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The role of granzyme B and other granule proteases as modulators of cytokine activity

Thesis submitted to Trinity College Dublin for the degree of Doctor of Philosophy

2012

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DECLARATION

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ABBREVIATIONS

AFC	7-amino-4-trifluoromethyl coumarin
AP-1	Activator protein-1
APAF	Apoptotic protease activating factor
ASC	Apoptosis-associated speck-like protein containing a CARD
ATP	Adenosine triphosphate
BALF	Bronchoalveolar lavage fluid
BCL-2	B cell lymphoma 2
BCR	B cell receptor
BH3	Bcl-2 homology region 3
BMDC	Bone marrow-derived dendritic cells
BSA	Bovine serum albumin
CAD	Caspase-activated DNase
CARD	Caspase activation and recruitment domain
CASPASE	Cysteinyl aspartate-specific protease
CF	Cystic fibrosis
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CHX	Cycloheximide
COPD	Chronic obstructive pulmonary disease
CREB	Cyclic AMP response-element binding
Crm	Cytokine response modifier
CTL	Cytotoxic lymphocyte
Cyt c	Cytochrome c
DAMP	Danger-associated molecular pattern
DBP	DNA binding protein
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent protein
DTT	Dithiothreitol
E:T	Effector:target
EBV	Epstein Barr virus
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FACS	Fluorescence-activated cell sorter
FBS	Fetal bovine serum
FGFR	Fibroblast growth factor receptor
FITC	Fluorescein isothiocyanate
FL	Full length
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	Granulocyte-monocyte colony-stimulating factor
Gzm	Granzyme

GluR	Glutamate receptor
HEK	Human embryonic kidney
HEPES	N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulphonic acid)
HIV	Human immunodeficiency virus
HMGB1	High-mobility group box I
HRP	Horse radish peroxidase
HSP	Heat-shock protein
HUVEC	Human umbilical vein endothelial cells
ICAD	Inhibitor of caspase-activated DNase
IETD	Isoleucine-glutamic acid-threonine-aspartic acid
IFN	Interferon
Ig	Immunoglobulin
IkB	Inhibitor of NFkB
IKK	IkB kinase
IL	Interleukin
IL-1R	Interleukin-1 receptor
IL-1RAcP	IL-1R accessory protein
IPTG	Isopropylthio-β-D-galactoside
IRAK	IL-1R-associated kinase
ITT	*In vitro* transcription and translation
JNK	Jun N-terminal kinase
kDa	kilodaltons
MALDI-TOF	Matrix-assisted laser desorption and ionisation time-of-flight
MDP	Muramyl dipeptide
MHC	Major histocompatibility complex
MS	Mass spectrometry
MSU	Monosodium urate
MyD	Myeloid differentiation factor
LPS	Lypopolysaccharide
LRR	Leucine-rich repeats
LTA	Lypoteichoic acid
NCBI	National centre for biotechnology information
NFkB	Nuclear factor kB
Ni-NTA	Nickel-nitrilotriacetic acid
NK	Natural killer cell
NKR	Natural killer receptor
NLR	Nod-like receptor
NLS	Nuclear localization signal
NOD	Nucleotide-binding oligomerisation domain
NP40	Nonidet P40
NUMA	Nuclear mitotic apparatus protein
ORF	Open reading frame
OVA	Ovalbumin
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen-associated molecular pattern
PAR	Protease-activated receptor

PARP	Poly(ADP-ribose) polymerase
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PI	Propidium iodide
PI-9	Proteinase inhibitor-9
PMA	Phorbol 12-myristate 13-acetate
PMSF	Phenylmethylsulphonylfluoride
PRB	Protease reaction buffer
PRR	Pattern recognition receptor
PYD	Pyrin domain
RING	Really interesting new gene
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
SDS	Sodium dodecyl sulphate
SLO	Streptolysin
TAB	TAK binding protein
TAK	Transforming growth factor-β-associated kinase
TIR	Toll/IL-1R
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRAF	TNF receptor-associated factor
UV	Ultraviolet irradiation
von WF	von Willebrand factor
WCEB	Wang Cell extract buffer
WT	Wild-type
XIAP	X-linked Inhibitor of Apoptotic Proteases

PUBLICATIONS

Afonina IS, Cullen SP, Martin SJ. Cytotoxic and non-cytotoxic roles of the CTL/NK protease granzyme B. Immunol Rev. 2010; 235:105-16.

Lüthi AU, Cullen SP, McNeela EA, Duriez PJ, Afonina IS, Sheridan C, Brumatti G, Taylor RC, Kersse K, Vandenabeele P, Lavelle EC, Martin SJ. Suppression of interleukin-33 bioactivity through proteolysis by apoptotic caspases. Immunity. 2009; 31:84-98.

Cullen SP, Afonina IS, Donadini R, Lüthi AU, Medema JP, Bird PI, Martin SJ. Nucleophosmin is cleaved and inactivated by the cytotoxic granule protease granzyme M during natural killer cell-mediated killing. J Biol Chem. 2009; 284:5137-47.

Afonina IS, Tynan GA, Logue SE, Cullen SP, Bots M, Lüthi AU, Reeves EP, McElvaney NG, Medema JP, Lavelle EC, Martin SJ. Granzyme B-dependent proteolysis acts as a switch to enhance the proinflammatory activity of IL-1α. Mol Cell. 2011; 44:265-78.

Chapter 1

Literature Review

1.1 Introduction

In all vertebrate organisms a properly functioning immune system is absolutely required for protection against infectious agents and also acts as a safeguard against tumour development. Over time we have developed a highly elaborate multifunctional immune system, where a variety of specialised cells recognise danger and respond adequately. How does the immune system eliminate danger? In response to pathogen recognition, immune cells secrete a range of different weapons: cytokines, antimicrobial peptides, cell death-inducing molecules, reactive oxygen species, and proteases, all of which contribute to the eradication of danger (Delves et al., 2011).

Of particular interest to this work are proteases; enzymes that cut proteins by catalysing hydrolysis of peptide bonds between amino acids. The family of serine proteases, characterised by a serine residue at their active sites, is the largest family of proteases. These enzymes prominently feature in the arsenal of immune cells and are capable of inducing cell death, regulating inflammation and inhibiting viral replication (Heutinck et al., 2010).

Neutrophils, the ground troops of the immune system, are one of the first to arrive at the site of danger, while mast cells participate predominantly in allergic reactions, but also take part in eliminating parasites, worms and bacteria. Both of these cells store a variety of serine proteases, which, upon secretion, promote bacterial and fungal killing via direct degradation of virulence factors or inhibition of protein synthesis and oxygen metabolism. Furthermore, neutophil and mast cell granule proteases coordinate immune responses via modulating proliferation,

differentiation and migration of immune cells or regulating the activity and production of cytokines and chemokines (Heutinck et al., 2010).

This work focuses on the subfamily of serine proteases, called granzymes. These enzymes are predominantly expressed by cytotoxic T lymphocytes (CTL) and natural killer (NK) cells and are best known for their ability to initiate a cell suicide programme, termed apoptosis, which ensures efficient, rapid and 'clean' elimination of defective or damaged cells (Cullen and Martin, 2008). The cytotoxic role of granzymes has been extensively explored and is now well established. However, an emerging body of evidence suggests that the function of these proteases is not strictly limited to cell death. Similarly to other serine proteases involved in inflammation, granzymes have been demonstrated to modulate leukocyte migration, cytokine activity and secretion, and blood coagulation (Afonina et al., 2010). This chapter will describe the nature of granzymes and their role in apoptosis, followed by a summary of the findings regarding the more unorthodox non-cytotoxic functions of granzymes, particularly their emerging role in the regulation of inflammatory processes.

1.2 Granzymes and apoptosis

1.2.1 Apoptosis

Apoptosis is a tightly regulated cell suicide programme that ensures the efficient and 'clean' removal of unwanted, damaged or defective cells and plays a major part in embryonic development, immune homeostasis and tissue remodelling (Jacobson et al., 1997). Various insults, which cause irreparable damage to cells,

initiate the apoptotic pathway that centers on mitochondrial membranes and is regulated by pro- and anti-apoptotic proteins of the Bcl-2 family. The Bcl-2 family orchestrates the downstream activation of the key executioner proteases, called caspases, which cleave a myriad of substrates and ensure rapid dismantling of the apoptotic cell and packaging of its cellular contents into apoptotic bodies for subsequent removal by phagocytes (Taylor et al., 2008). However, mutation can subvert the apoptosis machinery, which can lead to cellular transformation and uncontrolled proliferation. In addition, viruses have been demonstrated to suppress the host cell death programme, thereby granting the valuable time for replication and escape from host cells (White et al., 1992, Huang et al., 2003). To overcome these threats, the immune system employs CTLs or NK cells that recognise and eliminate virus-infected, non-self or transformed cells (Figure 1.1).

1.2.2 CTL/NK cells.

CTLs belong to the adaptive branch of the immune system and recognise foreign proteins, called antigens, subsequently mounting a specific immune response designed to remove cells carrying a particular non-self antigen. Antigen-presenting cells, primarily dendritic cells (DCs), capture antigens or phagocytose infected cells and then digest these proteins, followed by the exposure of the peptides on the cell surface via specific class I major histocompatibiliy complex (MHC) proteins. Exposed peptides are then presented to CTLs, along with co-stimulatory molecules in order to educate killer cells as to the foreign nature of the antigen. Once activated, CTLs recognise the same antigen on the surface of infected cells and then undergo clonal expansion over several days, which

dramatically increases the number of CTLs capable of recognising this specific antigen and destroying antigen-expressing cells. Some of these 'antigen-educated' CTLs subsequently differentiate into memory T cells, which facilitates a much faster and more aggressive T cell-mediated immune response upon another encounter with the same antigen.

Unlike CTLs, NK cells do not require pre-activation by DCs and are able to eliminate dangerous cells on first contact. NK cells are activated when their cell surface receptors are triggered by the presence or absence of specific NK ligands on host cells (Moretta et al., 2008).

Unsurprisingly, viruses and tumour cells have evolved several strategies to evade CTL-mediated recognition and have been shown to down-regulate MHC-I expression on the surface of infected cells (Petersen et al., 2003). Importantly, NK cells complement the function of CTLs by continually monitoring MHC-I expression using NK receptors (NKRs), rapidly activating in the absence of MHC-I proteins on the cell surface (Moretta and Moretta, 2004). In short, CTLs act as the special agents searching for 'known criminals', while NK cells constantly police surroundings for any signs of misbehaviour in the general cell population.

Despite their differences in target cell recognition, both CTLs and NK cells have one major common feature: both cell types utilise similar ammunition to rid the host of harmful or infected cells. This includes secretion of pro-apoptotic cytokines (e.g., TNF-α and IFN-γ), engagement of cell death receptors (e.g., Fas) or secretion of granzymes (Russell and Ley, 2002), all of which promote the cell death programme.

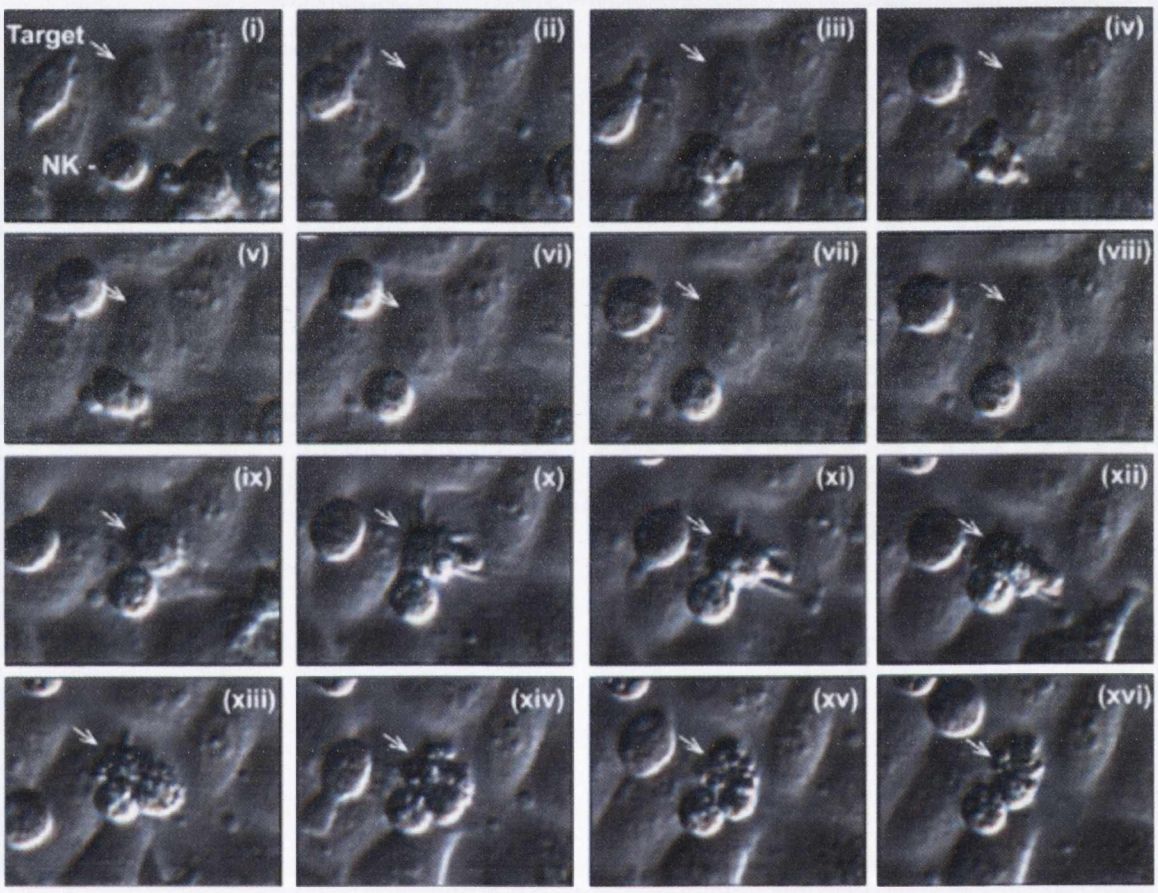


Figure 1.1

Time course of NK-mediated killing

Images of NK-mediated killing of HeLa cells. The frames shown represent 20-min intervals between the initial encounter of the YT cell (NK cell lene) and its HeLa target. Thus, the time elapsed from frame (i) to frame (xvi) is 300 min (R. Taylor and SJ. Martin).

1.2.3 Classification and structure of granzymes

Granzymes, delivered to target cell cytosols by CTLs and NK cells, induce apoptosis in the targets. They belong to a family of highly homologous serine proteases, characterised by a conserved catalytic triad of histidine, aspartic acid and serine at the active site (Trapani, 2001). 5 granzymes have been identified in humans to date (A, B, H, K and M), of which granzymes A and B are the most abundant, and, therefore, the most extensively studied. However, the nature and functions of the so-called 'orphan' granzymes have also been explored in recent years (Zhao et al., 2007; Andrade et al., 2007; Cullen et al., 2009).

Granzymes are most closely related to chymotrypsin in structure, although their functions and substrate specifities are divergent, and can be divided into three subgroups: tryptase-like (granzymes A and K), chymase-like (granzymes B and H) and metase-like (granzyme M) (Trapani, 2001), with most granzymes acting as monomers except for granzyme A, which acts as a heterodimer. Table 1.1 summarises information regarding the expression profile and substrate specifity of granzymes and provides a list of known granzyme substrates.

Granzymes are powerful weapons and safety measures exist to prevent granzymes from destroying effector cells themselves. Initially, granzymes are synthetised as inactive precursors containing an N-terminal dipeptide that must be removed for activation and are safely stored in cytotoxic granules. Cytotoxic granules are specialised secretory lysosomes, within which another protease, cathepsin C, is responsible for removing the N-terminal dipeptides and activating granzymes (Kummer et al., 1996). Furthermore, the acidic pH of cytotoxic

granules is suboptimal for granzyme activity and this acts as an additional safety mechanism to prevent premature activation of granzymes within effector cells.

1.2.4 Delivery of granzymes

How do granzymes gain entry to target cells where they can exert their destructive functions? Upon CTL/NK cell activation, secretory lysosomes move toward the contact site between target and effector cells, termed the immunological synapse, where granule membranes fuse with the effector cell membrane and release their deadly contents into the synapse (Figure 1.2). The subsequent delivery of granzymes into target cells is mediated by the pore-forming granule protein perforin, although the exact mechanism is unclear (Pipkin and Lieberman, 2007, Cullen and Martin 2008, Thiery et al., 2011). Originally, it was proposed that perforin forms pores in the plasma membrane of target cells through which granzymes may enter, however, there is little experimental evidence supporting this model. Alternatively, it has been suggested that perforin-generated pores are responsible for calcium influx, which subsequently triggers endocytosis of perforin and granzymes, followed by the perforin-mediated release of granzymes from endocytotic vesicles into the target cell cytosol (Thiery et al., 2010, Thiery et al., 2011).

While the fine details of how perforin facilitates granzyme delivery have yet to be universally accepted, knockout studies have unequivocally established the importance of perforin for granzyme function. Importantly, perforin-deficient mice exhibit profound defects in their ability to clear viral infections and tumour cells (Kagi et al., 1994). Moreover, these mice display an increased susceptibility to

Granzyme	Activity(specifity)	Expression	Substrates
Granzyme A	Tryptase (Arg/Lys)	Cytolytic CD4/CD8 T cells NK cells	SET Ape1 HMGB2 Ku70 histone H1 PARP-1 IL-1β
Granzyme B	Aspase (Asp/Glu)	Cytolytic CD4/CD8 T cells Regulatory CD4 T cells NK cells Mast cells Basophils Macrophages Keratinocytes B lymphocytes Spermatocytes Trophoblast cells Articular chondrocytes Plasmacytoid dendritic cells	Bid Caspase-3, -7, -8 ICAD NuMa PARP-1 DNA-PKcs Lamin B RockII Mcl-1 Topoisomerase I α-tubulin
Granzyme H	Chymotryptase (Phe/Leu)	Cytolytic CD8 T cells NK cells	Adenoviral protein 100K Adenoviral DBP
Granzyme K	Tryptase (Arg/Lys)	Cytolytic CD8 T cells	SET Ape1 HMGB2 Bid
Granzyme M	Metase (Met/Leu)	NK cells	ICAD PI-9 Nucleophosmin

Table 1.1

Human granzymes

A list of human granzymes with details of their substrate specifities, expression and non-exhaustive list of known substrates. Note that extracellular granzyme B substrates are listed separately (Table 1.2).

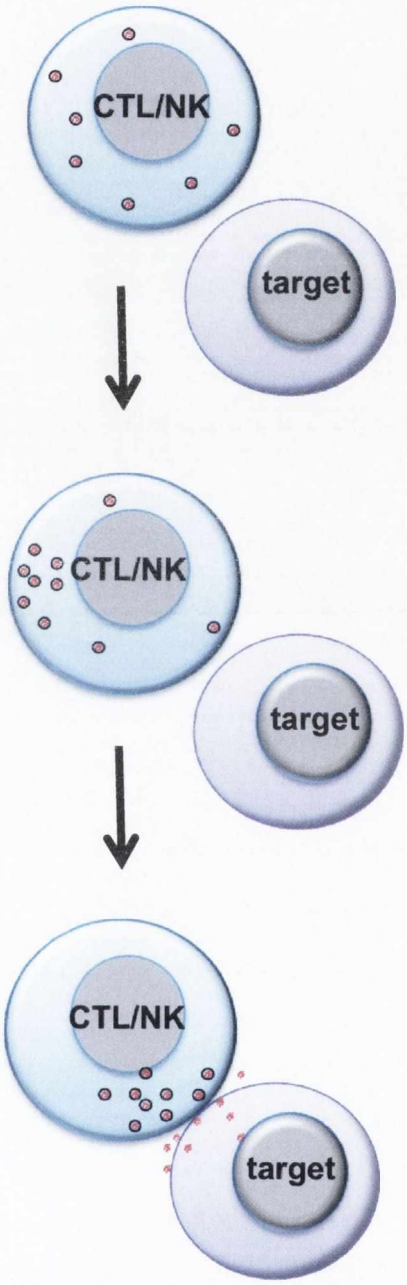


Figure 1.2

Schematic representation of cytotoxic granule exocytosis

Upon recognition of a target cell, cytotoxic granules of CTLs/NK cells cluster and move towards the contact site, followed by release of the granules into the synapse between effector and target cells.

viral or chemical carcinogenesis and develop spontaneous lymphomas, emphasising the critical importance of perforin in mediating the cytotoxic effects of granzymes (van den Broek et al., 1996, Smyth et al., 2000, Street et al., 2004). Surprisingly, mice deficient in individual granzymes display only minor deficiencies in clearing viral infections, suggesting a substantial redundancy of functions in the granzyme family (Simon et al., 1997, Pardo et al., 2004).

1.2.5 Granzyme B

Granzyme B is unique among serine proteases because of the strong preference to cleave its substrates after an aspartate acid residue (Asp), and most optimally after the tetrapeptide sequence Ile/ValGluProAsp, which is reminiscent of caspases (Thornberry et al., 1997). Unsurprisingly, granzyme B and apoptotic caspases cleave many of the same substrates (described below). Furthermore, granzyme B has been shown to induce apoptosis via direct activation of caspase-3, -7 and -8 (Martin et al., 1996; Medema et al., 1997).

Although granzyme B knockout mice do not display any dramatic defects, studies exploring CTL/NK-mediated killing showed that granzyme B-deficient effector cells induced target cell death with much slower kinetics, accompanied by attenuated DNA fragmentation and lack of caspase activation (Heusel et al., 1994, Pardo et al., 2004). This suggests that granzyme B is required for rapid and efficient target cell killing.

Granzyme B-initiated cell death pathway

Direct activation of apoptotic caspases by granzyme B is dispensable for the induction of apoptosis, as several reports have demonstrated that blocking caspase activation with specific inhibitors slows but does not entirely prevent granzyme B-initiated cell death (Sarin et al., 1997, Trapani et al., 1998, Cullen et al., 2007). On the other hand, overexpression of anti-apoptotic Bcl-2 proteins rescued cells from granzyme B-mediated apoptosis, suggesting that granzyme B initiates an alternative cell death pathway centering on mitochondria (Sutton et al., 1997, Davis et al., 2000). Subsequently, the pro-apoptotic Bcl-2 family protein Bid was identified as a granzyme B substrate (Barry et al., 2000). Cleavage of Bid facilitates its translocation to the mitochondrial membrane where it mediates oligomerisation of two other pro-apoptotic Bcl-2 family members BAX and/or BAK. Activation of the latter promotes mitochondrial outer membrane permeabilisation, followed by the release of cytochrome c and other mitochondrial intermembrane space proteins (Heiben et al., 2000, Kuwana et al., 2002). Release of cytochrome c into the cytosol is a key apoptosis event as it facilitates the formation of the apoptosome, a caspase-9 activation platform consisting of apoptotic protease activating factor-1 (APAF-1) and caspase-9 (Jiang et al., 2000). Activated caspase-9 then cleaves downstream effector caspases initiating a proteolytic cascade, which leads to cell death, characterised by chromatin condensation, oligonucleosomal DNA fragmentation, mitochondrial depolarisation and rapid loss of membrane integrity (Slee et al., 1999). This mitochondrial cell death pathway is regulated by the anti-apoptotic Bcl-2 family members, which form heterodimers with BAX/BAK proteins preventing their oligomerisation and inhibiting apoptosis

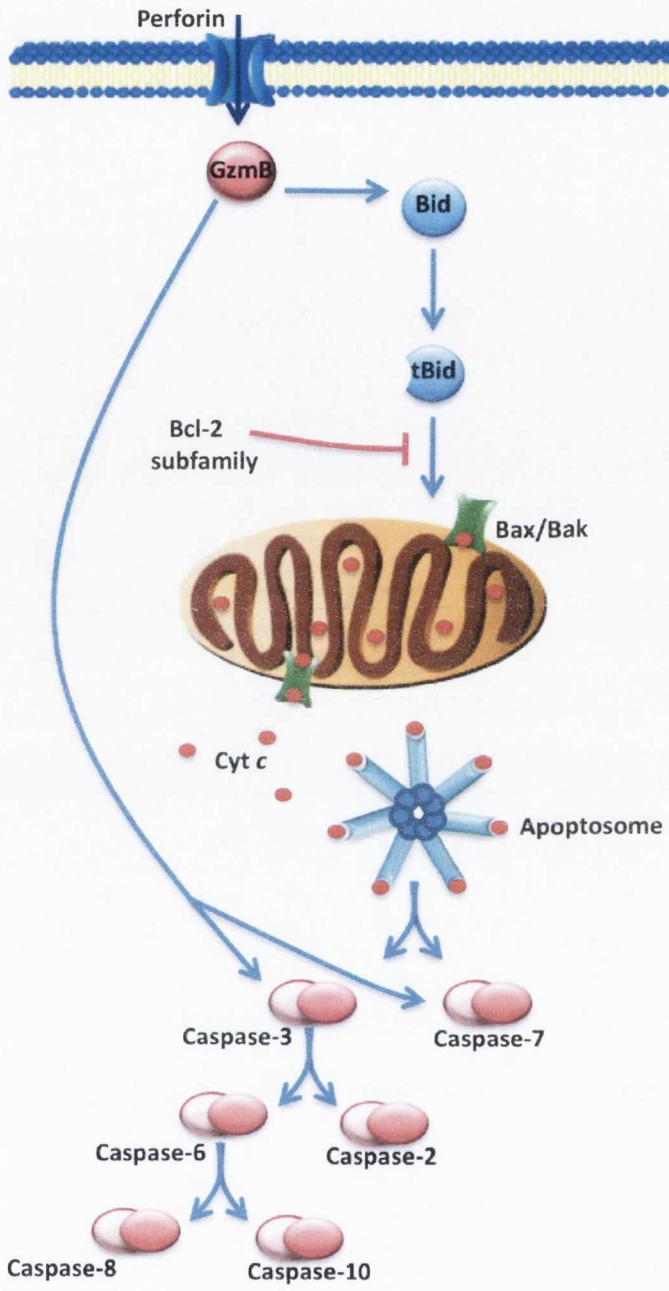


Figure 1.3.

Pathways to granzyme B-mediated cell death

Granzyme B, enters the target cell by a perforin-dependent mechanism. Once in the target cell cytosol, granzyme B processes Bid. Truncated BID then translocates to mitochondria where it induces the oligomerization of BAX and/or BAK in the outer mitochondrial membrane. This facilitates cytochrome c release into the cytosol, assembly of the apoptosome, with subsequent caspase-9 activation and the ensuing caspase cascade. Note that antiapoptotic Bcl-2 family members can inhibit cytochrome c release and thus block this pathway. Granzyme B can also directly process the effector caspases-3 and -7.

(Kluck et al., 1997). Figure 1.3 schematically depicts granzyme B-mediated killing pathways.

Interestingly, murine granzyme B has been demonstrated to cleave Bid very inefficiently and this granzyme displays a marked preference towards processing effector caspases (Cullen et al., 2007). Furthermore, addition of caspase inhibitors rescued cells from murine but not human granzyme B-mediated killing, suggesting that murine granzyme B may rely more heavily on direct caspase activation (Cullen et al., 2007). Collectively, these data showed that human and murine granzyme B exhibit divergent substrate preferences and rely on distinct killing pathways.

Other apoptotic substrates

Granzyme B has been shown to cleave other important apoptotic substrates, like inhibitor of caspase-activated DNAse (ICAD) (also cleaved by caspase-3), releasing the DNAse CAD to mediate internucleosomal DNA degradation characteristic of apoptosis (Thomas et al., 2000, Enari et al., 1998). Interestingly, α-tubulin was also recently identified as a granzyme B substrate, proteolysis of which enhances microtubule polymerisation rates *in vitro*, suggesting that granzyme B may perturb the microtubule network and affect the mitotic potential of target cells (Adrain et al., 2006). Aside from these, granzyme B directly targets other downstream caspase substrates, including poly (ADPribose) polymerase (PARP, Froelich et al., 1996), the catalytic subunit of DNA-dependent protein kinase (DNA-PK, Andrade et al., 1998), the nuclear mitotic apparatus protein (NUMA, Andrade et al., 1998), the nuclear envelope intermediate filament protein,

lamin B (Zhang et al., 2001) and the actin-binding protein, filamin (Browne et al., 2000). However, the significance of these proteolytic events for cellular demolition is unclear.

Additionally, granzyme B has also been shown to cleave a number of extracellular proteins, including cartilage proteoglycan (Froelich et al., 1993), the extracellular matrix proteins vimentin, fibronectin, and laminin (Buzza et al., 2005), cell surface receptors (Loeb et al., 2006) and pro-hemostatic molecules (Buzza et al., 2008). The significance of the proteolysis of these substrates will be discussed below.

1.2.6 Other granzymes

Granzyme A is a tryptase, which cleaves after arginine or lysine residues and induces caspase-independent cell death characterised by rapid loss of membrane integrity, mitochondrial depolarisation, chromatin condensation and formation of single-stranded DNA nicks. Importantly, granzyme A-induced cell death is not blocked by over-expression of Bcl-2 or by caspase inhibitors, suggesting that granzyme A does not utilise the intrinsic mitochondrial pathway and is not dependent on caspase activation (Beresford et al., 1999). Instead, granzyme A moves to the nucleus, where it cleaves the components of the endoplasmic reticulum (ER)-associated SET complex, interfering with the DNA repair machinery and activating endo- and exonucleases, which work in concert to degrade DNA and induce cell death (Beresford et al., 2001; Chowdhury et al., 2006; Fan et al., 2003).

Granzyme K is also a tryptase, which similarly to granzyme A, targets the SET complex within the nucleus (Zhao et al., 2007) and induces caspase-independent cell death, also characterised by single-stranded DNA nicks, mitochondrial depolarisation and loss of membrane integrity. Additionally, Bid was identified as an *in vitro* granzyme K substrate, suggesting that granzyme K potentially uses the mitochondrial pathway to induce apoptosis in target cells (Zhao et al., 2007b). However, the significance of this cleavage event is unclear as high concentrations of recombinant granzyme K were required to process Bid, while granzyme K-induced cell death was dependent on the production of reactive oxygen species and mitochondrial damage (Zhao et al., 2007b). Interestingly, recent work suggests that physiological concentrations of granzymes A and K lack cytotoxic potential and instead participate in the regulation of inflammatory cytokine secretion (discussed below, Metkar et al., 2008, Joeckel et al., 2011).

Granzyme H, which may have evolved from a recombination event between granzyme B and mast cell chymase, has chymotryptic activity and cleaves substrates after aromatic residues (Haddad, 1991, Fellows et al., 2007). No cellular granzyme H substrates have been found to date, however, granzyme H has been shown to cleave two adenoviral proteins: DNA binding protein (DBP, essential for viral DNA replication) and 100K protein (required for viral assembly and also acts as a granzyme B inhibitor) (Andrade et al., 2007; Andrade et al., 2001). These data suggest that granzyme H participates in the antiviral immune response by blocking viral replication and also indirectly promotes cell death by releasing granzyme B from its inhibitor.

Granzyme M, predominantly expressed by NK cells, preferentially cleaves after methionine or leucine residues (Smyth et al., 1996, Sayers et al., 2001). It induces

rapid cell death, characterised by swelling and extensive cell lysis, which cannot be blocked by the addition of caspase inhibitors or by overexpressing anti-apoptotic Bcl-2 proteins (Cullen et al., 2009). Similarly to granzyme B, granzyme M has been demonstrated to cleave ICAD, inducing DNA fragmentation (Lu et al., 2006). Moreover, granzyme M has also been shown to bind and inactivate proteinase inhibitor-9 (PI-9, the endogenous inhibitor of granzyme B) *in vitro*, suggesting that granzyme M may potentiate granzyme B activity by neutralising this inhibitor (Mahrus et al., 2004). Additionally, our laboratory has recently identified nucleophosmin, a nucleolar phosphoprotein essential for cell viability, as a novel granzyme M substrate, inactivation of which may contribute to target cell eradication (Cullen et al., 2009).

1.2.7 Granzyme inhibitors

Endogenous inhibitors

The activity of proteolytic enzymes must be tightly regulated to prevent any undesirable damage and there are several safety mechanisms which cooperate to keep granzymes in their inactive state while inside CTL/NK cells. Nevertheless, there are additional possibilities of active enzymes targeting 'innocent bystander' cells or leaking back to the effector cell cytoplasm during granule exocytosis. Endogenous granzyme inhibitors, belonging to a family of serpins (serine protease inhibitors), protect against such events and act as granzyme pseudosubstrates, covalently and irreversibly binding to the active sites of these proteases (Silverman et al., 2001).

PI-9 is the only intracellular inhibitor of human granzyme B and is expressed in various cells, including lymphocytes (Sun et al., 1996), dendritic cells, cells in immune privileged sites (Bladegroen et al., 2001), mast cells (Bladegroen et al., 2005), endothelial cells and mesothelial cells (Buzza et al., 2001), suggesting that PI-9 is capable of protecting not only effector cells themselves but also other cells in the vicinity.

Viral and synthetic inhibitors

Not surprisingly, as viruses have co-evolved with their hosts, they have also developed granzyme inhibitors, which allow postponement or complete inhibition of cell death, providing viruses with valuable time to complete the replication cycle and escape. For example, a pox virus-encoded serpin, cytokine response modifier (CrmA), directly binds to and inhibits granzyme B (Quan et al., 1995). Another viral inhibitor, adenoviral assembly protein Ad5-100K, acts as a granzyme B substrate with a very slow proteolysis rate (Andrade et al., 2001). Therefore, the abundance of 100K protein ensures saturation of available granzyme B, preventing the killer-protease from destroying infected cells. As was described earlier, granzyme H helps granzyme B to overcome this block by cleaving 100K (Andrade et al., 2001).

Synthetic granzyme inhibitors (isocoumarin derivatives, peptide chloromethyl ketones and peptide phosphonates) are also being developed for research and therapeutic purposes (Kam et al., 2000; Chowdhury and Lieberman, 2008). Unfortunately, the available synthetic inhibitors generally lack specificity and require further improvement.

1.3 Extracellular granzymes

Over the past 20 years the apoptotic functions of granzymes have attracted much attention and are well documented. However, detection of extracellular granzymes in plasma from patients with inflammatory conditions suggests that, under certain circumstances, these enzymes might have functions outside the cell (Spaeny-Decking et al., 1998, Tak et al., 1999). Several extracellular granzyme B substrates have been identified to date (see below). Moreover, expression of granzyme B has been repeatedly reported in non-cytotoxic cells of the immune system (reviewed in Afonina et al., 2010), further suggesting that granzymes are enzymes with a dual nature and, in addition to inducing apoptosis, might possibly perform extracellular functions.

1.3.1 Extracellular granzymes in inflammatory conditions

The development of sensitive assays has allowed investigators to detect low picogram quantities of granzyme A and B in plasma from healthy individuals (Spaeny-Dekking et al., 1998). Interestingly, levels of granzyme A and/or B were elevated in plasma from patients with EBV or HIV-1 infections, in plasma and synovial fluid from patients with rheumatoid arthritis and in bronchoalveolar lavage of atopic asthma patients challenged with an allergen (Spaeny-Dekking et al., 1998, Tak et al., 1999, Bratke et al., 2004).

Rheumatoid arthritis is a destructive disease, accompanied by bone and cartilage degradation in articular joints, which is largely mediated by extracellular proteases secreted by a variety of cells present at the inflamed site. Notably, the levels of

granzyme A and B in synovial fluid from rheumatoid arthritis patients were elevated up to low nanogram quantities, several hundred fold higher than in plasma from healthy individuals (Spaeny-Dekking, 1998; Tak et al., 1999). Moreover, Granzyme B levels in synovial fluid were significantly higher than in plasma from the same patients, suggesting that concentrations of extracellular granzymes are not elevated systemically, but increased as a result of the local production (Spaeny-Dekking et al., 1999). Indeed, granzyme B positive cytotoxic cells (mainly NKs) have been shown to infiltrate rheumatoid synovial tissue (Tak et al., 1994, Smeets et al., 1998), while expression of granzyme B was also detected in macrophages in the lesion areas of atherosclerosis and rheumatoid arthritis, further underscoring the possible role of granzyme B in inflammatory diseases (Kim et al., 2007). Importantly, granzyme B was reported to cleave several extracellular matrix components (see below) and is, therefore, thought to contribute to the pathogenesis of rheumatoid arthritis.

Elevated concentrations of both granzymes were found in plasma during experimental human endotoxemia and in patients with melioidosis (infection due to gram-negative bacteria), while whole blood stimulation with different gram-positive or -negative bacteria and lipopolysaccharide (LPS) also induced secretion of granzyme A and B (Lauw et al., 2000). Notably, based on the kinetics of granzyme release, the authors proposed that bacterial stimulation sends dual signals and induces an early release of pre-synthesized granzymes, followed by de novo synthesis of granzymes with secretion (Lauw et al., 2000). Collectively, these data imply that granzymes have important immunoregulatory functions during bacterial infection.

How do granzymes escape into extracellular millieu?

Several mechanisms have been suggested to explain how granzymes end up in the extracellular space, including leakage from the immunological synapse during granule-mediated killing, or non-specific secretion, or CTL/NK degranulation in response to inflammatory cytokines, chemokines and other signals (Buzza and Bird, 2006). Importantly, it has been shown that both CTLs and NK cells constitutively release granzyme B in the absence of stimulation or target cell engagement (Prakash et al., 2009). Moreover, various non-cytotoxic cells of the immune system were found to express and secrete granzyme B upon activation (see below), which, coupled with increased extracellular levels of this protease in inflammatory diseases, points to the existence of extracellular granzyme B functions.

The presence of the low levels of extracellular granzymes in plasma of healthy individuals is intriguing. It should be noted that granzyme B does not require perforin to enter cells and induce apoptosis in the presence of endosomolytic agents, such as adenovirus or pore-forming bacterial toxins (Froelich et al., 1996; Browne et al., 1999). Interestingly, treatment of vesicular stomatitis virus-infected monocytic cells with granzyme B resulted in the degradation of viral RNA without concomitant cell lysis, suggesting that constitutive secretion of granzyme B by the cytotoxic cells might serve as an immunosurveillance mechanism, where extracellular granzymes act as a first line of defence against invaiding viruses (Hommel-Berrey et al., 1997).

1.3.2 Extracellular functions of granzyme B

Proteolysis of extracellular matrix proteins

Detection of extracellular granzymes has prompted investigators to look for possible extracellular granzyme B functions and substrates. However, the first evidence for the existence of such functions comes from much earlier work, which reported the purification of a cytostatic factor from the rat natural killer cell line (RNK) (Sayers et al., 1992). The purified protein, structurally most closely related to murine granzyme B, inhibited growth of several adherent tumor cell lines and affected their morphology, inducing rounding of the cells and formation of large aggregates (Sayers et al., 1992).

This phenomenon was further investigated by Buzza and colleagues, who showed that exposure of primary endothelial cells to granzyme B in the absence of perforin leads to cell detachment followed by detachment-induced cell death (anoikis) (Buzza et al., 2005). Subsequently, extracellular matrix proteins vitronectin, fibronectin and laminin were identified as granzyme B substrates (Buzza et al., 2005), which was further supported by another study demonstrating that granzyme B, purified from UV-A irradiated keratinocytes, processed fibronectin *in vitro* (Hernandez-Pigeon et al., 2007). In addition, Pardo and colleagues found that treatment of endothelial cells with low concentrations of recombinant granzyme B led to disorganisation of cell-to-cell contacts, suggesting that granzyme B might also affect vascular permeability (Pardo et al., 2007). Thus, by cleaving extracellular matrix proteins, granzyme B disrupts integrin-dependent adhesion of cells and may induce anoikis, restrict motility of tumor cells and facilitate lymphocyte migration (Buzza et al., 2005).

Based on these data, granzyme B was suggested to contribute to the pathogenesis of inflammatory vascular diseases via induction of anoikis of smooth muscle cells (Choy et al., 2004). In fact, granzyme B was found in abundance in the blood vessels with advanced atherosclerosis or transplant vascular disease and its presence was associated with increased disease severity and cell death (Choy et al., 2003).

Granzyme B has been also shown to cleave extracellular matrix structural proteoglycan, aggrecan, and mediate proteoglycan degradation in the cartilage explants or the extracellular matrix newly synthesised by chondrocytes (Froelich et al., 1993, Ronday et al., 2001). This fact further encourages the idea that granzyme B plays an important role in the pathogenesis of rheumatoid arthritis by degrading cartillage aggrecan and remodelling extracellular matrix.

In addition, Buzza and colleagues have recently identified pro-hemostatic molecules, von Willebrand factor and fibrinogen, as granzyme B substrates, which were efficiently processed in a conformation-dependent manner: neither was processed in the soluble form, while presentation of these factors in a matrix configuration induced their proteolysis (Buzza et al., 2008). Once again, these proteolytic events had inhibitory effects on platelet aggregation and spread on the extracellular matrix, suggesting that granzyme B may regulate blood coagulation during inflammation (Buzza et al., 2008).

Cleavage of cell surface receptors

Granzyme B has also been shown to influence cell fate by cleaving cell surface receptors essential for proliferation and survival. Fibroblast growth factor receptor-1 (FGFR1) and Notch1 proved to be susceptible to granzyme B-mediated cleavage in an *in vitro* cDNA expression screen (Loeb et al., 2006) and more recently cleavage of Notch1 has been shown to inhibit its transcriptional activity, suggesting that proteolysis of these receptors renders them unable to transduce pro-survival signals (van Tetering et al., 2011).

Cleavage of neuronal glutamate receptor (GluR) by granzyme B was reported by Gahring and colleagues and is proposed to generate a potent autoantigen (Gahring et al., 2001). Moreover, conditioned medium from activated T cells was shown to remove GluR from the cell surface of normal human T cells, which was dependent on the activity of granzyme B in the medium, suggesting that granzyme B functions in the autocrine/paracrine fashion to down-regulate glutamate receptor on the surface of T cells (Ganor et al., 2007). Interestingly, Ganor and colleagues reported that glutamate mediates cell adhesion to laminin and fibronectin via GluR3 with loss of the receptor resulting in decreased adhesion of activated T cells to laminin (Ganor et al., 2003, Ganor et al., 2007). Thus, granzyme B may regulate T-lymphocyte migration not only through extracellular matrix remodelling, but also via inhibiting cell adhesion to the matrix itself. Figure 1.4 schematically summarises some of the extracellular granzyme B functions, while table 1.2 offers a list of extracellular granzyme B substrates and the functional consequences of proteolysis events.

One should keep in mind, however, that cleavage of extracellular substrates by granzyme B has not been confirmed *in vivo*. It is possible that processed fragments might not be detectable in healthy individuals, due to the insufficient quantities of extracellular granzymes or their substrates (low picogram range) *in vivo*. The dramatic elevation of extracellular granzyme B concentrations in inflammatory conditions should facilitate further investigation of the cleavage of extracellular substrates.

1.3.3 Granzyme B expression in non-cytotoxic cells

Extracellular functions of granzymes might explain probably the most intriguing aspect of granzyme biology - the expression of granzymes in non-cytotoxic cells. Surprisingly, granzyme B expression has been detected in plasmacytoid dendritic cells (Rissoan et al., 2002), a subset of human primary breast carcinomas (Hu et al., 2003), human articular chondrocytes (Horiuchi et al., 2003) and in the cells of the reproductive system (Hirst et al., 2001; Sasson et al., 2003). Moreover, activated mast cells (Strik et al., 2007), basophils (Tschopp et al., 2006), B lymphocytes (Hagn et al., 2009), macrophages (Kim et al., 2007), UV-irradiated keratinocytes (Hernandez-Pigeon et al., 2006; Hernandez-Pigeon et al., 2007) and septic platelets (Freishtat et al., 2009) have all been reported to express granzyme B, mostly in the absence of perforin co-expression (Table 1.3).

