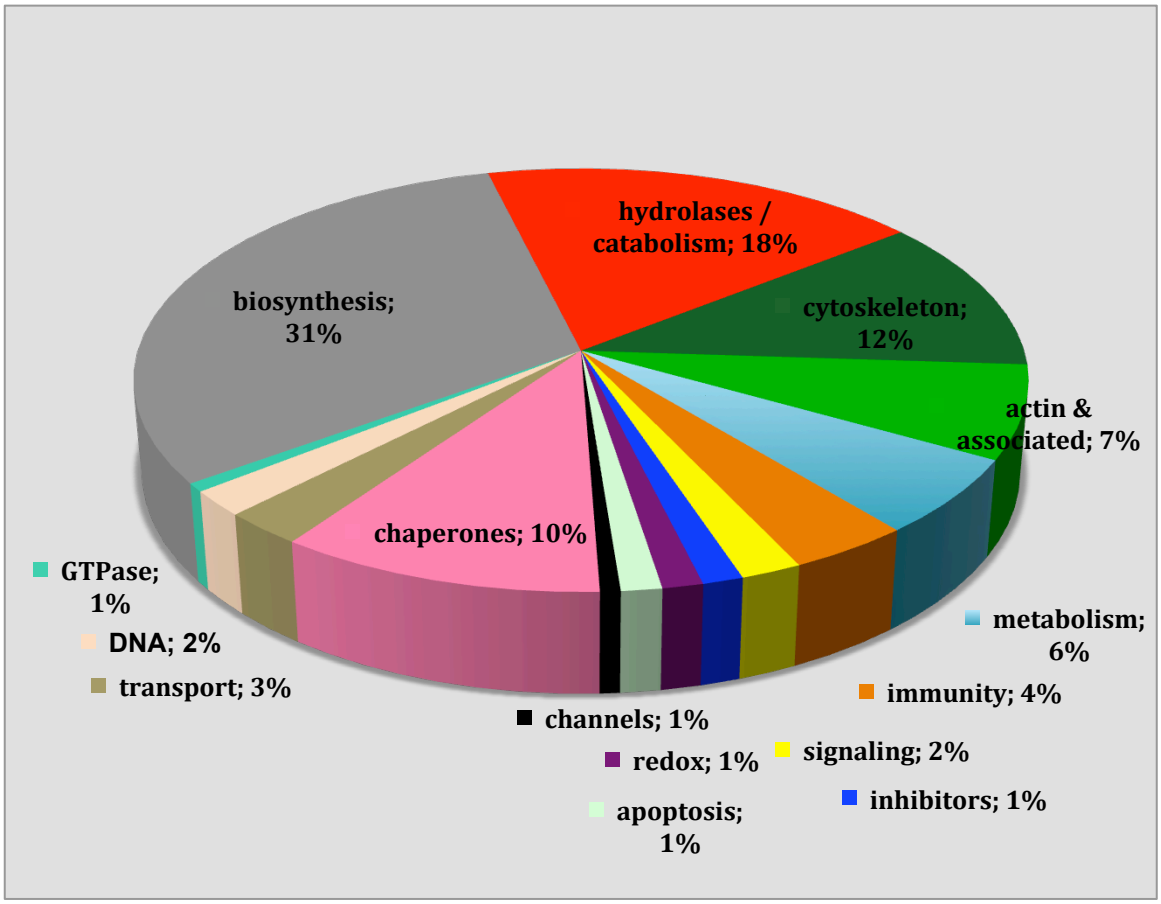
Appendix Figure 2 : Characterization of Subcellular Fractions isolated from a human CD8$^+$ T cell line: Detection of Cytotoxic Proteins. A) Lytic activity against Jurkat target cells. B) Granzyme B enzymatic activity. C) Perforin, D) GrH, and E) FasL levels detected by ELISA. Data is an average of the analysis of 2 independent subcellular fractionation experiments.