Freshly isolated polymorphonuclear neutrophils have been shown to express granzyme B and perforin (Wagner et al., 2004; Wagner et al., 2008). However, others were not able to reproduce this result (Metkar and Froelich, 2004; Grossman and Ley, 2004, Tschopp et al., 2006), possibly due to the use of

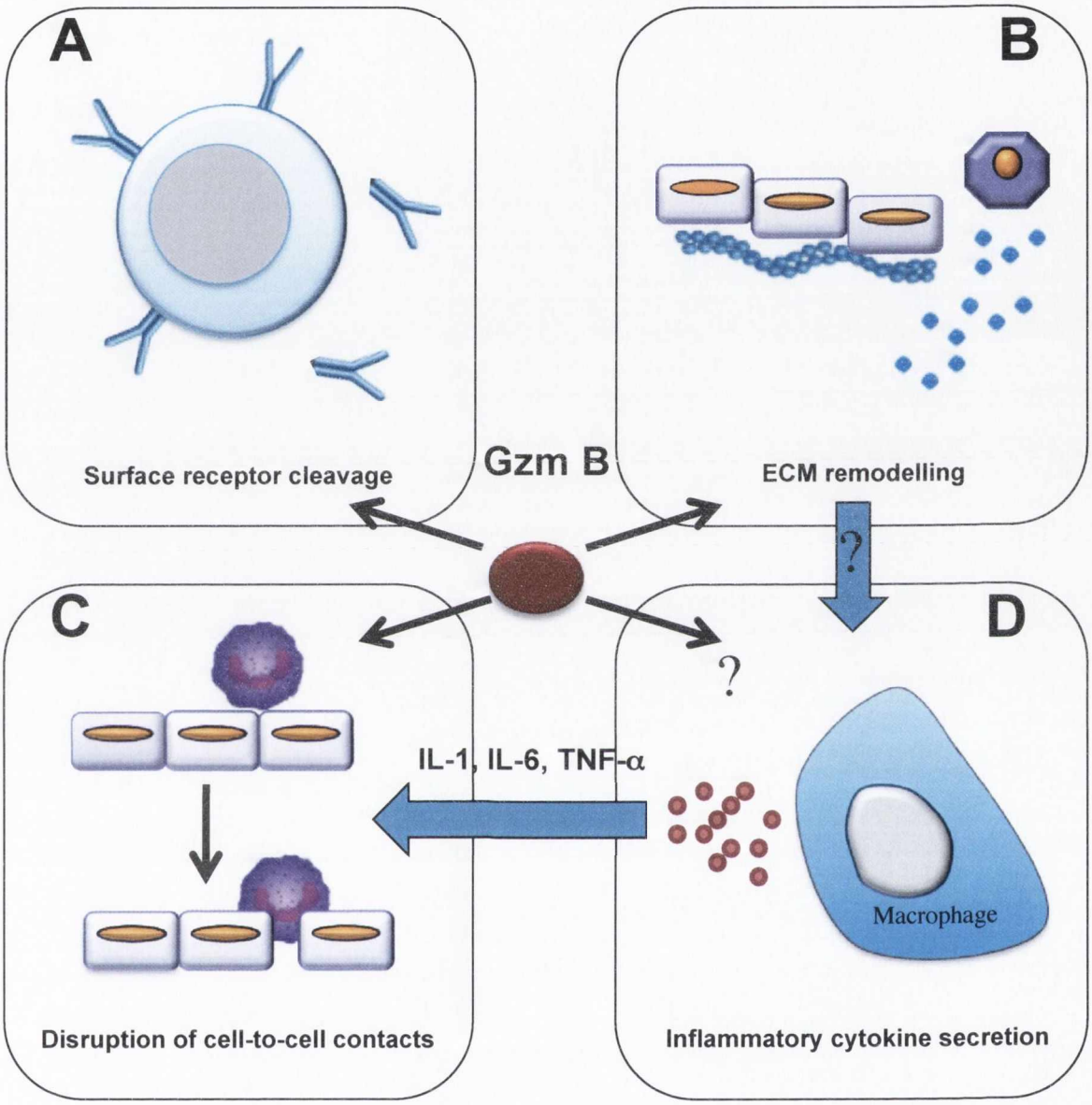


Figure 1.4

Extracellular roles of granzyme B

Extracellular granzyme B has been demonstrated to cleave cell-surface receptors (a) and ECM proteins (b). Cleavage of ECM proteins may result in cell detachment and death (b) or cause disruption of cell-cell contacts, which may increase vascular permeabilisation and, thus, facilitate leukocyte extravasation (c). Some ECM protein cleavage fragments have been reported to act as danger signals and activate macrophages to secrete proinflammatory cytokines (d). Like granzyme A, granzyme B may also directly activate macrophages. However, this requires further investigation.

Functional group	Substrate	Cleavage consequences
ECM proteins	Vitonectin Laminin Fibronectin	ECM remodelling Regulates cell attachment and migration Potential implication in "danger" signalling and neutrophil chemotaxis
	Aggrecan	ECM remodelling
Cell surface receptors	FGFR-1	Inhibits pro-survival signals
	Notch1	Inhibits pro-survival signals
	GluR3	Production of auto-antigen Inhibits glutamate-induced lymphocyte attachment to ECM and migration
Serum proteins	von WF	Inhibits platelet attachment, aggregation and migration
	Fibrinogen	Unknown, potential implication in "danger" signalling
	Plasminogen	Potential anti-angiogenic effect

Table 1.2.

Extracellular granzyme B substrates

List of extracellular granzyme B substrates and possible functional consequences of granzyme B-mediated proteolysis.

Cell type	Stimulus	Perforin expression
CTL/NK cells	Receptor-mediated activation	+
Mast cells	Ionomycin/PMA	-/+
Basophils	IL-3	-
B-cells	BCR stimulation	-
Keratinocytes	UV-A/UV-B	-/+
Macrophages		?
Platelets	Sepsis	?
Articular chondrocytes		+
Plasmacytoid dendritic cells	Freshly isolated, IL-3	-
Breast carcinoma cells		?

Table 1.3.

Granzyme B-expressing cells

Non-exhaustive list of cells expressing granzyme B, with details of perforin co-expression and inducing stimuli.

different detection antibodies and Fc receptor blocking techniques. Further studies are required to completely resolve this issue.

In the case of basophils, IL-3 or supernatants from activated mast cells induce expression of granzyme B, which is then sorted to cytoplasmic granules and released upon treating cells with exocytosis triggering stimuli (Tschopp et al., 2006). Secretion of granzyme B by basophils was also confirmed *in vivo* after experimental allergen challenge of asthmatic patients, suggesting a potential role for this protease in asthma (Tschopp et al., 2006).

Human and mouse mast cells also express and secrete granzyme B, which was subsequently shown to induce detachment-induced death of mouse embryonic fibroblasts (Strik et al., 2007; Pardo et al., 2007). Interestingly, PMA/ionomycin-stimulated HMC-1 cells, which were shown to express both granzyme B and perforin, also dramatically up-regulated expression of granzyme B inhibitor, PI-9, in this way, acquiring protection from their own granzymes (Bladegroen et al., 2005).

UV-B irradiation induces expression of both granzyme B and perforin in keratinocytes, which provides these cells with some degree of cytotoxic potential against a variety of targets *in vitro*, including keratinocytes themselves, melanocytes and lymphocytes (Hernandez-Pigeon et al., 2006). UV irradiation causes DNA damage and it is, therefore, plausible that UV-induced cytotoxicity enables keratinocytes to eliminate potentially damaged skin cells that reside in the close proximity within the epidermis.

In contrast to UV-B, UV-A fails to contribute to keratinocyte killing potential and induces granzyme B expression in the absence of perforin (Hernandez-Pigeon et

al., 2007). As was described earlier, granzyme B is proposed to induce cell detachment and inhibit migratory potential of cells via degradation of extracellular matrix proteins. Consequently, ablation of granzyme B expression with antisense oligonucleotide inhibited UV-A induced cell detachment and enhanced keratinocyte motility upon UV-A exposure (Hernandez-Pigeon et al., 2007). Taken together, these data implicate granzyme B in photoaging and photocarcinogenesis, where the protease potentially protects skin cells against UV stress.

Additionally, analysis of platelet mRNA expression profiles revealed more than a hundred fold up-regulation of granzyme A and granzyme B in platelets isolated from septic mice, which appeared to be cytotoxic against CD4+ splenocytes in an ex vivo setting, while septic platelets from granzyme B-deficient mice lacked cytotoxic activity (Freishtat et al., 2009). However, the physiological significance of the killing potential of non-cytolytic cells is unclear as the studies described above used extremely high effector to target ratios, which may be difficult to achieve *in vivo*.

Finally, B lymphocytes have been shown to express and secrete active granzyme B after IL-21 and B cell receptor (BCR) stimulation (Hagn et al., 2009). Interestingly, the latter authors also found that B cells, isolated from individuals previously vaccinated against viral diseases, secreted higher amounts of granzyme B upon viral antigen re-stimulation than the cells from unvaccinated individuals (Hagn et al., 2009). This finding suggests that B cells participate in the early phase of the immune response to viral infections and may explain elevated concentrations of granzyme B in plasma from patients with EBV or HIV-1 infections (Spaeny-Dekking et al., 1998).

It remains largely unclear why non-cytotoxic cells express granzyme B, especially in the absence of perforin. Possibly granzyme B expression confers on these cells a direct cytotoxic potential or ability to induce anoikis in certain circumstances. This may allow for the immediate elimination of dangerous cells without the need for activation and recruitment of cytotoxic T lymphocytes. Additionally, release of granzymes at inflammatory sites may help to increase vascular permeability, facilitating extravasation of lymphocytes from the blood, suggesting that granzymes may regulate inflammatory processes.

1.4 Inflammation

Inflammation is the orchestrated response of an organism to harmful stimuli, such as microbial invasion, damaged cells or irritants, and is accompanied by immune cell activation with the release of inflammatory mediators (cytokines, chemokines, prostaglandins etc.), vasodilation and lymphocyte extravasation to the affected area. Proinflammatory mediators are primarily released by the cells of the innate immune system as a response to activating stimuli, or directly liberated from the intracellular stores of damaged cells, consequently inducing the vascular endothelium to express chemokines and adhesion molecules. This, in turn, attracts leukocytes and permits these cells and other blood-borne defences, such as antibodies or complement proteins, to enter the damaged tissue and destroy injurious agents, clear dead cells and induce tissue repair.

1.4.1 How does the immune system sense danger?

Pathogens possess highly conserved invariant molecular motifs, distinct from the host, termed pathogen associated molecular patterns (PAMPs) that, being critical cellular components (e.g., LPS, dsRNA, flagellin), serve as a microbial 'fingerprint' and allow the immune system to identify the invaders. However, certain host molecules can also act as signals of danger, and are, thus, called danger associated molecular patterns (DAMPs). These self-molecules are normally sequestered within cells and released primarily as a result of cell injury and death, alerting the immune system about possible infection. They are thought to include ATP, uric acid, galectins, thioredoxin, high-mobility group box I protein (HMGB1) and IL-1α (Bianchi, 2007, Chen et al., 2007).

In order to be able to identify danger signals, the immune system is equipped with a set of germline-encoded pattern-recognition receptors (PRR), which are predominantly expressed by the cells that patrol the borders, like macrophages, monocytes, dendritic cells, neutrophils and epithelial cells. These receptors are triggered by microbial PAMPs or DAMPs and mobilise different mechanisms of defense.

Toll-like receptors (TLRs) are highly conserved transmembrane proteins that function either on the plasma membrane or the membranes of endosomal compartments and, therefore, are responsible for scanning the extracellular space for the signs of bacterial or viral invaders and activating signal transduction cascades, which culminate in the secretion of inflammatory cytokines, chemokines and antimicrobial peptides. TLR signalling pathways are largely similar to IL-1 signalling, which will be described below.

Unlike TLRs, nucleotide-binding oligomerisation domain (NOD)-like receptors (NLRs) are cytosolic sensors of bacterial infection and, upon ligand recognition, initiate formation of the multiprotein complex, termed the inflammasome (see Figure 1.5). The ultimate goal of inflammasome assembly is the proteolytic processing and activation of a major pro-inflammatory protease, caspase-1, achieved via proximity-induced auto-processing between the large and small subunits of caspase-1 (Schroeder and Tschopp, 2010). Activated caspase-1 then processes the major pro-inflammatory cytokines, IL-1β and IL-18, which may also facilitate their secretion into the extracellular space (Thornberry et al., 1992, Gu et al., 1997). Figure 1.5 depicts NALP3 inflammasome assembly and the consequences of caspase-1 activation. Importantly, activating mutations in NLRs have been described in patients with autoinflammatory diseases, who suffer from recurrent inflammatory attacks in the absence of infection, possibly due to the excessive production of IL-1β, implying that controlled activation of the inflammasome is crucial for maintaining efficient homeostasis of the immune system (Schroeder and Tschopp, 2010).

1.4.2 Caspase-1 functions

Caspase-1 is also known as IL-1β-converting enzyme (ICE) for its ability to convert inactive 31 kDa IL-1β precursor into the mature 17 kDa active form (Thornberry et al., 1992, see below). Additionally, another inflammatory cytokine, IL-18, has been identified as a physiological caspase-1 substrate (Gu et al., 1997). The central role caspase-1 plays in the regulation of inflammatory processes has been demonstrated in caspase-1-deficient mice, which are highly resistant to LPS-

induced endotoxic shock and exhibit profound defects in the production and secretion of IL-1β, IL-18 and, more surprisingly, another IL-1 family member, IL-1α (Li et al., 1995, Kuida et al., 1995). Importantly, regulation of apoptosis in caspase-1 knockout mice is normal, suggesting that, contrary to initial thinking, caspase-1 is dispensable for the control of cell death (Kuida et al., 1995).

Despite several attempts to identify novel caspase-1 substrates, IL-1β and IL-18 remain the only two physiological substrates for this protease, which is in striking contrast to over 600 apoptotic caspase substrates discovered to date (Lüthi and Martin, 2007). In addition, it appears that the half-life of caspase-1 within the cell is limited, as this protease appears to be extremely unstable and is rapidly inactivated, which may ultimately restrict its availability within the cell and reduce substrate proteolysis (Walsh et al., 2011).

The functions of other inflammatory caspases are less well defined. Activation of caspase-5 was reported as a result of its recruitment to the NALP1 inflammasome (Martinon et al., 2002), although no physiological substrates have been reported for caspase-4 or -5. Similarly to caspase-1 deficiency, it is interesting to note that the absence of caspase-11 (murine analog of caspase-5) in mice resulted in increased resistance to endotoxic shock and decreased serum concentrations of IL-1 in response to LPS-stimulation, implying that caspase-11 (and possibly caspase-5 in humans) is involved in caspase-1 activation *in vivo* (Wang et al., 1998).

Recently, a range of bacterial pathogens have been reported to induce caspase-1-dependent cell death, termed pyroptosis, characterised by rapid membrane rupture and release of intracellular contents (Bergsbacken et al., 2009). Although

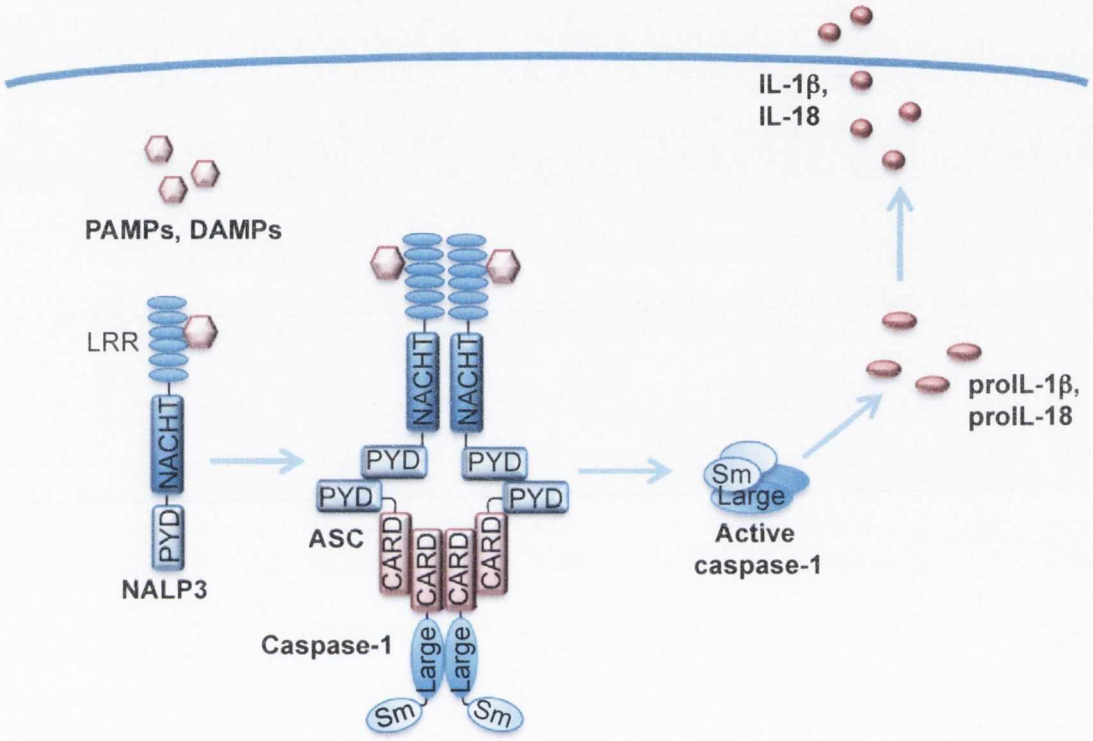


Figure 1.5

Assembly of the NALP3 inflammasome

Upon ligand binding via LRR domain, NALP3 oligomerises and initiates inflammasome formation via recruitment of the adaptor protein, apoptosis-assocciated speck-like protein containing CARD (ASC), which in turn brings caspase-1 to the complex. Caspase-1 then undergoes proximity-induced autoactivation, followed by the processing of inflammatory cytokines. LRR, leucine-rich repeats; NACHT, NACHT domain; PYD, pyrin domain; CARD, caspase recruitment domain; Large, large subunit; Sm, small subunit.

the dependence of pyroptosis on caspase-1 has been demonstrated in caspase-1-deficient cells, the exact pathway leading to cell death is obscure (Lamkanfi et al., 2008). As the apoptotic caspase, caspase-7, has been demonstrated to be a novel caspase-1 substrate, it is appealing to speculate that the activation of the former mediates the execution of pyroptosis (Lamkanfi et al., 2008). However, caspase-7 deficiency did not protect from Salmonella typhimurium-induced caspase-1-dependent cell death (Lamkanfi et al., 2008). Thus, the physiological significance of this cleavage event and the exact role that caspase-1 plays in pyroptosis require further investigation.

1.4.3 Interleukin-1

As described in chapter 4 of this work, our research group has recently tested cleavage of cytokines by a panel of apoptotic proteases and identified IL-1α as a granzyme B substrate. Accordingly, the following section will focus on the biology of this major pro-inflammatory cytokine. There are two forms of IL-1; IL-1α and IL-1β. Both share only 26% homology but exhibit similar bioactivities, affecting almost every cell type either alone or in concert with other cytokines and inflammatory mediators. The basis for the range of biological effects of IL-1 is the influence this cytokine has on the rest of the immune system, namely, the expression of other cytokines and their receptors, proinflammatory mediators, hepatic acute phase proteins, colony-stimulating, growth, clotting and tissue remodelling factors, adhesion molecules and many others (Dinarello, 1996). Therefore, IL-1 promotes vasodilation, hematopoiesis, lymphocyte activation (hence, the alternative name lymphocyte activating factor (LAF)), leukocyte

attraction and extravasation, angiogenesis and antibody synthesis (adjuvant effect).

IL-1α

Primary cells and some cell lines express constitutive levels of a 31 kDa IL-1α precursor, however, IL-1α is rarely found in body fluids and remains mostly intracellular (Hacham et al., 2002, Hurgin et al., 2007, Werman et al., 2004). Processing of the IL-1α precursor by a calcium-activated neutral protease, calpain, has been reported, although the effect of proteolysis on the bioactivity of this cytokine was not investigated (Carruth et al., 1991; Kobayashi et al 1990). As both precursor and mature forms of IL-1α are able to bind the IL-1 receptor (IL-1R) and elicit biological activity, cleavage of IL-1α by calpain was proposed to affect the selective secretion of the mature form, although this phenomenon requires further investigation (Mosley et al., 1987a; Watanabe and Kobayashi, 1994).

Interestingly, the IL-1α precursor possesses a nuclear localisation signal (NLS) in its N-terminus and was shown to translocate to the nucleus upon inflammatory stimulation, suggesting an intracellular signalling role (Wessendorf et al., 1993; Werman et al., 2004). In support of this hypothesis, overexpression of full-length IL-1α resulted in the activation of transcription factors nuclear factor κB (NFκB) and activator protein-1 (AP-1) in a surface IL-1R-independent manner (Werman et al., 2004). Furthermore, stable transfection of precursor IL-1α, but not the mature form lacking the NLS, has been demonstrated to regulate morphology, growth rate and migratory potential of human endothelial cells (Maier et al., 1994, McMahon et al., 1997).

Additionally, IL-1α was reported to exist in a membrane-bound form, where biologically active precursor IL-1α binds to the plasma membrane via lectin-like interactions and exhibits potent thymocyte stimulatory activity (Kurt-Jones et al., 1985, Kurt-Jones et al., 1987, Brody and Durum, 1989). Moreover, LPS-stimulated human peripheral blood mononuclear cells (PBMC) were shown to induce IL-8 secretion from endothelial cells via IL-1α-dependent juxtacrine mechanism (Kaplanski et al., 1994). Most interestingly, the severity of arthritis in IL-1α transgenic mice was found to correlate with membrane-bound IL-1α activity, rather than the soluble form of this cytokine, underscoring the important role that membrane-bound IL-1α plays in the regulation of inflammatory processes (Niki et al., 2004).

IL-1β

Unlike IL-1α, IL-1β is synthesised as inactive 31 kDa precursor, unable to bind the IL-1 receptor, and activation of which requires removal of 116 N-terminal amino acids by caspase-1 (Mosley et al., 1987a, Black et al., 1988; Thornberry et al., 1992). Additionally, IL-1β was reported to be susceptible to cleavage by extracellular proteases: trypsin, cathepsin G (Hazuda et al., 1991), collagenase, elastase (Hazuda et al., 1990), mast cell chymase (Mizutani et al., 1991) and granzyme A (Irmler et al., 1995) *in vitro*.

The importance of IL-1β processing by these proteases was demonstrated in a mouse model of neutrophil-dominated arthritis, in which disease severity was strongly correlated with serum IL-1β concentrations (Joosten et al., 2009, Guma et al., 2009). Interestingly, Joosten and colleagues found that arthritis progression

was similar in caspase-1 knockout and wild type mice in several models of arthritis (Joosten et al., 2009). Moreover, the concentration of IL-1β in synovial tissue explants from caspase-1-deficient mice was even slightly higher than in wild type, suggesting that caspase-1 is dispensable for IL-1β processing in certain disease models.

Guma and colleagues reported a similar observation in a serum transfer model of arthritis and monosodium urate (MSU)-induced peritonitis (Guma et al., 2009). Importantly, the authors showed that treatment of caspase-1 knockout mice with elastase or chymase synthetic inhibitors reduced joint swelling in serum-induced arthritis model, which correlated with decreased concentrations of IL-1β in tissue samples. Collectively, these data convincingly show that IL-1β is processed and activated by multiple proteases *in vivo*.

IL-1-deficient mice

Mice deficient in IL-1α or IL-1β develop normally and are healthy and fertile, which suggests that IL-1 is dispensable for normal embryonic and postnatal development (Zheng et al., 1995, Horai et al., 1998). However, IL-1β, but not IL-1α, knockout mice have been demonstrated to mount an impaired febrile and acute-phase inflammatory response to stimulation with turpentine (a model of local inflammation), implying that IL-1β plays a more important role in certain inflammatory scenarios and, despite having identical biological functions, IL-1α and IL-1β are not functionally redundant (Zheng et al., 1995, Horai et al., 1998). Data regarding the febrile response to LPS stimulation in IL-1β-deficient mice are conflicting (Kozak et al., 1995, Alheim et al., 1997). However, several groups have

shown that IL-1β knockout mice display normal susceptibility to LPS challenge and have normal serum levels of inflammatory cytokines, possibly due to IL-1α compensating for the lack of IL-1β in this model of systemic inflammation (Zheng et al., 1995, Fantuzzi and Dinarello, 1996). In support of the hypothesis, mice deficient in caspase-1, which exhibit reduced serum levels of both forms of IL-1, are resistant to endotoxic shock (Li et al., 1995).

1.4.4 IL-1 secretion

IL-1α and IL-1β are expressed without a signal peptide and secreted through an unconventional, ER/Golgi-independent pathway, which is poorly characterised. However, exocytosis of secretory lysosomes, microvesicle shedding from plasma membrane, transporter protein-facilitated efflux and passive release as a result of cell lysis have all been suggested as possible mechanisms of IL-1 secretion (reviewed in Eder, 2009). Interstingly, inflammasome assembly and caspase-1 activation were shown to be absolutely required for IL-1β secretion from murine macrophages (Qu et al., 2007). Moreover, Keller and colleagues demonstrated that IL-1α secretion is also dependent on caspase-1, as caspase-1-deficient keratinocytes failed to secrete IL-1α in response to UV-treatment (Keller et al., 2008).

Importantly, caspase-1 activity was required for the secretion of IL-1α and overexpression of a catalytically inactive caspase-1 mutant resulted in the reduced IL-1α release from COS-1 cells (Keller et al., 2008). Considering that IL-1α is not a substrate for caspase-1, the latter observation was surprising. However, these data support an earlier observation reported by Li and colleagues, who found

reduced levels of serum IL-1α in LPS-challenged caspase-1 knockout mice (Li et al., 1995), which, due to the fact that IL-1 has been demonstrated to induce its own synthesis (Dinarello et al., 1987, Horai et al., 1998), was initially explained by the absence of mature IL-1β. However, serum IL-1α levels in LPS-challenged IL-1β-deficient mice were normal, suggesting that secretion of IL-1α is, indeed, dependent on the presence of caspase-1 itself (Fantuzzi and Dinarello, 1996).

1.4.5 IL-1 receptor signalling

IL-1 exerts its extracellular activities via binding to a high affinity receptor, IL-1RI, which is an 80kDa transmembrane glycoprotein expressed on the surface of a wide range of cells. The extracellular domain of the receptor is composed of three immunoglobulin-like domains, while the intracellular Toll/IL-1R (TIR) domain bears homology to the corresponding domain of the Toll-like receptors. Upon ligand binding, IL-1R aggregates with IL-1 receptor accessory protein (IL-1RAcP) and triggers the formation of a signalling complex (Greenfeder et al., 1995, Huang et al., 1997). Figure 1.6 represents the IL-1R signalling pathway.

The first step in the signalling cascade requires the involvement of the myeloid differentiation protein MyD88, which is an adaptor protein, composed of two domains (Muzio et al., 1997). A C-terminal TIR domain mediates interaction with the receptor complex, while an N-terminal death domain facilitates the recruitment of downstream effector molecules (Figure 1.6a, Burns et al., 1998). The indispensable role of MyD88 in IL-1R signalling was demonstrated in MyD88-deficient mice, which exhibit complete loss of inflammatory responses to IL-1 and IL-18 treatment (Adachi et al., 1998).

Within seconds of ligand binding, IL-1 receptor-associated kinase-1 (IRAK-1) is recruited to the IL-1R complex and becomes phosphorylated by another member of the IRAK family, IRAK-4, followed by active autophosphorylation, which increases its affinity to another adaptor protein, TNF receptor-associated factor 6 (TRAF6) (Figure 1.6a, Cao et al., 1996a,b; Jiang et al., 2002; Burns et al., 2003). Importantly, mice deficient in IRAK-1 have impaired cytokine signalling in response to IL-1 (Kanakaraj et al., 1999).

TRAF6 is characterised by an N-terminal RING domain, which posesses ubiquitin ligase activity, generating the lysine (Lys)-63-linked polyubiquitin chains, absolutely required for IL-1-mediated signalling (Cao et al, 1996b, Deng et al., 2000, Lamothe et al., 2007). The IRAK-1/TRAF6 complex dissociates from IL-1R and interacts at the membrane with the preformed complex consisting of transforming growth factor-β (TGF-β)-activated kinase 1 (TAK1) and TAK binding proteins, TAB1 and TAB2/3 (Takaesu et al., 2000, Jiang et al., 2002). This interaction results in the activation of TAK1 and the translocation of the TRAF6/TAK1/TAB complex to the cytosol (Figure1.6b, Jiang et al., 2002; Wang et al., 2001).

The signalling pathway then diverges at activated TAK1, which phosphorylates and activates the IκB kinase (IKK) complex, mediating degradation of NFκB inhibitor, IkB (Figure 1.6c, Wang et al., 2001). In unstimulated cells, IκB binds the key transcription factor NFκB and prevents it from being transported to the nucleus (reviewed in Karin and Ben-Neriah, 2000). Thus, degradation of IκB results in the release of NFκB from the cytoplasm and translocation to the nucleus, where it can induce expression of numerous inflammatory mediators. Additionally, IKKβ has also been reported to induce activation of extracellular signal-regulated kinase-1

and -2 (ERK1/2) via phosphorylation and degradation of another NFκB family member, NFκB1/p105 (Waterfield et al., 2004).

Alternatively, TAK1 activates mitogen-activated protein kinase MAPK pathways, followed by the mobilisation of p38 and Jun N-terminal kinase (JNK) (Figure 1.6d), which culminates in the activation of cAMP response-element binding (CREB) and AP-1 transcription factors (Wang et al., 2001, Dong et al., 2002). Collectively, activation of these transcription factors results in the induction of pro-inflammatory gene expression.

1.4.6 Sterile inflammation

In addition to infection, a variety of host substances are capable of inducing inflammatory processes. For example, deposition of MSU crystals in the joints of patients with hyperuricemia initiates an inflammatory condition, known as gout (Nuki and Simkin, 2006), while inhalation of external silicate particles causes lung inflammation, leading to pulmonary fibrosis (Mossman and Churg, 1998). These conditions are characterised by IL-1-dependent neutrophil-driven responses (Chen et al., 2006, So et al., 2007). Importantly, neutralisation of IL-1 with specific antibodies inhibited neutophil infiltration in response to MSU in a mouse model, while IL-1R blockade relieved the symptoms of MSU-induced gout in human patients, underscoring the importance of IL-1 in the neutrophil-driven sterile inflammation (Chen et al., 2006, So et al., 2007).

Unlike apoptotic cells, necrotic cells and their debris also induce inflammatory responses *in vivo*. Interestingly, neutrophil infiltration induced by necrotic cells was

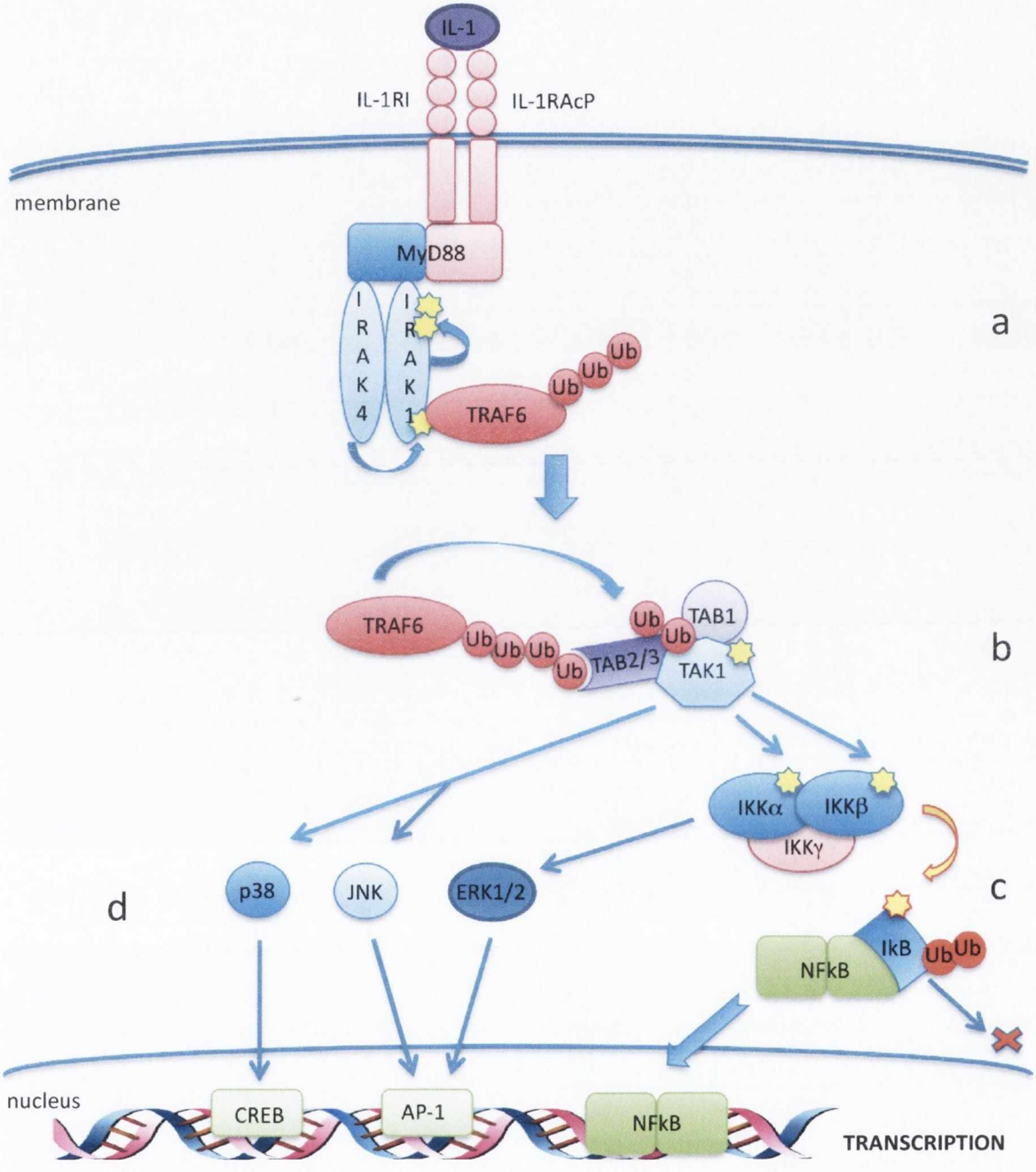


Figure 1.6

IL-1R signalling pathway

(a) Upon ligand (IL-1) binding IL-1R dimerises with IL-1RAcP and recruits adaptor proteins MyD88, IRAKs and TRAF6. (b) Consequently, TRAF6 binds and activates TAB/TAK1 complex in the cytosol. (c, d) The signalling pathway then diverges at TAK1, leading to the activation of NF-kB (c) and MAPK (d) pathways, which results in the nuclear translocation of transcription factors and activation of transcription.

found to be dependent on MyD88 and IL-1R and was inhibited by a neutralising antibody to IL-1α (Chen et al., 2007). Although several studies have demonstrated that, upon release from dying cells, IL-1α precursor initiated proinflammatory signalling (Eigenbrod et al., 2008, Cohen et al., 2010), Kono and colleagues reported that resident host macrophages, and not necrotic cells, are the primary source of IL-1α, releasing the cytokine in response to dying cells (Kono et al., 2010). Thus, IL-1α has been shown to act both as a danger signal, released from dying cells, and as a downstream messenger, secreted in response to as yet undefined danger signals.

1.5 Granzymes and inflammation

1.5.1 Granzymes and danger signals

Danger signals, or alarmins, released from host cells as a result of cell injury, activate antigen-presenting cells via stimulation of conserved cell surface receptors and initiate immune responses (Bianchi, 2007). Interestingly, two extracellular substrates of granzyme B, fibrinogen and fibronectin, were recently implicated in danger signalling and shown to induce pro-inflammatory cytokine secretion from mouse splenocytes and human monocytes (Kuhns et al., 2007, Okamura et al., 2001). It is, therefore, interesting to speculate that cleavage of these substrates by granzyme B results in the liberation of protein fragments, which subsequently mediate pro-inflammatory signal transduction. Alternatively, proteolysis by granzyme B might destroy the immunostimulatory capacities of these extracellular matrix proteins and dampen the immune response. Further

studies are required to understand the functional consequences of granzyme B-mediated cleavage of fibronectin and fibrinogen on the pro-inflammatory signalling.

Heat-shock proteins (HSPs) are also speculated to function as danger signals. Besides their ability to facilitate the internalisation of antigenic peptides by APCs, HSP were shown to activate APCs via TLR stimulation, although the observed TLR-pathway activation may have been caused by contaminating LPS (Osterloch and Breloer, 2008). Of note, Hsp70 is capable of binding granzyme B and mediating its cellular internalisation in the absence of perforin (Gross et al., 2003). Moreover, stimulation of NK cells with Hsp70 resulted in a dramatic enhancement of granzyme B expression in NK cells and increased cytotoxicity against tumor cells, expressing membrane form of Hsp70 (Gross et al., 2003). However, the impact of the Hsp70 and granzyme B interaction on the immune system activation is still unclear and requires further investigation.

1.5.2 Granzymes and pro-inflammatory cytokines

The current literature regarding the extracellular roles of granzymes suggests a firm link between granzymes and inflammatory cytokine production. For example, Sower and colleagues showed that addition of granzyme A to various primary human cells induced secretion of pro-inflammatory cytokines (IL-1β, IL-6, IL-8, TNF-α), which was accompanied by increased phagocytic activity of monocytes (Sower et al., 1996a,b, Metkar et al., 2008). Moreover, intact cytotoxic cells (NK-92) induced IL-1β and TNF-α secretion by adherent PBMC (Metkar et al., 2008). These effects were proposed to be dependent on the granzyme A-mediated cleavage of the thrombin receptor, which was also shown to induce IL-6

production in human lung fibroblasts upon cleavage with thrombin (Sower et al., 1995). Alternatively, Metkar and colleagues reported that cytokine induction was dependent on granzyme A internalisation and was blocked by a specific caspase-1 inhibitor (Metkar et al., 2008). Notably, granzyme A has been previously implicated in IL-1β processing (Irmler et al., 1995), while granzyme B was shown to cleave IL-18, albeit inefficiently (Omoto et al., 2010).

Similarly to granzyme A, recombinant granzyme K has recently been reported to induce IL-1β secretion from mouse macrophages as well as a range of pro-inflammatory cytokines from human lung fibroblasts (Joeckel et al., 2011, Cooper et al., 2011). Again, this effect was proposed to be mediated by proteolytic activation of protease-activated receptor-1 (PAR-1), as an antibody blocking PAR-1 cleavage sites efficiently inhibited granzyme K-induced cytokine secretion (Cooper et al., 2011). However, the suggested cleavage of PAR-1 by granzyme K was not investigated in any detail and requires further exploration.

Most interestingly, granzyme A-deficient mice appear to be more resistant to LPS-induced mortality when compared to wild type mice. Although the pro-inflammatory activity of granzyme B was not directly explored in this study, granzyme B deficient mice showed even greater resistance to LPS (Metkar et al., 2008), suggesting that granzyme B may also drive the response of the immune system to LPS. Collectively, these data strongly suggest that aside from their well-established role in granule-mediated cytotoxicity, granzymes may have an important function in the regulation of inflammatory processes.

1.6 Aims of this study

A growing body of evidence suggests that granzymes are not just cytotoxic proteases, but also perform other non-cytotoxic, extracellular functions and participate in inflammatory processes via as yet undefined pathways (Metkar et al., 2008, Joeckel et al., 2011). In this work, we decided to explore whether granzyme B was capable of directly cleaving proinflammatory cytokines. Indeed, we identified IL-1α, a potent inflammatory mediator, as a novel granzyme B substrate. We have subsequently examined the functional consequences of IL-1α processing by granzyme B and other inflammatory proteases, which have previously been suggested to process IL-1α. The relevance of granzyme B-mediated proteolysis of IL-1α *in vivo* was also assessed in the course of this work.

Chapter 2

Materials and Methods

2.1 Reagents

Recombinant caspase-1, -3, -4, -5, -7 were expressed and purified from bacteria by other members of the lab as described previously (Walsh et al., 2008; Lüthi et al., 2009). PI-9 was expressed in bacteria by K.Viikov (Martin lab). Mouse granzyme B was expressed in Pichia pastoris (Cullen et al., 2007). cDNA clones encoding full-length human or mouse IL-1α were purchased from Origene. Human calpain-1 and human mast cell chymase were purchased from Calbiochem, human leukocyte elastase was purchased from Serva. Human IL-6, IL-8 and GM-CSF DuoSet ELISA kits were purchased from R&D systems. Unless otherwise stated all other reagents were purchased from Sigma Ltd. (Ireland).

Antibodies

Anti-IL-1α antibody was purchased from AbD Serotec, anti-transferrin antibody was from Bethyl Laboratories, anti-Bid, anti-caspase-7 and anti-XIAP antibodies were from BD Pharmingen, anti-calpastatin antibody was from Cell Signalling, anti-cochaperone-p23 was from Affinity Bioreagents, anti-actin antibody was from MP Biomedicals, goat anti-rabbit and goat anti-mouse antibodies were from Jackson ImmunoResearch, donkey anti-sheep antibody was from Santa Cruz.

2.2 Plasmids and recombinant proteins

2.2.1 Cloning

Cloning was carried out according to standard procedures. Briefly, the insert sequence was amplified from respective cDNA by polymerase chain reaction (PCR) using specific primers, designed to add restriction enzyme digest sites at the ends of the resultant PCR product. The insert and the vector plasmid were digested with the appropriate restriction enzymes (NEB, UK) overnight at 37°C. DNA fragments were then separated on agarose gel and isolated by silica based capture, followed by overnight ligation with T4 DNA ligase (NEB, UK) at 16°C. Cloning was verified by sequencing.

pET45b-hBid and pET45b-hBidD60A plasmids were kindly provided by Dr.Xiao-Ming Yin (University of Pittsburg, PA), pCDNA3-GAPDH plasmid was kindly provided by Dr. Douglas Green (St. Jude Children's Research Hospital, Memphis), pCDNA3-hIL-1β FL was constructed by Dr. Lisa Boucher-Hayes (Martin lab), other expression plasmids were cloned by the author.

2.2.2 Site-directed mutagenesis

Stratagene Quickchange protocol was used to mutate aspartic acid in position 103 of full-length IL-1α to alanine with the help of specific mutagenic primers and proofreading polymerase Pfu (Promega, UK). After amplification step DNA was digested with DpnI (NEB, UK), which cleaves only methylated template DNA, and then transformed into the high competent Escherichia coli DH5a strain. Mutagenesis was confirmed by sequencing.

2.2.3 Expression and purification of recombinant human granzyme B in Pichia pastoris

Recombinant human granzyme B wild type (Gzm BWT) or catalytically inactive mutant (Gzm BSA) were expressed and purified from Pichia pastoris as described previously (Cullen et al., 2007). Briefly, P. pastoris yeast clones stably harboring respective human granzyme B expression plasmid were grown in 500 ml cultures for 3 days at 30°C. For induction of granzyme B expression methanol was added every 24 h to the final concentration of 0.5%. His-tagged granzyme B was subsequently purified from culture supernatants using Ni-NTA agarose (Qiagen, UK), followed by elution with 500 mM imidazole. Protein was then extensively washed in PBS, pH7.2, followed by concentration in the microconcentrator units (Sartorius).

Granzyme B activity was measured by diluting protein into protease reaction buffer (PRB, 50 mM HEPES, pH7.4, 75 mM NaCl, 0.1% CHAPS, 2 mM DTT) containing 50 μM fluorescent substrate Ac-IETD-AFC (Bachem). Hydrolysis of the peptide was determined in an automated fluorimeter (Spectrafluor Plus, TECAN, UK). For PI-9 inhibition assay, granzyme B was preincubated with 5 fold molar excess of PI-9 and incubated for 30 min at 37°C before measuring residual activity by fluorimetry.

2.2.4 Expression and purification of recombinant proteins in Escherichia coli

His-tagged IL-1α was generated by cloning the human IL-1α encoding sequence in frame with the polyhistidine tag sequence in the bacterial expression plasmid

pET45b (Novagen, UK). Protein was expressed by addition of 600 μM IPTG to the exponentially growing BL21 E. coli strain for 3 hours at 24°C. Cells were lysed by sonication and IL-1α was captured using Ni-NTA agarose, followed by elution with 100 mM imidazole in PBS, pH7.2. Truncated IL-1α$^{104-271}$ and full-length Bid were expressed in BL21 strain for 3 h at 28°C and 37°C, respectively. Proteins were purified using Ni-NTA agarose, as described above.

To improve solubility of full-length IL-1α the protocol was modified and IL-1α was expressed for 3 h 37°C. The anionic detergent sarcosyl was then added to the resuspended bacterial pellet (final volume 0.25%) prior the sonication step. In order to sequester sarcosyl, Triton X-100, a nonionic detergent, was added to the sarcosyl-solubilised bacterial supernatants (final volume 0.5%) and protein was further purified using Ni-NTA, as described above. IL-1α^{D103A} was expressed and purified in a similar way.