Appendix Figure 3 : Characterization of Subcellular Fractions isolated from a human CD8$^+$ T cell line: Markers of various cellular compartments. Fractions separated by a density gradient were evaluated for the presence of numerous cellular compartments; A & B) mitochondrial markers Cox IV and cytochrome C, C) golgi marker GM130, D) ER marker calnexin, E) endosomal marker EEA1, F) and nuclear marker Histone H1. Immunoblots are representative of 2 independent experiments.

mass spectrometry, and categorized by function (Appendix Figure 4). Proteins with scores of log (e) under -10 were deemed as confident identification, as this ensured that most proteins were identified by at least 2 unique peptide sequences.

Proteins related to the synthesis of purine/pyrimidines, amino acids, or proteins (transcription and translation) were classified under "Biosynthesis". "Hydrolases and catabolism" include proteins with peptidase activity or those involved in protein degradation. "DNA" includes proteins involved in DNA replication, repair, and the structure of nucleosomes. Proteins classified as "Immunity" include HLA molecules, anti-viral proteins, and cytokine/ cytokine-like mediators. "Apoptosis" proteins included perforin and Apoptosis Inducing Factor. "Channel" proteins are involved in proton and solute transport across cellular membranes. "Transport" are proteins are those involved in vesicular and protein trafficking. "Cytoskeleton" proteins include keratins, tubulin molecules, and vimentin, while "actin and associated" are cytoskeleton proteins that interact with actin specifically.



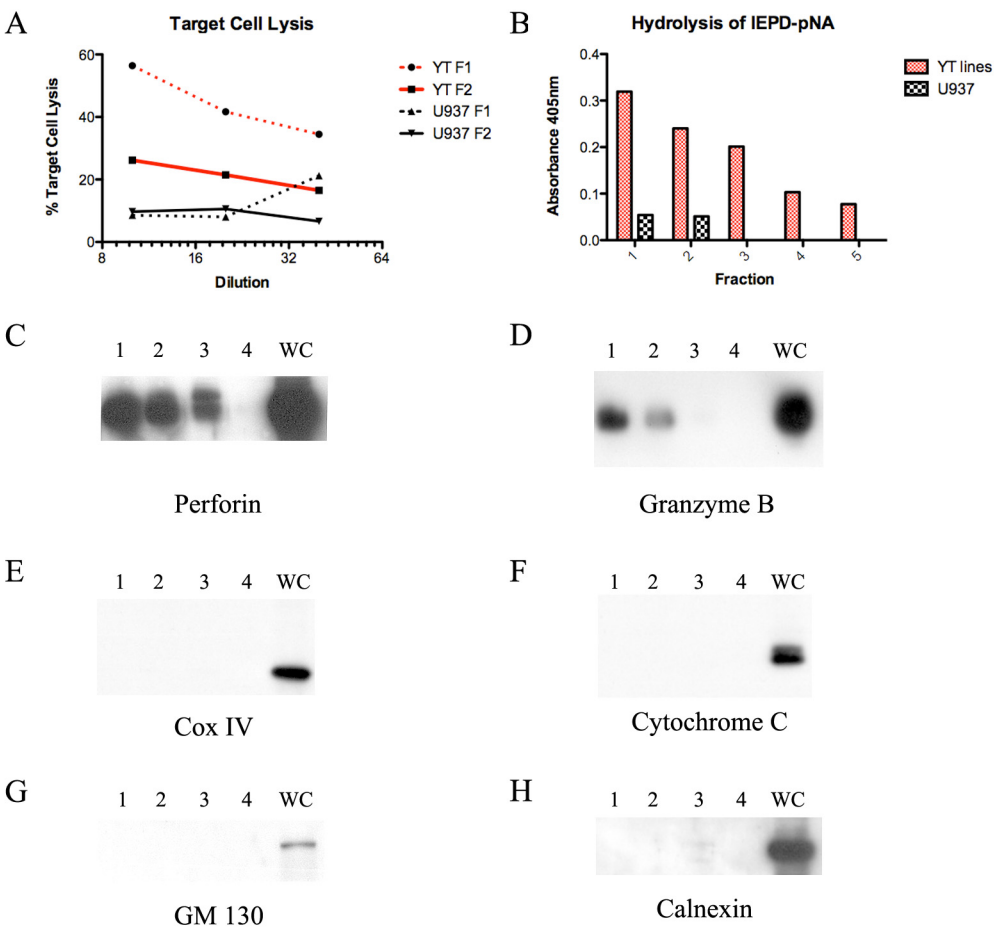
Appendix Figure 4 – Proteomic Analysis of Secretory Lysosomes isolated from a human CD8$^+$ T cell line. A total of 153 proteins were identified and classified according to function.

Human NK cells – YT Indy cell line

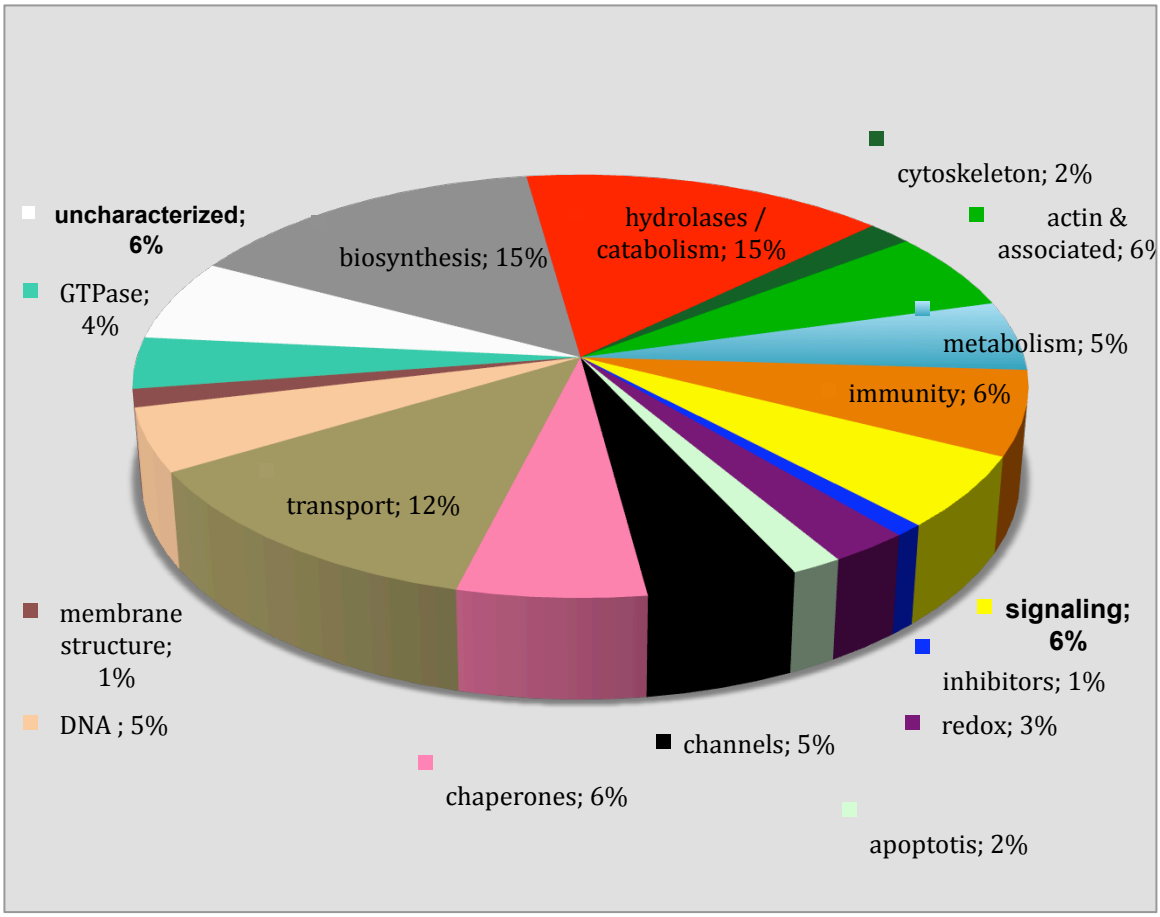
Fractions isolated from YT Indy cells and U937 (a monocytic control cell line) were assessed for lytic activity against Jurkat target cells (Appendix Figure 5-A). Fraction 1 from YT Indy cells contained the most significant cytotoxic activity, while fractions from a non-cytotoxic line did not induce cell lysis. Levels of GrB activity present in YT Indy and U937 fractions were assessed by hydrolysis of Ac-IEPD-pNA (Appendix Figure 5-B). Perforin and GrB (protein) were evaluated by immunoblot, and indicated highest levels were present in fraction 1 (Appendix Figure 5-C &D). Immunoblot analysis also indicated that Fraction 1 was free of mitochondria markers Cox IV and cytochrome C (Appendix Figure 5-E&F), golgi marker GM130 (Appendix Figure 5-G), and ER marker Calnexin (Appendix Figure 5-H).