2.2.5 *In vitro* transcription/translation

^{35}S-labeled proteins were synthetised using a coupled *in vitro* transcription/translation system according to the manufacturer's instructions (Promega, UK). Plasmid template was added to the reaction containing rabbit reticulocyte lysate, amino acid mix (minus methionine), T7 RNA polymerase and ^{35}S-methionine (Amersham). The reaction was carried out for 2 h at 30°C.

2.3 Protein analysis

2.3.1 SDS-PAGE electophoresis

Proteins were typically subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) for analysis. Protein samples were denatured by boiling for 7 minutes in SDS loading buffer (2 % SDS, 50 mM Tris-HCl, pH 6.8, 10 % glycerol, 2.5 % β-mercaptoethanol) and loaded onto 12 % polyacrylamide gels. Electrophoresis was carried out at 55 V through the stacking gel, and 75 V through the separating gel.

In order to visualise proteins with Coomassie Blue staining, SDS-PAGE gels were incubated in Coomassie Blue staining solution (45 % methanol, 10 % acetic acid, 0.25 % Coomassie Brilliant Blue) overnight. Gels were then incubated in destain solution (45 % methanol, 10 % acetic acid) to remove background staining.

Methionine-labelled proteins were visualised by enhanced fluorography using Amplify reagent (Amersham). Gels were fixed in fixative solution (45 % methanol, 10 % acetic acid) for 2 hours followed by incubation for 25 minutes in Amplify and vacuum drying onto blotting paper (Whatman) for 2 hours at 80°C. Gels were exposed to autoradiography film (HyperfilmMP, Amersham) at –70°C for 12 - 48 hours.

2.3.2 Immunoblot

After protein separation using SDS-PAGE, proteins were transferred onto 0.45 μM nitrocellulose membranes (Schleicher and Schuell) at 35 mA overnight in transfer buffer (39 mM glycine, 48 mM Tris-HCL, pH 8.3, 0.037% SDS, 20% methanol).

Membranes were next blocked with TBST (10 mM Tris-HCL, pH8.3, 150 mM NaCl, 0.05% Tween-20) containing 5% (w/v) non-fat dried milk for 1 h and then probed with the primary antibody at a typical concentration of 250 ng/ml for 2 h. Blots were then washed for 30 min in TBST and incubated with the appropriate horseradish peroxidase (HRP)-coupled secondary antibody for 1 h. Blots were washed again and incubated with Supersignal West, Peroxide and Enhancer solutions (Pierce). Proteins were visualised by exposing blots to autoradiography film.

2.3.3 Maldi-TOF mass spectrometry

For protein identification by mass spectrometry, protein spots were manually excised from gels, destained and dried in a SpeedVac (ThermoSavant). Proteins were then in-gel digested with trypsin (Boehringer, sequencing grade) for 12 - 16 hours at 37°C. Following digestion, peptides were extracted from the gel pieces and identified by matrix-assisted laser desorption and ionization (MALDI-TOF) mass spectrometry using Voyager DE Pro MALDI mass spectrometer. Data from about 1000 laser shots was collected for each sample. Spectra were deisotoped and internally calibrated based on the known m/z values of autolytic tryptic peptides. Peptide mass data was submitted to MASCOT and MS-Fit search engines for protein identification based on comparison with theoretical trypsin digests of proteins in the NCBI database.

2.3.4 Protease cleavage reactions

In vitro transcribed/translated or recombinant proteins were incubated with specified proteases in PRB or PBS and incubated for 2-4 hours at 37°C. 1 mM CaCl2 was added to the cleavage reactions with murine granzyme B and human calpain-1. Cleavage was analysed by SDS-PAGE electrophoresis followed by either autoradiography or Coomassie Blue staining.

2.3.5 Bronchoalveolar lavage fluid analysis.

Bronchoalveolar lavage fluid (BALF) samples from control individuals or patients with cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD) and bronchiectasis were generously provided by Dr. Emer Reeves (Reeves et al., 2010). Informed patient consent was obtained and ethical approval was granted by the Beaumont Hospital Institutional Review Board. Recombinant IL-1α (100 ng) was incubated with 10 µl of BALF sample for 20 min at 37°C. IL-1α processing was analysed by immunoblot.

2.4 Cell-based assays

2.4.1 Cell culture

HeLa (human cervical epithelial carcinoma) cells were cultured in RPMI media, supplemented with 5% fetal calf serum. THP-1 and U937 (human monocytic cell lines), and YT cells (human NK-cell line) were cultured in RPMI media, supplemented with 10% fetal calf serum. NK-92 cells (human NK-cell line) were

cultured in RPMI media, supplemented with 10% fetal calf serum and 100 U/ml of recombinant IL-2. HUVEC (human umbilical vein endothelial cells) were cultured in endothelial cell growth media with added growth supplement (PromoCell). Cells were split twice a week and cultured at 37°C in humidified atmosphere with 5% CO2.

2.4.2 Preparation of cell-free extracts

For preparation of cell-free extracts, THP-1 cells were treated with 1 µg/ml LPS for 4h. Approximately 0.5-1*109 cells were pelleted at 400 g and washed twice with 50 ml of PBS, pH7.2. Cells were transferred to a 2 ml Dounce-type homogeniser in 1 ml of Wang cell extract buffer (WCEB; 20mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 100 µM PMSF, 10 µg/ml leupeptin, 2 µg/ml aprotinin) and pelleted at 800 g. Two volumes of WCEB were then added to the cell pellet and cells were allowed to swell on ice for 20 min. Cells were lysed by 20-30 strokes of a B-type pestle. Lysates were clarified by centrifugation at 15,000 g for 15 min to remove nuclei, mitochondria, and other cellular debris; the resulting supernatant was aliquoted and stored at -70°C.

2.4.3 IL-1 bioactivity assay

Cells were plated at a density of $4*10^4$ (HeLa) or $2*10^4$ (HUVEC) cells per well in a 24-well plate (Sarstedt). The next day cells were incubated with the specified concentrations of IL-1α and supernatants were collected for quantitative cytokine

analysis by ELISA. Pro-inflammatory activity of Bid was analysed in a similar way using bone marrow-derived dendritic cells ($6.25*10^5$ cells per treatment).

2.4.4 ELISA

ELISAs were performed according to the manufacturer's instructions. Briefly, 96-well polysterene plates were coated with corresponding capture antibody and incubated overnight at room temperature. Next day, plates were extensively washed and blocked with 1% BSA in PBS, pH 7.2. After blocking, plates were incubated with samples or standard cytokines, followed by the appropriate biotin-conjugated detection antibody. Finally, plates were incubated with HRP-conjugated streptavidin, after which HRP substrate was added. Reactions were stopped with 2N H_2SO_4 and optical density was measured on the automated plate reader (wavelength 430nm).

2.5.5 NK-killing assay

HeLa cells were transiently transfected with the IL-1α expression plasmid using GeneJuice transfection reagent (Merck, Ireland) according to the manufacturer's instructions. For NK-mediated killing, HeLa cells were incubated with YT or NK-92 cells at the varying effector to target ratios. NK cells were taken off after 3-5 h, and HeLa cells were incubated further for a total of 24 h. Cell lysates were subsequently generated at $2*10^7$ cells/ml and analysed by western blotting. Cell death was analysed by flow cytometry using Annexin V/PI staining.

For the extracellular cleavage assay, recombinant IL-1α was added to YT cells in the presence of HeLa cells for 4 h at 37°C. IL-1α processing was analysed by immunoblot.

2.4.6 LPS-induced cell death

THP-1 or U937 cells were adjusted to a concentration of 106 cells/ml and treated with 100 ng/ml of LPS either alone or in the presence of 1 µg/ml cycloheximide (CHX) for 24-48 h at 37°C. Cell death was quantitated by annexin V/propidium iodide staining.

2.5.7 Annexin V/propidium iodide staining

To quantitate apoptosis, cells were resuspended in annexin V staining buffer (10 mM HEPES-NaOH, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$) containing 1 µg/ml of annexin V-FITC. Propidium iodide was added prior analysis to the final concentration of 10 µg/ml. Cell fluorescence was measured on a flow cytometer (FACSCalibur, Becton Dickinson, CA) and analysed using CellQuest software.

2.5 *In vivo* experiments

2.5.1 LPS depletion

Triton X-114 was added to recombinant proteins to a final concentration of 1%. Proteins were incubated for 30 min at 4°C, followed by a 10 min incubation at 30°C. The protein fraction was then separated by centrifugation at 20 000 g for 10 min at room temperature. The procedure was repeated three times.

2.5.2 Animals and *in vivo* treatment

C57BL/6 mice were purchased from Harlan (UK). Mice were injected intraperitoneally (i.p.) with ovalbumin (OVA) either alone or in combination with different forms of IL-1α as described in the Chapter 5. Mice were boosted with the same combinations after two weeks. Spleens, peritoneal lavage and peripheral blood were collected for analysis on day 21. Animal experiments were conducted in collaboration with Adjuvant Research Group (School of Biochemistry and Immunology, TCD). Granzyme B null mice, on the C57BL/6 background, were bred at Academic Medical Center (AMC), Amsterdam, the Netherlands.

2.6.3 Re-stimulation assay

Peritoneal lavage cells and splenocytes were plated at a concentration of 2*106 cells/ml on the ovalbumin (OVA), anti-CD3 or anti-CD3/PMA coated 96-well plates. 72 h later supernatants were collected and IL-4, IL-5, IL-10 and IFN-γ secretion was determined by ELISA using paired antibodies (IL-4, IL-5, IFN-γ were

purchased from BD Pharmingen, IL-10 was from R&D Systems) as described above.

2.6.4 Determination of OVA-specific IgG levels

96-well ELISA plates were coated with 50 µg/ml OVA and incubated overnight at 4°C. Next day, plates were extensively washed and blocked with 3% BSA in PBS, pH 7.2. Blocked plates were incubated with the serial dilutions of serum samples, followed by the appropriate biotin-conjugated detection antibody (anti-IgG was purchased from Sigma, IgG1, $IgG2_{a,b}$ were from BD Pharmingen), HRP-streptavidin and peroxidase substrate. Reactions were stopped with 2N H_2SO_4 and the optical density was measured on the automated plate reader.

Chapter 3

Analysis of caspase-1-mediated Bid cleavage *in vitro*

3.1 Introduction.

Caspase family members are classified based on their respective functions. Apoptotic caspases orchestrate cellular demolition during programmed cell death, while inflammatory caspases participate in immune responses to infection and injury. Surprisingly, while over 600 substrates are cleaved by apoptotic caspases and granzymes during programmed cell death (Lüthi and Martin, 2007), caspase-1, a major pro-inflammatory protease, has only two physiological substrates: cytokines IL-1β and IL-18 (Thornberry et al. 1992, Gu et al., 1997). Although another pro-inflammatory cytokine IL-33 was recently described as a novel substrate for caspase-1 (Schmitz et al., 2005), work in our lab subsequently demonstrated that IL-33 is not cleaved and activated by caspase-1 but is, in fact, inactivated via processing by apoptotic caspase-3 and -7 (Lüthi et al., 2009). Moreover, CTL/NK granzymes have been implicated in the regulation of inflammation via modulation of cytokine processing and secretion (Irmler et al., 1995, Sower et al., 1996a,b, Metkar et al., 2008, Omoto et al., 2010). Therefore, we decided to search for additional granzyme or caspase substrates within the interleukin family. We tested IL-1α and IL-1β against a panel of caspases and granzyme B, using the known granzyme B substrate Bid as a control (see Chapter 4.2.1). To our surprise, we observed that recombinant caspase-1 efficiently processed Bid *in vitro*.

Bid is a key mediator of the intrinsic apoptotic pathway and, upon cleavage, promotes mitochondrial permeabilisation leading to cytochrome c release, apoptosome formation and activation of effector caspases (Wang et al., 1996, Luo et al., 1998, Kuwana et al., 2002). Interestingly, processing of Bid has been

previously demonstrated under conditions where caspase-1 was concomitantly activated (Zhang et al., 2003, Wang et al., 2007). Moreover, Li and colleagues have reported that, in addition to caspase-3 and caspase-8, recombinant caspase-1 was capable of directly cleaving murine Bid (Li et al., 1998), although this observation was not investigated further in any detail. Importantly, Wang and colleagues showed that Bid-deficient mice were protected from LPS-induced lung damage when compared to wild type mice (Wang et al., 2007). Collectively, these data suggest the possibility that Bid is a physiological caspase-1 substrate.

The apparent scarcity of caspase-1 substrates is puzzling, prompting several groups to undertake extensive screens in an attempt to identify novel caspase-1 substrates (Shao et al., 2007, Lamkanfi et al., 2008, Agard et al., 2010). However, the physiological relevance of the few substrates identified in these screens remains unclear. Therefore, in an attempt to identify new caspase-1 substrates, we decided to explore caspase-1-mediated cleavage of Bid.

3.2 Results

3.2.1 Bid is cleaved by caspase-1 *in vitro*

To determine whether Bid is cleaved by inflammatory caspases, we subjected ^{35}S-labeled Bid to proteolysis by recombinant caspases-1, -4 and -5. We also used caspase-3, granzyme B and caspase-7 as positive and negative controls, respectively. IL-1β was used as a control to test the activity of inflammatory caspases, while the activity of apoptotic caspases and granzyme B was verified independently (data not shown). Figure 3.1 shows that at the concentration

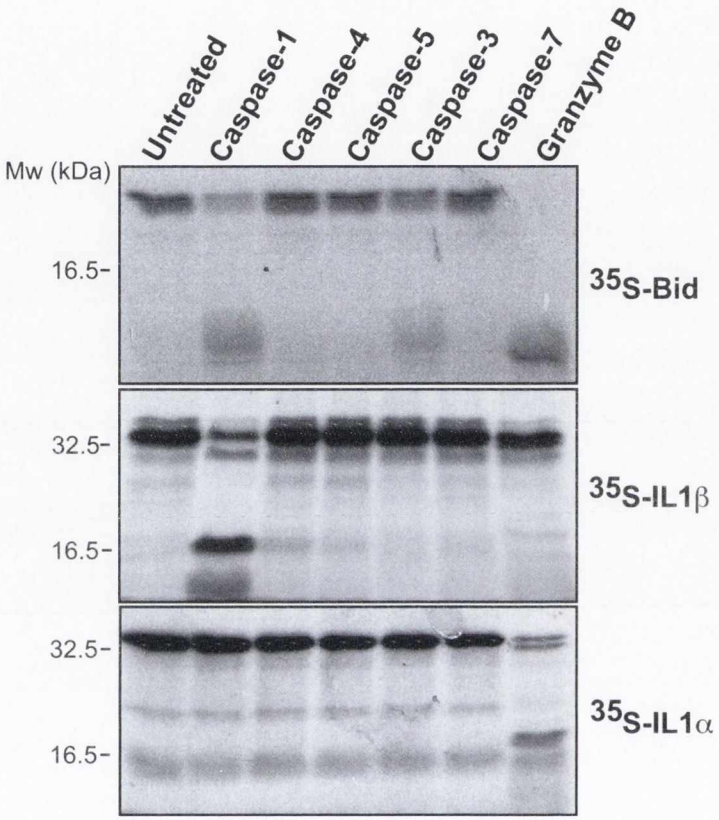


Figure 3.1

Bid is processed by caspase-1, -3 and granzyme B

^{35}S-labeled Bid, IL-1β and IL-1α were incubated at 37^{o}C for 2 h, either alone, or in the presence of recombinant caspase-1, -4, -5 (20 nM), caspase-3, -7 or granzyme B (200 nM), followed by analysis by SDS-PAGE/fluorography.

sufficient to cleave a well-known substrate, IL-1β, caspase-1 also efficiently processed Bid (note, we have not used saturating concentrations of caspase-1 as determined by incomplete IL-1β proteolysis). In agreement with previous reports, caspase-3 and granzyme B also cleaved Bid, while caspase-7 failed to so (Figure 3.1 and Walsh et al., 2008). The cleavage pattern generated by caspase-1 was similar to that produced by caspase-3, while granzyme B produced cleavage fragments of different size, as described previously (Figure 3.1 and Li et al., 1998). Caspase-4 and -5 did not cleave Bid at the concentration tested, even though some degree of IL-1β proteolysis was detected (Figure 3.1).

To further characterise proteolysis of Bid by caspase-1, we titrated recombinant caspase-1 onto *in vitro* transcribed/translated Bid, using IL-1β as a positive control (Figure 3.2A). We chose a range of caspase-1 concentrations (1-20 nM), within which proteolysis of IL-1β was incomplete (Figure 3.2). Importantly, treatment of Bid with recombinant caspase-1 resulted in a considerable degree of proteolysis at the concentrations tested (Figure 3.2A). Densitometric analysis of Bid and IL-1β cleavage showed that, although proteolysis of IL-1β appeared to be more efficient, caspase-1 cleaved a significant amount of Bid within the concentration range between 5-20 nM (Figure 3.2B). Moreover, at the highest concentration of caspase-1 used (20 nM) proteolysis of Bid was almost as efficient as that of IL-1β (Figure 3.2B).

We next decided to compare the efficiency of Bid proteolysis by caspase-1 with the other known Bid-processing proteases, caspase-3 and granzyme B (Figure 3.3A). While both caspase-1 and granzyme B efficiently processed Bid, caspase-3 failed to do so at the low nanomolar concentrations tested. Again, the quantitation of proteolysis efficiency (Figure 3.3B) shows, that although granzyme

B processed Bid more readily at the lowest concentrations, caspase-1 exhibited a similar degree of proteolysis at the highest concentrations (10-20 nM).

Recently, Shao and colleagues have identified glycolysis pathway enzymes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in particular, as caspase-1 substrates (Shao et al., 2007). However, the amount of recombinant caspase-1 necessary to induce a significant degree of GAPDH proteolysis was approximately 50 times higher than the amount required to induce a similar degree of IL-1β processing. Therefore, we decided to compare the ability of caspase-1 to cleave Bid and GAPDH. As Figure 3.4 shows, Bid was effectively processed by caspase-1 at all concentrations tested, while GAPDH failed to be cleaved within the same caspase-1 concentration range. Collectively, these data strongly suggest that Bid is an efficient caspase-1 substrate *in vitro*.

3.2.2 Caspase-1 cleaves Bid at D60

Sequence analysis of human Bid suggests that this protein has two potential caspase cleavage sites: aspartic acids (D) 60 and 75 (Figure 3.5A). Previous studies have identified D60 to be the cleavage site for caspase-3 and -8, while granzyme B cleaves Bid after D75 (Li et al., 1998, Luo et al., 1998). Li and colleagues demonstrated that mutation of D59 in murine Bid (caspase cleavage site) renders the mutant resistant to caspase-3, -8 and also to caspase-1-mediated proteolysis (Li et al., 1998). Therefore, we asked if human Bid is cleaved by caspase-1 after D60 and subjected *in vitro* transcribed/translated human Bid, both wild type and D60A mutant (aspartic acid mutated to alanine), to proteolysis by recombinant caspase-1. As Figure 3.5B illustrates, wild type Bid was efficiently

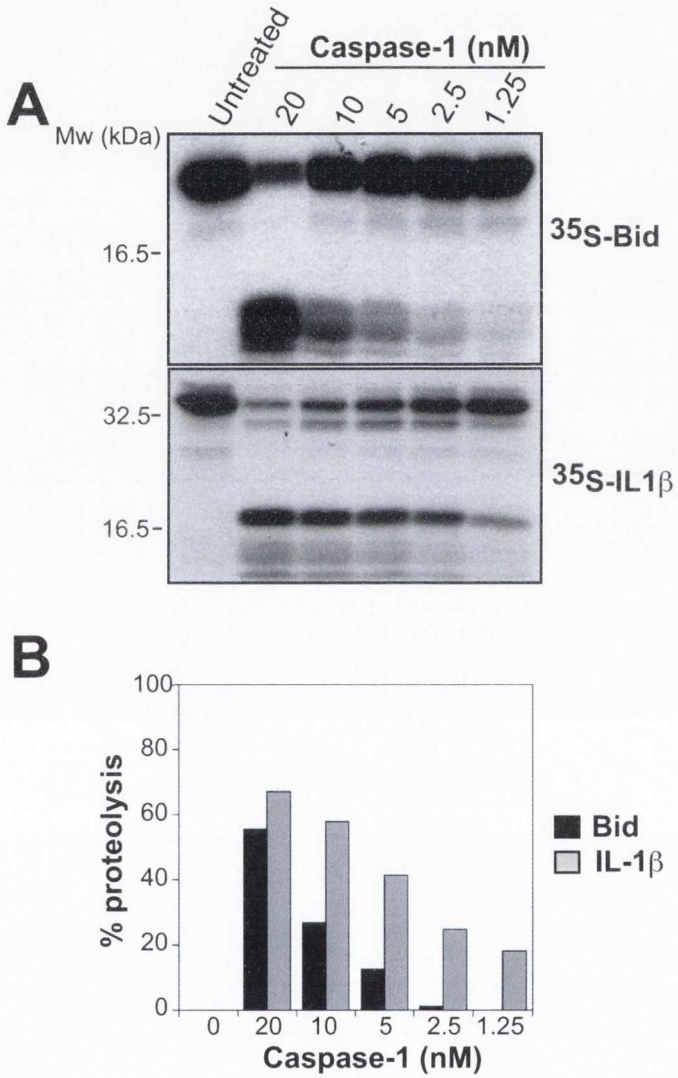


Figure 3.2

Bid is a caspase-1 substrate

(A) ^{35}S-labeled Bid and IL-1β were incubated for 2 h at 37°C with the indicated concentrations of caspase-1 followed by analysis by SDS-PAGE/fluorography.

(B) Densitometric analysis of data presented in (A).

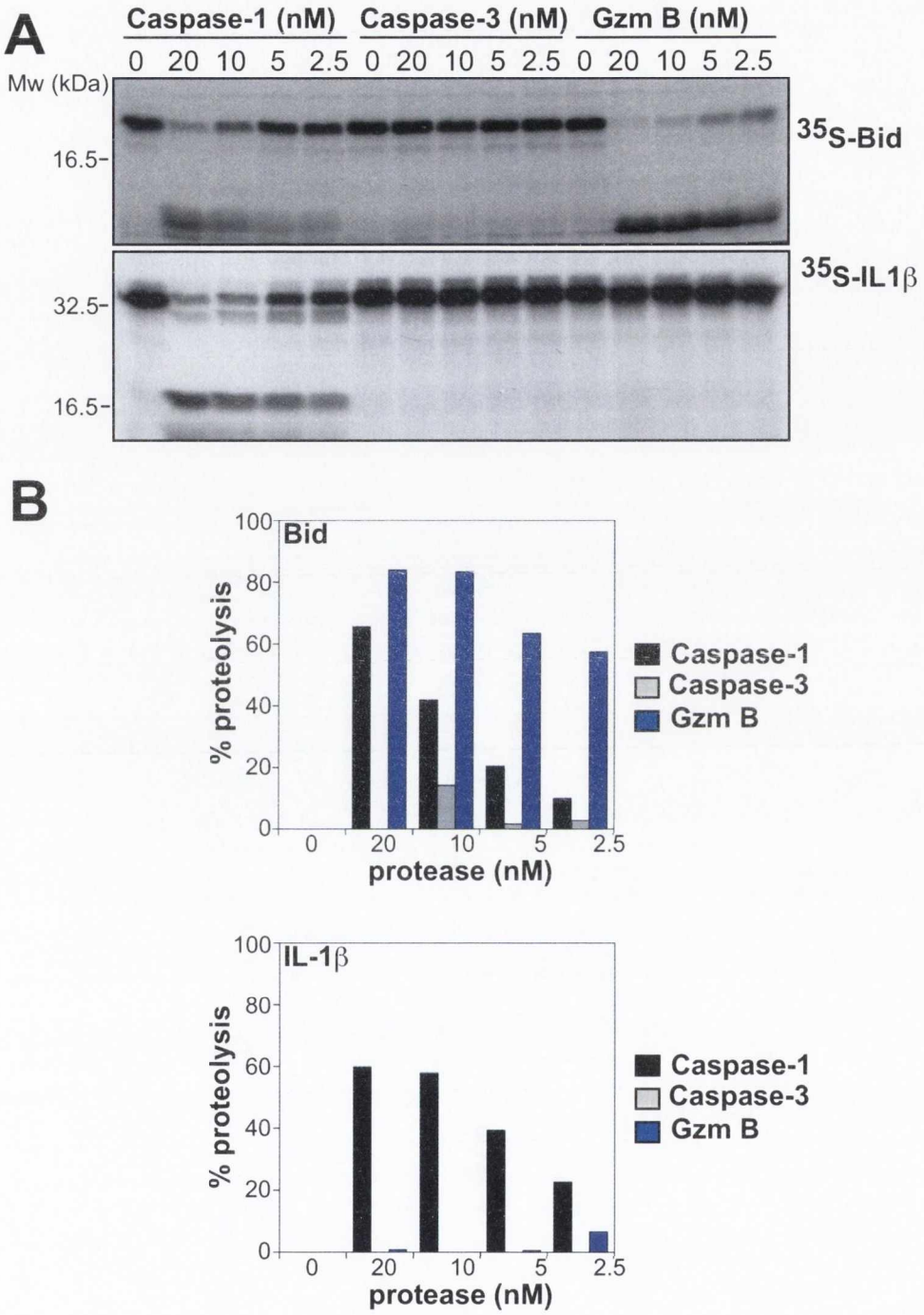


Figure 3.3

Caspase-1 processes Bid more efficiently than caspase-3

(A) ^{35}S-labeled Bid and IL-1β were incubated for 2 h at 37°C with the indicated concentrations of caspase-1, caspase-3 or granzyme B, followed by analysis by SDS-PAGE/fluorography.

(B) Densitometric analysis of data presented in (A).

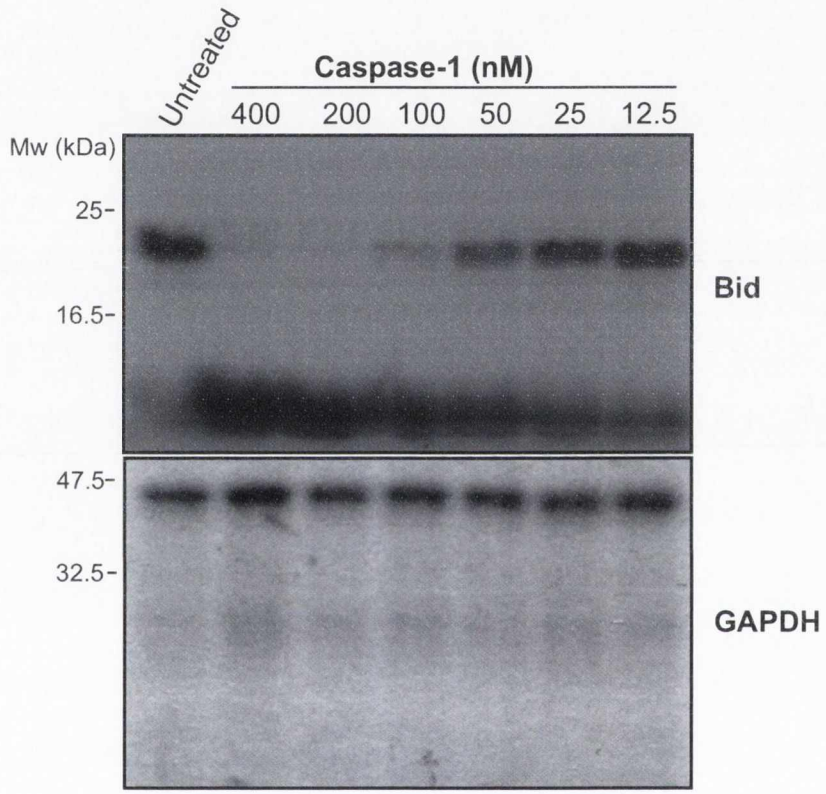


Figure 3.4

Bid is a more efficient caspase-1 substrate than GAPDH

^{35}S-labeled Bid and GAPDH were incubated for 2 h at 37^{o}C with the indicated concentrations of caspase-1, followed by analysis by SDS-PAGE/fluorography.

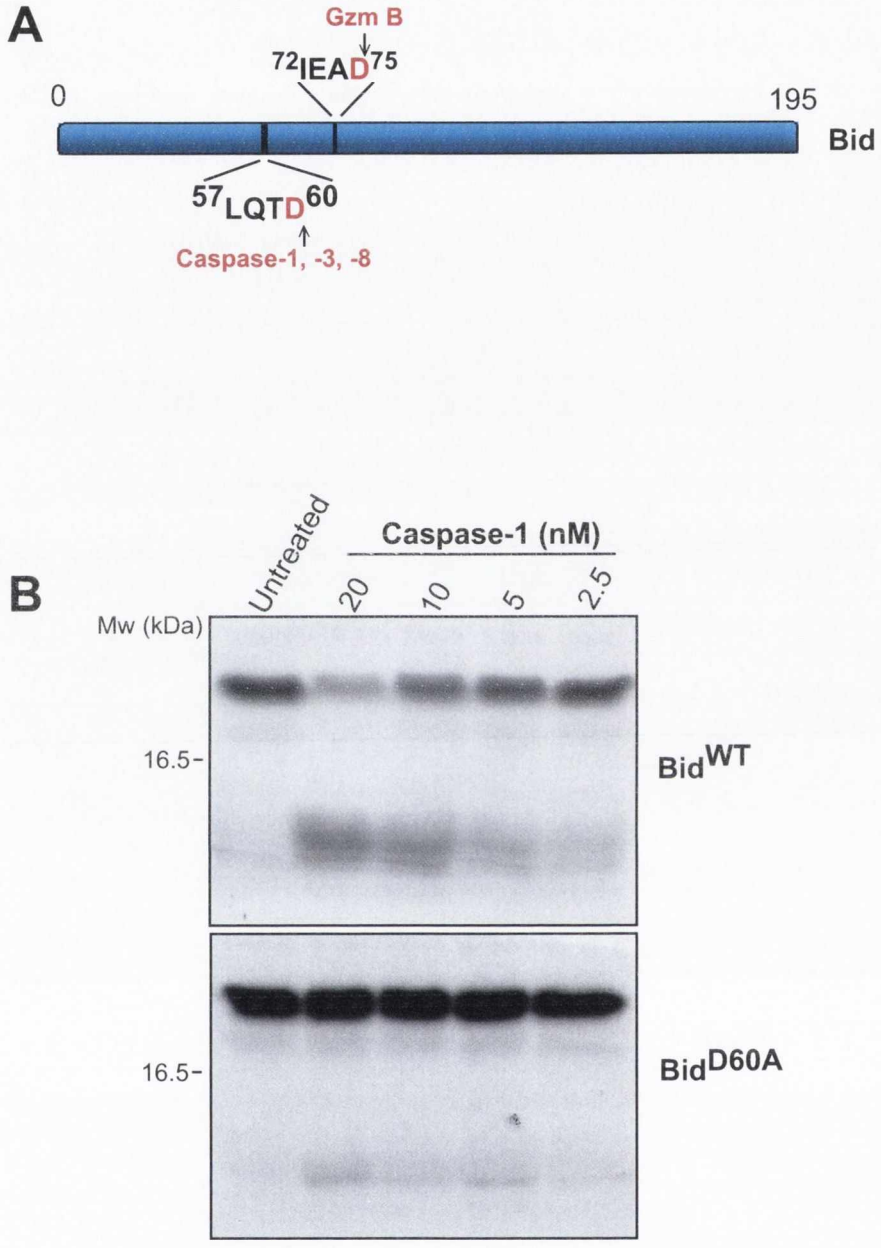


Figure 3.5

Caspase-1 processes Bid at D60

(A) Schematic representation of Bid indicating caspase and granzyme B cleavage sites.

(B) ^{35}S-labeled Bid^{WT} and Bid^{D60A} were incubated for 2 h at 37^{o}C with the indicated concentrations of caspase-1, followed by analysis by SDS-PAGE/fluorography.

processed by caspase-1 at all concentrations tested, while the D60A mutant was completely resistant to proteolysis, confirming that caspase-1 processes human Bid at the caspase cleavage site after D60.

3.2.3 Bid does not induce inflammatory cytokine production

Although the apoptotic role of Bid is well documented, a recent report by Yeretssian and colleagues demonstrated that Bid knock down in HT-29 cells resulted in decreased IL-8 production in response to NOD1 receptor agonist, suggesting Bid involvement in the pro-inflammatory signalling (Yeretssian et al., 2011). This finding is in agreement with previous studies reporting the increased survival of Bid-deficient mice in an experimental sepsis model when compared to wild type mice. This was reflected by reduced systemic and local pro-inflammatory cytokine levels in Bid null mice (Chung et al., 2010).

Moreover, Bid was also identified amongst secreted proteins in a caspase-1 secretome analysis (Keller et al., 2008). The authors of the latter work reported caspase-1-dependent release of full-length Bid in both LPS-treated monocytic cells and UV-irradiated keratinocytes (Keller et al., 2008), which when coupled to the caspase-1-mediated processing of Bid *in vitro*, suggested the exciting possibility that Bid is a pro-inflammatory mediator.

To explore if Bid has a direct pro-inflammatory function, we expressed His-tagged full-length recombinant Bid in E.coli BL21 strain. Human Bid is readily expressed in bacteria and was easily purified from bacterial lysates using Ni-NTA beads, as shown in Figure 3.6A. The purified protein was extensively dyalised to remove

imidazole used in the purification procedure and subjected to several rounds of LPS depletion. The purified protein was then either left untreated or subjected to proteolysis with recombinant caspase-1 (Figure 3.6B). Importantly, full-length Bid preparations received an equal amount of heat-inactivated caspase-1 to account for possible bacterial contaminants in the protease fraction (such as residual LPS), which might induce an inflammatory response.

We then decided to compare full-length and cleaved forms of Bid in a cellular assay. To this end, LPS-depleted Bid was added to mouse bone marrow-derived dendritic cells (BMDCs) and pro-inflammatory cytokine secretion was measured 24 hours later by ELISA. As shown in Figure 3.7A, treatment of BMDCs with full-length Bid resulted in the secretion of IL-6 in a stimulus-dependent manner, while the caspase-1-cleaved form of Bid induced a comparable, albeit slightly weaker, response. However, it was still possible that residual bacterial-derived LPS was responsible for the observed effects on IL-6 production. To control for this possibility, we treated BMDCs with Bid in the presence of polymixin B and found that cytokine-inducing activities of Bid and LPS were completely inhibited in the presence of this LPS inhibitor, while IL-6 secretion in response to CpG, the TLR9 agonist, was unaltered (Figure 3.7B).

Finally, we used BMDCs, derived from TLR-4 (LPS receptor) defective mice (HeJ), and treated these cells with recombinant Bid or control PAMPs. Figure 3.7C shows that TLR-4-deficient BMDCs were completely unresponsive to Bid or LPS treatment, while IL-6 secretion in response to CpG was normal. Collectively, these data suggest that the observed effects of Bid on cytokine production were entirely attributable to the residual LPS contamination. Thus, although an exciting

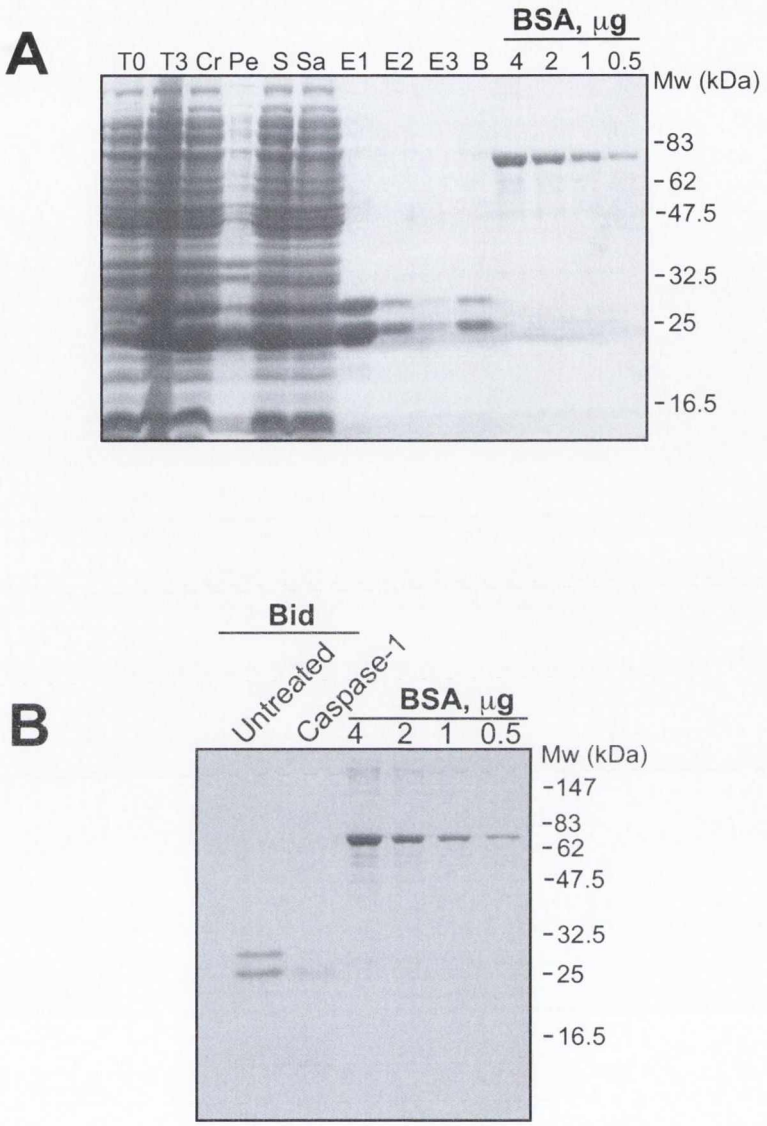


Figure 3.6

Expression and purification of recombinant Bid

(A) Bid expression was induced in *E.coli* BL21. Protein was purified using Ni-affinity chromatography. T0 and T3 - bacterial culture samples before induction and 3h post-induction, respectively, Cr - sample of sonicated lysate, Pe - insoluble fraction, S and Sa - supernatant prior and post-incubation with Ni-NTA beads, respectively, E1-E3 - elution fractions (1/50 of total elution volume), Be - sample of beads post-elution. Indicated amounts of BSA were added to facilitate estimation of purified Bid concentration.

(B) Recombinant Bid was incubated with 20 nM of heat-inactivated or active caspase-1 for 2h at 37^{o}C, followed by SDS-PAGE analysis.

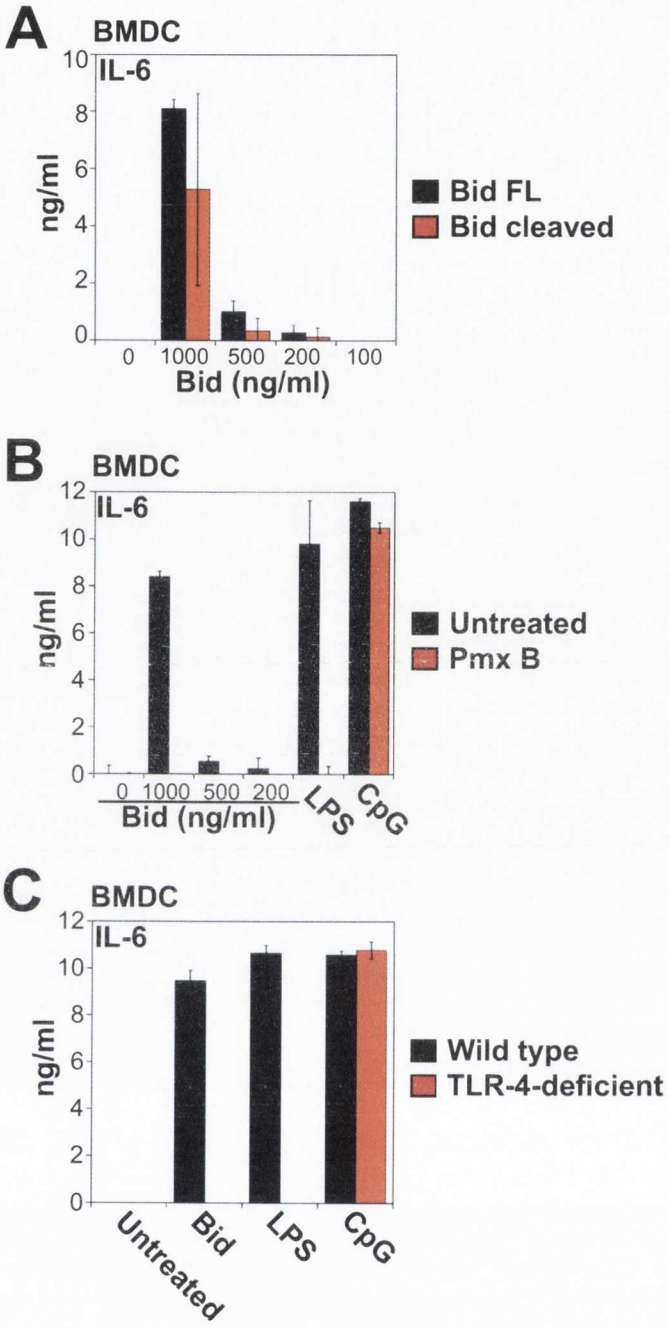


Figure 3.7

Bid does not act as a pro-inflammatory stimulus in bone marrow-derived dendritic cells (BMDC)

(A) BMDCs were incubated with the indicated concentrations of full-length or caspase-1-cleaved Bid for 24 h. Concentrations of IL-6 in the culture supernatants were determined by ELISA.

(B) BMDC were incubated with the indicated concentrations of full-length Bid for 24 h in the presence or absence of 100 μg/ml of PmxB. LPS (10 ng/ml) or CpG (4 μg/ml) were used as controls. Concentrations of IL-6 in the culture supernatants were determined by ELISA.

(C) BMDC derived from wild type or TLR-4-deficient mice were either left untreated or incubated with full-length Bid (1000 ng/ml), or LPS (10 ng/ml), or CpG (4 μg/ml) for 24 h. Concentrations of IL-6 in the culture supernatants were determined by ELISA.

possibility, Bid does not have a direct pro-inflammatory effect, at least in the chosen experimental setting.

3.2.4 Endogenous Bid is not cleaved in THP-1 cell-free extracts under inflammatory conditions

To explore caspase-1-mediated Bid cleavage in a more physiological setting, we used the monocytic cell lines U937 and THP-1, which express endogenous caspase-1. First, we hypothesised that Bid cleavage might be more readily detectable in a cell death scenario. Previous studies have demonstrated that in the presence of CHX, a protein synthesis inhibitor, LPS treatment induced caspase-1-dependent cell death in human endothelial cell lines (Pohlman and Harlan 1989, Wang et al., 2007). Therefore, we treated monocytic cells with LPS and CHX either alone or in combination and determined cell viability after 24 h and 48 h. To measure cell death, we stained cells with annexin V, a protein that binds to phosphatidylserine exposed on the plasma membranes of apoptotic cells, in combination with the vital dye propidium iodide (Martin et al., 1995).

In U937 cells, LPS treatment alone did not induce any significant cell death at any time point, while CHX alone induced a considerable increase in cell death (24%) (Figure 3.8A and 3.8B). As expected, the combination of LPS and CHX resulted in a dramatic increase in cell death (53%). In THP-1 cells, LPS alone induced 17% of cell death, while CHX-induced cell death was negligible (8%) (Figure 3.8C and 3.8D). Again, the combination of the two stimuli had the biggest effect on cell death (29%) (note that prolonged treatment (48 h) did not result in any significant augmentation of cell death at 24 h). However, despite a dramatic increase in cell

death, we were not able to detect Bid processing in the lysates from U937 or THP-1 under the conditions tested (data not shown).

Finally, to explore Bid cleavage in a more controlled setting, we decided to use a THP-1 cell-free system, which is routinely used to identify caspase substrates, as it allows controlled activation of apoptotic or inflammatory caspase cascades (Cullen et al., 2008). We incubated THP-1 cell-free extracts at 37°C to initiate caspase-1 activation, which we monitored via processing of the physiological caspase-1 substrate, IL-1β. As Figure 3.9 shows, caspase-1 was efficiently activated within 15 min, with over 50% of IL-1β cleaved at 30 min (note that XIAP and calpastatin were used as controls for apoptotic caspase activation and loading control, respectively). However, we did not observe proteolysis of Bid at any time-point tested (Figure 3.9). These data show that Bid remains unprocessed under caspase-1 activation conditions, suggesting that Bid is not a physiological caspase-1 substrate.