Proteomic analysis of Fraction 1 isolated from YT Indy cells resulted in the identification of 433 proteins with log (e) values less than -10. Proteins were categorized as described above, and the findings are summarized in Appendix Figure 6.

Proteins that were identified in both human $CD8^+$ T cells and NK secretory lysosome fraction are summarized in Appendix Table 1.



Appendix Figure 5 : Characterization of Subcellular Fractions isolated from a human NK cell line, YT Indy. Fractions separated by a density gradient were assessed for markers of secretory lysosomes: A) Lytic Activity against Jurkat target cells. B) GrB enzymatic activity. C) Perforin immunoblot D) GrB protein by immunoblot. In A & B, fractions from a human monocytic cell line, U937, were used as negative controls. The presence of numerous other cellular compartments were assessed by immunoblots to E&F) mitochondrial markers Cox IV and cytochrome C, G) golgi marker GM130, and H) ER marker calnexin, Data is representative of at least 2 independent experiments.



Appendix Figure 6 – Proteomic Analysis of Secretory Lysosomes isolated from human NK cell line. A total of 433 proteins were identified and classified according to function.

Appendix Table 1 – Common proteins identified by Mass Spectrometry of Secretory Lysosmes from hCD8+ T cells and YT Indy NK cells

Hydrolases/ carbohydrate/ acid protease	26S protease regulatory subunit 7 26S proteasome non-ATPase regulatory subunit 2 Acid ceramidase Arylsulfatase A Beta-hexosaminidase beta chain Beta-hexosaminidase subunit alpha Cathepsin C Cathepsin D Cytosol aminopeptidase Dipeptidyl-peptidase 2 precursor Gamma-glutamyl hydrolase Granzyme B Granzyme H Lysosomal alpha-mannosidase N-acetylglucosamine-6-sulfatase Palmitoyl-protein thioesterase 1 Phospholipase D3 Proteasome subunit alpha type 3 Proteasome subunit alpha type-5 Proteasome subunit beta type-3
Actin & associated	Annexin A2 Coronin 1A Filamin-A Myosin regulated light chain Myosin-9 (mysoin heavy chain) Plastin-2 (L-plastin)
Metabolism	Alpha-enolase Ferratin light chain Fructose-bisphosphate aldolase A Glucose-6-phosphate isomerase Glyceraldehyde-3-phosphate dehydrogenase Nucleoside diphosphate kinase A (aka NM23-H1) Pyruvate kinase isozymes M1/M2 Transaldolase Triosephosphate isomerase
Immunity	59 kDa 2'-5'-oligoadenylate synthetase-like protein HLA class I histocompatibility antigen, B-7 alpha chain HLA class II histocompatibility antigen, DQB1*0602 beta chain
Signaling & Receptors	14-3-3 protein zeta/delta Receptor of activated protein kinase C 1 (RACK1)
Inhibitors	Leukocyte elastase inhibitor (precursor of acid DNase)
Redox	Superoxide dismutase Thioredoxin-dependent peroxide reductase
Apoptosis	Perforin-1

Chaperones	Heat shock 70 kDa protein 1 Heat shock cognate 71 kDa protein Heat shock protein HSP 90-alpha Heat shock protein HSP 90-beta T-complex protein 1 subunit beta T-complex protein 1 subunit eta T-complex protein 1 subunit gamma T-complex protein 1 subunit theta
Transport/ Vesicular Trafficking	Clathrin heavy chain 1 Major vault protein Transitional endoplasmic reticulum ATPase
GTPases	Ras-related protein Rab-7a
Biosynthesis	60S acidic ribosomal protein P0 Heterogeneous nuclear ribonucleoprotein Q Heterogeneous nuclear ribonucleoproteins C1/C2 Interleukin enhancer-binding factor 2 Polyadenylate-binding protein 1 Proliferation-associated protein 2G4

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