3.3 Discussion

3.3.1 Search for inflammatory caspase substrates

During the process of programmed cell death, a wide range of different proteins are processed to ensure orderly dismantling of the apoptotic cell, and over 600 substrates for apoptotic caspases have been identified to date (Lüthi and Martin, 2007). Astonishingly, only two physiological substrates, IL-1β and IL-18, have been described for inflammatory caspase-1 (Thornberry et al., 1992, Gu et al., 1997), although inflammatory processes are no less labyrinthine and sophisticated

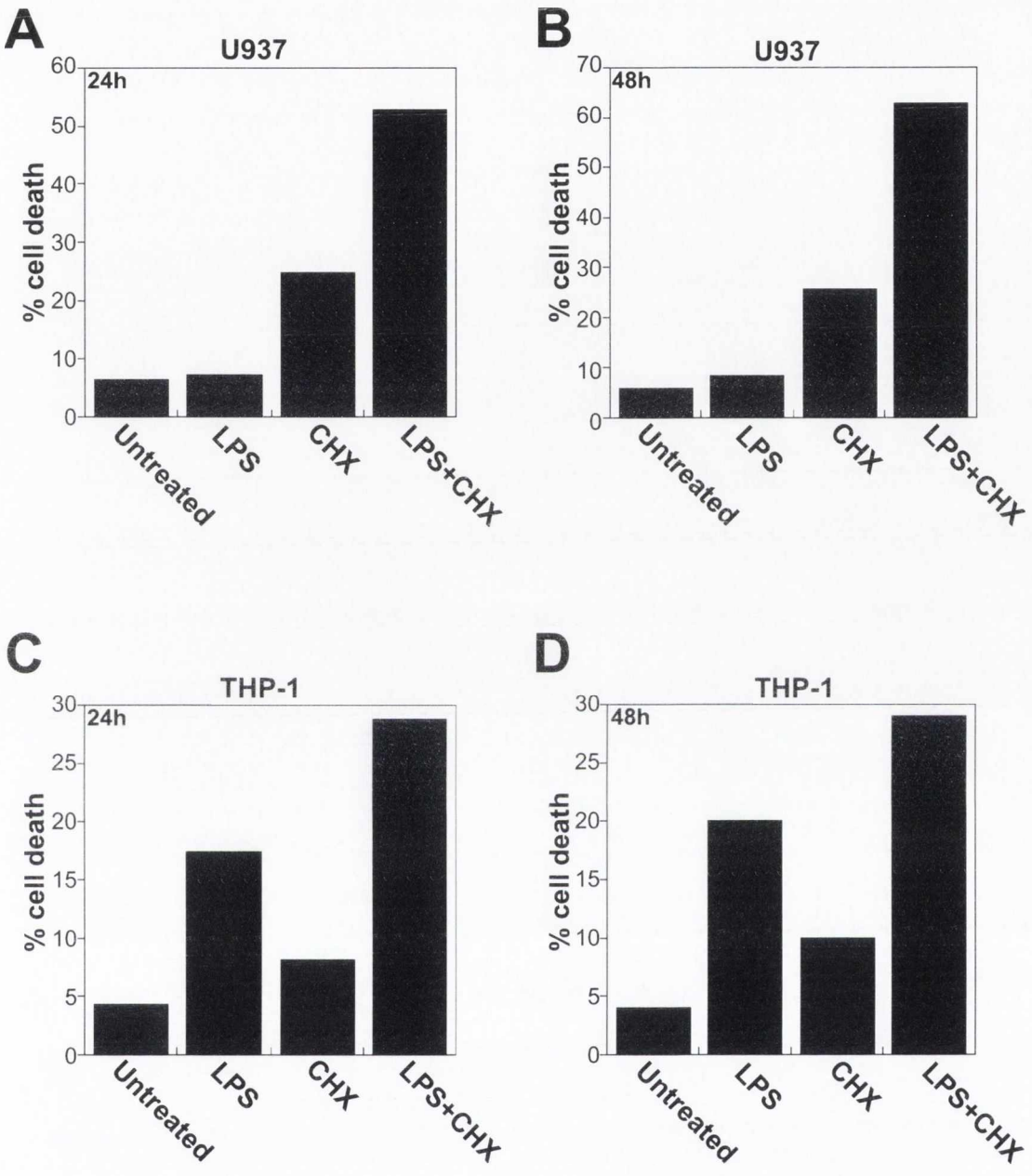


Figure 3.8

LPS/CHX treatment induces cell death in human monocytic cell lines

U937 (A, B) or THP-1 (C, D) were incubated either alone, or with LPS (100 ng/ml), or CHX (1 μg/ml) as indicated for 24 h (A, C) or 48 h. Cells were stained with Annexin V/PI and analysed by flow cytometry.

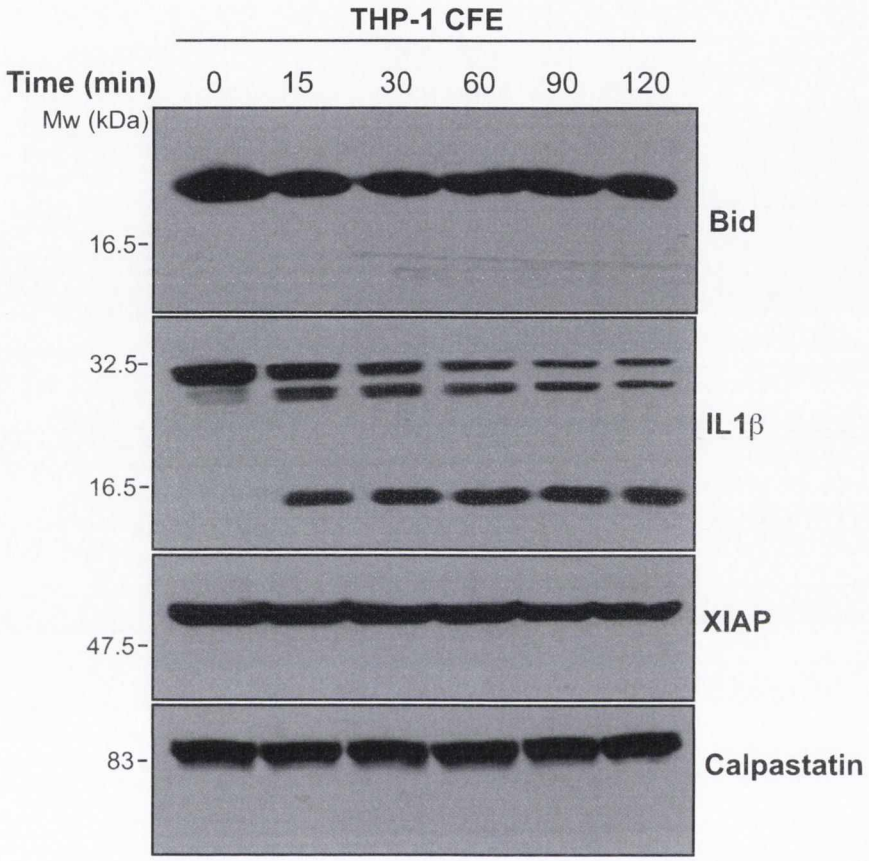


Figure 3.9

Bid is not cleaved in THP-1 cell-free extracts (CFE) under inflammatory conditions

THP-1 CFE were incubated at 37^{o}C to activate the inflammasome. Samples were taken at the indicated time-points and analysed by immunoblot.

than the process of apoptosis. Therefore, identification of novel caspase-1 substrates will serve as an invaluable asset in helping scientists to unravel inflammatory pathways. Furthermore, the description of the caspase-1 digestome will assist in understanding the roles played by caspase-1 in pathogen-induced cell death and non-classical protein secretion (Monack et al., 2001, Keller et al., 2008).

The apparent paucity of caspase-1 substrates is highly puzzling. Consequently, a lot of interest in recent years has been concentrated on the search for inflammatory caspase substrates (Shao et al., 2007, Lamkanfi et al., 2008, Agard et al., 2010). However, the major drawback of these screens is the use of excessively high concentrations (up to 400 nM) of recombinant caspase-1.

It is vitally important to use physiologically achievable concentrations of the enzyme under investigation, as using abnormally high amounts of the protease may produce erroneous conclusions. For example, initial work by Schmitz and colleagues suggested that caspase-1 activates pro-inflammatory cytokine IL-33 via internal proteolysis (Schmitz et al., 2005). However, closer investigation demonstrated that caspase-1, used at the physiological concentrations, was not capable of processing IL-33 (Lüthi et al., 2009).

Importantly, another report from our lab has recently shown that, although high concentrations of recombinant caspase-1 cleaved a wide range of substrates, the activity of caspase-1 is tightly regulated within the cell, permitting only a brief burst of proteolytic activity (Walsh et al., 2011). These data again emphasize the importance of careful evaluation of *in vitro* caspase-1 substrates in a cellular setting.

Nonetheless, several compelling candidates for caspase-1 processing have been identified in the previous studies, among which were enzymes of the glycolysis pathway and apoptotic caspase-7 (Shao et al., 2007, Lamkanfi et al., 2008). Subsequently, caspase-7 knockout mice were demonstrated to be resistant to endotoxin-induced mortality, suggesting that caspase-1-mediated activation of caspase-7 is instrumental in mediating the lethal effects of endotoxin (Lamkanfi et al., 2009, Lamkanfi et al., 2008). However, the precise biological roles of these proteolytic events remain largely elusive and require further investigations.

3.3.2 Bid is an *in vitro* caspase-1 substrate

In this work, we identified the apoptotic protein Bid as an *in vitro* caspase-1 substrate. Proteolysis of Bid by caspase-3, caspase-8 and granzyme B has been previously extensively characterized (Li et al., 1998, Slee et al., 2000, Barry et al., 2000). In agreement with Li and colleagues, we also demonstrated that Bid is processed by caspase-1 *in vitro*. Moreover, unlike the previous studies, we showed that the cleavage of Bid takes place at the non-saturating physiological concentrations of caspase-1, as judged from cleavage of its physiological substrate, IL-1β.

We also compared proteolysis of Bid by caspase-1 with proteolysis by caspase-3 and granzyme B. Proteolysis rates of Bid by caspase-1 and granzyme B were comparable, with granzyme B being slightly more efficient. Interestingly, caspase-3 failed to process Bid at similar concentrations, although previous work in our laboratory showed that human monocytic cell lines have similar amounts of endogenous caspase-1 and caspase-3 (Walsh et al., 2011). This suggests that

Bid is a more efficient substrate for caspase-1 than for caspase-3. Finally, we compared the cleavage efficiency of Bid with the novel caspase-1 substrate GAPDH. Again, we observed efficient *in vitro* processing of Bid, while GAPDH was not affected by caspase-1 treatment at the same concentrations. Collectively, these data suggest that Bid is an efficient *in vitro* caspase-1 substrate.

3.3.3 Functional consequences of Bid cleavage

Bid is a pro-apoptotic Bcl-2 family protein that participates in the initiation of the mitochondrial apoptosis pathway by mediating mitochondrial membrane permeabilisation and subsequent release of cytochtome c. Cleavage of Bid is a critical step in regulating this cell death pathway, permitting the translocation of truncated Bid to the mitochondrial fraction, where this protein facilitates the formation of the Bax/Bak channel in the mitochondrial outer membrane (Wang et al., 1996, Luo et al., 1998, Kuwana et al., 2002). Most interestingly, recent data implicate Bid in inflammation and innate immunity, showing that macrophages from Bid knockout mice displayed profound defects in cytokine production in response to NOD agonists (Yeretssian et al., 2011). Furthermore, Bid-deficient mice were demonstrated to be resistant to septic shock and exhibit a blunted immune response in experimental models of sepsis and NOD receptor stimulation (Zhao et al., 2001, Chung et al., 2010, Yeretssian et al., 2011).

Based on the aforementioned findings and on the fact that pro-inflammatory caspase-1 processed Bid *in vitro*, we speculatively explored the idea that Bid may possess direct pro-inflammatory activity. In support of this idea, previous studies have implicated several normally intracellular molecules in danger signaling. For

example, HMGB1 was reported to induce inflammatory cytokine secretion from human monocytes (Andersson et al., 2000), which was particularly surprising, considering that under normal conditions HMGB1 is a chromatin binding nuclear transcription factor, sequestered deep within the cell (Bianchi and Beltrame, 2000). Similarly, IL-1α acts as a danger signal upon release from necrotic cells (Chen et al., 2007, Cohen et al., 2010). Taking into account caspase-1-dependent secretion of Bid, this protein was a possible candidate as an inflammatory mediator. However, we could not detect any pro-inflammatory activity of recombinant Bid protein on BMDCs above the contaminating endotoxin background.

Recently, Bid was suggested to have a structural role in NOD1 signaling acting as a bridge between NOD1 receptor and downstream effector molecules (Yeretssian et al., 2011). However, the survival of Bid-deficient mice in the models of septic shock is most likely explained by the inability of Bid-deficient cells to respond to apoptotic stimuli and the resultant reduction in tissue damage (Zhao et al., 2001).

Therefore, we decided to explore caspase-1-mediated Bid cleavage in a cell death scenario initiated by LPS and CHX treatment, which was previously demonstrated to induce caspase-1-dependent cell death in human endothelial cells (Wang et al., 2007). We also demonstrated that LPS in combination with CHX induced apoptosis in human monocytes. How does CHX affect LPS signalling and redirects it to the cell death pathway? CHX is a protein synthesis inhibitor and, hence, would inhibit LPS-induced synthesis of inflammatory mediators and other pro-survival proteins. Importantly, LPS was shown to induce apoptosis resistance in tumour cells via upregulation of Bcl-XL – an anti-apoptotic Bcl-2 family protein, which sequesters Bid in the cytosol (Sun et al., 2008, Cheng et al., 2001). Thus,

blocking LPS-induced Bcl-XL expression by CHX could enable caspase-1-processed Bid to translocate to mitochondria. Importantly, LPS/CHX-induced cell death in endothelial cells was inhibited by the overexpression of Bcl-XL (Wang et al., 2007), further implicating a Bid-regulated mitochondrial pathway in LPS/CHX-induced apoptosis. However, we could not detect Bid cleavage in the lysates of LPS/CHX-treated monocytes, suggesting that other pathways regulate cell death in response to LPS in these cells.

We were also unable to detect Bid cleavage in THP-1 cell free extracts under inflammatory conditions. Therefore, Bid does not appear to be an efficient caspase-1 substrate under physiological conditions. Nevertheless, we cannot exclude the possibility that Bid is processed by caspase-1 under conditions where caspase-1 is robustly activated. Therefore, several caspase-1 activating stimuli (e.g., MSU, bacterial infection, DNA) should be tested in the future experiments. These stimuli engage distinct NLRs and may result in a differential degree of caspase-1 activation. In support of this, Agard and colleagues recently demonstrated cleavage of a divergent set of proteins upon stimulation of THP-1 cells with various inflammasome-activating stimuli: LPS/ATP, MSU and DNA (Agard et al., 2010).

In conclusion, in this chapter we demonstrated that Bid is an efficient caspase-1 substrate *in vitro*. However, we could not demonstrate cleavage of Bid by endogenous caspase-1 nor implicate Bid in the regulation of inflammatory responses within a cellular context. Clearly, the question of whether Bid is processed under inflammatory conditions requires further investigation.

Chapter 4

IL-1α is a novel granzyme B substrate

4.1 Introduction

The importance of granzymes in the induction of apoptosis is widely accepted and many granzyme-initiated pathways leading to cell death are now well described (Cullen and Martin, 2009). Interestingly, several groups have found elevated levels of extracellular granzyme A and granzyme B in inflammatory conditions, such as rheumatoid arthritis (Tak et al., 1999, Lauw et al., 2000). Furthermore, accumulating evidence suggests that a wide range of non-cytotoxic cells express and secrete granzyme B, often in the absence of perforin (reviewed in Afonina et al., 2010), while Prakash and colleagues demonstrated that CTLs and NK cells constitutively secrete granzyme B in the absence of target cell engagement (Prakash et al., 2009).

Recent data examining extracellular functions of granzymes suggest that these proteases may have a pro-inflammatory role via the induction of cytokine secretion. In this way, granzyme A has been shown to induce IL-1β, IL-8 and TNF-α release from different cell lines or PBMCs and directly process the major pro-inflammatory cytokine IL-1β (Sower et al., 1996a,b, Metkar et al., 2008, Irmler et al., 1995). Interestingly, both IL-1α and IL-1β were found to be processed and secreted during CTL-induced apoptosis (Hogquist et al., 1991). Moreover, Metkar and colleagues also showed that granzyme A- or granzyme B-deficient mice are resistant to LPS-induced endotoxic shock (Metkar et al., 2008).

Based on this evidence, we hypothesised that granzyme B might have a pro-inflammatory function and process cytokines. To explore this hypothesis, we performed a screen testing a wide range of interleukins for susceptibility to

granzyme B-mediated proteolysis. Several potential substrates were identified in the screen (data not shown), with IL-1α being the focus of this work.

4.2 Results

4.2.1 IL-1α is a substrate for granzyme B

To explore whether granzyme B is capable of cleaving IL-1α or IL-1β, we incubated *in vitro* transcribed/translated cytokines with recombinant human granzyme B along with a panel of recombinant inflammatory (caspase-1, -4, -5) and apoptotic (caspase-3, -7) caspases. As figure 4.1 shows, granzyme B efficiently cleaved IL-1α producing a 17.5 kDa fragment, while all other caspases examined failed to cleave this cytokine. The activity of recombinant caspase-1, –3 and granzyme B was confirmed via cleavage of their respective substrates, IL-1β and Bid, while the activity of the remaining caspases was controlled via their ability to cleave synthetic tetrapeptide substrates (data not shown).

To confirm the specifity of granzyme B-mediated proteolysis of IL-1α, we titrated granzyme B over a range of concentrations from 12.5 nM to 100 nM, again incorporating Bid as a positive control for granzyme B activity. Importantly, granzyme B cleaved IL-1α at any concentration tested, albeit slightly less efficiently than it cleaved Bid (Figure 4.2A). Densitometric analysis using ImageJ software was performed to estimate the percentage of substrate cleaved and showed that, although proteolysis of Bid was more efficient, approximately 40% of IL-1α was cleaved by 100 nM granzyme B (Figure 4.2B). Notably, granzyme B failed to cleave IL-1β at the concentrations tested (Figure 4.2A and B).

4.2.2 Recombinant IL-1α purification

To obtain recombinant IL-1α for further experiments, we cloned IL-1α cDNA into pET45b vector and expressed His6-tagged protein in E.coli BL21 strain. Unfortunately, standard purification methods yielded only small quantities of this cytokine. Analysis of protein purification steps revealed that IL-1α is expressed as a highly insoluble protein in E.coli (Figure 4.3A, compare Pe and SN1 fractions). To improve protein solubility, we tried several modifications to the purification protocol. To this end, we varied the expression temperature and induction time, expressed the protein in E.coli BL21 DE3 RIL (a bacterial strain which facilitates expression of eukaryotic genes in bacteria) or in the yeast P.Pastoris, and also attempted to solubilise the protein using an acid extraction method (data not shown). However, all of these attempts were unsuccessful.

The sodium salt of the alkyl anionic detergent N-laurylsarcosine (sarcosyl) has been demonstrated to improve the solubility of a variety of GST-tagged proteins and is easily sequestrated by the non-ionic detergent Triton X-100 (Frangioni and Neel, 1993). Adaptation of this method using minimal amounts of detergents greatly improved solubility of IL-1α (Figure 4.3B, compare Pe, SN1 and Be fractions in A and B), yielding ~40-50 μg of protein from 500 ml of bacterial culture. Figure 4.3C shows a representative SDS-PAGE analysis of IL-1α expression and purification using sarcosyl. This method was used throughout the project to purify full-length IL-1α for further experiments.

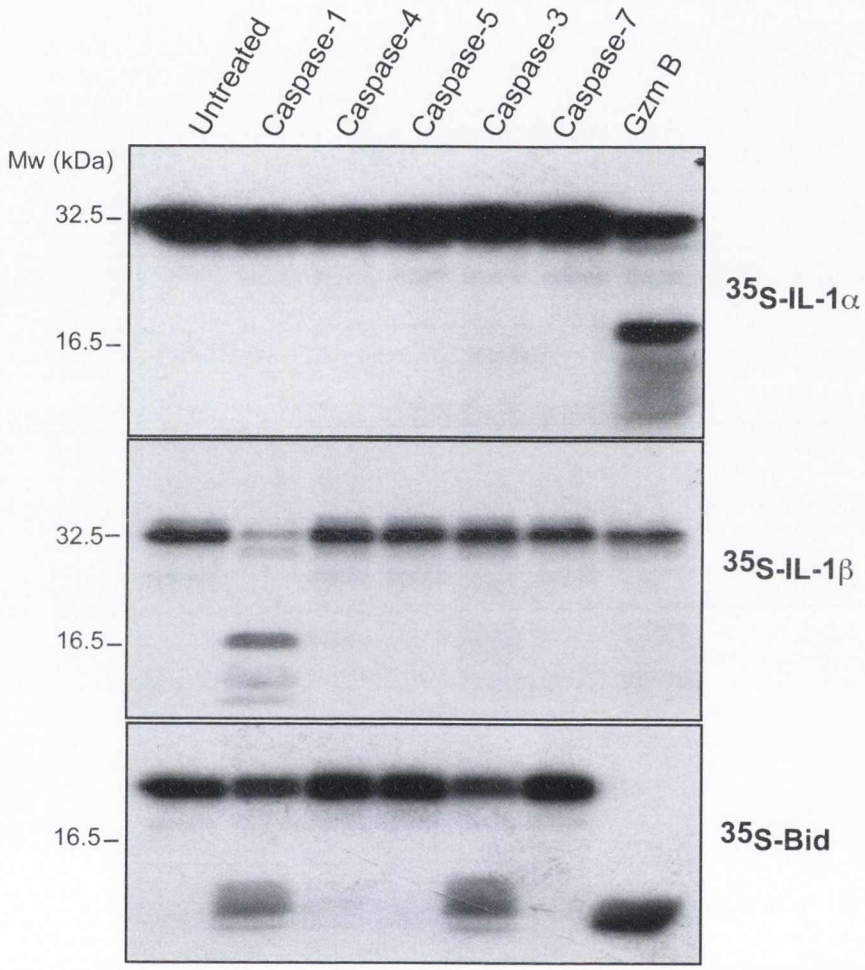


Figure 4.1

IL-1α is processed by granzyme B

In vitro transcribed/translated IL-1α, IL-1β and Bid were incubated with 20 nM recombinant caspase-1, -4, -5 and 200 nM recombinant caspase-3, -7 and granzyme B. Reactions were carried out for 2 h at 37°C and cleavage was assessed by SDS-PAGE, followed by autoradiography.

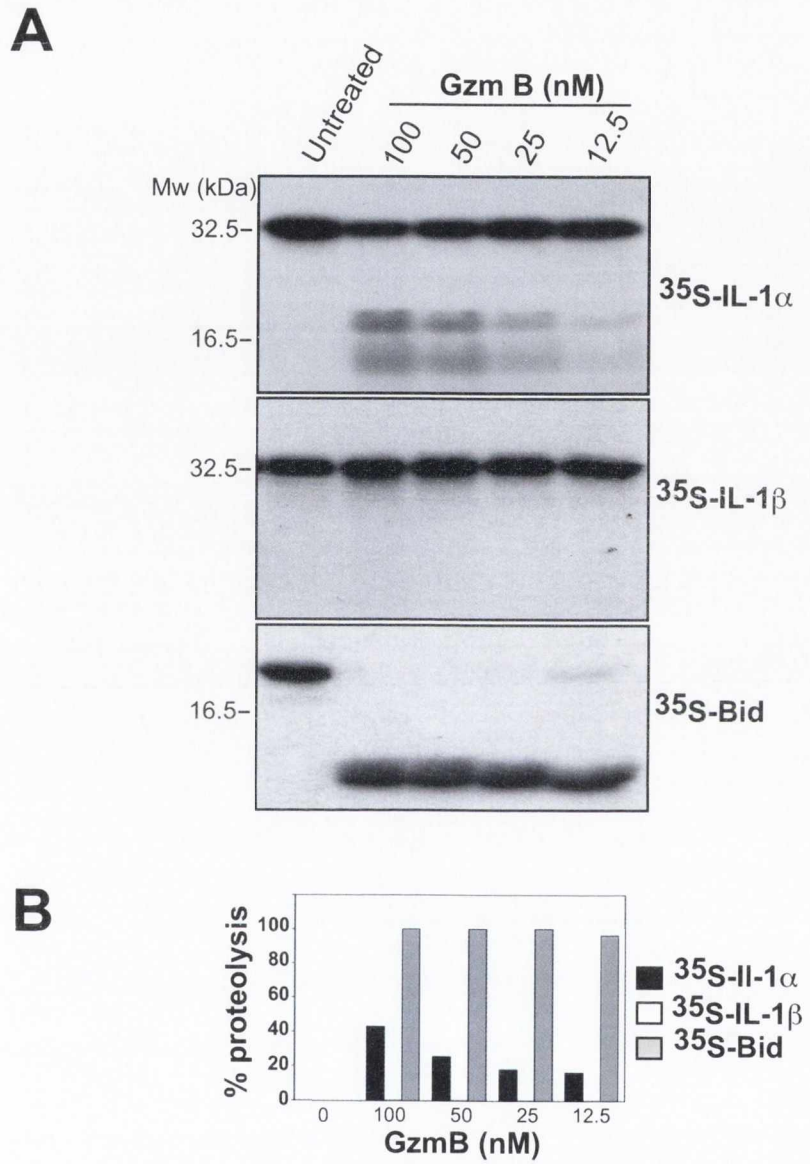


Figure 4.2

IL-1α is a granzyme B substrate

(A) *In vitro* transcribed/translated IL-1α, IL-1β and Bid were incubated with the indicated concentrations of recombinant granzyme B for 2 h at 37°C. Samples were resolved by SDS-PAGE and visualised by autoradiography. (B) Densitometric analysis of data presented in (A). Results are represented as graph bellow the gels.

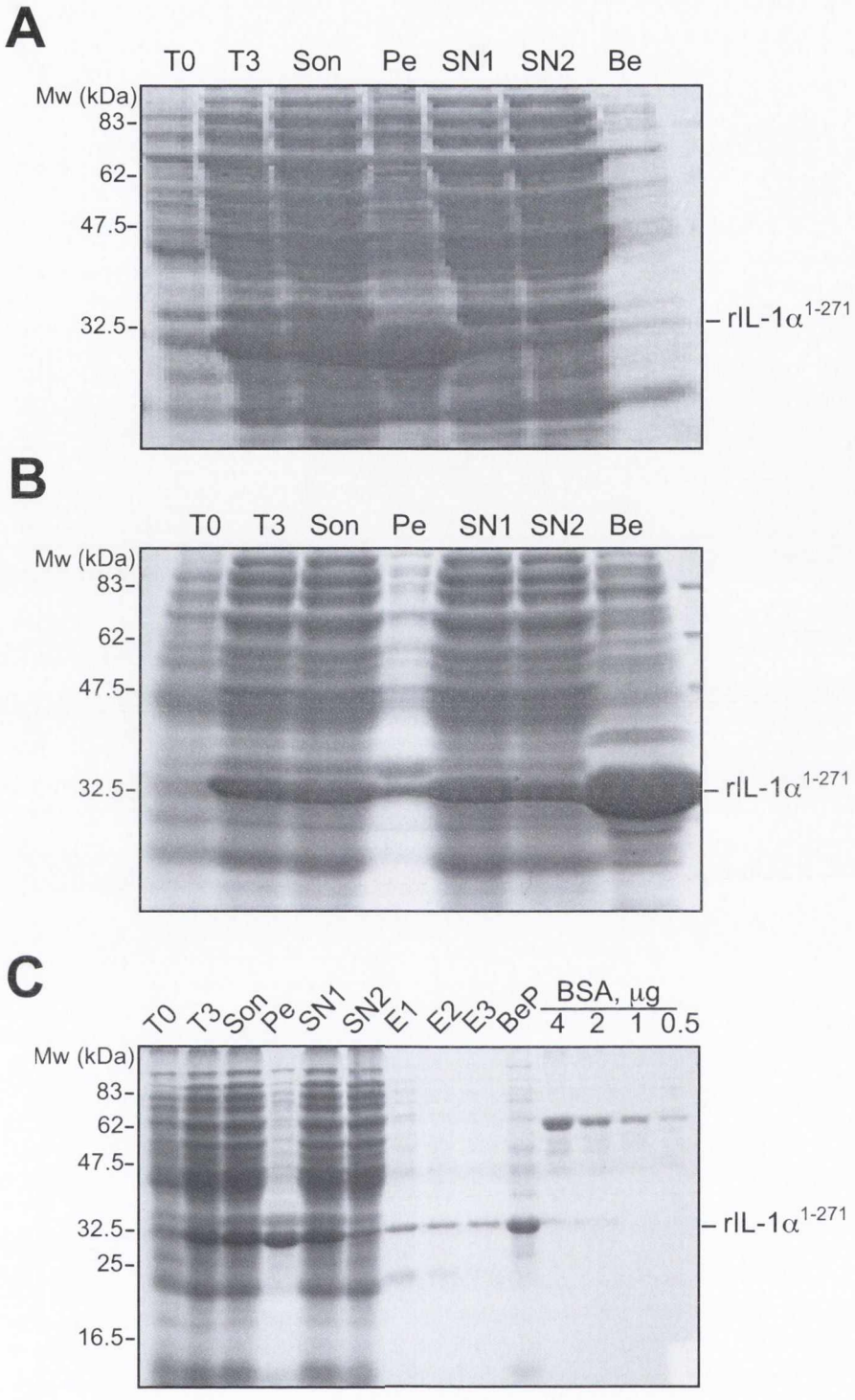


Figure 4.3

Expression and purification of recombinant IL-1α

IL-1α expression was induced in *E.coli* BL21. T0 and T3 - bacterial culture samples before induction and 3 h post-induction, respectively, Son - sample of sonicated lysate, Pe - insoluble fraction, SN1 and SN2 - supernatant prior and post-incubation with Ni-NTA beads, respectively, E1-E3 - elution fractions (1/50 of total elution fraction), Be and BeP - sample of beads prior and post-elution, respectively. (A) Standard purification protocol. (B) Sarcosyl was added to bacterial lysate prior to sonication step. (C) SDS-PAGE analysis of IL-1α expression and purification using sarcosyl. Known amounts of BSA were loaded on the gel to help estimate concentration of purified IL-1α.

4.2.3 IL-1α is cleaved by granzyme B at D103

To map the granzyme B cleavage site within IL-1α, we subjected recombinant full-length IL-1α to proteolysis by granzyme B and analysed the resulting cleavage fragments by mass-spectrometry. This suggested that the granzyme B cleavage site is located between amino acids 80 and 112 of IL-1α (Figure 4.4).

Granzyme B exhibits an absolute preference for cleavage after an aspartic acid residue and typically cleaves after the tetrapeptide motif IXXD, where X represents any amino acid. Therefore, based on the analysis of the protein sequence, this suggested that granzyme B processed IL-1α after the tetrapeptide motif 100IAND103. Importantly, alignment of the IL-1α sequence from different mammalian species showed that this cleavage site is conserved (Figure 4.5A).

Figure 4.5B depicts a schematic representation of IL-1α, placing the putative granzyme B cleavage site, IAND, within the context of other important sequence motifs (calpain cleavage site, NLS). Thus, granzyme B-mediated proteolysis generates a slightly larger product than the calpain-cleaved, 'mature' form of IL-1α (see below), suggesting that IL-1α would retain its activity after processing with granzyme B. Also, as Figure 4.5B shows, proteolysis of IL-1α by both proteases might remove the NLS, rendering the protein unable to enter the nucleus.

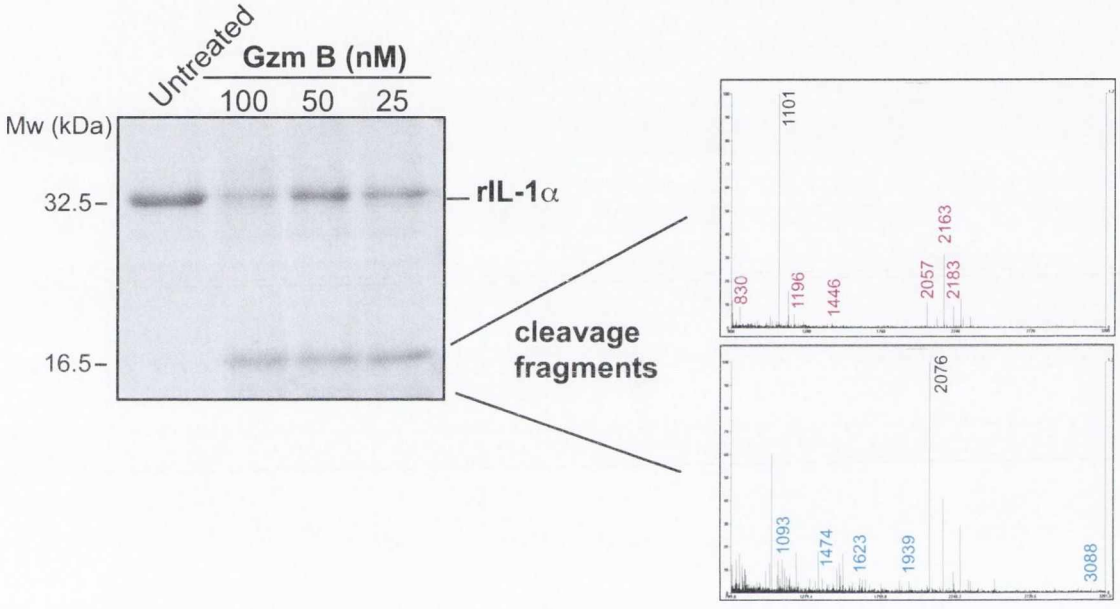
To confirm that D103 is indeed the granzyme B cleavage site, we mutated the aspartic acid at position 103 to alanine by site-directed mutagenesis. This single-point mutant was *in vitro* transcribed/translated and subjected to processing by granzyme B. As demonstrated in Figure 4.5C, the IL-1α^{D103A} mutant was completely resistant to proteolysis by granzyme B, while the wild type form was processed under the same conditions.

We also generated a truncated version of IL-1α (IL-1α$^{104-271}$), representing the fragment created by granzyme B cleavage (Figure 4.6A-B). We then incubated recombinant IL-1α$^{104-271}$ with granzyme B at the concentrations used in the experiments described above, but observed no further cleavage of IL-1α$^{104-271}$ by granzyme B (Figure 4.6C). Taken together, these data clearly show that IL-1α is cleaved by granzyme B at position D103.

Calpain, a calcium activated neutral protease, has been shown to cleave IL-1α after F118, a position close to the granzyme B cleavage site (Carruth et al., 1991; Kobayashi et al., 1990). We next wanted to confirm that granzyme B and calpain processed IL-1α at different sites and incubated *in vitro* transcribed/translated wild type IL-1α and the IL-1α^{D103A} mutant with various concentrations of granzyme B or calpain. As figure 4.7 shows, IL-1α was efficiently cleaved by both proteases at nanomolar concentrations, but the two enzymes produced different cleavage patterns. Importantly, the IL-1α^{D103A} mutant was still processed by calpain but not granzyme B, confirming that calpain and granzyme B cleave IL-1α at distinct sites.

4.2.4 Cleavage of IL-1α by granzyme B is an evolutionary conserved event

As murine and human granzyme B have been shown to exhibit divergent substrate preferences (Cullen et al., 2007), we decided to test whether cleavage of IL-1α is an evolutionary conserved event. To this end, we cloned *in vitro* transcribed/translated mouse IL-1α and subjected it to proteolysis with human and murine granzyme B. As demonstrated in Figure 4.8, both human (Figure 4.8A) and murine (Figure 4.8B) granzyme B cleaved mouse IL-1α, confirming that

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1   makvpdmfed lkncysenee dsssidhlsl nqksfyhvsy gplhegcmdq
51  svslsisets ktskltfkes mvvvatngkv lkkrrlslsq sitdddleai
101 andseeeiik prsapfsfls  nvkynfmrii kyefilndal  nqsiirandq
151 yltaaalhnl deavkfdmga yksskddaki tvilrisktq lyvtaqdedq
201 pvllkempei pktitgsetn llffwethgt knyftsvahp nlfiatkqdy
251 wvclaggpps itdfqilenq a
```

Figure 4.4

Mass spectrometric analysis of IL-1α cleavage fragments

Recombinant IL-1α was incubated with recombinant granzyme B (200 nM) for 2 h at 37°C. Samples were resolved by SDS-PAGE then visualised by Coomassie Blue staining. The bands, representing N- and C-terminal cleaved forms of IL-1α, were excised and analysed by mass-spectormetry.

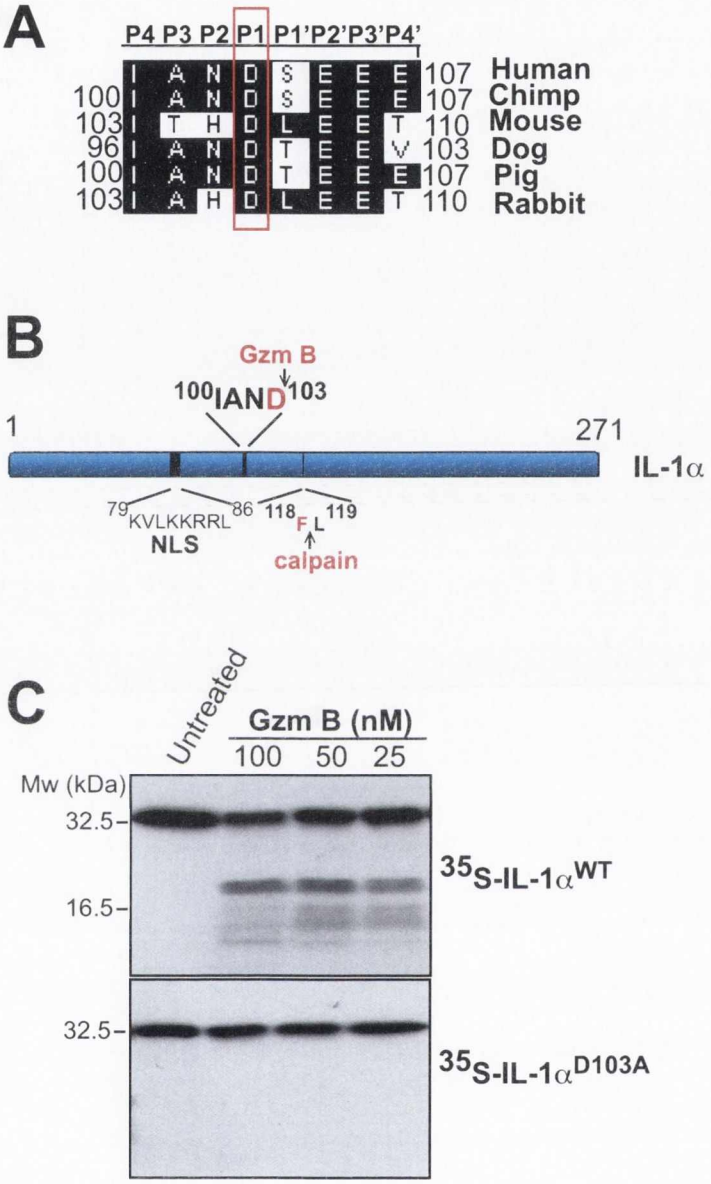

Figure 4.5

IL-1α is cleaved by granzyme B at D103

(A) A sequence alignment of the putative granzyme B cleavage site in IL-1α from a number of mammals. The P1 Asp residue is indicated. (B) Schematic representation of IL-1α indicating nuclear localisation signal and granzyme B and calpain cleavage sites. (C) *In vitro* transcribed/translated IL-1α^{WT} and IL-1α^{D103A} were incubated with the indicated concentrations of recombinant granzyme B for 2 h at 37°C. Samples were resolved by SDS-PAGE and visualised by autoradiography.

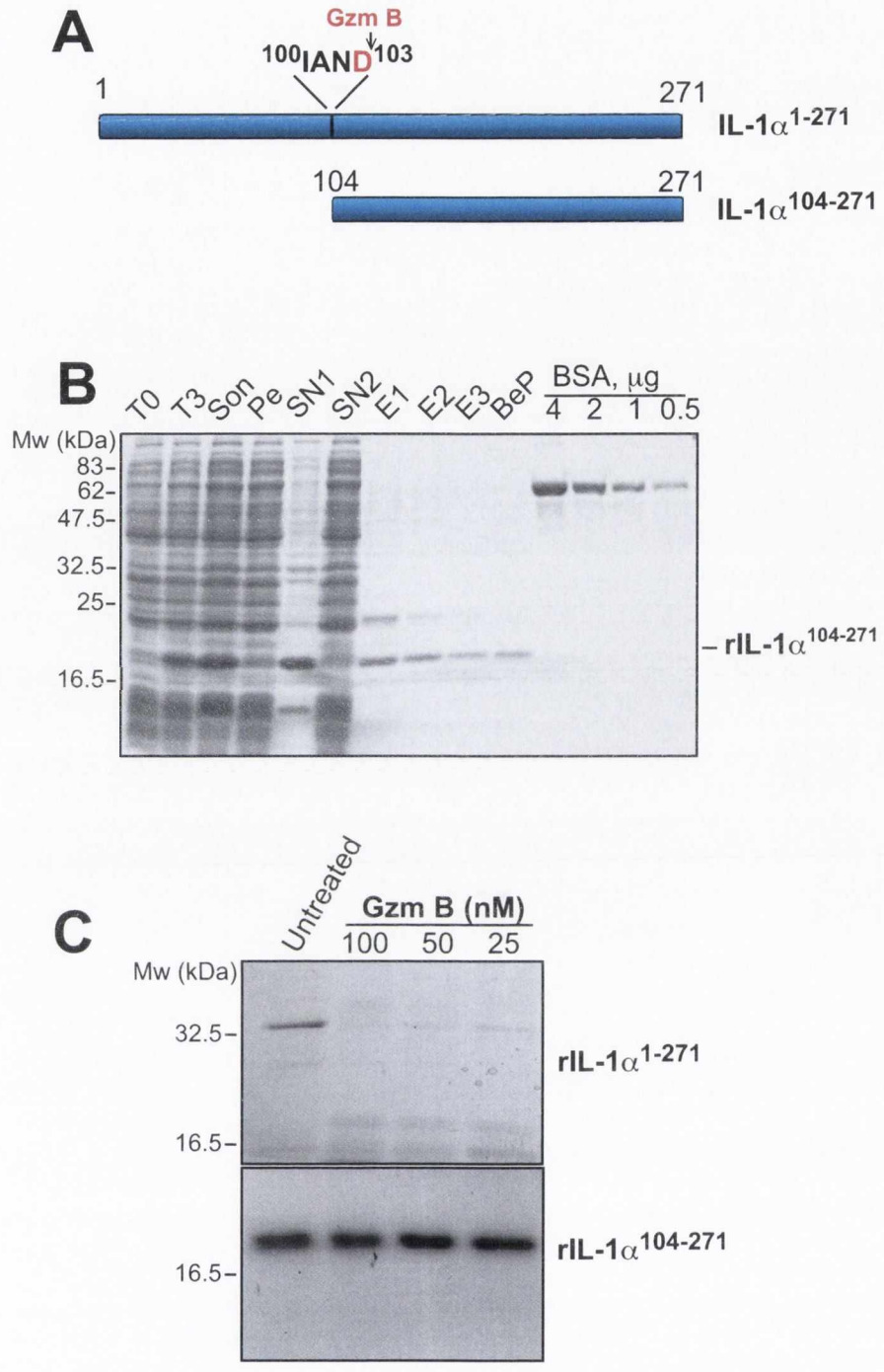

Figure 4.6

IL-1α$^{104-271}$ is not cleaved by granzyme B

(A) Schematic representation of IL-1α constructs, IL-1α$^{1-271}$ and IL-1α$^{104-271}$. (B) SDS-PAGE analysis of IL-1α$^{104-271}$ expression and purification. T0 and T3 - bacterial culture samples before induction and 3 h post-induction, respectively, Son - sample of sonicated lysate, Pe - insoluble fraction, SN1 and SN2 - supernatant prior and post-incubation with Ni-NTA beads, respectively, E1-E3 - elution fractions (1/50 of total elution fraction), Be - sample of beads post-elution. Known amounts of BSA were loaded on the gel to help estimate concentration of purified IL-1α. (C) Recombinant IL-1α and IL-1α$^{104-271}$ were incubated with the indicated concentrations of recombinant granzyme B for 2 h at 37°C. Samples were resolved by SDS-PAGE and visualised by Coomassie Blue staining.

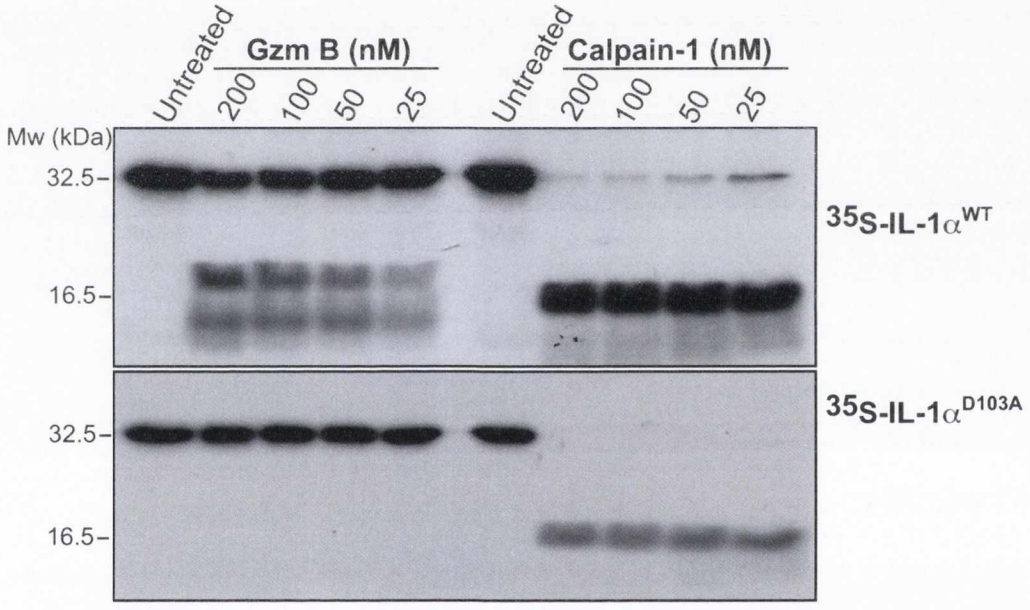

Figure 4.7

Calpain-1 and granzyme B cleave IL-1α at distinct sites

In vitro transcribed/translated IL-1α and IL-1α^{D103A} were incubated with the indicated concentrations of granzyme B or calpain-1 for 2 h at 37°C. Samples were resolved on SDS-PAGE and visualised by autoradiography.

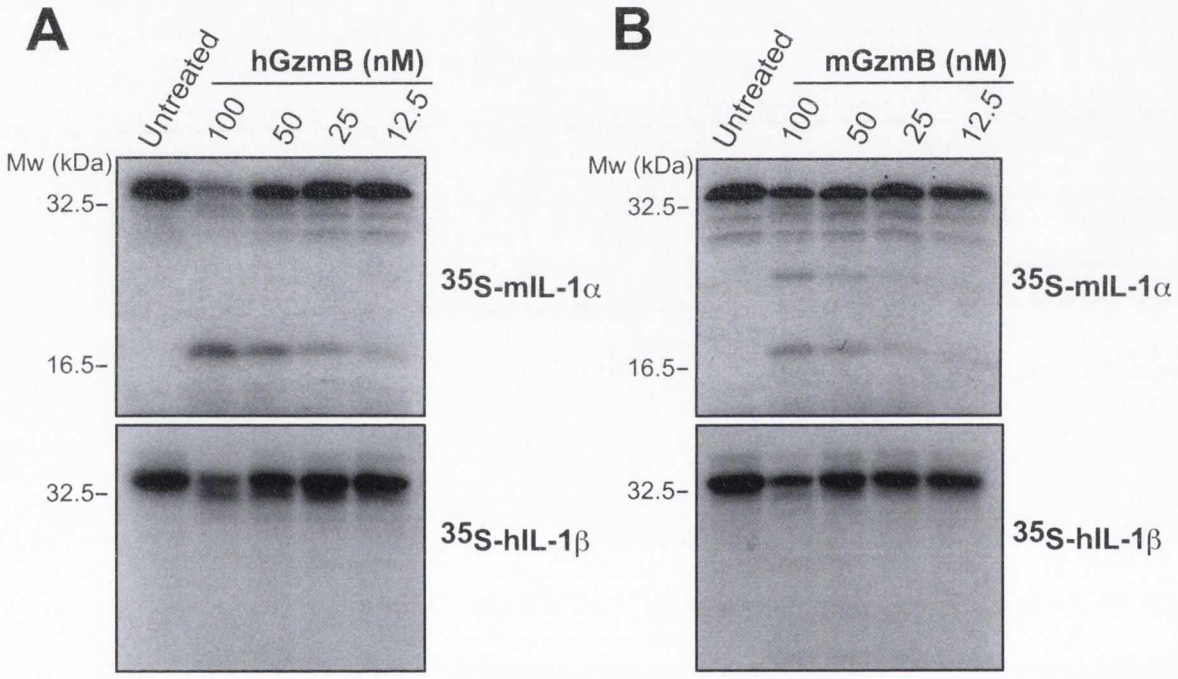

Figure 4.8

IL-1α is cleaved by mouse granzyme B

In vitro transcribed/translated mouse IL-1α and human IL-1β were incubated with the indicated concentrations of human (A) or mouse (B) granzyme B for 2 h at 37°C. Samples were resolved on SDS-PAGE and visualised by autoradiography.

granzyme B-mediated cleavage of IL-1α is conserved from mouse to man. However, note that processing of mouse IL-1α by murine granzyme B generated an extra cleavage fragment, suggesting the existence of an additional cleavage site for murine granzyme B within the mouse IL-1α protein sequence.

4.2.5 IL-1α is processed during NK-mediated killing

Previous studies have showed that CTLs and NK cells constitutively release both proform and active granzyme B into the extracellular millieu (Prakash et al., 2009). Moreover, non-cytotoxic cells, such as mast cells, keratinocytes and basophils, express and secrete granzyme B in the absence of perforin (Strik et al., 2007, Hernandez-Pigeon et al., 2006, Tschopp et al., 2006). To explore whether endogenous granzyme B cleaves IL-1α in the extracellular space, we permeabilised the NK cell line, YT, either with the pore-forming protein, streptolysin O, or by repeated freeze-thaw cycles and then incubated recombinant IL-1α with the granzyme B-containing supernatants. As Figure 4.9A shows, recombinant IL-1α was efficiently processed by the supernatants of permeabilised YT cells, with the cleavage product running at a size identical to the cleavage fragment produced by recombinant granzyme B. Moreover, this cleavage product was even detected when IL-1α was incubated with non-permeabilised YT supernatant, demonstrating the constitutive release of granzyme B by YT cells.

During NK-attack, granzyme B is secreted into the immunological synapse, defined as the contact space between the target and NK cell. However, it is possible that some granzyme B may escape the immunological synapse. To explore whether IL-1α is processed extracellularly during NK-mediated killing, we

incubated recombinant IL-1α with YT cells in the presence or absence of target HeLa cells, as schematically represented in Figure 4.9B. Importantly, proteolysis of IL-1α was readily detected over a range of effector-to-target ratios (Figure 4.9C), suggesting that secreted granzyme B processed IL-1α extracellularly. We also performed time-course analysis of IL-1α proteolysis in the same experimental setting and found that IL-1α was efficiently cleaved over a 24 h period, with the processed fragment already apparent after a 1 h incubation with YT cells (Figure 4.9D). Importantly, addition of IL-1α to HeLa cells alone did not result in IL-1α processing.

However, it is possible that other proteases capable of cleaving IL-1α are released from YT cells along with granzyme B. To test whether granzyme B is indeed the protease responsible for IL-1α cleavage, we incubated recombinant IL-1α with YT cells and their target HeLa cells in the presence of the granzyme B inhibitor, PI-9 (Figure 4.10A top panel). As Figure 4.10A (bottom panel) shows, addition of recombinant PI-9 efficiently blocked proteolysis of IL-1α in a concentration-dependent manner, with 0.5 μM PI-9 completely inhibiting IL-1α processing. We also compared processing of wild type IL-1α and the granzyme B cleavage mutant IL-1α^{D103A} in the same system (Figure 4.10B). Notably, IL-1α^{D103A} was completely resistant to proteolysis, while wild type IL-1α was efficiently cleaved. Collectively, these data demonstrate that granzyme B is responsible for the processing of extracellular IL-1α in the context of NK cell attack.

Finally, we explored the ability of granzyme B to cleave intracellular IL-1α during NK-mediated killing. To this end, we subjected HeLa cells overexpressing full-length IL-1α to cytotoxic attack by YT and NK-92 (another NK cell line) cells. As Figures 4.11A and B show, intracellular IL-1α was efficiently processed during

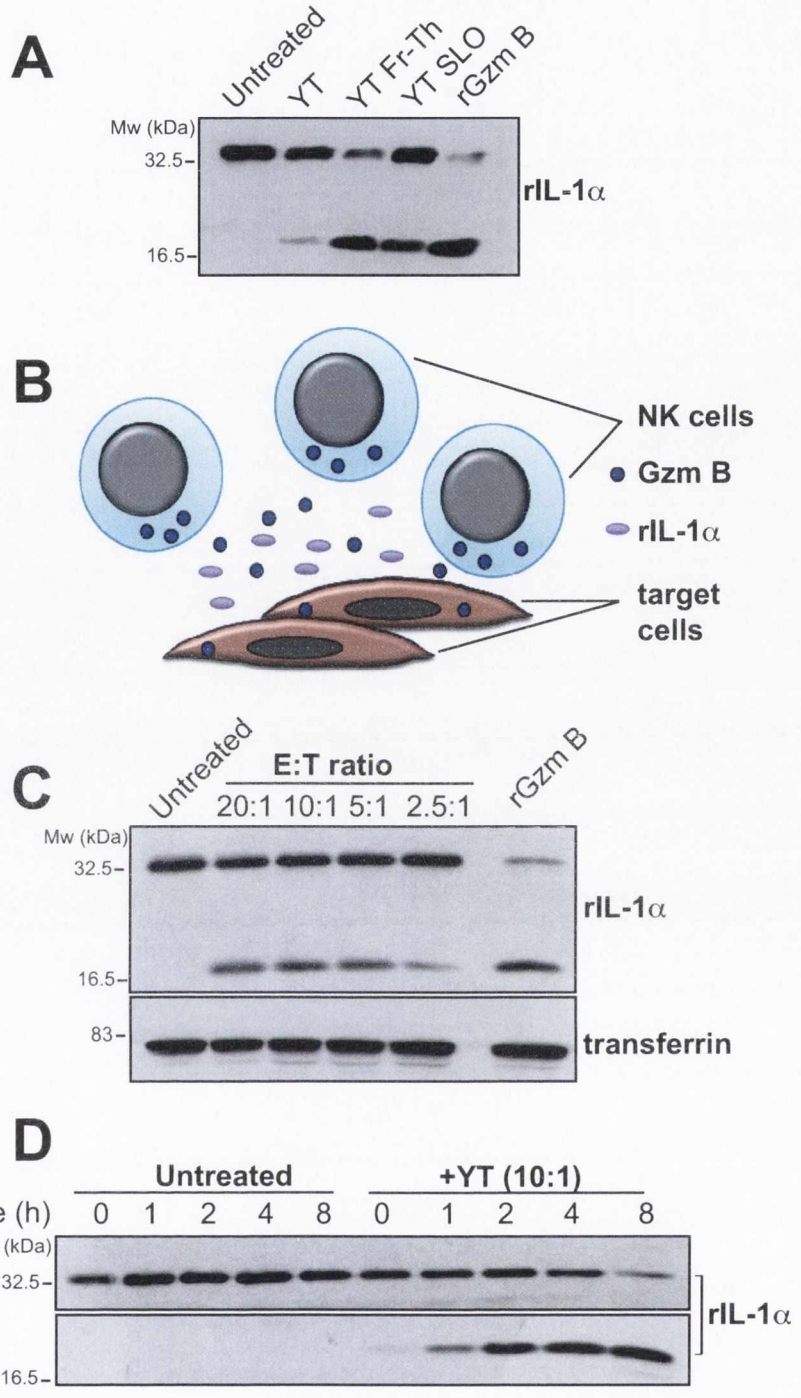

Figure 4.9

IL-1α is processed in the supernatants of NK cells

(A) YT cells were either left untreated or were permeabilized via 2 cycles of freeze-thaw (Fr-Th), or addition of 10 µg/ml of streptolysin O (SLO) for 90 min. Recombinant IL-1α was added to the supernatants, which were further incubated for 2 h at 37°C and analysed for IL-1α processing by immunoblotting. (B) Schematic representation of the extracellular IL-1α cleavage assay. (C) YT cells were incubated for 18 h with HeLa target cells at the indicated effector:target ratios in the presence of recombinant IL-1α. Culture supernatants were analysed by immunoblot for IL-1α processing. Serum-derived transferrin served as a loading control. (D) HeLa cells were incubated with recombinant extracellular IL-1α for the indicated times, in the presence or absence of YT cells, as indicated. Supernatants were then analysed for IL-1α processing by immunoblot.

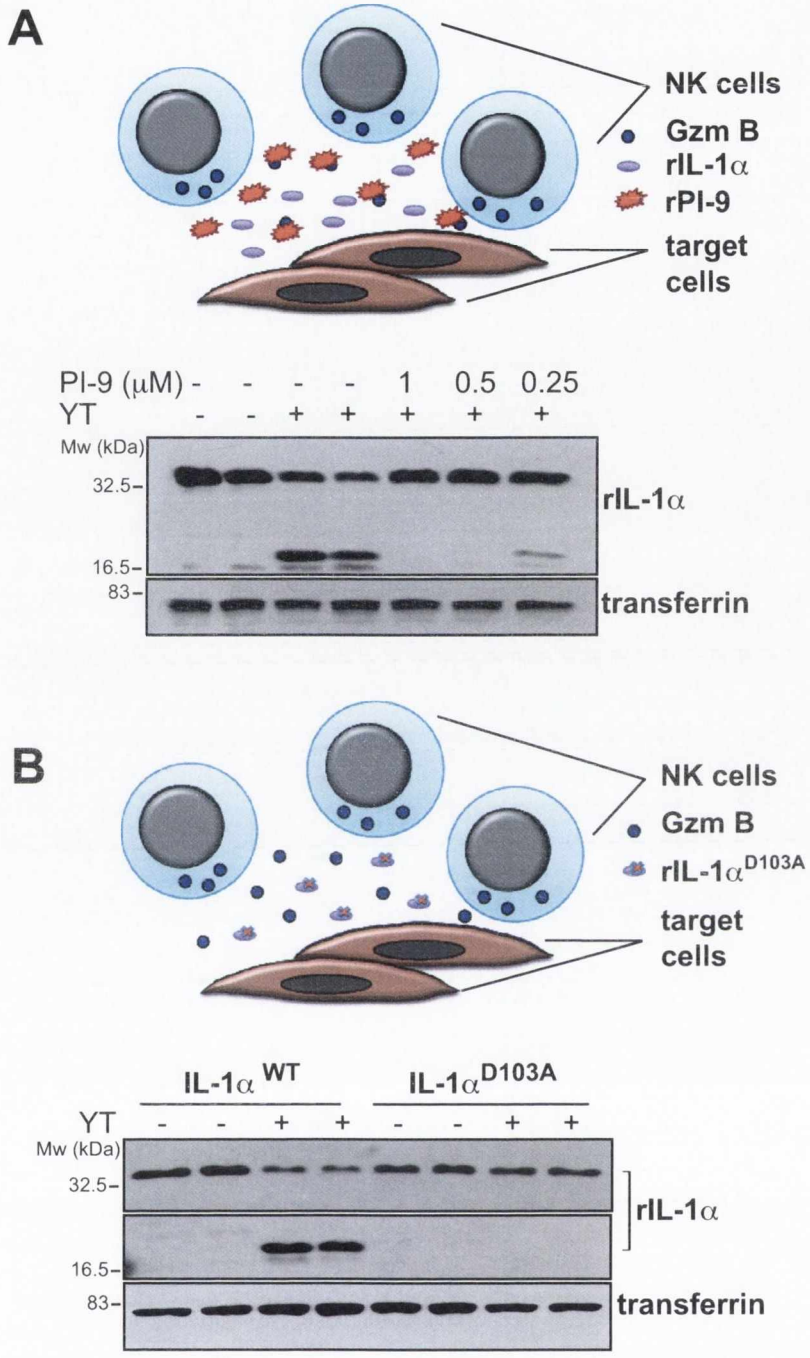

Figure 4.10

IL-1α is processed by granzyme B in the supernatants of NK cells

(A) YT cells were incubated for 6 h with HeLa target cells at an E:T ratio of 10:1 in the presence of recombinant IL-1α and PI-9, as indicated. Supernatants were then analysed for IL-1α processing by immunoblot. Serum-derived transferrin served as a loading control. Schematic representation of the extracellular IL-1α cleavage assay is shown above the data. (B) YT cells were incubated for 6 h with HeLa target cells at an E:T ratio of 10:1 in the presence of either recombinant IL-1α^{WT} or IL-1α^{D103A} mutant, as indicated. Supernatants were then analysed for IL-1α processing by immunoblot. Serum-derived transferrin served as a loading control. Schematic representation of the extracellular IL-1α cleavage assay is shown above the data.

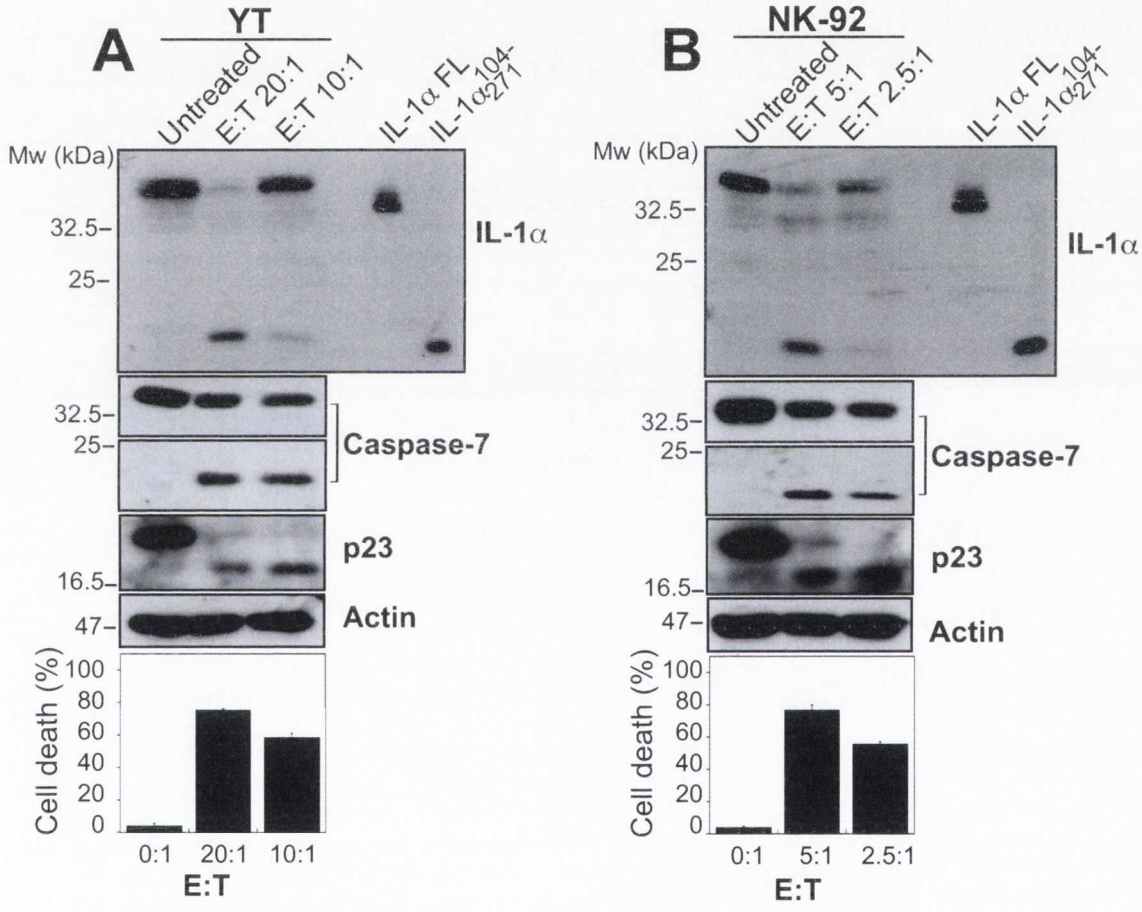

Figure 4.11

IL-1α is processed during NK-mediated killing

(A, B) HeLa cells were transfected with IL-1α expression plasmid. 24 h later cells were exposed to YT cells (A) or NK-92 cells (B) cells at the indicated E:T ratios. NK cells were removed 3-5 h later and HeLa cells were further incubated for the total of 24 h, after which cell lysates were generated and immunoblotted for the indicated proteins. Cell death was assessed by annexin V/propidium iodide binding, measured by flow cytometry.

target cell apoptosis (initiation of apoptosis was confirmed via cleavage of caspase-7 and its substrate, p23, as well as annexin V/PI staining).

4.2.6 IL-1α is not an inhibitor of granzyme B

It has been reported that recombinant IL-1α and TNF-α can protect specific cell lines (HeLa, WISH) from NK cell-mediated cytotoxicity (Reiter and Rubinstein, 1990). Therefore, we decided to test whether IL-1α is an inhibitor of granzyme B. To this end, we simultaneously co-incubated granzyme B with its biological substrate Bid and IL-1α. If IL-1α acts as a granzyme B inhibitor we would expect to see decreased cleavage of Bid. However, Bid was still efficiently cleaved by granzyme B even in the presence of IL-1α (Figure 4.12), indicating that IL-1α does not act as a direct granzyme B inhibitor.

4.3 Discussion

4.3.1 IL-1α is a novel granzyme B substrate

Granzyme B plays an established role in apoptosis, cleaving a well described set of proteins, which, in turn, leads to the demolition of cells (Cullen and Martin, 2008). However, several extracellular granzyme B substrates have been described, including extracellular matrix proteins and growth factor receptors (Buzza et al., 2005; Loeb et al., 2006). Moreover, elevated levels of both granzyme A and granzyme B were found in synovial fluid of patients with rheumatoid arthritis and plasma of patients with bacterial infections (Spaeny-

Dekking et al, 1998; Lauw et al., 2000). Even more intriguingly, several groups reported granzyme B expression in non-cytotoxic immune cells, such as plasmacytoid dendritic cells, macrophages, mast cells, basophils, B lymphocytes, keratinocytes and platelets (reviewed in Afonina et al., 2010). These puzzling phenomena imply that granzymes might have additional non-cytotoxic functions.

In this study, we establish a direct link between granzyme B and inflammation, identifying the major inflammatory cytokine IL-1α as a novel granzyme B substrate. Several earlier studies support our observation. Thus, granzymes A and K have been proposed to have a proinflammatory function and induce cytokine secretion by a variety of different cells (Sower et al., 1996a,b, Metkar et al, 2008, Joeckel et al., 2011). Furthermore, granzyme A was shown to directly cleave the proinflammatory cytokine IL-1β, although the biological consequences of this proteolytic event are not fully understood (Irmler et al., 1995).

Additionally, recent work by Omoto and colleagues has identified IL-18 as a granzyme B substrate (Omoto et al., 2010). There, authors showed that granzyme B-mediated cleavage of IL-18 resulted in the activation of the latter, as determined by induction of IL-18-dependent IFN-γ secretion by responder cells. However, unlike the proteolysis of IL-1α demonstrated in our work, cleavage of IL-18 by granzyme B was extremely inefficient with minimal proteolysis being detected after 6h (Omoto et al., 2010). These data suggest that IL-1α is a more efficient substrate for granzyme B than IL-18 is. Notably, an interleukin screen conducted in our lab identified several additional interleukins (IL-6, IL-12, IL-17) as potential granzyme B substrates. However, only IL-1α proved to be an efficient substrate for granzyme B, underlining the high specifity of this proteolytic event (data not shown).

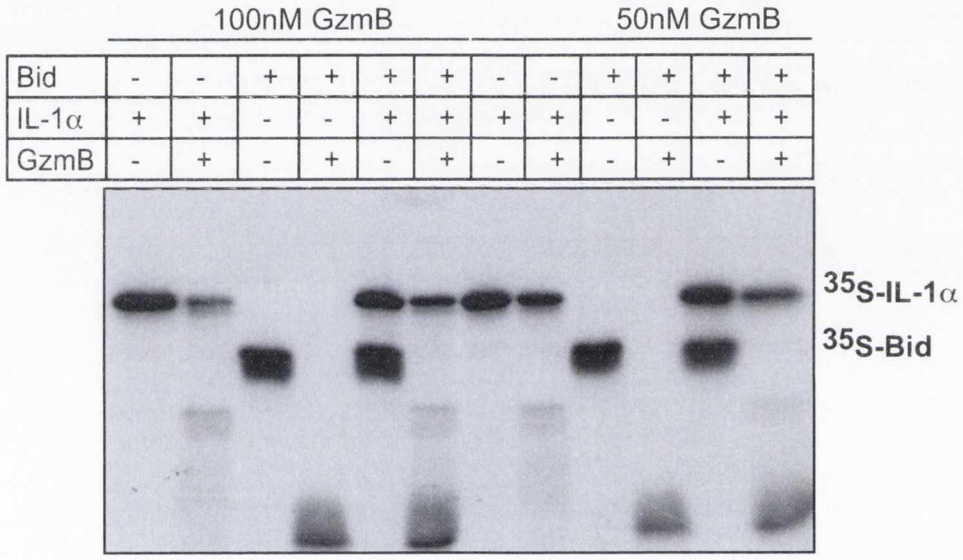

Figure 4.12

IL-1α is not an inhibitor of granzyme B

In vitro transcribed/translated Bid and IL-1α were incubated as indicated with granzyme B for 2 h at 37°C. Samples were resolved by SDS-PAGE and visualised by autoradiography.

As was discussed earlier, it is important to use physiological concentrations of any protease under investigation, as excessive concentrations of such enzymes are likely to produce spurious results. Therefore, candidate substrates have to be validated in a cellular setting. Most importantly, we demonstrated here that endogenous IL-1α is efficiently processed during NK-mediated attack.

We also showed that in the presence of target cells, NK cells leak some of their granzyme B into extracellular space, which is in agreement with previous data (reviewed in Buzza and Bird, 2006). Intriguingly, upon escaping the immunological synapse, granzyme B was capable of cleaving IL-1α. Thus, our experiments suggest an alternative scenario, where granzyme B may encounter IL-1α or IL-18 extracellularly and regulate inflammatory response via proteolysis of these cytokines.

Considering that NK cells store other granzymes and proteases in their granules, we confirmed the specifity of IL-1α proteolysis using recombinant granzyme B inhibitor PI-9 and uncleavable IL-1α^{D103A} mutant. Collectively, these data establish that IL-1α is a bona fide granzyme B substrate.

4.3.2 Evolutionary conservation of IL-1α proteolysis

Data generated previously in our lab demonstrated that human and mouse granzyme B have divergent substrate specifities and differ in their ability to process the key substrate, Bid, and other common granzyme B substrates, such as ICAD and caspase-8 (Cullen et al., 2007). Therefore, we were interested to explore whether pro-inflammatory function of granzyme B is evolutionary

conserved. Importantly, sequence analysis of IL-1α from different species showed that the putative granzyme B cleavage site is absolutely conserved across species (see Figure 4.5A). Moreover, we also demonstrated that mouse granzyme B is capable of processing mouse IL-1α, although with somewhat different efficiency and cleavage patterns. Thus, while human granzyme B only produced a 17.5 kDa fragment, mouse granzyme B generated an additional 25 kDa cleavage fragment, suggesting the existence of an additional cleavage site within the N-terminus of mouse IL-1α. Interestingly, proteolysis of mouse IL-1α by human granzyme B produced the 17.5 kDa fragment only, which further confirms the previous data reporting the differences in the substrate specifity between the two granzymes (Cullen et al., 2007).

4.3.3 Functional consequences of IL-1α proteolysis: redistribution and secretion of IL-1α

What could be the functional consequences of granzyme B-mediated proteolysis of IL-1α? Firstly, cleavage of IL-1α might abolish the biological activity of this cytokine, as was previously shown for caspase-mediated inactivation of pro-inflammatory cytokine IL-33 (Lüthi et al., 2009). However, Mosley and colleagues previously analysed the activity of N-terminal truncations of IL-1α and showed that the core biologically active protein sequence is located between amino acids 128-267 (Mosley et al., 1987b). Considering that the granzyme B cleavage site (D103) is located upstream of this critical boundary (Figure 4.13), we predict that granzyme B-mediated proteolysis of IL-1α would not abrogate the bioactivity of

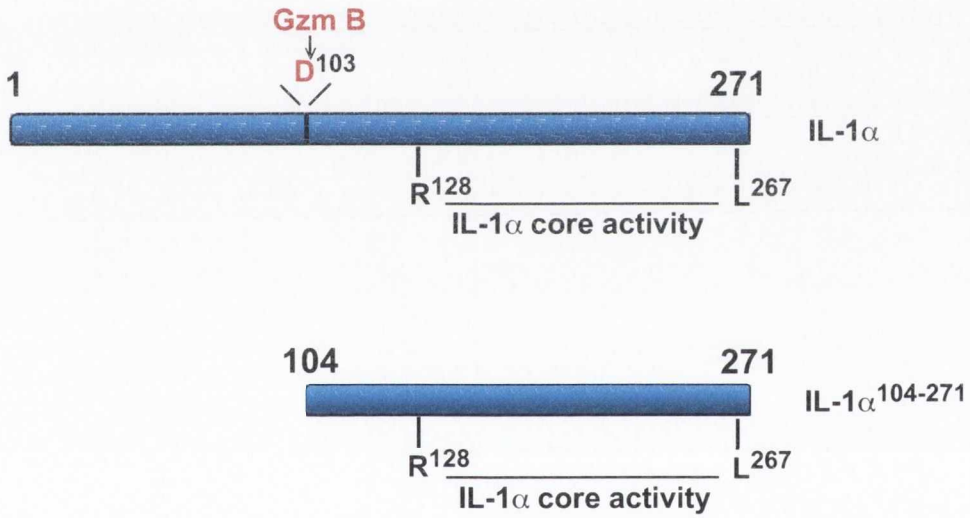

Figure 4.13

IL-1α schematic

Schematic representation of IL-1α$^{1-271}$ and IL-1α$^{104-271}$ indicating granzyme B cleavage site and the core bilogically active IL-1α sequence (Mosley et al., 1987b).

this cytokine. However, this question will be formally addressed in the following chapter.

Interestingly, IL-1α and TNF-α have been previously shown to protect specific cell lines from NK cell-mediated cytotoxicity (Reiter and Rubinstein, 1990). The observed effect was shown to be dependent on reduced target cell sensitivity to NK cytotoxic factors and not due to reduced target-effector conjugate formation or release of cytotoxic factors by NK cells (Reiter and Rubinstein, 1990). Hypothetically, similarly to PI-9 or adenoviral 100K protein, IL-1α might act as a granzyme B pseudosubstrate and exert its cytoprotective effect via inhibition of the protease. However, our data indicate that IL-1α does not act as a direct granzyme B inhibitor. Consequently, the observed protection from NK cell-mediated cytotoxicity was due to indirect effects of IL-1α on the transcription of pro-survival genes.

IL-1α secretion from cells is a rare event, however, IL-1α is present on the cell surface in a membrane-associated form (Kurt-Jones et al., 1985). Cell surface associated IL-1α is biologically active and has been shown to act as a co-stimulator of thymocyte proliferation (Kurt-Jones et al., 1985, Kurt-Jones et al., 1987) and induce gene expression in neighbouring cells in a juxtacrine fashion (Sasu et al., 2001). Since IL-1α does not have any hydrophobic trans-membrane domains, it is thought to be anchored to the cell-surface via lectin-like interactions (Brody and Durum, 1989) and can be removed from the cell surface by mild trypsin or plasmin digestion, while retaining its biological activity (Matsuhima et al., 1986; Niki et al., 2004).

One can hypothesise that granzyme B-mediated cleavage would release membrane-bound IL-1α in a similar manner and allow the cytokine to enter the circulation. In its membrane-bound form, IL-1α serves as a persistent localised stimulus for surrounding cells at the inflammatory site, while secreted IL-1α has been reported to mediate systemic responses, like fever and synthesis of acute phase proteins. Therefore, granzyme B possibly acts as a switch allowing IL-1α transition from a localised, membrane-bound form to a more systemically available, free IL-1α. Notably, Tak and colleagues found a positive correlation between granzyme B expression and serum levels of acute-phase proteins in rheumatoid arthritis patients (Tak et al., 1994).

Interestingly, a study by Niki and colleagues has shown that membrane-associated IL-1α contributed to chronic synovitis and cartilage destruction in human IL-1α transgenic mice (Niki et al., 2004). Thus, mice overexpressing human pro-IL-1α developed severe polyarthritic phenotype at 4 weeks of age, while mice overexpressing mature IL-1α, lacking the sequence necessary for interactions with the membrane, showed significantly lower histological and macroscopic scores of arthritis severity (Niki et al., 2001, Niki et al., 2004). Moreover, the activity of membrane-associated IL-1α displayed a significant correlation with the disease scores, while soluble IL-1α activity and serum IL-1α concentrations had no correlation with either score (Niki et al., 2004). These data suggest that localised membrane-bound IL-1α plays a key role in the development of rheumatoid arthritis. The potential ability of granzyme B to remove IL-1α from the cell membrane might, therefore, offer protection from harmful effects of membrane-associated cytokines.

Granzyme B could additionally affect distribution of IL-1α by affecting its secretion. Similarly to IL-1β, processed IL-1α has been shown to be selectively secreted from cells, while the full-length form stays trapped within the cell and its release is correlated with cell damage (Watanabe and Kobayashi, 1994). The release of IL-1α and IL-1β in response to cell injury and induction of apoptosis has also been investigated (Hogquist et al., 1991). Necrotic cell death was found to result in the predominant release of full-length IL-1α and IL-1β, while activation of the inflammasome or CTL-mediated killing led to the secretion of processed forms. Importantly, full-length IL-1α has been reported to have intracellular functions, controlling gene expression, cell proliferation and differentiation (Werman et al., 2004; Maier et al., 1990). Thus, granzyme B-mediated processing of IL-1α might facilitate secretion of cell-trapped cytokine, changing its status from an intracellular transcription factor to a systemic proinflammatory mediator.

Interestingly, a recent study has found that IL-1α is retained in the nucleus of apoptotic cells, while necrotic cells release the cytokine into extracellular space (Cohen et al., 2010). Considering that IL-1α transport to the nucleus is facilitated by the N-terminal NLS, which was found between amino acids 79 to 86 (Wessendorf et al., 1993), we suggest that cleavage of IL-1α by granzyme B may remove the NLS and help to override the nuclear retention of this cytokine, although this hypothesis requires further investigation.

In conclusion, we have shown here that the proinflammatory cytokine IL-1α is a direct substrate for granzyme B, which is capable of cleaving IL-1α both intra- and extracellularly. Cleavage of IL-1α is an evolutionary conserved event, since both human and mouse IL-1α are cleaved by granzyme B from corresponding species.

This is one of the first reports showing that granzyme B cleaves a proinflammatory cytokine, suggesting a role for the former in regulating the immune response.

Interestingly, the effects of IL-1α cleavage by calpain on its biological activity have never been investigated. Therefore, in the next chapter of this study we formally explore the consequences of IL-1α proteolysis on the biological activity of this cytokine.

Chapter 5

Proteolysis of IL-1α enhances its biological activity both *in vitro* and *in vivo*

5.1 Introduction

In the previous chapter, we described the proinflammatory cytokine IL-1α as a novel substrate for the apoptotic protease granzyme B. Interestingly, Mosley and colleagues have reported that both proform and N-terminally truncated forms of IL-1α bind the IL-1R in an *in vitro* binding assay and exhibit biological activity (Mosley et al., 1987a,b). Although previous studies have implicated calpain in the processing of IL-1α, the biological consequences of this proteolytic event have never been resolved (Kobayashi et al., 1990, Carruth et al., 1991). Unlike IL-1α, only the mature form of IL-1β was shown to bind the IL-1R and display bioactivity (Mosley et al., 1987a), but the molecular basis for these differences remains unclear.

Previous work in our lab has shown that processing of the pro-inflammatory cytokine IL-33 by apoptotic caspases, caspase-3 and caspase-7, resulted in the loss of bioactivity of this cytokine (Lüthi et al., 2009). However, based on the work of Mosley and colleagues, who mapped the core IL-1α activity between amino acids 128-267, we suggest that granzyme B-mediated cleavage would not inactivate IL-1α (Mosley et al., 1987b). Interestingly, Omoto and colleagues showed that proteolysis of IL-18 by granzyme B activates this cytokine (Omoto et al., 2010).

As the consequences of IL-1α cleavage have never been formally explored, we decided to compare the activity of full-length and cleaved forms of IL-1α. In this chapter we show that contrary to the common perception, proteolytic processing of IL-1α dramatically potentiates its bioactivity both *in vitro* and *in vivo*.

5.2 Results

5.2.1 IL-1α activity assay

To determine if granzyme B-mediated processing of IL-1α modulates its activity, we took advantage of the ability of IL-1α to induce expression and secretion of proinflammatory cytokines in HeLa cells (Bertelsen and Sanfridson, 2007). First of all, we performed a time-course analysis of cytokine secretion in response to the IL-1α treatment, where we compared IL-1α with an arbitrary control protein, Annexin V. Addition of recombinant Annexin V failed to induce any cytokine release from HeLa cells, while IL-1α clearly induced secretion of IL-6 and IL-8 in a time-dependent manner (Figure 5.1A). Additionally, we added truncated IL-1α to HUVEC cells to confirm that primary cells respond to this cytokine, as was previously shown (Rhim et al., 2008). Figure 5.1B shows that in response to truncated IL-1α HUVEC cells also produced IL-6, IL-8 and GM-CSF.

Bacterially expressed proteins are often contaminated with bacterial PAMPs, predominantly LPS. Therefore, to control for possible PAMP-induced cytokine release, we treated HeLa cells with a range of bacterial pro-inflammatory stimuli and analysed supernatants for IL-6 and IL-8 secretion. Importantly, HeLa cells did not release any of the cytokines tested in response to various bacterial or yeast PAMPs (Figure 5.2), confirming that the observed effects on cytokine secretion was IL-1-dependent.

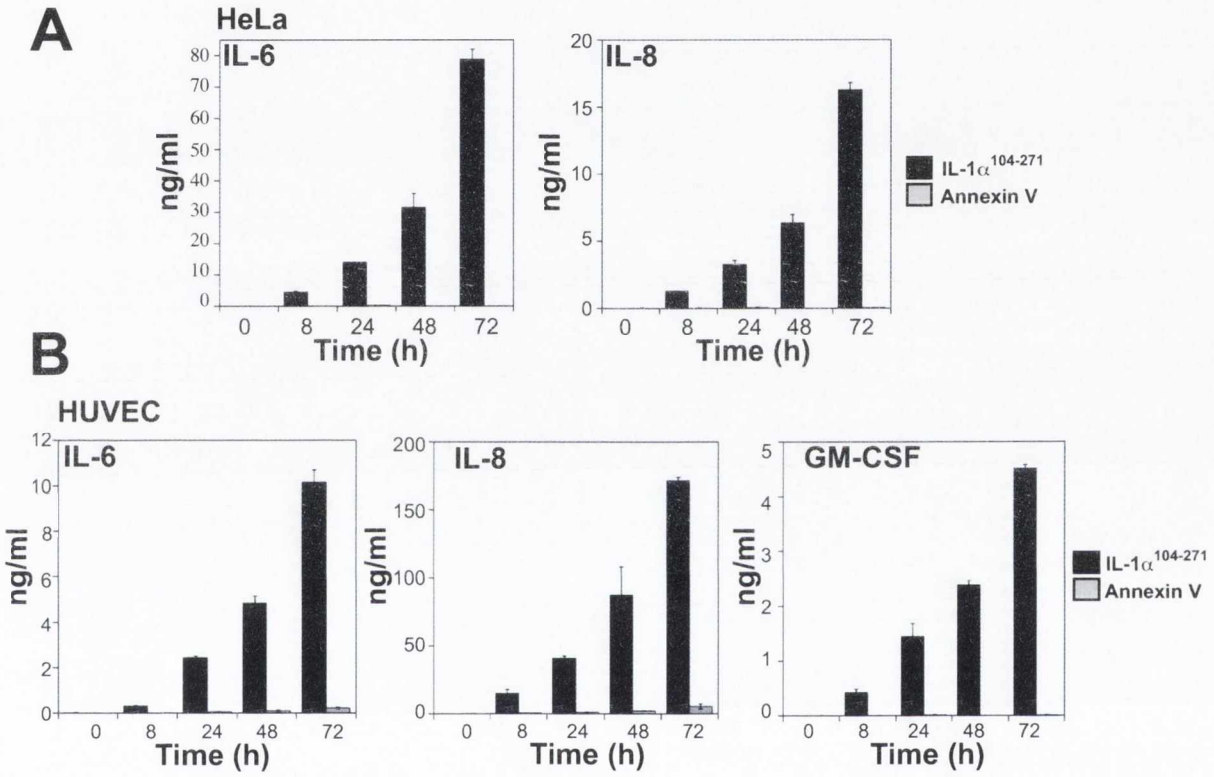

Figure 5.1

IL-1α induces release of pro-inflammatory cytokines

HeLa (A) or HUVEC (B) cells were incubated with 1 nM of recombinant IL-1α$^{104-271}$ or AnnexinV. Supernatants were collected at the indicated time-points and IL-6, IL-8 and GM-CSF concentrations were determined by ELISA. Error bars represent the mean ±SEM of determinations from three independent experiments.

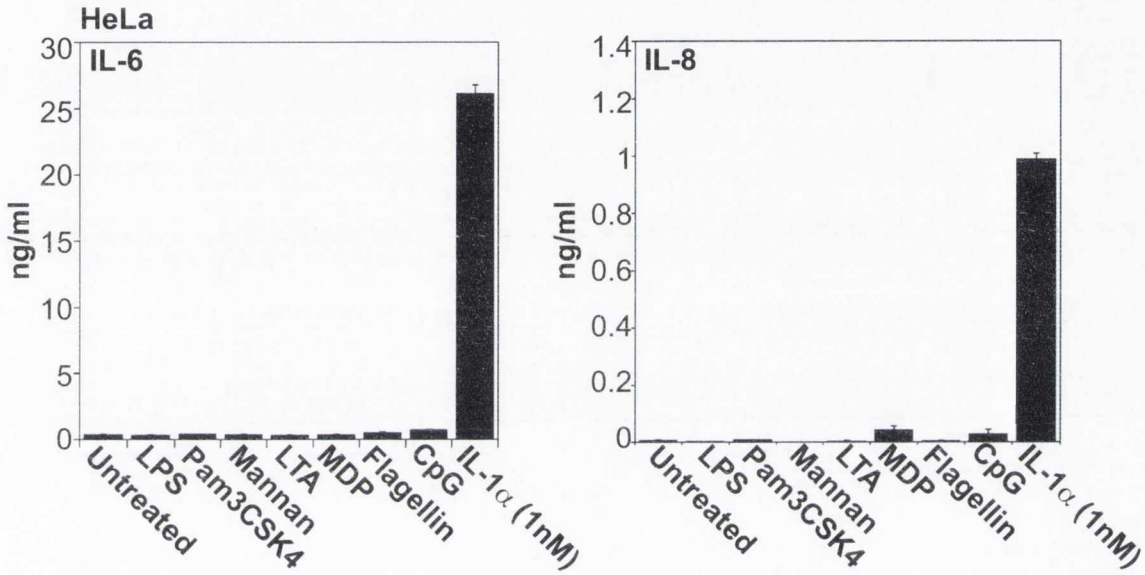

Figure 5.2

HeLa cells do not respond to bacterial or yeast stimuli

HeLa cells were incubated for 18 h with LPS (10 µg/ml), Pam3CSK4 (10 µg/ml), Mannan (10µg/ml), LTA (10 µg/ml), MDP (10 µg/ml), Flagellin (1 µg/ml), CpG (200 nM) or IL-1α (1 nM). IL-6 and IL-8 levels in culture supernatants were determined by ELISA. Error bars represent the mean ±SEM of determinations from three independent experiments.

5.2.2 Granzyme B-mediated proteolysis of IL-1α increases its bioactivity *in vitro*

To explore the functional consequences of granzyme B-mediated cleavage of IL-1α, we treated recombinant full-length IL-1α with either active or heat-inactivated granzyme B, which was added as a control to compensate for any factors present within the granzyme B fraction that may affect cytokine production. HeLa cells were incubated with different concentrations of either full-length or granzyme B-cleaved IL-1α and their effects on cytokine secretion were examined by ELISA. Surprisingly, we found that, even though full-length IL-1α was biologically active and induced significant production of IL-6 and IL-8 in a concentration-dependent manner, granzyme B-mediated cleavage of IL-1α enhanced its bioactivity 3-8 fold (Figure 5.3A). A similar effect of granzyme B-mediated proteolysis on IL-1α-induced secretion of IL-6, IL-8 and GM-CSF was also observed in primary HUVEC cells (Figure 5.3B).

We also performed time-course experiments comparing the full-length and cleaved forms of IL-1α and demonstrated that IL-1α cleavage by granzyme B significantly enhanced IL-1-dependent cytokine secretion when compared to full-length IL-1α at every time-point tested (Figure 5.4A). We then compared the potency of full-length IL-1α and IL-1α$^{104-271}$, which mimics the granzyme B cleavage fragment. Once again, we found that IL-1α$^{104-271}$ was more efficient in inducing cytokine release from HeLa cells than full-length IL-1α (Figure 5.4B).

To eliminate any possibility that the granzyme B present in IL-1α preparation might contribute to the cytokine production by HeLa cells, we decided to selectively inhibit this protease upon completion of IL-1α cleavage. To this end, we incubated

IL-1α with granzyme B for 3 hours, after which we added the granzyme B inhibitor PI-9 to the reaction and analysed the inhibition of granzyme B by measuring the hydrolysis of its fluorogenic substrate Ac-IETD-AFC. Addition of PI-9 completely inhibited any residual activity of granzyme B in the IL-1α preparations (Figure 5.5A), which was also confirmed by monitoring the cleavage of Bid by active granzyme B and granzyme B pre-incubated with PI-9. As Figure 5.5B shows, cleavage of Bid by granzyme B was entirely prevented by the pre-incubation of the enzyme with PI-9. We then compared the biological activity of full-length and cleaved IL-1α preparations, where residual granzyme B activity was inhibited. Inhibition of granzyme B had no effect on the secretion of either IL-6 or IL-8 by HeLa cells, demonstrating that cleaved IL-1α was still more potent than the full-length protein at any concentration tested (Figure 5.5C). Finally, as Figure 5.5D shows, incubation of truncated IL-1α$^{104-271}$ with granzyme B had no additional effect on the activity of this form of the cytokine, as expected. Taken together, these data demonstrate that processing of IL-1α by granzyme B enhances its activity *in vitro*.

5.2.3 IL-1α is cleaved by other granule proteases

Extracellular proteases, present at sites of inflammation, have been previously shown to regulate inflammation via cleavage of a variety of cytokines (Bank and Ansorge, 2001). For example, elastase, cathepsin G (Hazuda et al., 1990) and chymase (Mizutani et al., 1991) were reported to cleave and activate IL-1β, which prompted us to test whether IL-1α can be cleaved by any of these proteases.

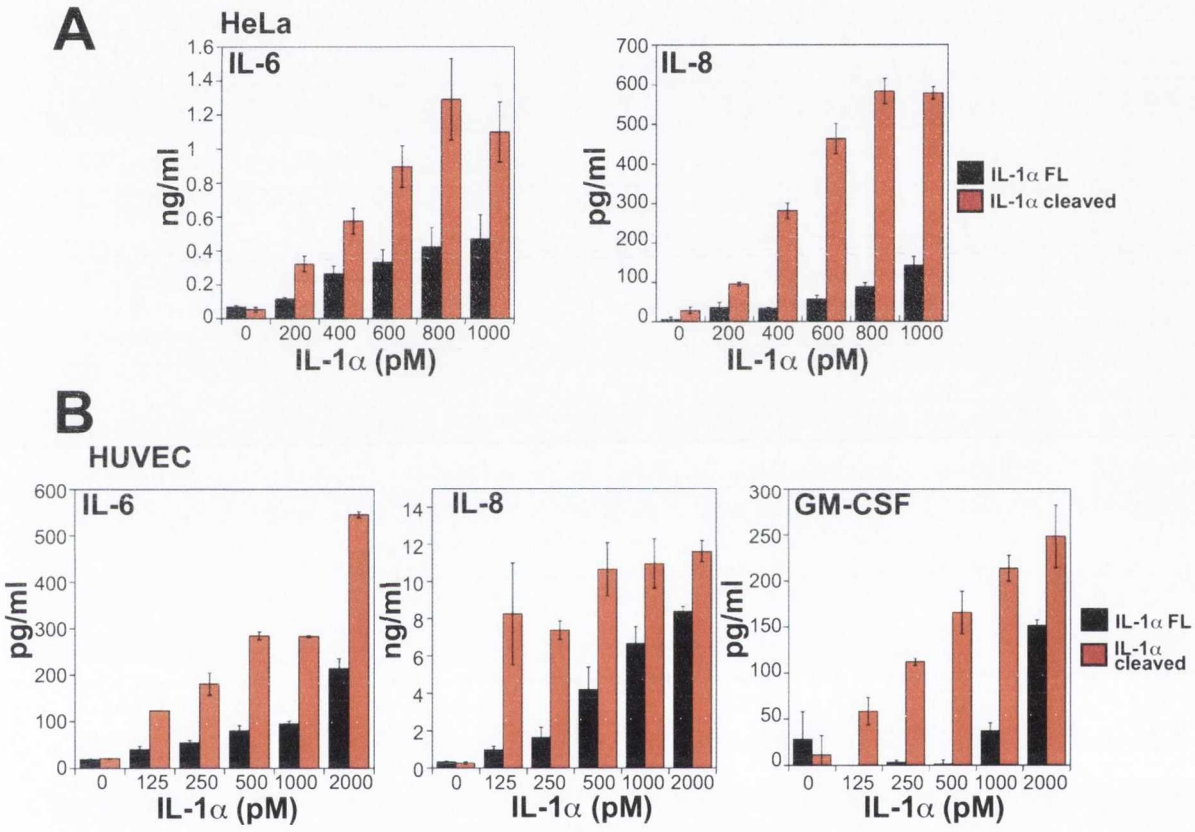

Figure 5.3

Granzyme B-mediated proteolysis enhances the biological activity of IL-1α
HeLa (A) or HUVEC (B) cells were incubated with the indicated concentrations of full-length or granzyme B cleaved forms of IL-1α. Supernatants were collected 8 h later and IL-6, IL-8 and GM-CSF concentrations were determined by ELISA. Error bars represent the mean ±SEM of determinations from three independent experiments.

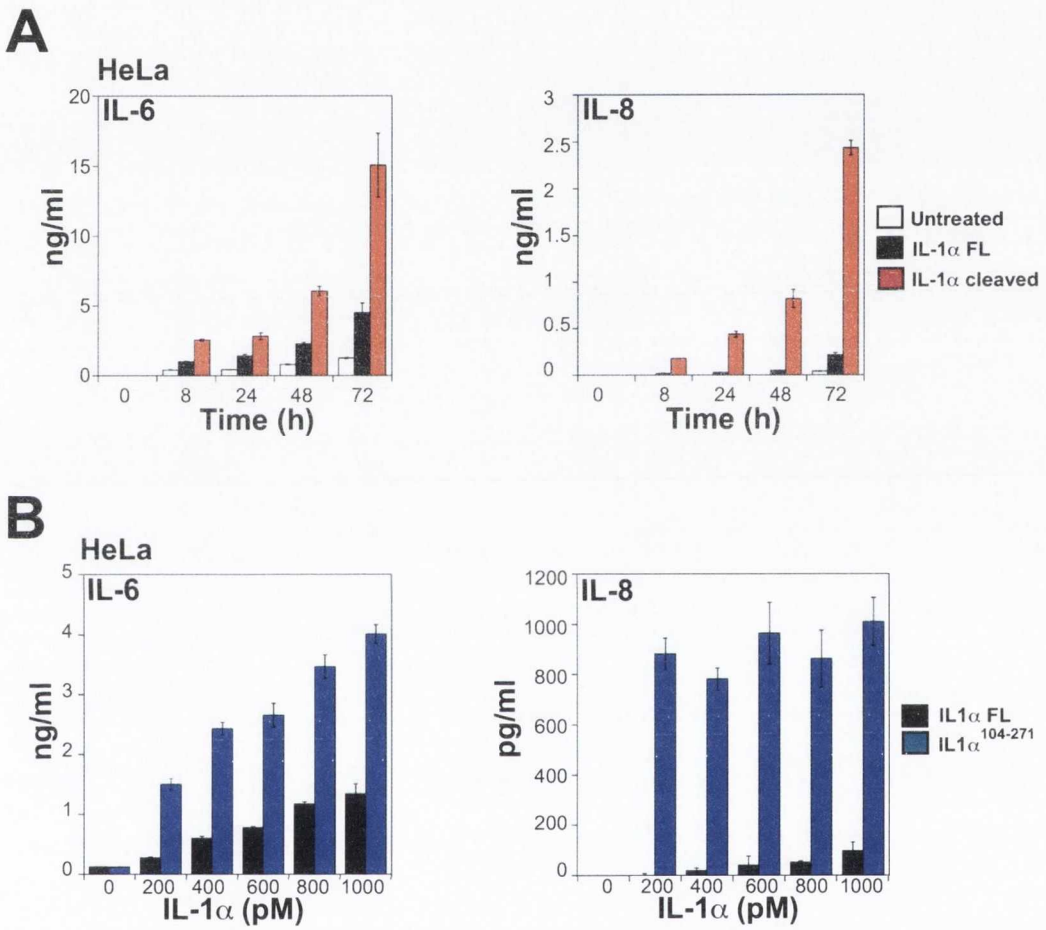

Figure 5.4

Granzyme B-mediated proteolysis enhances the bioactivity of IL-1α (2)

(A) HeLa cells were left untreated or incubated with 1 nM of full-length or granzyme B-cleaved IL-1α. Supernatants were collected at the indicated time-points. (B) Cells were incubated with the indicated concentrations of full-length IL-1α or IL-1α$^{104-271}$. Supernatants were collected 8 h later. IL-6 and IL-8 concentrations were determined by ELISA. Error bars represent the mean ±SEM of determinations from three independent experiments.

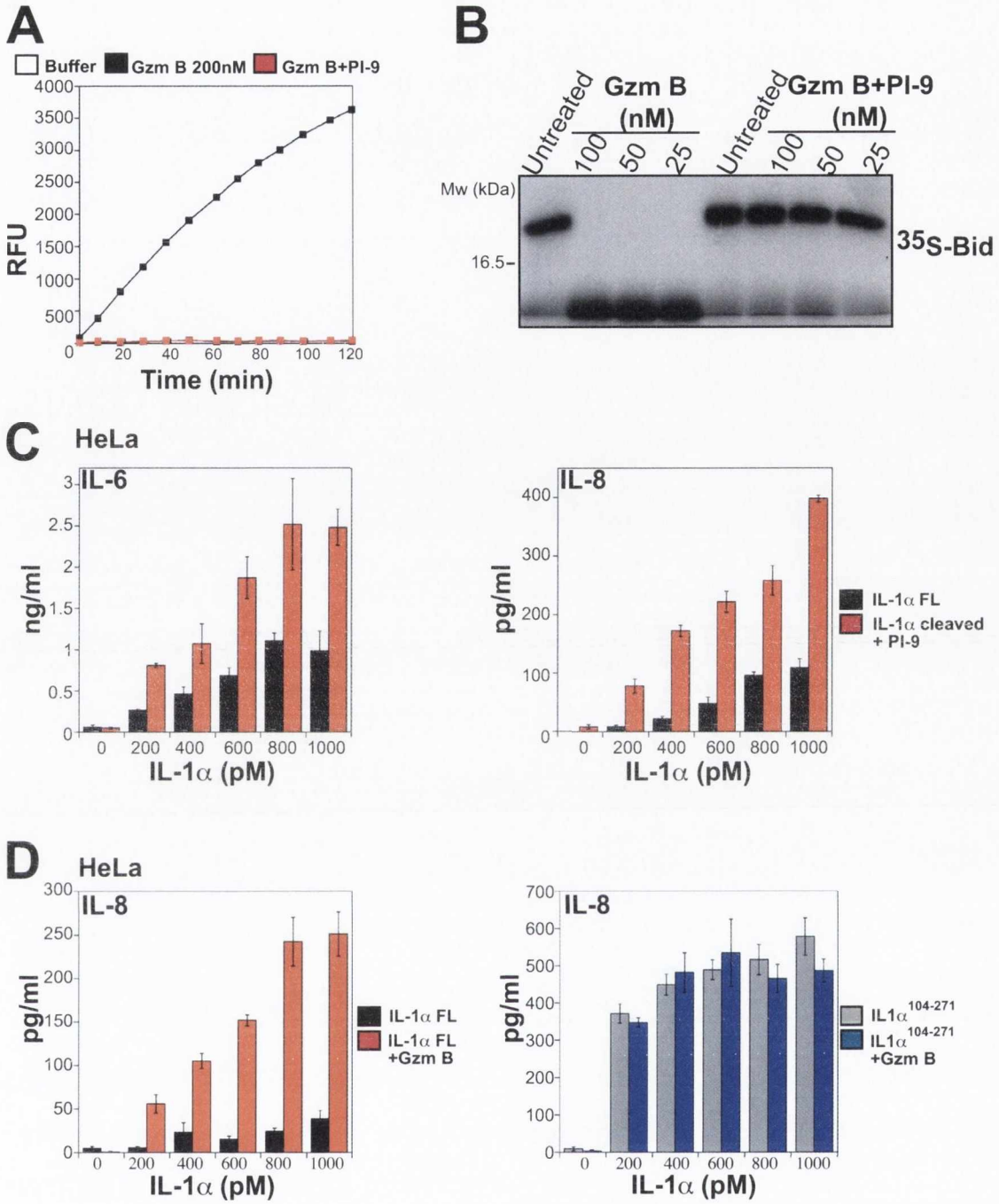

Figure 5.5

Granzyme B activity is not responsible for IL-1α-induced cytokine production by HeLa cells

(A-B) Recombinant IL-1α was incubated with granzyme B (200 nM) for 3 h at 37°C. PI-9 (1 μM) was then added to the reaction mix for further 30 min. Granzyme B inhibition was confirmed by measuring hydrolysis rate of granzyme B substrate Ac-IETD-AFC by fluorometry (A) and monitoring cleavage of *in vitro* transcribed/translated Bid (B). (C) HeLa cells were incubated for 8 h with the indicated concentrations of full-length or granzyme B-cleaved IL-1α, where granzyme B was inhibited by PI-9. IL-6 and IL-8 concentrations were determined by ELISA. (D) HeLa cells were incubated for 8 h with the indicated concentrations of IL-1α^{FL}, granzyme B-treated IL-1α^{FL}, IL-1$\alpha^{104-271}$, or granzyme B-treated IL-1$\alpha^{104-271}$, as indicated. IL-6 and IL-8 levels were determined by ELISA. Error bars represent the mean ±SEM of determinations from three independent experiments.

In vitro transcribed/translated IL-1α and IL-1β (used as a control) were incubated with either human neutrophil elastase (Figure 5.6A), cathepsin G (Figure 5.6C), proteinase 3 (Figure 5.6D) or human skin mast cell chymase (Figure 5.6B). All of these proteases readily cleaved IL-1α and IL-1β, although proteolysis by elastase and proteinase 3 was more efficient at the concentrations tested.

Cleavage of IL-1α by low concentrations of elastase initially generated a 24 kDa product, which was further processed upon increasing the concentration of the protease, with the final cleavage product running at approximately the same size as granzyme B-cleaved IL-1α (~17.5kDa) (Figure 5.6A). This migration pattern suggests that IL-1α has two elastase cleavage sites, one of which is located close to D103. Chymase-mediated proteolysis generated one major cleavage product with a molecular weight of 16.5 kDa, close in size to calpain-cleaved IL-1α, suggesting that chymase cleaves IL-1α after F116 (Figure 5.6B).

Cathepsin G cleavage patterns resembled that of elastase (compare Figure 5.6A and C), although the second cleavage product ran slightly faster than granzyme B-cleavage fragment, indicating that proteolysis takes place downstream of D103. Proteinase 3-mediated processing resulted in a single 25 kDa fragment, implying that cleavage takes place within the N-terminus of the protein (Figure 5.6D). Notably, all the proteases efficiently cleaved IL-1α at the concentrations tested.

5.2.4 Cleavage of IL-1α by other inflammatory proteases enhances its bioactivity *in vitro*

Calpain, a calcium activated neutral protease, has been previously shown to cleave IL-1α after F118, positioned close to the granzyme B cleavage site, although the functional consequences of this proteolytic event have never been investigated (Carruth et al., 1991; Kobayashi et al., 1990). Therefore, we decided to test whether calpain-mediated cleavage of IL-1α enhances the bioactivity of this cytokine.

We first confirmed that calpain cleaves IL-1α. Figure 5.7A shows that incubation of *in vitro* transcribed/translated IL-1α with calpain results in the efficient proteolysis of this cytokine. Furthermore, similarly to granzyme B, processing of IL-1α by calpain resulted in increased IL-6 and IL-8 secretion in both HeLa (Figure 5.7B) and HUVEC cells (Figure 5.7C) when compared to the full-length form. Moreover, processing by calpain enhanced IL-1α bioactivity to the same extent as granzyme B-mediated proteolysis, which was confirmed in both HeLa and HUVEC cells.

We then decided to test the effect of IL-1α cleavage by elastase and chymase. Unsurprisingly, proteolysis of IL-1α by either of these proteases enhanced its bioactivity to the same degree as granzyme B-mediated cleavage (Figure 5.8A and Figure 5.8B). Collectively, these data provide strong evidence that proteolytic processing of IL-1α by these inflammatory proteases results in the increased biological activity of the cytokine.

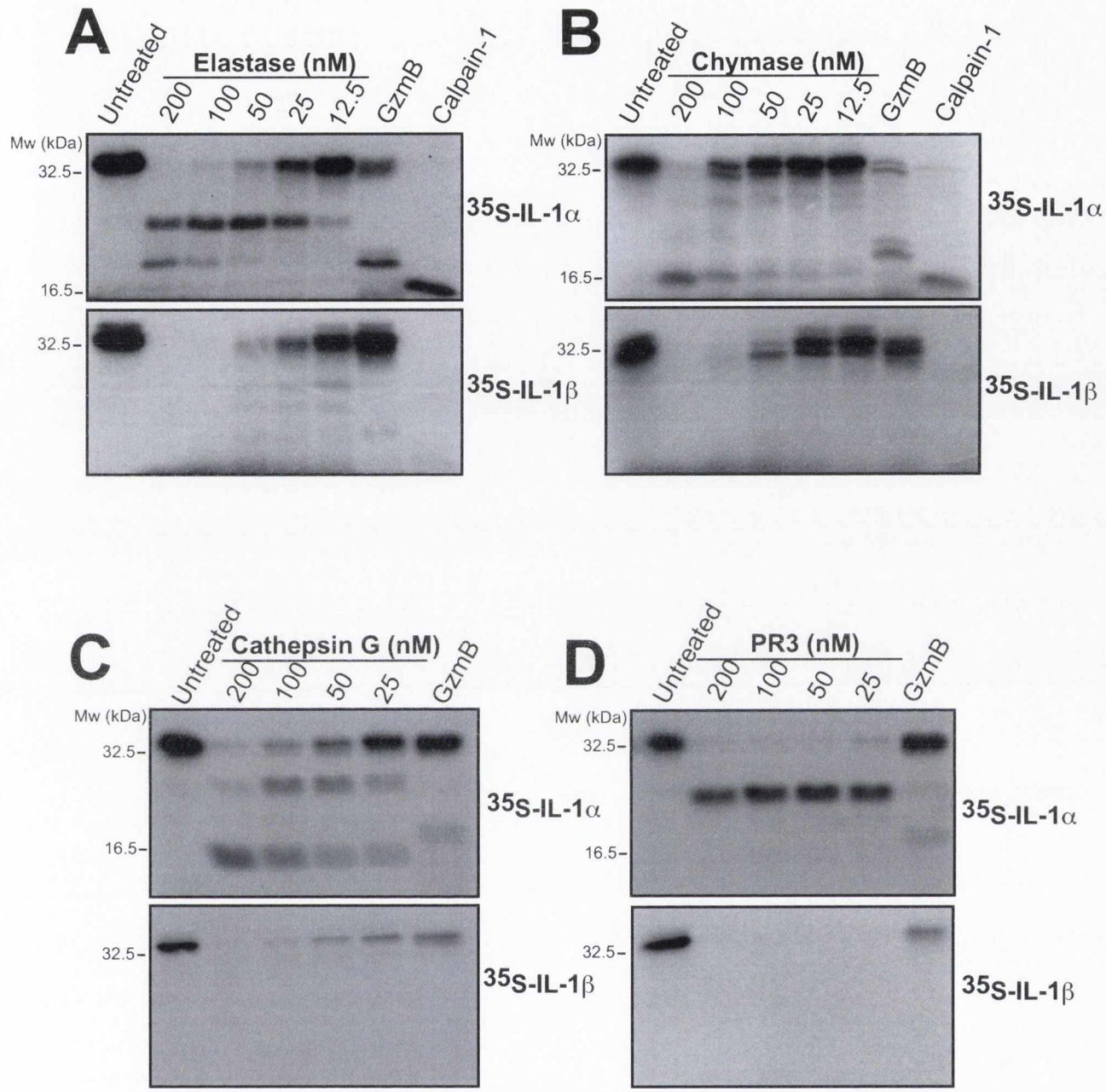

Figure 5.6

IL-1α is cleaved by other serine proteases

In vitro transcribed/translated IL-1α was incubated with the indicated concentrations of elastase (A), chymase (B), cathepsin G (C), proteinase-3 (D) or granzyme B (200nM), or calpain-1 (200nM) for 2 h at 37°C. IL-1α proteolysis was analysed by SDS/PAGE, followed by fluorography.

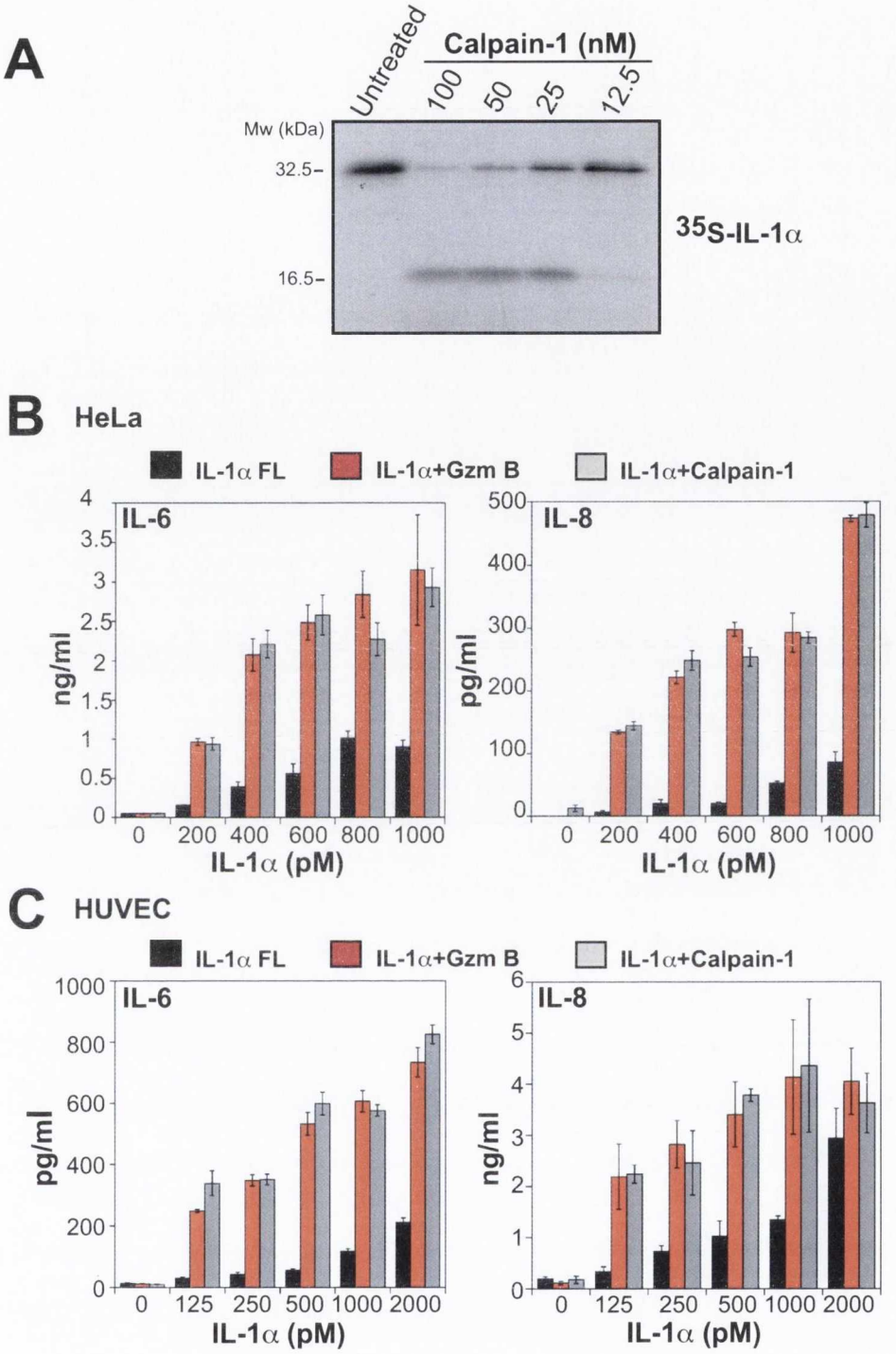

Figure 5.7

Calpain-mediated cleavage enhances the bioactivity of IL-1α

(A) *In vitro* transcribed/translated IL-1α was incubated with the indicated concentrations of calpain-1 for 2 h at 37°C. IL-1α proteolysis was analysed by SDS/PAGE, followed by fluorography. (B, C) HeLa (B) or HUVEC (C) cells were incubated for 8 h with the indicated concentrations of full-length, granzyme B- or calpain-1-cleaved IL-1α. IL-6 and IL-8 concentrations in the culture supernatants were determined by ELISA. Error bars represent the mean ±SEM of determinations from three independent experiments.

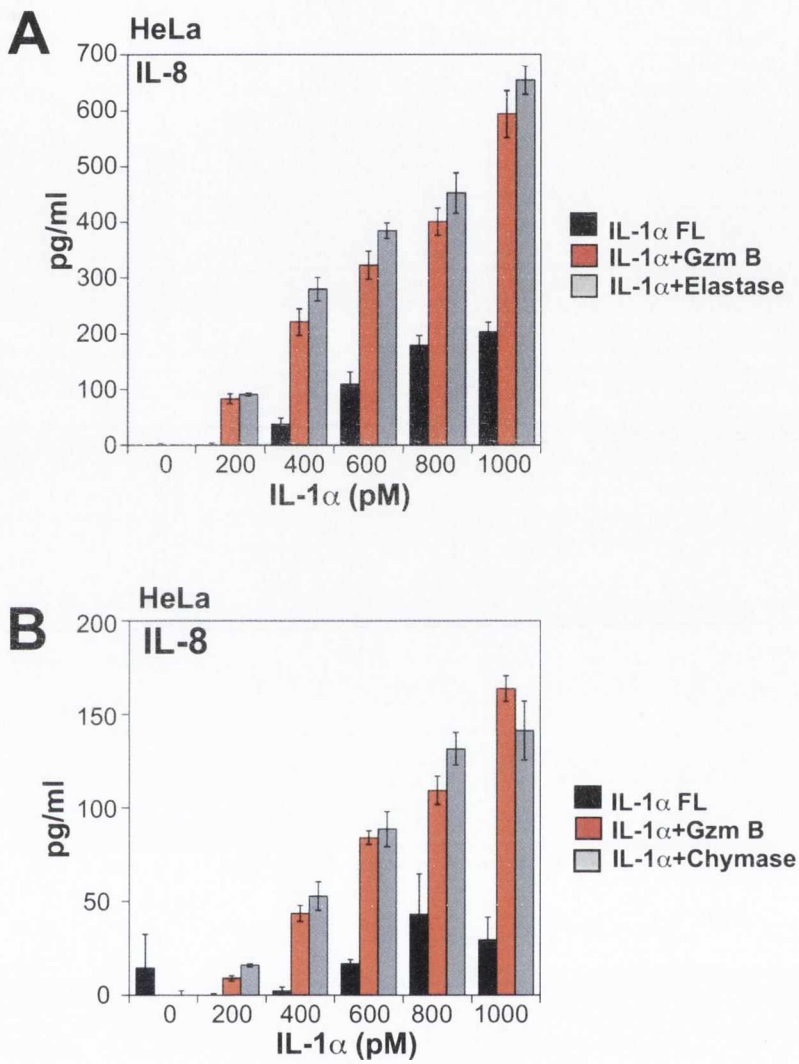

Figure 5.8

Elastase- and chymase-mediated cleavage enhances the bioactivity of IL-1α

HeLa cells were incubated for 8 h with the indicated concentrations of full-length, granzyme B-, elastase- (A) or chymase-cleaved IL-1α (B). IL-8 concentration in the culture supernatants was determined by ELISA. Error bars represent the mean ±SEM of determinations from three independent experiments.

5.2.5 IL-1α processing activity in inflammatory conditions

Persistent inflammatory lung conditions, such as cystic fibrosis, are characterised by massive neutrophil invasion and as a result by high levels of pulmonary proteases, in particular elastase (Vega-Carrascal et al., 2011, Hayes et al., 2011). To explore whether IL-1α is processed during inflammatory conditions, we obtained bronchoalveolar lavage fluids (BALF) from patients with cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD) or non-CF bronchiectasis and added recombinant IL-1α directly to BALF samples. Although we could not detect cleavage of IL-1α in COPD samples tested, possibly due to the lower quantities of the proteases present in these fluids (Vega-Carrascal et al., 2011), IL-1α was efficiently processed in several samples from patients with CF and bronchiectasis but not in control samples (Figure 5.9). Notably, the clevage fragments ran at the same size as recombinant granzyme B/elastase-generated cleavage fragments. Thus, proteases capable of processing IL-1α are present in the extracellular milieu under inflammatory conditions *in vivo*.

5.2.6 Granzyme B-cleaved IL-1α displays enhanced bioactivity *in vivo*

In vitro studies are conducted in a highly controlled environment and may lack crucial factors present *in vivo*. Therefore, we decided to examine whether cleavage of IL-1α by granzyme B resulted in enhanced bioactivity of this cytokine *in vivo*. To this end, we prepared recombinant full-length IL-1α for injection into mice and depleted it of contaminating LPS. Recombinant IL-1α was then incubated with either heat-inactivated or active granzyme B and the bioactivity of both forms of IL-1α was confirmed using HeLa cells (data not shown). We then

explored the effects of the full-length and cleaved forms of IL-1α in a specific, antigen-driven mouse model.

Generally, in the absence of adjuvant, immunisation of mice with an antigen induces only minimal humoral and cellular immune responses, while adjuvants (e.g., mineral salts, bacteria-derived molecules, liposomes and cytokines) greatly improve the immunogenicity of antigens. As it was previously shown that co-administration of IL-1 along with an antigen dramatically enhanced antigen-specific immune responses (Ben-Sasson et al., 2009), we decided to compare the adjuvant activity of full-length versus cleaved IL-1α using ovalbumin (OVA) as an antigen. BALB/c mice received OVA peptide either alone, or in combination with full-length or granzyme B-cleaved IL-1α, followed by boosting with the same combinations 2 weeks later. At 3 weeks, blood samples, peritoneal lavage and spleens were collected for further analysis (Figure 5.10).

To determine the effect of IL-1α on the humoral immune response, OVA-specific antibodies (total IgG, IgG1, $IgG2_a$ and $IgG2_b$) were measured in the serum samples. As Figure 5.11 shows, injection of OVA alone resulted in minimal antibody production, while both forms of IL-1α significantly boosted antibody responses to OVA, as expected. Importantly, the cleaved form of IL-1α exhibited considerably greater potency in this regard.

Next, we measured OVA-specific T-cell responses in the peritoneal lavage and spleens of the immunised mice. To this end, freshly isolated cells were re-stimulated with different OVA concentrations and the secretion of cytokines in response to antigen was measured by ELISA. Although immunisation with OVA alone resulted in an antigen-specific cytokine secretion in the re-stimulation

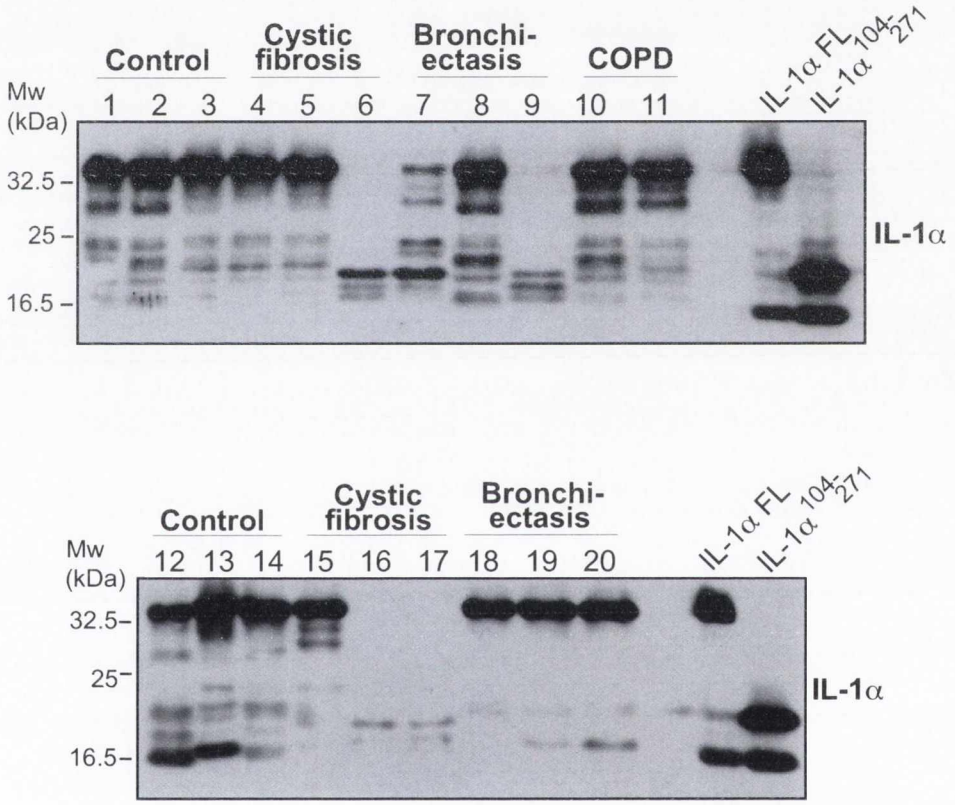

Figure 5.9

IL-1α is processed in the BALF samples from patients with cystic fibrosis and bronchiectasis

Recombinant purified full length IL-1α was incubated at 37°C for 20 min with 10 µl of BALF samples from control patients or patients with cystic fibrosis, bronchiectasis or chronic obstructive pulmonary disease (COPD). IL-1α processing was analysed by immunoblot. As controls, full-length IL-1α and IL-1α$^{104-271}$ were expressed in HEK293T cells and were included to facilitate size comparison of cleavage products.

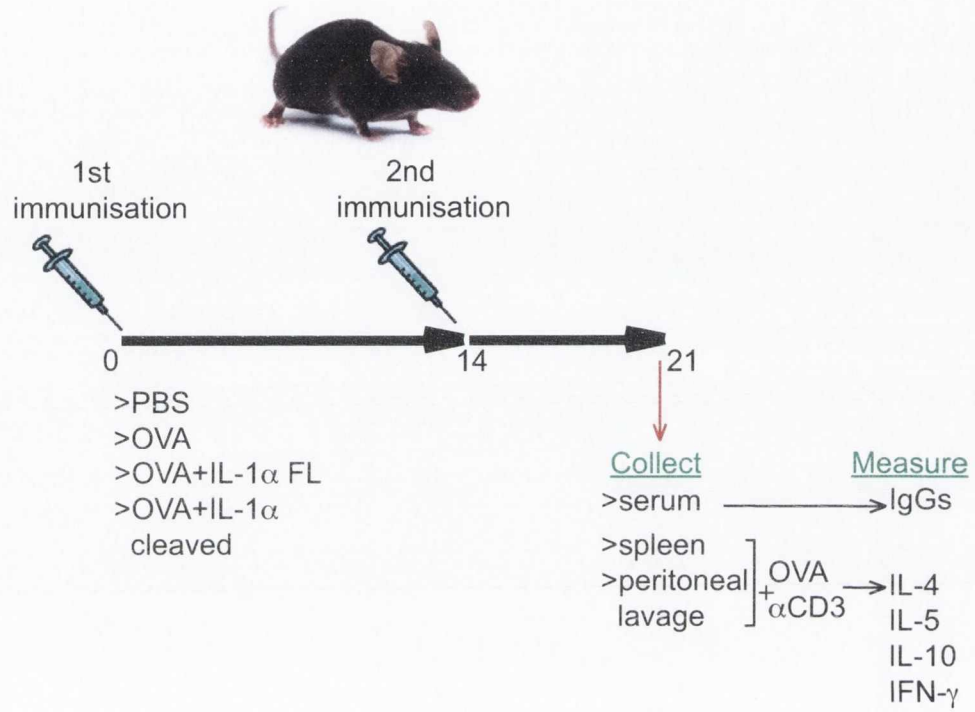

Figure 5.10

Comparison of IL-1α adjuvant activity in a mouse model

Balb/c mice (5 mice per group) were immunised either with ovalbumin (OVA) alone (200 µg), OVA in combination with full-length IL-1α (5 µg per mouse), or OVA in combination with granzyme B-cleaved IL-1α (5 µg per mouse). All mice were boosted with the same combinations on day 14. Peripheral blood, spleens and peritoneal lavages were collected on day 21 for analysis. OVA-specific IgGs were measured in serum samples. Cells from peritoneal lavages and spleens were re-stimulated for 3 days with OVA or anti-CD3. IL-4, IL-5, IL-10 and IFN-γ concentrations in the culture supernatants were determined by ELISA.

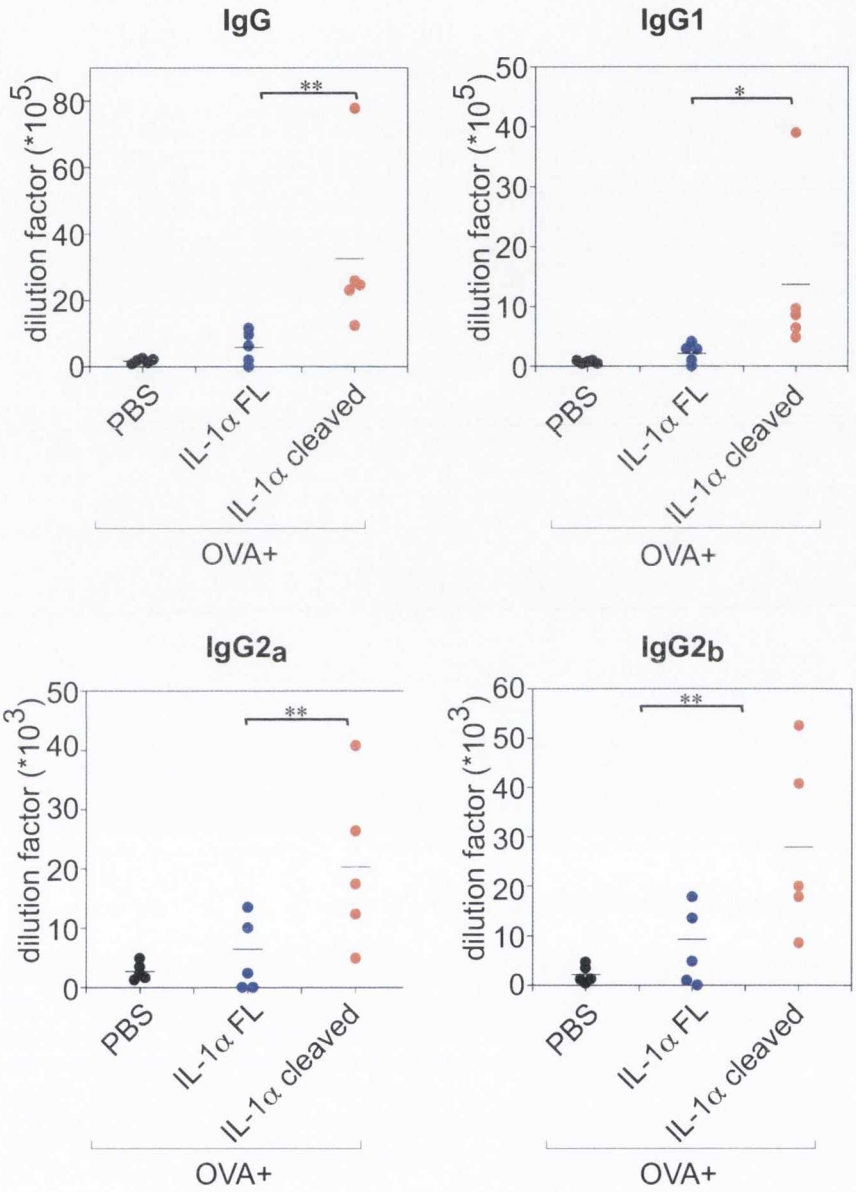

Figure 5.11

Granzyme B-cleaved IL-1α displays enhanced bioactivity *in vivo*. OVA-specific antibody response

C57BL/6 mice (5 mice per group) were immunised with either OVA alone (200μg), or OVA in combination with full-length IL-1α (5 μg), or OVA with granzyme B-cleaved IL-1α (5 μg). All mice were boosted with the same combinations on day 14. Blood was collected via tail bleed on day 21 and OVA-specific total IgG, IgG1, $IgG2_a$ and $IgG2_b$ in plasma samples were determined by ELISA. Significance levels, ***= p<.001, **= p<.05, *= p<.01, by students t-test.

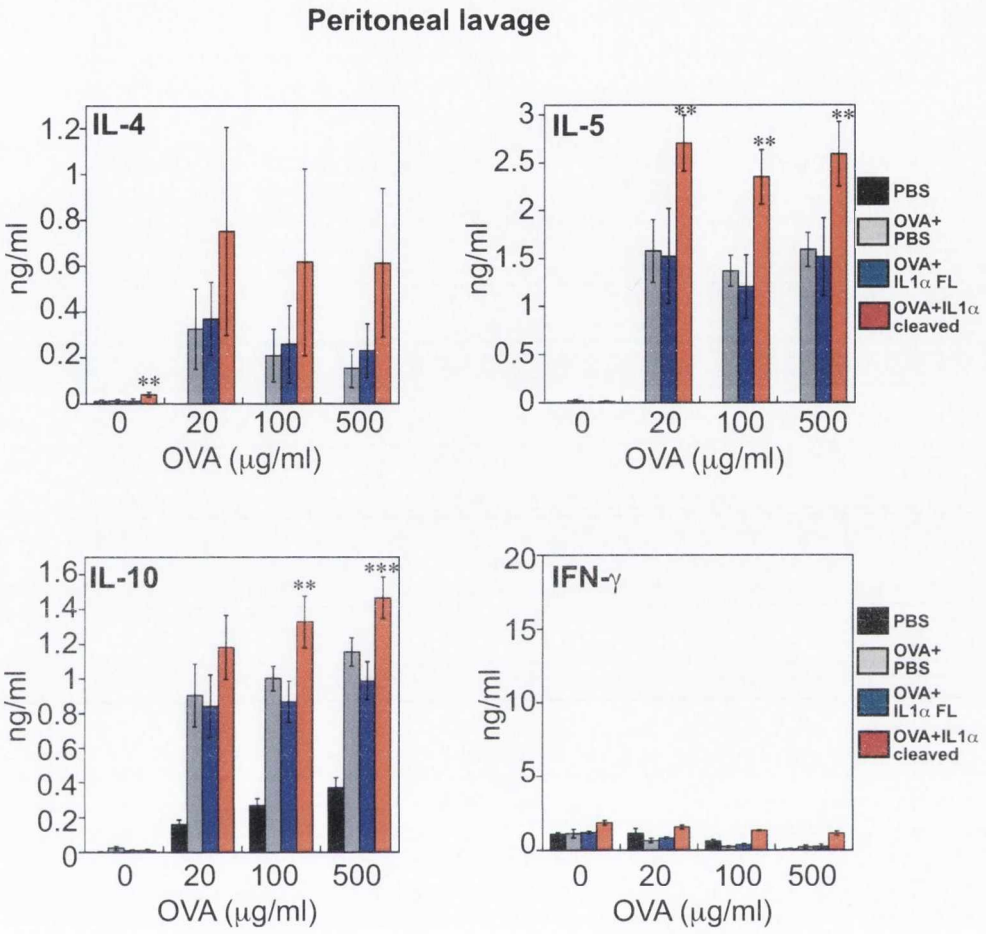

Figure 5.12

Granzyme B-cleaved IL-1α displays enhanced bioactivity *in vivo*. Re-stimulation of peritoneal lavage cells

Balb/c mice (5 mice per group) were immunised either with OVA alone (200 μg), OVA in combination with full-length IL-1α (5 μg per mouse), or OVA in combination with granzyme B-cleaved IL-1α (5 μg per mouse). All mice were boosted with the same combinations on day 14. Peritoneal lavages were collected on day 21 for analysis. Cells from peritoneal lavages were re-stimulated for 3 days with the indicated concentrations of OVA. IL-4, IL-5, IL-10 and IFN-γ concentrations in the culture supernatants were determined by ELISA. All measurements were taken in triplicate. Error bars represent the mean ± SEM from each group of five mice. Significance levels, ***= p<.001, **= p<.05, *= p<.01, by students t-test.

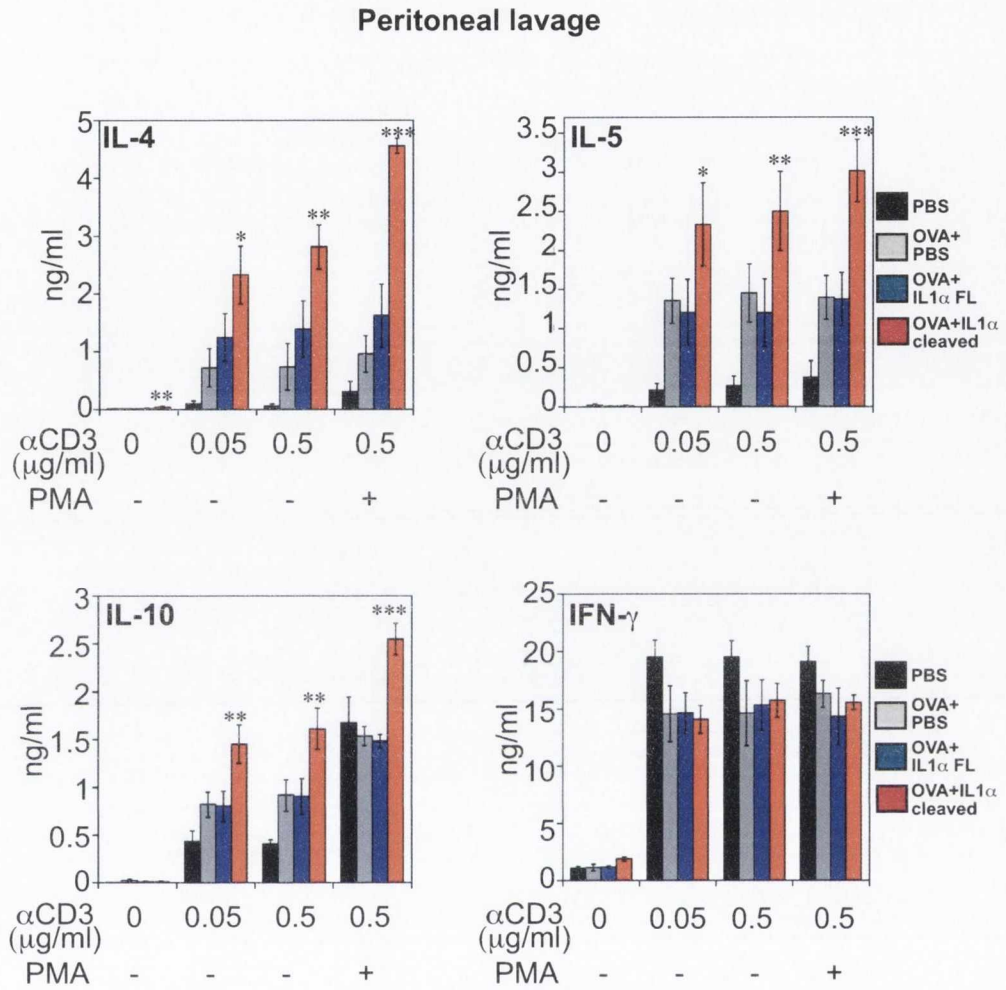

Figure 5.13

Granzyme B-cleaved IL-1α displays enhanced bioactivity *in vivo*. Re-stimulation of peritoneal lavage cells (2)

Balb/c mice (5 mice per group) were immunised either with OVA alone (200 μg), OVA in combination with full-length IL-1α (5 μg per mouse), or OVA in combination with granzyme B-cleaved IL-1α (5 μg per mouse). All mice were boosted with the same combinations on day 14. Peritoneal lavages were collected on day 21 for analysis. Cells from peritoneal lavages were re-stimulated for 3 days with the indicated concentrations of anti-CD3 and PMA (25ng/ml), as indicated. IL-4, IL-5, IL-10 and IFN-γ concentrations in the culture supernatants were determined by ELISA. All measurements were taken in triplicate. Error bars represent the mean ± SEM from each group of five mice. Significance levels, ***= p<.001, **= p<.05, *= p<.01, by students t-test.

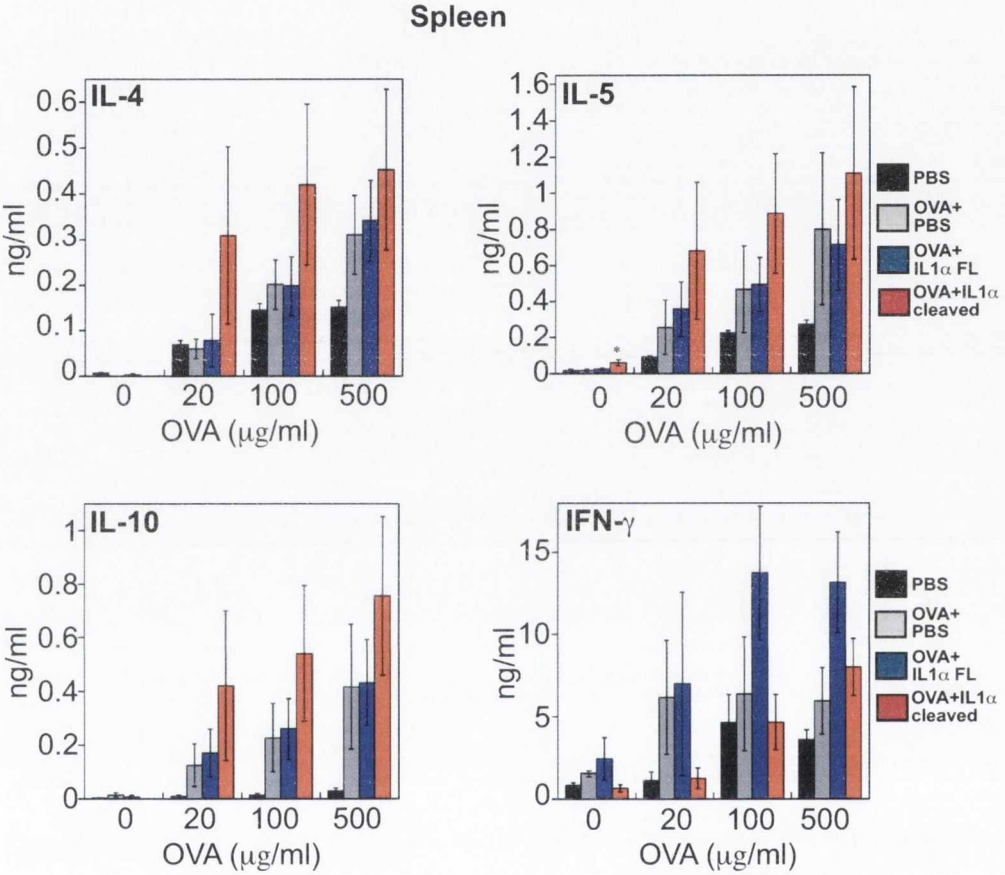

Figure 5.14

Granzyme B-cleaved IL-1α displays enhanced bioactivity *in vivo*. Re-stimulation of splenocytes

Balb/c mice (5 mice per group) were immunised either with ovalbumin (OVA) alone (200 µg), OVA in combination with full-length IL-1α (5 µg per mouse), or OVA in combination with granzyme B-cleaved IL-1α (5 µg per mouse). All mice were boosted with the same combinations on day 14. Spleens were collected on day 21 for analysis. Splenocytes were re-stimulated for 3 days with the indicated concentrations of OVA. IL-4, IL-5, IL-10 and IFN-γ concentrations in the culture supernatants were determined by ELISA. All measurements were taken in triplicate. Error bars represent the mean ± SEM from each group of five mice. Significance levels, ***= p<.001, **= p<.05, *= p<.01, by students t-test.

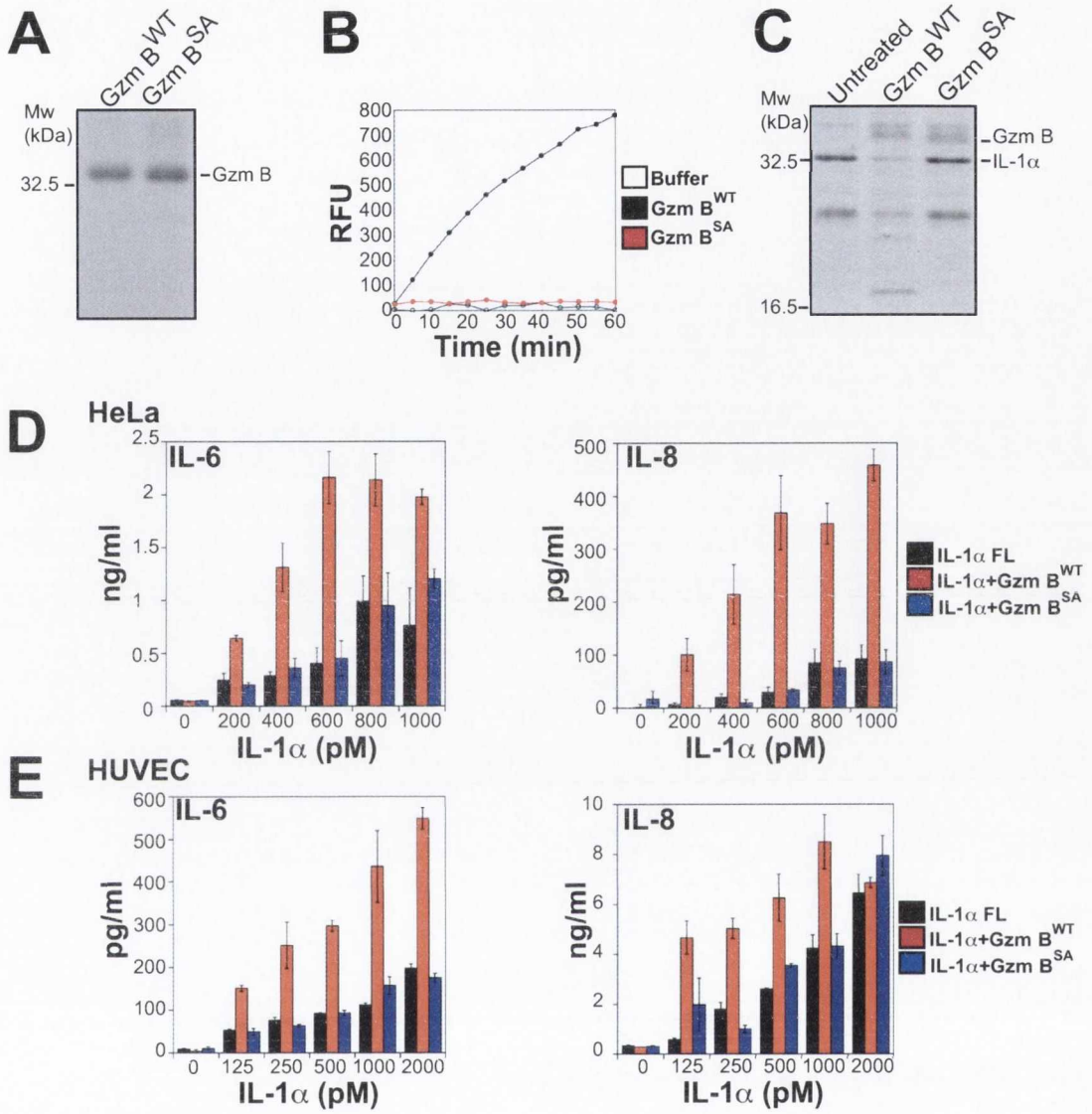

Figure 5.15

Catalytically inactive Gzm B^{SA} does not enhance the bioactivity of IL-1α *in vitro*

(A) Equal amounts of recombinant granzyme B^{WT} and granzyme B^{SA} were analyzed by SDS-PAGE followed by Coomassie staining. (B) Catalytic activity of granzyme B^{WT} or granzyme B^{SA} was measured by monitoring hydrolysis of the synthetic granzyme B substrate IETD-AFC by fluorimetry. (C) Recombinant IL-1α was incubated for 4 h at 37°C, either alone, or in the presence of 200 nM granzyme B^{WT} or granzyme B^{SA}, followed by SDS-PAGE and Coomassie staining. (D) HeLa or (E) HUVEC cells were incubated for 8 h with the indicated concentrations of full-length IL-1α, that had been pre-treated with buffer alone, or granzyme B^{WT} (200 nM) or granzyme B^{SA} (200 nM) for 4 h. IL-6 and IL-8 levels in culture supernatants were determined by ELISA. Error bars represent the mean ±SEM of determinations from three independent experiments.

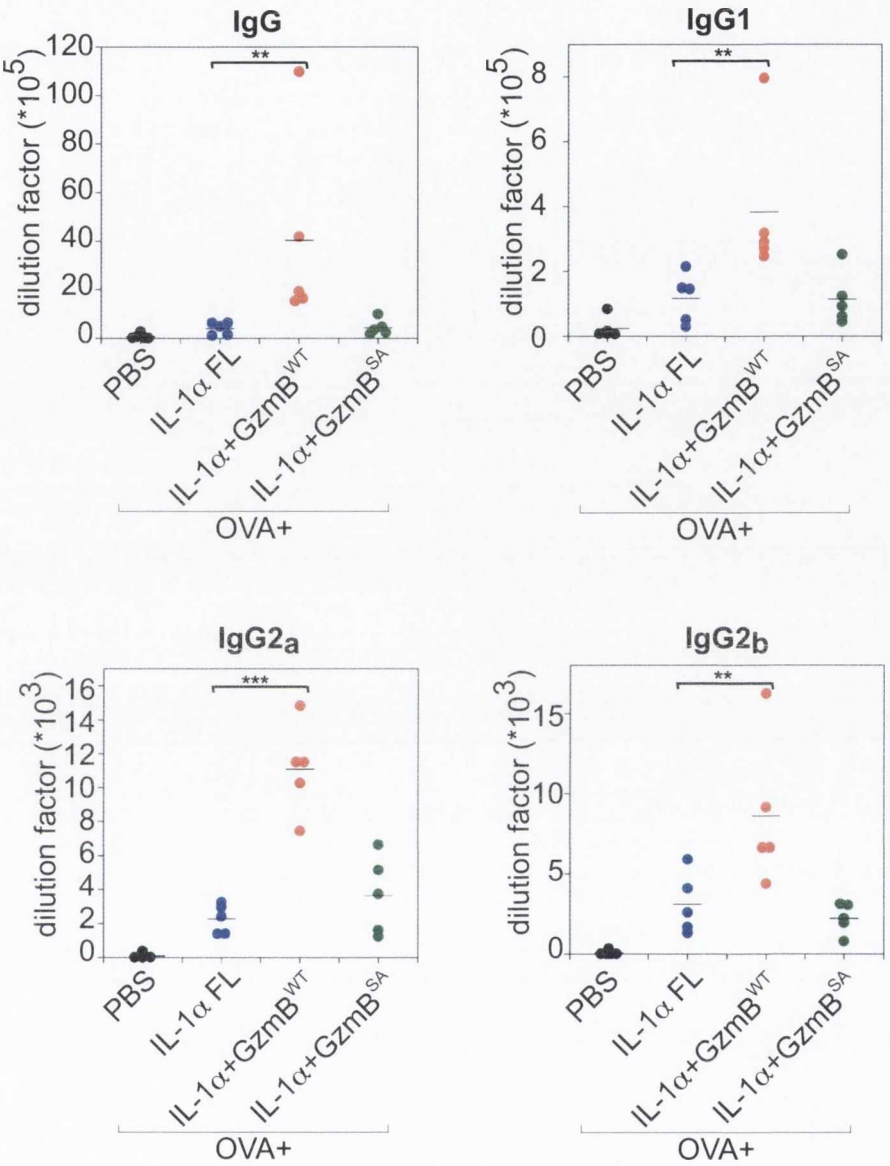

Figure 5.16

Catalytically inactive Gzm B^{SA} does not enhance the biological activity of IL-1α *in vivo*

C57BL/6 mice (5 mice per group) were immunised with either OVA alone (200 μg), or OVA in combination with full-length IL-1α (5 μg), or granzyme B^{WT}-treated IL-1α (5 μg), or granzyme B^{SA}-treated IL-1α (5 μg). All mice were boosted with the same combinations on day 14. Blood was collected via tail bleed on day 21 and OVA-specific total IgG, IgG1, $IgG2_a$ and $IgG2_b$ in plasma samples were determined by ELISA. Significance levels, ***= p<.001, **= p<.05, *= p<.01, by students t-test.

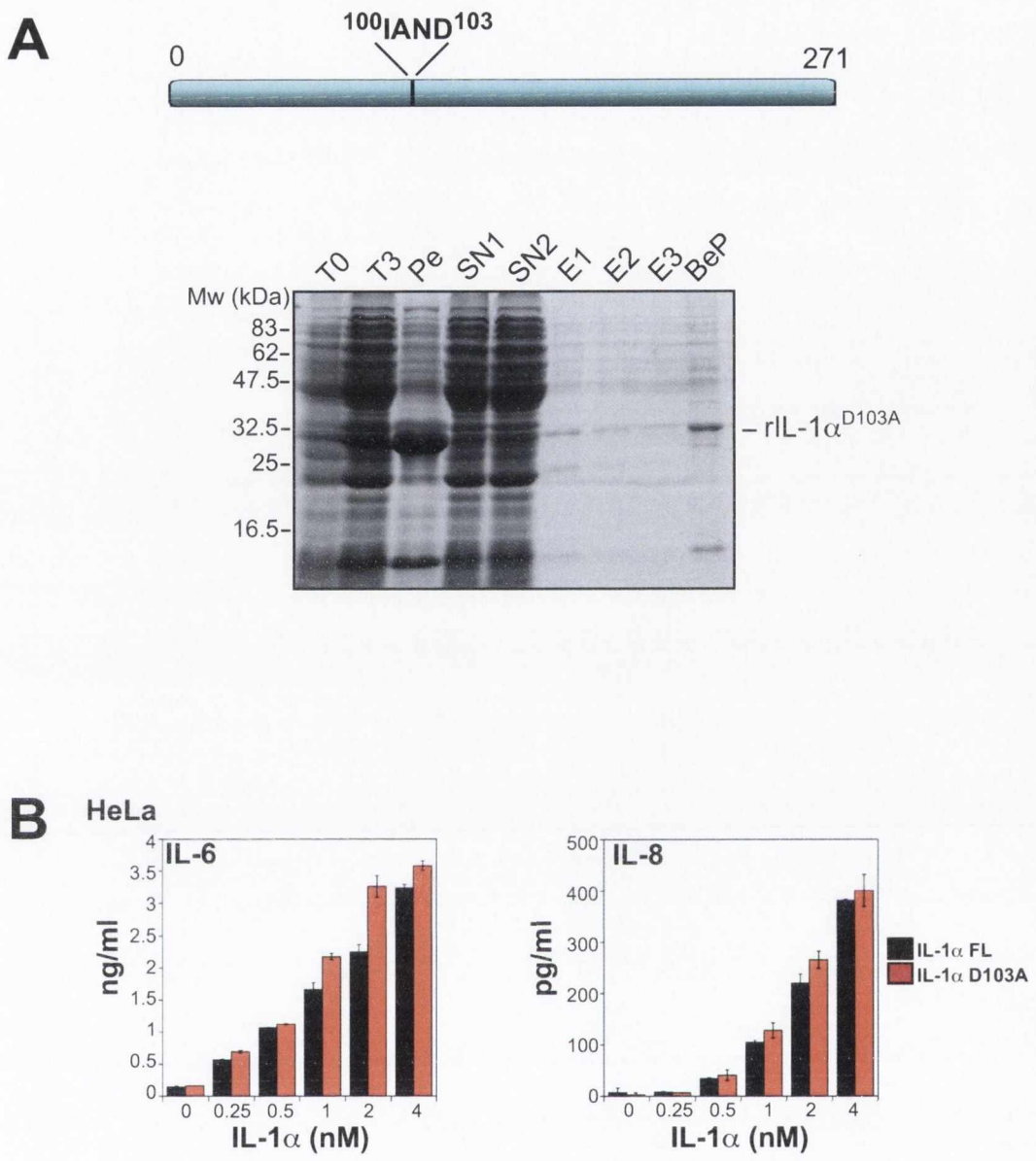

Figure 5.17

Expression and purification of granzyme B-uncleavable IL-1α^{D103A}

(A) Schematic representation of IL-1α indicating the granzyme B cleavage site (top panel). SDS-PAGE analysis of IL-1α^{D103A} expression and purification. T0 and T3 - bacterial culture samples before induction and 3 h post-induction, respectively, Pe - insoluble fraction, SN1 and SN2 - supernatant prior and post-incubation with Ni-NTA beads, respectively, E1-E3 - elution fractions (1/50 of total elution fraction), Be - sample of beads post-elution. (B) HeLa cells were treated for 8 h with the indicated concentrations of full-length IL-1α or IL-1α^{D103A}. IL-6 and IL-8 concentrations in the culture supernatants were measured by ELISA. Error bars represent the mean ±SEM of determinations from three independent experiments.

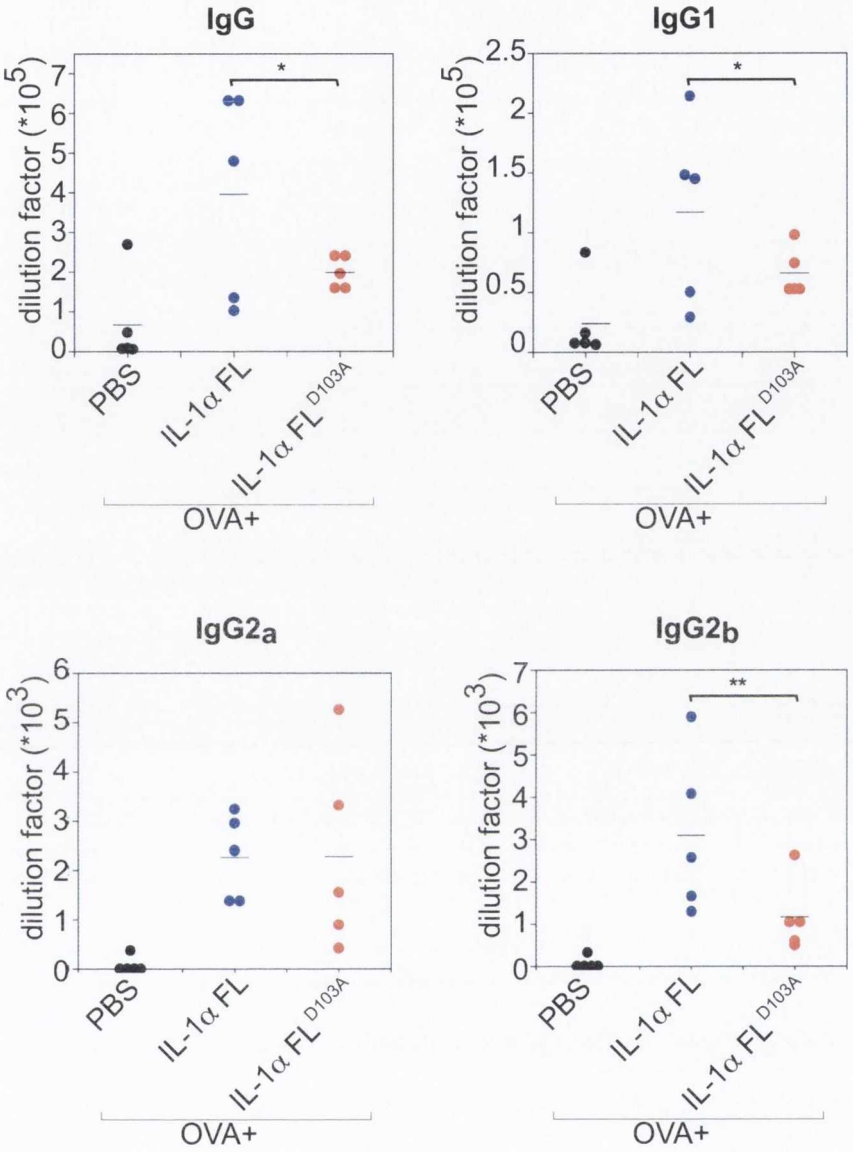

Figure 5.18

IL-1α^{D103} exhibits diminished activity *in vivo* compared to wild type IL-1α

C57BL/6 mice (5 mice per group) were immunised with either OVA alone (200 μg), or OVA in combination with full-length IL-1α (5 μg) or IL-1α^{D103A} (5 μg). All mice were boosted with the same combinations on day 14. Blood was collected via tail bleed on day 21 and OVA-specific total IgG, IgG1, $IgG2_a$ and $IgG2_b$ in plasma samples were determined by ELISA. Significance levels, ***= p<.001, **= p<.05, *= p<.01, by students t-test.

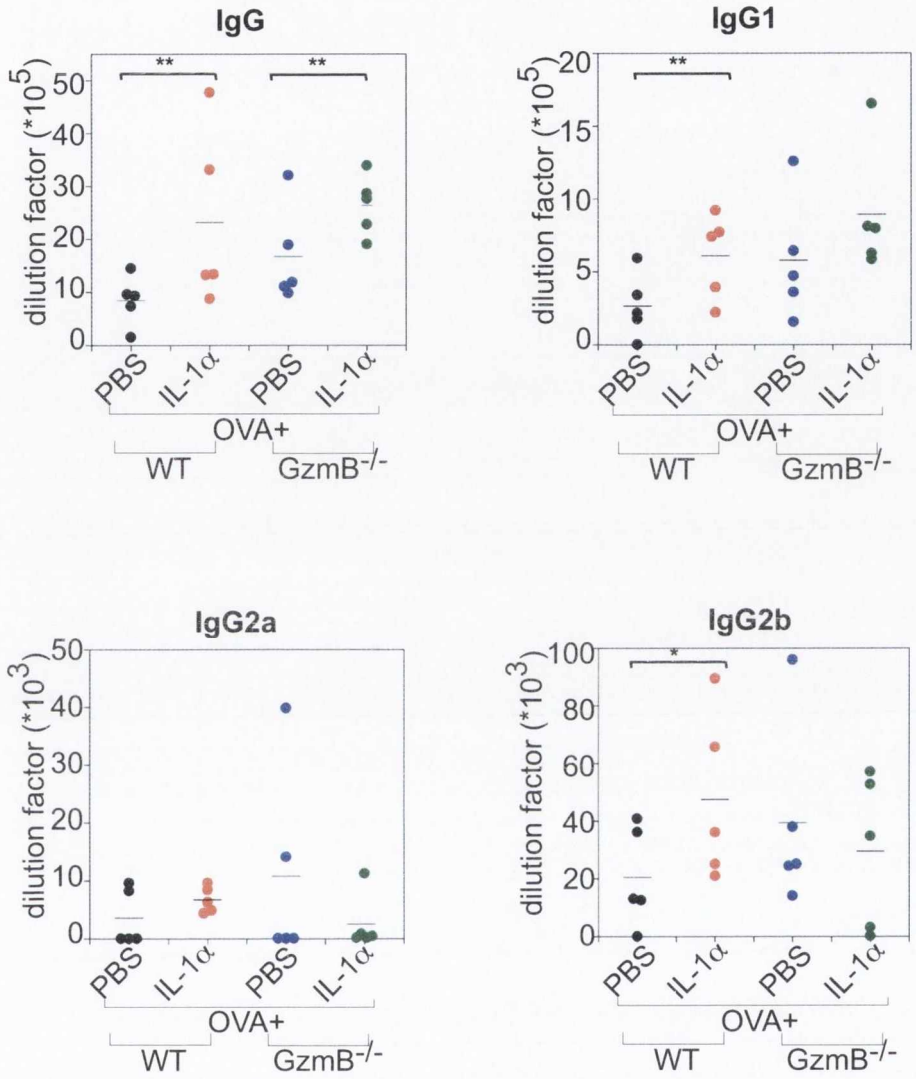

Figure 5.19

Granzyme B contributes to IL-1α processing *in vivo*

WT or Granzyme $B^{-/-}$ C57BL/6 mice (5 mice per group) were immunised either with OVA alone (200 µg), or with OVA in combination with full-length IL-1α (5 µg per mouse). All mice were boosted with the same combinations on day 14. On day 21, peripheral bloods were collected and OVA-specific total IgG, IgG1, $IgG2_a$ and $IgG2_b$ in plasma samples were determined by ELISA. Significance levels, ***= p<.001, **= p<.05, *= p<.01, by students t-test.

experiment, OVA-specific production of IL-4, IL-5, and IL-10 by peritoneal lavage cells was significantly greater when mice received the granzyme B-cleaved form of IL-1α when compared to the full-length cytokine, which did not enhance antigen-specific responses to OVA alone (Figure 5.12). Non-specific polyclonal stimulation with anti-CD3 antibody, either alone or in combination with PMA (a potent stimulator of protein kinase C), also produced more robust cytokine responses in cells isolated from mice that received cleaved IL-1α (Figure 5.13). A similar trend towards stronger OVA-specific responses was also observed in splenocytes isolated from mice immunised with processed IL-1α, but this was less statistically significant (Figure 5.14). Note that IL-1α mainly enhanced production of the type 2 cytokines (IL-4, IL-5), while secretion of IFN-γ (type 1) was largely unaffected.

Again, to rule out the possibility that yeast-derived contaminants in the granzyme B preparation might influence the activity of IL-1α, we expressed and purified catalytically inactive recombinant granzyme B, where the critical serine residue was mutated to alanine (Figure 5.15A-B). We verified that granzyme BSA was unable to cleave recombinant IL-1α (Figure 5.15C) and then used this protein in our *in vitro* assay, demonstrating that IL-1α pre-incubated with granzyme BSA exhibited only basal activity, while wild type enzyme dramatically potentiated IL-1α-dependent cytokine secretion by both HeLa cells and HUVECs (Figure 5.15D-E). We then used this granzyme B mutant in the antigen-driven mouse model. As figure 5.16 shows, pre-incubation with catalytically inactive granzyme BSA failed to enhance the adjuvant activity of IL-1α, while wild type granzyme B clearly enhanced the production of OVA-specific IgGs when compared to full-length IL-1α. Again, antigen alone induced minimal antibody production. Collectively, these data lend further support to our *in vitro* observations, confirming that Gzm B-

dependent proteolysis of IL-1α acts to enhance the pro-inflammatory activity of this cytokine.

5.2.7 Granzyme B contributes to IL-1α processing *in vivo*

To explore whether granzyme B contributes to IL-1α processing *in vivo*, we first of all asked if the granzyme B-uncleavable mutant IL-1α^{D103A} exhibited reduced potency *in vivo*, compared to wild type IL-1α. To this end, we purified recombinant IL-1α^{D103A} (Figure 5.17A) and normalised the bioactivity of wild type IL-1α and IL-1α^{D103A} using HeLa cells (Figure 5.17B), followed by comparison of the adjuvant activity of both forms in the mouse model. As Figure 5.18 shows, full-length IL-1α significantly boosted OVA-specific production of antibodies when compared to antigen alone. Importantly, granzyme B-uncleavable IL-1α mutant was significantly less potent in enhancing OVA-specific IgG, IgG1 and IgG2$_b$ responses than wild type IL-1α. These data suggest that IL-1α is processed by granzyme B *in vivo* and that this proteolysis event results in the significant enhancement of IL-1α bioactivity.

Finally, we decided to investigate whether the absence of granzyme B has an impact on IL-1α activity *in vivo*. To address this question, we compared the adjuvant activity of IL-1α in wild type and granzyme B-deficient mice. As expected, IL-1α boosted production of all tested IgG antibodies in wild type mice, while it failed to boost production of OVA-specific IgG2$_a$ and IgG2$_b$ in granzyme B-deficient mice (Figure 5.19). Although IL-1α enhanced production of total IgG and IgG1 in granzyme B knockout mice, note that baseline antibody responses to OVA alone in these mice were generally higher than in wild type for the reasons that

remained unclear. Therefore, we determined the fold increase in IL-1α-iduced OVA-specific antibody production in wild type and granzyme B-deficient mice (Figure 5.20). Importantly, IL-1α induced considerably higher fold increases in the production of all tested IgG types in wild type mice, when compared to the mice deficient in granzyme B, which again underscores the role of endogenous granzyme B in the processing of IL-1α *in vivo*.

Collectively, the data presented in this chapter unveil a hitherto unappreciated role for granzyme B as a modulator of pro-inflammatory cytokine activity. Moreover, contrary to previous understanding, we showed that proteolytic processing of IL-1α to the mature 17 kDa form has a significant impact on the bioactivity of this cytokine.

5.3 Discussion

5.3.1 Activation of IL-1α via proteolytic processing

Proteolysis of several cytokines was previously demonstrated to have dramatic effects on their biological activity. For example, limited internal processing of IL-1β and IL-18 by caspase-1 is indispensable for unlocking their biological activities (Thornberry et al. 1992, Gu et al., 1997). On the other hand, previous work in our lab established that proteolytic processing of another IL-1 family member, IL-33, by apoptotic caspases inactivates this cytokine (Lüthi et al., 2009). Thus, we decided to formally investigate the consequences of the proteolytic processing of IL-1α by granzyme B and other inflammatory proteases.

It has been previously shown that, unlike IL-1β, full-length IL-1α binds to the IL-1R and is biologically active (Mosley et al., 1987a,b). These studies also suggested that processing of IL-1α has no major impact on the bioactivity of this cytokine. However, as Mosley and colleagues were using an insensitive assay system, the question regarding the functional consequences of IL-1α proteolysis had not been properly addressed. In this chapter we explored the consequences of IL-1α proteolysis on the bioactivity of this cytokine in both *in vitro* and *in vivo* assays.

Contrary to current thinking, we demonstrated that cleavage of IL-1α by granzyme B and other proteases, including calpain, elastase and chymase, results in robust enhancement of IL-1α bioactivity by up to 8 fold *in vitro*. We also confirmed that observation in vivo, comparing adjuvant activities of full-length and cleaved IL-1α in an OVA-driven model. In accordance with a previous report (Mosley et al., 1987a), both forms of IL-1α displayed significant adjuvant activity in vivo. However, proteolysis of IL-1α by granzyme B resulted in a remarkable enhancement of the bioactivity of IL-1α, supporting our *in vitro* observations. Speculations on the underlying mechanistic phenomena, explaining why proteolysis affects the activity of IL-1α, are discussed below.

5.3.2 How does cleavage of IL-1α result in the enhancement of its bioactivity?

As proteolysis of a protein hydrolyses the peptide bond between two amino acids, it is possible that cleavage of IL-1α by inflammatory proteases induces a conformational change, which enhances IL-1 receptor binding and, hence, the bioactivity of the cytokine. This hypothesis is consistent with a previous study that

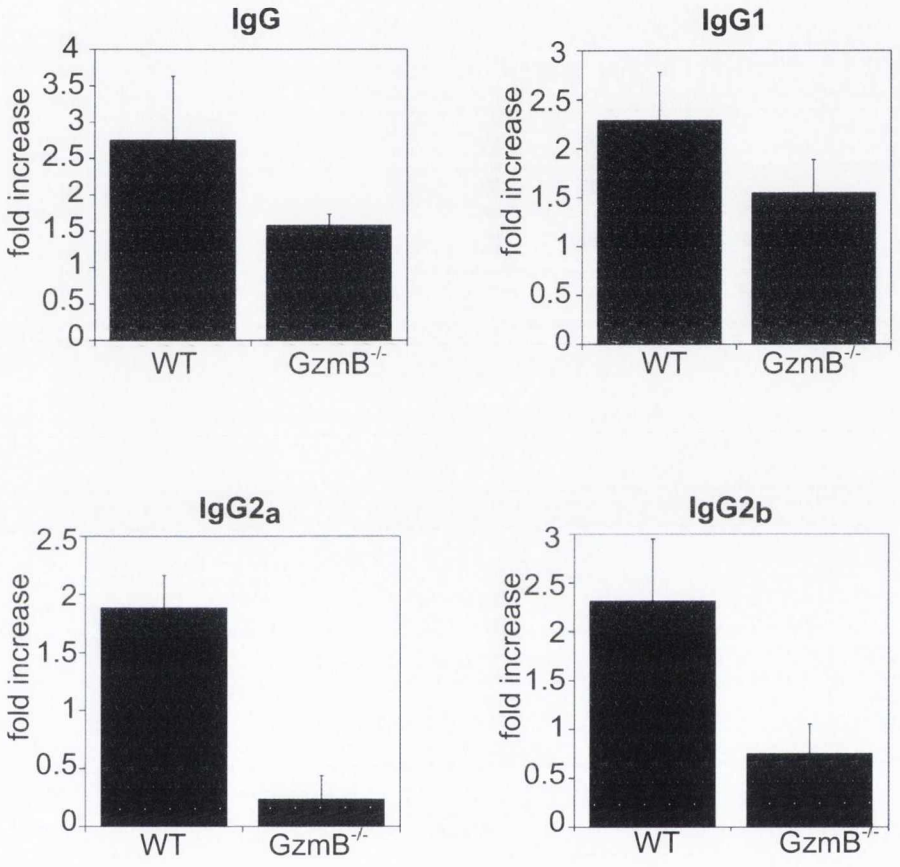

Figure 5.20

Granzyme B contributes to IL-1α processing *in vivo* (2)

Fold increase in antibody production in WT versus Granzyme $B^{-/-}$ mice treated with OVA plus IL-1α , compared with OVA alone. The analysis shown is based on the data presented in Figure 5.19. Error bars represent the mean ± SEM from each group of five mice.

utilised sensitivity to proteinase K to explore whether IL-1α underwent a conformational change as a consequence of limited internal proteolysis (Hazuda et al., 1991). Although the latter study did not explore the functional effects of IL-1α processing in detail, the authors showed that both pro-IL-1α and pro-IL-1β were extremely sensitive to proteolytic degradation by proteinase K, while mature forms were highly resistant to degradation. Therefore, it was suggested that proteolysis of IL-1 initiates a profound conformational change in the structure of the molecule from a proteinase K-sensitive to a proteinase K-insensitive state (Hazuda et al., 1991).

Based on these findings and on our own observations, we hypothesise that this conformational change results in an increased IL-1 receptor affinity. It is worth noting that previous work from our laboratory has reported that proteolytic processing of IL-33 by apoptotic caspases altered both resistance of this cytokine to serum proteases and receptor binding efficiency (Lüthi et al., 2009), suggesting that limited internal proteolysis results in changes in the protein structure and receptor binding affinity of the cytokines.

Alternatively, the N-terminal end of proIL-1α might occlude the receptor binding site or affect the optimal binding of IL-1R accessory protein. Thus, removal of the N-terminal domain would permit a more stable interaction of the ligand with IL-1R or facilitate ligand-dependent receptor heterodimerisation with accessory protein.

Based on the work of Hazuda and colleagues, it is interesting to speculate that processing of IL-1α by any of the inflammatory proteases results in the stabilisation of this cytokine in the extracellular milieu (Hazuda et al., 1991), which would allow more time for the otherwise vulnerable cytokine to fulfill its mission.

Thus, cleavage of IL-1α by granzyme B and other extracellular ptoteases, could lead to the combined activation and stabilisation of the cytokine, enhancing the strength of the inflammatory response.

5.3.3 IL-1α is processed by pro-inflammatory proteases *in vivo*

In this work, we extensively demonstrated that IL-1α is an efficient granzyme B substrate *in vitro*. But does granzyme B process IL-1α in a physiological setting? We provided strong evidence that this proteolytic event does indeed happen *in vivo*.

First, we compared the adjuvant activities of wild type and mutant IL-1α^{D103A} in a mouse model. Although mutation of the granzyme B cleavage site did not abrogate bioactivity of IL-1α in our *in vitro* assay, the adjuvant activity of the mutant cytokine was dramatically reduced in the *in vivo* experiments when compared to the wild type protein. To explain this result, we suggest that both wild type and mutant IL-1α induce infiltration of granulocytes and other immune cells to the peritoneal cavity, where recruited cells secrete their granule proteases, which, in turn, process and enhance the activity of wild type IL-1α, providing a positive feedback loop. Unlike wild type cytokine, mutant IL-1α cannot be processed by extracellular granzyme B and, hence, its activity stays at the basal state. However, we cannot exclude the possibility that mutation of the important aminoacid residue might affect stability of the protein. Although we carefully normalised the activities of both forms of IL-1α, the observed decrease in the adjuvant activity of IL-1α^{D103A} could also be attributable to reduced stability of this form *in vivo*.

Therefore, we explored the processing of IL-1α by granzyme B in another model, where we compared the adjuvant activity of full-length IL-1α in wild type versus granzyme B knockout mice. Again, if endogenous extracellular granzyme B enhances the adjuvant activity of IL-1α via proteolytic processing, the absence of granzyme B in the knockout mice should result in reduced adjuvant activity of IL-1α when compared to wild type mice. We found that this was indeed the case, which is in agreement with the work of Metkar and colleagues, who showed previously that the absence of granzyme B renders mice more resistant to LPS-induced endotoxic shock, implicating granzyme B in the regulation of inflammatory responses (Metkar et al., 2008). Collectively, our data suggest that endogenous granzyme B contributes to the proteolysis of IL-1α *in vivo*.

Finally, we obtained BALF samples from patients with persistent inflammatory conditions such as cystic fibrosis, bronchiectasis and COPD, which were previously reported to have high levels of extracellular neutrophil granule proteases (Vega-Carrascal et al., 2011, Hayes et al., 2011). Initially, we attempted to detect endogenous IL-1α in the BALF samples. However, we were not successful in detecting IL-1α by direct western blot, immunoprecipitation or ELISA (data not shown), which was not suprising, as IL-1α is normally found in minuscule quantities in body fluids. Therefore, we directly added recombinant full-length IL-1α to the BALF samples and demonstrated robust IL-1α-processing activity in several samples from patients with cystic fibrosis and bronchiectasis, which was absent in the samples from healthy donors. Note that different samples displayed different levels of proteolytic activity, which explains the different degree of proteolytic efficiency seen (Vega-Carrascal et al., 2011). Although the size of the cleavage fragments suggests the involvement of elastase or cathepsin G, the

usage of specific inhibitors will help to ultimately define the proteases involved. However, we would suspect the participation of all the proteases described in this work, with the relative input being determined by the abundance of the particular protease in the extracellular space.

In conclusion, we have demonstrated here that IL-1α is efficiently cleaved by granzyme B and other granule serine proteases both *in vitro* and *in vivo*. Furthermore, proteolytic processing enhances the biological activity of IL-1α protein. Thus, contrary to current thinking, the N-terminus of IL-1α acts as a sensor of inflammation and regulates the activity of this cytokine.

Chapter 6

General Discussion

6. Discussion

6.1 Does caspase-1 process Bid *in vivo*?

The search for substrates of the inflammatory and apoptotic proteases is still ongoing. Two major approaches are generally employed to help investigatorss identify potential substrates. In one approach, a set of candidate substrates is directly tested *in vitro*. Alternatively, recombinant protease of interest is added to differentially labelled cell lysates, following which proteolysis-related changes are tracked and identified with the help of various analytical techniques, such as mass spectrometry. However, the major drawback of these approaches is the use of arbitrary amounts of recombinant proteases by many investigators.

The work by Agard and colleagues nicely illustrates the importance of using physiologically relevant concentrations of the protease under investigation (Agard et al., 2010). The authors show that treatment of labeled cell lysates with high concentrations of recombinant caspase-1 (400 nM) resulted in the identification of 82 candidate substrates, while activation of endogenous caspase-1 with pro-inflammatory stimuli allowed detection of only 11 substrates. Therefore, due caution should be applied when interpreting the results of *in vitro* proteolysis screens.

In the first chapter of this work, we set out to explore whether Bid is a caspase-1 substrate. The possibility that the major pro-inflammatory caspase processes a key mediator of the apoptotic pathway is very exciting, suggesting that under certain conditions caspase-1 is capable of initiating a cell death programme. Alternatively, these data implies that Bid is a potential regulator of the inflammatory responses, which is another exciting possibility. Indeed, recent work

by Yeretssian and colleagues implicated Bid in NOD receptor signalling, while several earlier studies demonstrated that Bid-deficient mice are resistant to septic shock (Yeretssian et al., 2011, Zhao et al., 2001, Chung et al., 2010). Moreover, Bid was shown to be secreted by human monocytic cells in a caspase-1-dependent manner upon stimulation with inflammatory stimuli (Keller et al., 2008). However, we were not able to confirm cleavage of endogenous Bid under inflammatory conditions, although recombinant caspase-1 efficiently processed Bid *in vitro* , once again underscoring the importance of verifying candidate substrates in the endogenous setting.

Despite the absence of Bid proteolysis in THP-1 cell-free extracts, we cannot rule out the possibility that endogenous Bid is cleaved under certain inflammatory conditions. In support of our observation, induction of neuronal cell death in a cerebral ischemia model was shown to be accompanied by caspase-1-dependent Bid processing (Zhang et al., 2003). Furthermore, cleavage of caspase-7 and GAPDH by caspase-1 was demonstrated predominantly upon infection of macrophages with S.typhimurium and accompanied by caspase-1-dependent pyroptosis, suggesting excessive activation of caspase-1 (Shao et al., 2007, Lamkanfi et al., 2008). It is worth noting that, similarly to Bid, we also failed to detect the cleavage of caspase-7 in THP-1 cell-free extracts under inflammatory conditions, although cleavage and activation by recombinant caspase-1 was readily observed (Walsh et al., 2011, data not shown). Importantly, recent work in our laboratory established that the specifity of caspase-1 is restricted by the abundance of the active form in the cell (Walsh et al., 2011). Indeed, caspase-1 exhibited a concentration-dependent promiscuity towards a range of synthetic and endogenous substrates, but the protease was rapidly inactivated in monocytic cell

lysates (Walsh et al., 2011). Thus, it is worth examining Bid cleavage in conditions, such as bacterial infection, where caspase-1 is efficiently maintained in its active state.

Alternatively, cleavage of endogenous Bid might be affected by post-translational modifications. For example, casein kinases have been previously reported to phosphorylate Bid in the vicinity of the caspase cleavage site, which was shown to prevent cleavage of this protein by caspase-3 or caspase-8, while treatment with phosphatases resulted in the increased processing of endogenous Bid by both caspases (Desagher et al., 2001, Degli Eposti et al., 2003). Thus, further studies are still required to address definitively the question of Bid processing by caspase-1.

6.2 Granzyme B is a pro-inflammatory protease

Granule proteases of neutrophils, mast cells, and cytotoxic lymphocytes belong to a large family of serine proteases that play a major function in the immune and inflammatory responses, with neutrophil and mast cell proteases participating in the removal of pathogens, regulation of cytokine activity, extracellular matrix remodelling and blood coagulation (Heutinck et al., 2010).

In contrast, the primary function of granzymes was for a long time considered to be the killing of virus-infected or tumour cells via inducing target cells to undergo apoptosis. In this way, granzymes differ from other serine proteases, which to a certain extent share similar substrates and functions. However, the accumulating evidence suggests that granzymes also have a range of non-cytotoxic functions

(reviewed in Afonina et al., 2010). In fact, granzymes A and K were shown to lack cytolytic potential when used at physiological concentrations (Metkar et al., 2008, Joeckel et al., 2011). Moreover, similar to other members of the serine protease family, granzymes have been implicated in cleaving extracellular matrix proteins, cytokines, blood coagulation factors and cell surface receptors (reviewed in Afonina et al., 2010), suggesting that granule proteases generally share their substrate preferences and perform broadly similar functions. In this work, we demonstrated that neutrophil and mast cell proteases, and, in particular, the CTL/NK protease, granzyme B, process the major pro-inflammatory cytokine IL-1α. Considering these similarities, it would be tempting to look for novel granzyme substrates among the already identified substrates of other granule serine proteases.

Proteases belonging to the caspase family also participate in the processing of cytokines. Accordingly, the enzymes of this family are broadly divided into two groups based on their functions: apoptotic and inflammatory. Similarly to serine proteases, the functions of these two groups and the respective choice of substrates were considered to be non-overlapping. However, apoptotic caspases were demonstrated to attenuate the inflammatory response by cleaving and inactivating pro-inflammatory cytokine IL-33 (Lüthi et al., 2009, Cayrol and Girard, 2009, Ali et al., 2010). Another example comes from the work of Kazama and colleagues, who reported caspase-dependent regulation of the oxidation status and the resultant neutralisation of the danger signal HMGB1 (Kazama et al., 2008). Notably, in both cases apoptotic caspases act to dampen inflammatory response, assuring 'clean' and 'quiet' removal of the apoptotic cell.

However, in this work, we extensively demonstrated that cleavage of IL-1α by granzyme B, also an apoptotic protease, resulted in a dramatic increase in the bioactivity of this cytokine *in vitro* and *in vivo*. Moreover, we also showed that adjuvant activity of full-length IL-1α was markedly reduced in granzyme B-deficient mice, which is in agreement with the report of Metkar and colleagues, who observed increased resistance to LPS-induced endotoxic shock in granzyme B knockout mice when compared to wild type mice (Metkar et al., 2008). We, thus, postulate that, in contrast to apoptotic caspases, granzyme B is a protease with a dual nature and plays an important role in both inflammatory and apoptotic processes.

A number of studies support our hypothesis. First, several groups reported processing of cytokines by the members of the granzyme family, although the physiological relevance of these proteolytic events is not fully elucidated (Irmler et al., 1995, Omoto et al., 2010). Secondly, granzymes have been detected extracellularly in a range of inflammatory conditions (Spaeny-Dekking et al., 1998, Tak et al., 1999, Lauw et al., 2000, Bratke et al., 2004), while CTLs and NK cells have been shown to constitutively release granzymes in the absence of antigen stimulation (Prakash et al., 2009). Moreover, CTLs and NK cells have been reported to secrete granzymes upon stimulation with pro-inflammatory stimuli, such as bacteria, endotoxin, cytokines and chemokines (reviewed in Buzza and Bird, 2006).

Finally, a number of non-cytotoxic immune cells also express and secrete granzyme B, often in the absence of perforin secretion (reviewed in Afonina et al., 2010). Although, in several cases, cytotoxic potential was ascribed to pro-inflammatory cells secreting granzyme B (Tschopp et al., 2006, Hernandez-Pigeon

et al., 2006, Freishtat et al., 2009), extremely high effector-to-target ratios were used in the aforementioned studies and, therefore, the validity of the cytotoxicity in a physiological setting is doubtful. Instead, the data suggest that secreted granzyme B performs a distinct role upon secretion from pro-inflammatory cells. We propose that granzyme B is released into the extracellular space by non-cytotoxic cells and regulates the immune response via proteolytic processing and activation of cytokines, such as IL-1α and IL-18. It would be interesting to investigate whether other granzymes also process pro-inflammatory cytokines, which would extrapolate our findings to the whole granzyme family.

Alternatively, granzyme B-mediated processing of IL-1α might affect secretion and localisation of this cytokine. Interestingly, a recent study reported that, unlike necrotic cells, apoptotic cells retained IL-1α in the nucleus, tightly bound to chromatin (Cohen et al., 2010). Therefore, by removing the NLS, granzyme B-mediated cleavage of IL-1α could override the nuclear retention of the latter and facilitate its secretion from the dying cell. In support of this idea, our preliminary experiments showed that overexpression of full-length IL-1α in HeLa cells resulted in the predominantly nuclear localisation of the protein, while truncated IL-1α$^{104-271}$, which mimicks the granzyme B cleavage fragment, was mostly cytoplasmic (data not shown). Furthermore, Hogquist and colleagues have previously demonstrated secretion of IL-1α during CTL-mediated killing (Hogquist et al., 1991). Thus, it would be interesting to further investigate cellular distribution of IL-1α during CTL/NK-mediated killing and explore whether granzyme B-mediated cleavage facilitates the secretion of IL-1α by apoptotic cells.

6.3 Cleavage-induced conformational change of IL-1α

Strikingly, all IL-1α proteolysis events described in this work take place within close proximity to each other (amino acids 100-120) (Figure 6.1). Apparently, cleavage within this region enables the re-orientation of the two halves of the protein, which affects the biological activity of IL-1α. Therefore, we propose that the N-terminal half of IL-1α (and possibly IL-1β) acts as a sensor of inflammation and allows the protein to undergo a bioactivity switch from a basal to a hyperactive state. Thus, similar to IL-1β, the biological activity of IL-1α is also regulated via limited proteolysis.

Notably, as IL-1β is processed by a variety of proteases within a similar restricted space (Hazuda et al., 1990), one would suspect that IL-1β undergoes a similar conformational change as a result of the proteolysis by caspase-1 and other inflammatory proteases. Indeed, it would be very interesting to see the crystal structures of the full-length and cleaved forms of IL-1 proteins, allowing us to analyse how proteolysis changes the structure of the protein. However, only the crystal structures of mature IL-1α/IL-1β have been published to date (Priestle et al., 1988, Graves et al., 1990).

It is unlikely that singular cleavage events would result in the absolute removal of a portion of the protein. Accordingly, our preliminary experiments suggest that upon proteolysis, the two fragments of IL-1α stay together (data not shown), confirming that cleavage results in a shift in the protein conformation.

6.4 IL-1α cleavage *in vivo*

There are a number of scenarios where IL-1α may encounter granzyme B *in vivo*. First of all, as we demonstrated here, granzyme B may process intracellular IL-1α during CTL/NK-mediated killing. Alternatively, we also showed that granzyme B is capable of processing IL-1α in the extracellular space.

How does granzyme B end up in the extracellular millieu? In this work, we demonstrated that during NK-mediated killing, granzyme B escaped the immunological synapse and was capable of processing extracellular IL-1α. Notably, a number of studies report that cytotoxic cells actively secrete granzymes into the extracellular millieu either constitutively or upon stimulation with pro-inflammatory stimuli (Prakash et al., 2009; Spaeny-Dekking et al., 1998). Finally, various non-cytotoxic, pro-inflammatory cells express and secrete granzyme B, thus serving as an additional source of extracellular granzyme B (reviewed in Afonina et al., 2010).

Importantly, we have also found that neutrophil- and mast cell-derived granule proteases also process IL-1α and demonstrated robust IL-1α-processing activity in BALF samples from patients with cystic fibrosis and bronchiectasis. Interestingly, Pseudomonas aeruginosa, the most common respiratory pathogen in the cystic fibrosis lung have been reported to upregulate IL-1α and IL-1β production in mast cells. This, in turn, facilitated neutrophil transendothelial migration (Lin et al., 2002), thus providing a scenario where neutrophil/mast cell-derived proteases encounter IL-1α *in vivo*.

Mast cells have also been implicated in arthritis via the production of IL-1 upon their activation in the arthritic joint (Nigrovic et al., 2007). Additionally, mast cells

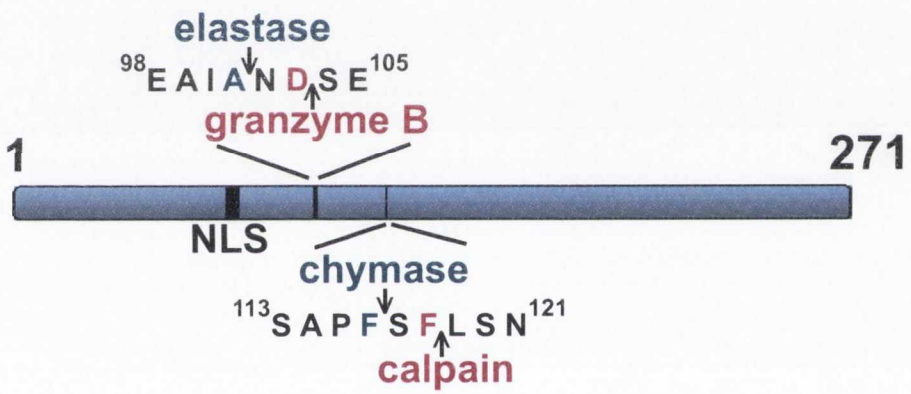

Figure 6.1

IL-1α schematic

Schematic representation of IL-1α indicating sites of granzyme B- and calpain I-mediated proteolysis and predicted sites of chymase- and elastase-mediated proteolysis

express and secrete granzyme B upon activation (Strik et al., 2007), suggesting that bioactivity of IL-1α, secreted by mast cells, could be enhanced through the cleavage by proteases simultaneously released by the same cells.

IL-1 is an important mediator of rheumatoid arthritis playing a major role in cartilage destruction and bone erosion (Abramson and Amin, 2002). Blocking the effects of IL-1 was shown to reduce joint destruction in mice with collagen-induced arthritis (Joosten et al., 1999). Notably, the levels of granzyme B are elevated in the plasma and synovial fluid of patients with rheumatoid arthritis (Tak et al., 1999). Furthermore, macrophages were shown to express granzyme B in the lesion areas of atherosclerosis and rheumatoid arthritis (Kim et al., 2007).

Granzyme B has already been proposed to play a part in the tissue destruction in rheumatoid arthritis and other inflammatory conditions via its extracellular matrix remodelling ability. Based on the data presented in this work, we hypothesise that granzyme B might also contribute to the progression of inflammatory diseases through proteolytic processing and activation of IL-1α. In support of this, we demonstrated that granzyme B-uncleavable IL-1α mutant exhibited diminished activity *in vivo* as compared to the wild type protein. Additionally, IL-1α elicited a significantly weaker immune response in granzyme B-deficient mice, suggesting that endogenous granzyme B contributes to IL-1α processing *in vivo*.

IL-1α is rarely found in body fluids, only in cases of severe inflammation. However, necrotic cells have been shown to release full-length IL-1α, which mediated the neutrophil-dominated sterile inflammatory response (Hogquist et al, 1991, Chen et al., 2007; Eigenbrod et al., 2008), implying that IL-1α is capable of acting as a danger signal and alert the immune system to the potential danger. Importantly,

we showed here that inflammatory proteases capable of processing IL-1α were also secreted into extracellular space during neutrophil-driven inflammatory conditions and could potentially further amplify the immune response via enhancing the bioactivity of IL-1α. Figure 6.2 schematically represents a number of cells secreting IL-1α and/or the proteases with IL-1α-processing ability.

Can inhibitors of granzyme B, calpain or other extracellular proteases have a therapeutic effect? A study by Yoshifuji and colleagues examined the biological effects of calpain-inhibitory compounds in a mouse model of experimental arthritis (Yoshifuji et al., 2005). The authors demonstrated that a high dose of the membrane-permeable cysteine proteinase inhibitor, E64d, was able to alleviate the clinical symptoms of experimental arthritis. Importantly, E64d inhibited the production of mature IL-1α in the fibroblast-like synovial cell line *in vitro* (Yoshifuji et al., 2005), which suggests that suppressing the conversion of full-length IL-1α to a more active mature form may enable specific protease inhibitors to ameliorate the progression of inflammatory diseases. Thus, granzyme B inhibitors may prove to be useful therapeutic agents in the fight against IL-1-dependent inflammatory conditions.

6.5 Conclusions

The data in this thesis support the following conclusions:

1. Bid is an *in vitro* caspase-1 substrate

2. IL-1α is a novel granzyme B substrate both *in vitro* and *in vivo*

3. Granzyme B processes IL-1α both intra- and extracellularly

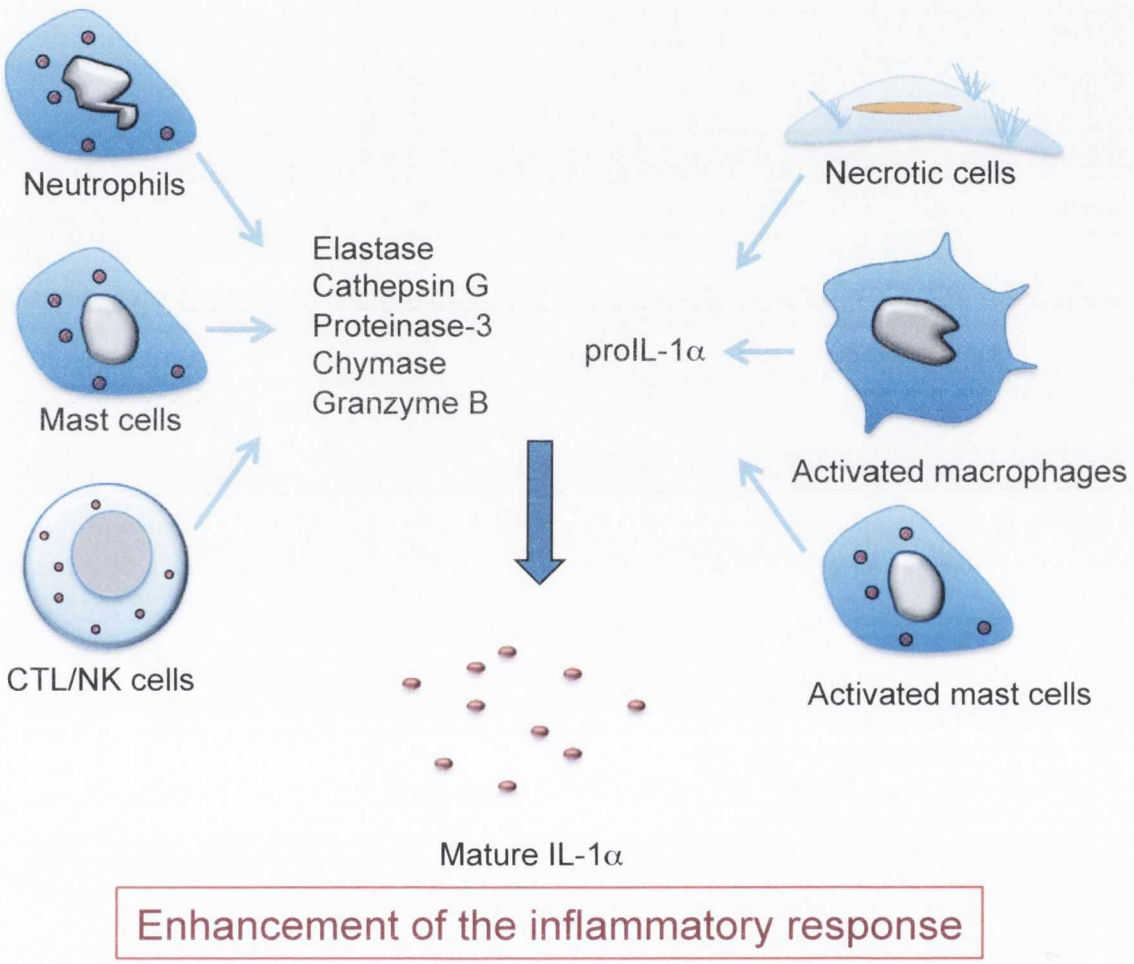

Figure 6.2

Granule serine proteases potentiate inflammatory response

Granule serine proteases, derived from neutrophils, mast cells, CTL/NK and other immune cells, enhance inflammatory response via proteolytic processing of IL-1α, released from necrotic cells, activated macrophages or mast cells.

4. IL-1α is processed by other neutrophil and mast cell granule proteases

5. Proteolysis of IL-1α by the proteases, described in this work, increases the bioactivity of this cytokine 3-8 fold

References

Abramson SB, Amin A. Blocking the effects of IL-1 in rheumatoid arthritis protects bone and cartilage. Rheumatology (Oxford). 2002; 41: 972-80.

Adachi O, Kawai T, Takeda K, Matsumoto M, Tsutsui H, Sakagami M, Nakanishi K, Akira S. Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. Immunity. 1998; 9:143-50.

Adrain C, Duriez PJ, Brumatti G, Delivani P, Martin SJ. The cytotoxic lymphocyte protease, granzyme B, targets the cytoskeleton and perturbs microtubule polymerization dynamics. J Biol Chem 2006; 281: 8118-81125.

Afonina IS, Cullen SP, Martin SJ. Cytotoxic and non-cytotoxic roles of the CTL/NK protease granzyme B. Immunol Rev. 2010; 235:105-16.

Agard NJ, Maltby D, Wells JA. Inflammatory stimuli regulate caspase substrate profiles. Mol Cell Proteomics. 2010; 9:880-93.

Alheim K, Chai Z, Fantuzzi G, Hasanvan H, Malinowsky D, Di Santo E, Ghezzi P, Dinarello CA, Bartfai T. Hyperresponsive febrile reactions to interleukin (IL) 1alpha and IL-1beta, and altered brain cytokine mRNA and serum cytokine levels, in IL-1beta-deficient mice. Proc Natl Acad Sci U S A. 1997; 94:2681-6.

Ali S, Nguyen DQ, Falk W, Martin MU. Caspase 3 inactivates biologically active full length interleukin-33 as a classical cytokine but does not prohibit nuclear translocation. Biochem Biophys Res Commun. 2010; 391:1512-6.

Andersson U, Wang H, Palmblad K, Aveberger AC, Bloom O, Erlandsson-Harris H, Janson A, Kokkola R, Zhang M, Yang H, Tracey KJ. High mobility group 1 protein (HMG-1) stimulates proinflammatory cytokine synthesis in human monocytes. J Exp Med. 2000; 192:565-70.

Andrade F, Roy S, Nicholson D, Thornberry D, Rosen A, Casciola-Rosen, L. Granzyme B directly and efficiently cleaves several downstream caspase substrates: implications for CTL-induced apoptosis. Immunity 1998; 8: 451–460.

Andrade F, Bull HG, Thornberry NA, Ketner GW, Casciola-Rosen LA, Rosen A. Adenovirus L4-100K assembly protein is a granzyme B substrate that potently inhibits granzyme B-mediated cell death. Immunity. 2001; 14: 751-61.

Andrade F, Fellows E, Jenne DE, Rosen A, Young CS. Granzyme H destroys the function of critical adenoviral proteins required for viral DNA replication and granzyme B inhibition. EMBO J 2007; 26: 2148-2157.

Bank U, Ansorge S. More than destructive: neutrophil-derived serine proteases in cytokine bioactivity control. J Leukoc Biol. 2001 69: 197-206.

Barry M, Heibein, JA, Pinkoski, MJ, Lee, SF, Moyer, RW, Green, DR and Bleackley RC. Granzyme B short-circuits the need for caspase 8 activity during granule-mediated cytotoxic T-lymphocyte killing by directly cleaving Bid. Mol Cell Biol. 2000; 20: 3781-94.

Ben-Sasson SZ, Hu-Li J, Quiel J, Cauchetaux S, Ratner M, Shapira I, Dinarello CA, Paul WE. IL-1 acts directly on CD4 T cells to enhance their antigen-driven expansion and differentiation. Proc Natl Acad Sci U S A. 2009; 106:7119-24.

Beresford PJ, Xia Z, Greenberg AH, Lieberman J. Granzyme A loading induces rapid cytolysis and a rapid form of DNA damage independently of caspase activation. Immunity 1999; 10: 585-594.

Beresford PJ, Zhang D, Oh DY, Fan Z, Greer EL, Russo ML et al. Granzyme A activates an endoplamsic reticulum associated caspse-independent nuclease to induce single strand DNA nicks. J Biol Chem 2001; 8: 357-368.

Bergsbaken T, Fink SL, Cookson BT. Pyroptosis: host cell death and inflammation. Nat Rev Microbiol. 2009; 7:99-109.

Bertelsen M, Sanfridson A. TAB1 modulates IL-1αlpha mediated cytokine secretion but is dispensable for TAK1 activation. Cell Signal. 2007; 19: 646-57.

Bianchi ME, Beltrame M. Upwardly mobile proteins. Workshop: the role of HMG proteins in chromatin structure, gene expression and neoplasia. EMBO Rep. 2000; 1:109-14.

Bianchi ME. DAMPs, PAMPs and alarmins: all we need to know about danger. J Leukoc Biol. 2007; 81: 1-5.

Bladergroen BA, Strik MC, Bovenschen N, van Berkum O, Scheffer GL, Meijer CJ, Hack CE, Kummer JA. The granzyme B inhibitor, protease inhibitor 9, is mainly expressed by dendritic cells and at immune-privileged sites. J Immunol. 2001; 166: 3218-25.

Bladergroen BA, Strik MC, Wolbink AM, Wouters D, Broekhuizen R, Kummer JA, Hack CE. The granzyme B inhibitor proteinase inhibitor 9 (PI9) is expressed by human mast cells. Eur J Immunol. 2005; 35: 1175-83.

Black RA, Kronheim SR, Cantrell M, Deeley MC, March CJ, Prickett KS, Wignall J, Conlon PJ, Cosman D, Hopp TP, et al. Generation of biologically active interleukin-1 beta by proteolytic cleavage of the inactive precursor. J Biol Chem. 1988; 263: 9437-42.

Bratke K, Böttcher B, Leeder K, Schmidt S, Küpper M, Virchow JC Jr, Luttmann W. Increase in granzyme B+ lymphocytes and soluble granzyme B in bronchoalveolar lavage of allergen challenged patients with atopic asthma. Clin Exp Immunol. 2004;136:542-8.

Brody DT, Durum SK. Membrane IL-1: IL-1 alpha precursor binds to the plasma membrane via a lectin-like interaction. J Immunol. 1989; 143:1183-7.

Browne KA, Blink E, Sutton VR, Froelich CJ, Jans DA, Trapani JA. Cytosolic delivery of granzyme B by bacterial toxins: evidence that endosomal disruption, in addition to transmembrane pore formation, is an important function of perforin. Mol Cell Biol. 1999; 19:8604-15.

Browne KA, Johnstone RW, Jans DA, Trapani JA. Filamin (280-kDa actin-binding protein) is a caspase substrate and is also cleaved directly by the cytotoxic T lymphocyte protease granzyme B during apoptosis. J Biol Chem 2000; 275: 39262-39266.

Burns K, Martinon F, Esslinger C, Pahl H, Schneider P, Bodmer JL, Di Marco F, French L, Tschopp J. MyD88, an adapter protein involved in interleukin-1 signaling. J Biol Chem. 1998; 273:12203-9.

Buzza MS, Hirst CE, Bird CH, Hosking P, McKendrick J, Bird PI. The granzyme B inhibitor, PI-9, is present in endothelial and mesothelial cells, suggesting that it protects bystander cells during immune responses. Cell Immunol. 2001; 210: 21-9.

Buzza MS, Zamurs L, Sun J, Bird CH, Smith AI, Trapani JA, Froelich CJ, Nice EC, Bird PI. Extracellular matrix remodeling by human granzyme B via cleavage of vitronectin, fibronectin, and laminin. J Biol Chem. 2005; 280: 23549-58.

Buzza MS, Bird PI. Extracellular granzymes: current perspectives. Biol Chem. 2006; 387:827-37.

Buzza MS, Dyson JM, Choi H, Gardiner EE, Andrews RK, Kaiserman D, Mitchell CA, Berndt MC, Dong JF, Bird PI. Antihemostatic activity of human granzyme B mediated by cleavage of von Willebrand factor. J Biol Chem. 2008 15: 22498-504.

Cao Z, Henzel WJ, Gao X. IRAK: a kinase associated with the interleukin-1 receptor. Science. 1996a; 271:1128-31.

Cao Z, Xiong J, Takeuchi M, Kurama T, Goeddel DV. TRAF6 is a signal transducer for interleukin-1. Nature. 1996b; 383:443-6.

Carruth LM, Demczuk S, Mizel SB. Involvement of a calpain-like protease in the processing of the murine interleukin 1 alpha precursor. J Biol Chem. 1991; 266: 12162-7.

Cassens U, Lewinski G, Samraj AK, von Bernuth H, Baust H, Khazaie K, Los M. Viral modulation of cell death by inhibition of caspases. Arch Immunol Ther Exp (Warsz). 2003; 51:19-27.

Cayrol C, Girard JP. The IL-1-like cytokine IL-33 is inactivated after maturation by caspase-1. Proc Natl Acad Sci U S A. 2009; 106:9021-6.

Chowdhury D, Beresford PJ, Zhu P, Zhang D, Sung JS, Demple B, Perrino FW, Lieberman J. The exonuclease TREX1 is in the SET complex and acts in concert with NM23-H1 to degrade DNA during granzyme A-mediated cell death. Mol Cell 2006; 23: 133-142.

Chowdhury D, Lieberman J. Death by a thousand cuts: granzyme pathways of programmed cell death. Annu Rev Immunol. 2008; 26:389-420.

Chen CJ, Shi Y, Hearn A, Fitzgerald K, Golenbock D, Reed G, Akira S, Rock KL. MyD88-dependent IL-1 receptor signaling is essential for gouty inflammation stimulated by monosodium urate crystals. J Clin Invest. 2006; 116:2262-71.

Chen CJ, Kono H, Golenbock D, Reed G, Akira S, Rock KL. Identification of a key pathway required for the sterile inflammatory response triggered by dying cells. Nat Med. 2007; 13: 851-6.

Cheng EH, Wei MC, Weiler S, Flavell RA, Mak TW, Lindsten T, Korsmeyer SJ. BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. Mol Cell 2001; 8:705-11.

Choy JC, McDonald PC, Suarez AC, Hung VH, Wilson JE, McManus BM, Granville DJ. Granzyme B in atherosclerosis and transplant vascular disease: association with cell death and atherosclerotic disease severity. Mod Pathol. 2003; 16: 460-70.

Choy JC, Hung VH, Hunter AL, Cheung PK, Motyka B, Goping IS, Sawchuk T, Bleackley RC, Podor TJ, McManus BM, Granville DJ. Granzyme B induces smooth muscle cell apoptosis in the absence of perforin: involvement of extracellular matrix degradation. Arterioscler Thromb Vasc Biol. 2004; 24: 2245-50.

Chung CS, Venet F, Chen Y, Jones LN, Wilson DC, Ayala CA, Ayala A. Deficiency of Bid protein reduces sepsis-induced apoptosis and inflammation, while improving septic survival. Shock. 2010; 34:150-61.

Cohen I, Rider P, Carmi Y, Braiman A, Dotan S, White MR, Voronov E, Martin MU, Dinarello CA, Apte RN. Differential release of chromatin-bound IL-1alpha discriminates between necrotic and apoptotic cell death by the ability to induce sterile inflammation. Proc Natl Acad Sci U S A. 2010; 107:2574-9.

Cooper DM, Pechkovsky DV, Hackett TL, Knight DA, Granville DJ. Granzyme K activates protease-activated receptor-1. PLoS One. 2011; 6:e21484.

Cullen SP and Martin SJ. Mechanisms of granule-dependent killing. Cell Death Differ. 2008; 15: 251-62.

Cullen SP, Martin SJ. Caspase activation pathways: some recent progress. Cell Death Differ. 2009; 16:935-8.

Cullen SP, Lüthi AU, Martin SJ. Analysis of apoptosis in cell-free systems. Methods. 2008; 44:273-9.

Cullen SP, Adrain, C, Lüthi, AU, Duriez, PJ and Martin, SJ, Human and murine granzyme B exhibit divergent substrate preferences. J Cell Biol. 2007; 176: 435-44.

Cullen SP, Afonina IS, Donadini R, Lüthi AU, Medema JP, Bird PI, Martin SJ. Nucleophosmin is cleaved and inactivated by the cytotoxic granule protease granzyme M during natural killer cell-mediated killing. J Biol Chem. 2009; 284: 5137-47.

Davis JE, Sutton VR, Smyth MJ, Trapani JA. Dependence of granzyme B-mediated cell death on a pathway regulated by Bcl-2 or its viral homolog, BHRF1. Cell Death Differ 2000; 7: 973-983.

Degli Esposti M, Ferry G, Masdehors P, Boutin JA, Hickman JA, Dive C. Post-translational modification of Bid has differential effects on its susceptibility to cleavage by caspase 8 or caspase 3. J Biol Chem. 2003; 278:15749-57.

Delves PJ, Martin SJ, Burton DR, Roitt IM. Roitt's essential immunology. Wiley-Blackwell. 2011; 12th edition.

Deng L, Wang C, Spencer E, Yang L, Braun A, You J, Slaughter C, Pickart C, Chen ZJ. Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. Cell. 2000; 103:351-61.

Desagher S, Osen-Sand A, Montessuit S, Magnenat E, Vilbois F, Hochmann A, Journot L, Antonsson B, Martinou JC. Phosphorylation of bid by casein kinases I and II regulates its cleavage by caspase 8. Mol Cell. 2001; 8:601-11.

Dinarello CA, Ikejima T, Warner SJ, Orencole SF, Lonnemann G, Cannon JG, Libby P. Interleukin 1 induces interleukin 1. I. Induction of circulating interleukin 1 in rabbits *in vivo* and in human mononuclear cells *in vitro*. J Immunol. 1987; 139:1902-10.

Dinarello CA. Biologic basis for interleukin-1 in disease. Blood. 1996; 87: 2095-147.

Dong C, Davis RJ, Flavell RA. MAP kinases in the immune response. Annu Rev Immunol. 2002; 20:55-72.

Eder C. Mechanisms of interleukin-1beta release. Immunobiology. 2009; 214:543-53.

Eigenbrod T, Park JH, Harder J, Iwakura Y, Núñez G. Cutting edge: critical role for mesothelial cells in necrosis-induced inflammation through the recognition of IL-1 alpha released from dying cells. J Immunol. 2008; 181: 8194-8.

Enari M, Sakhira H, Yokoyama H, Okawa K, Iwasmatsu A, Nagata S. A caspse-activated DNase that degrades DNA during apoptosis, and its inhibitor, ICAD. Nature 1998; 391: 43-50.

Fan Z, Beresford PJ, Zhang D, Xu Z, Novina CD, Yoshida A, Pommier Y, Lieberman J. Cleaving the oxidative repair protein Ape1 enhances cell death mediated by granzyme A. Nat Immunol. 2003; 4: 145-53.

Fantuzzi G, Dinarello CA. The inflammatory response in interleukin-1 beta-deficient mice: comparison with other cytokine-related knock-out mice. J Leukoc Biol. 1996; 59:489-93.

Fellows E, Gil-Parrado S, Jenne DE, Kurschus FC. Natural killer cell-derived human granzyme H induces an alternative, caspase-independent cell-death program. Blood. 2007; 110: 544-52.

Frangioni JV, Neel BG. Solubilization and purification of enzymatically active glutathione S-transferase (pGEX) fusion proteins. Anal Biochem. 1993 210: 179-87.

Freishtat RJ, Natale J, Benton AS, Cohen J, Sharron M, Wiles AA, Ngor WM, Mojgani B, Bradbury M, Degnan A, Sachdeva R, Debiase LM, Ghimbovschi S, Chow M, Bunag C, Kristosturyan E, Hoffman EP. Sepsis alters the

megakaryocyte-platelet transcriptional axis resulting in granzyme B-mediated lymphotoxicity. Am J Respir Crit Care Med. 2009;179:467-73.

Froelich CJ, Zhang X, Turbov J, Hudig D, Winkler U, Hanna WL. Human granzyme B degrades aggrecan proteoglycan in matrix synthesized by chondrocytes. J Immunol. 1993; 151: 7161-71.

Froelich CJ, Orth K, Turbov J, Seth P, Gottlieb R, Babior B, Shah GM, Bleackley RC, Dixit VM, Hanna W. New paradigm for lymphocyte granule-mediated cytotoxicity. Target cells bind and internalize granzyme B, but an endosomolytic agent is necessary for cytosolic delivery and subsequent apoptosis. J Biol Chem. 1996; 271:29073-9.

Froelich CJ, Hanna WL, Poirier GG, Duriez PJ, D'Amours D, Salvesen GS, Alnemri ES, Earnshaw WC, Shah GM. Granzyme B/perforin-mediated apoptosis of Jurkat cells results in cleavage of poly(ADP-ribose) polymerase to the 89-kDa apoptotic fragment and less abundant 64-kDa fragment. Biochem Biophys Res Commun 1996; 227: 658-665.

Gahring L, Carlson NG, Meyer EL, Rogers SW. Granzyme B proteolysis of a neuronal glutamate receptor generates an autoantigen and is modulated by glycosylation. J Immunol. 2001;166:1433-8.

Ganor Y, Besser M, Ben-Zakay N, Unger T, Levite M. Human T cells express a functional ionotropic glutamate receptor GluR3, and glutamate by itself triggers integrin-mediated adhesion to laminin and fibronectin and chemotactic migration. J Immunol. 2003; 170:4362-72.

Ganor Y, Teichberg VI, Levite M. TCR activation eliminates glutamate receptor GluR3 from the cell surface of normal human T cells, via an autocrine/paracrine granzyme B-mediated proteolytic cleavage. J Immunol. 2007;178:683-92.

Graves BJ, Hatada MH, Hendrickson WA, Miller JK, Madison VS, Satow Y. Structure of interleukin 1 alpha at 2.7-A resolution. Biochemistry. 1990; 29:2679-84.

Greenfeder SA, Nunes P, Kwee L, Labow M, Chizzonite RA, Ju G. Molecular cloning and characterization of a second subunit of the interleukin 1 receptor complex. J Biol Chem. 1995; 270:13757-65.

Gross C, Koelch W, DeMaio A, Arispe N, Multhoff G. Cell surface-bound heat shock protein 70 (Hsp70) mediates perforin-independent apoptosis by specific binding and uptake of granzyme B. J Biol Chem. 2003; 278:41173-81.

Grossman WJ, Ley TJ. Granzymes A and B are not expressed in human neutrophils. Blood. 2004; 104:906-7; author reply 907-8.

Gu Y, Kuida K, Tsutsui H, Ku G, Hsiao K, Fleming MA, Hayashi N, Higashino K, Okamura H, Nakanishi K, Kurimoto M, Tanimoto T, Flavell RA, Sato V, Harding MW, Livingston DJ, Su MS. Activation of interferon-gamma inducing factor mediated by interleukin-1beta converting enzyme. Science. 1997; 275:206-9.

Guma M, Ronacher L, Liu-Bryan R, Takai S, Karin M, Corr M. Caspase 1-independent activation of interleukin-1beta in neutrophil-predominant inflammation. Arthritis Rheum. 2009; 60:3642-50.

Hacham M, Argov S, White RM, Segal S, Apte RN. Different patterns of interleukin-1alpha and interleukin-1beta expression in organs of normal young and old mice. Eur Cytokine Netw. 2002; 13: 55-65.

Haddad P, Jenne D, Tschopp J, Clément MV, Mathieu-Mahul D, Sasportes M. Structure and evolutionary origin of the human granzyme H gene. Int Immunol. 1991; 3: 57-66.

Hagn M, Schwesinger E, Ebel V, Sontheimer K, Maier J, Beyer T, Syrovets T, Laumonnier Y, Fabricius D, Simmet T, Jahrsdörfer B. Human B cells secrete granzyme B when recognizing viral antigens in the context of the acute phase cytokine IL-21. J Immunol. 2009; 183: 1838-45.

Hayes E, Pohl K, McElvaney NG, Reeves EP. The cystic fibrosis neutrophil: a specialized yet potentially defective cell. Arch Immunol Ther Exp (Warsz). 2011; 59:97-112.

Hazuda DJ, Strickler J, Kueppers F, Simon PL, Young PR. Processing of precursor interleukin 1 beta and inflammatory disease. J Biol Chem. 1990 265: 6318-22.

Hazuda D, Strickler J, Simon P, Young PR. Structure-function mapping of interleukin 1 precursors. Cleavage leads to a conformational change in the mature protein. J Biol Chem. 1991; 266: 7081-6.

Heibein JA, Goping IS, Barry M, Pinkoski MJ, Shore GC, Green DR, Bleackley RC. Granzyme B-mediated cytochrome c release is regulated by the Bcl-2 family members bid and Bax. J Exp Med 2000; 192: 1391-1402.

Hernandez-Pigeon H, Jean C, Charruyer A, Haure MJ, Titeux M, Tonasso L, Quillet-Mary A, Baudouin C, Charveron M, Laurent G. Human keratinocytes acquire cellular cytotoxicity under UV-B irradiation. Implication of granzyme B and perforin. J Biol Chem. 2006; 281: 13525-32.

Hernandez-Pigeon H, Jean C, Charruyer A, Haure MJ, Baudouin C, Charveron M, Quillet-Mary A, Laurent G. UVA induces granzyme B in human keratinocytes through MIF: implication in extracellular matrix remodeling. J Biol Chem. 2007; 282: 8157-64.

Heusel JW, Wesselschmidt RL, Shresta S, Russell JH, Ley TJ. Cytotoxic lymphocytes require granzyme B for the rapid induction of DNA fragmentation and apoptosis in allogeneic target cells. Cell 1994; 76: 977-987.

Heutinck KM, ten Berge IJ, Hack CE, Hamann J, Rowshani AT. Serine proteases of the human immune system in health and disease. Mol Immunol. 2010; 47:1943-55.

Hirst CE, Buzza MS, Sutton VR, Trapani JA, Loveland KL, Bird PI. Perforin-independent expression of granzyme B and proteinase inhibitor 9 in human testis

and placenta suggests a role for granzyme B-mediated proteolysis in reproduction. Mol Hum Reprod. 2001; 7: 1133-42.

Hogquist KA, Nett MA, Unanue ER, Chaplin DD. Interleukin 1 is processed and released during apoptosis. Proc Natl Acad Sci U S A. 1991; 88: 8485-9.

Hommel-Berrey GA, Bochan MR, Montel AH, Goebel WS, Froelich CJ, Brahmi Z. Granzyme B independently of perforin mediates noncytolytic intracellular inactivation of vesicular stomatitis virus. Cell Immunol. 1997; 180:1-9.

Horai R, Asano M, Sudo K, Kanuka H, Suzuki M, Nishihara M, Takahashi M, Iwakura Y. Production of mice deficient in genes for interleukin (IL)-1alpha, IL-1beta, IL-1alpha/beta, and IL-1 receptor antagonist shows that IL-1beta is crucial in turpentine-induced fever development and glucocorticoid secretion. J Exp Med. 1998; 187:1463-75.

Horiuchi K, Saito S, Sasaki R, Tomatsu T, Toyama Y. Expression of granzyme B in human articular chondrocytes. J Rheumatol. 2003;30:1799-810.

Hu SX, Wang S, Wang JP, Mills GB, Zhou Y, Xu HJ. Expression of endogenous granzyme B in a subset of human primary breast carcinomas. Br J Cancer. 2003; 89: 135-9.

Huang J, Gao X, Li S, Cao Z. Recruitment of IRAK to the interleukin 1 receptor complex requires interleukin 1 receptor accessory protein. Proc Natl Acad Sci U S A. 1997; 94:12829-32.

Huang Q, Petros AM, Virgin HW, Fesik SW, Olejniczak ET. Solution structure of the BHRF1 protein from Epstein-Barr virus, a homolog of human Bcl-2. J Mol Biol. 2003; 332:1123-30.

Hurgin V, Novick D, Werman A, Dinarello CA, Rubinstein M. Antiviral and immunoregulatory activities of IFN-gamma depend on constitutively expressed IL-1alpha. Proc Natl Acad Sci U S A. 2007; 104:5044-9.

Irmler M, Hertig S, MacDonald HR, Sadoul R, Becherer JD, Proudfoot A, Solari R, Tschopp J. Granzyme A is an interleukin 1 beta-converting enzyme. J Exp Med. 1995; 181: 1917-22.

Jacobson MD, Weil M, Raff MC. Programmed cell death in animal development. Cell. 1997; 88:347-54.

Jiang X, Wang X. Cytochrome c promotes caspase-9 activation by inducing nucleotide binding to Apaf-1. J Biol Chem 2000; 275: 31199-31203.

Jiang Z, Ninomiya-Tsuji J, Qian Y, Matsumoto K, Li X. Interleukin-1 (IL-1) receptor-associated kinase-dependent IL-1-induced signaling complexes phosphorylate TAK1 and TAB2 at the plasma membrane and activate TAK1 in the cytosol. Mol Cell Biol. 2002; 22:7158-67.

Joeckel LT, Wallich R, Martin P, Sanchez-Martinez D, Weber FC, Martin SF, Borner C, Pardo J, Froelich C, Simon MM. Mouse granzyme K has pro-inflammatory potential. Cell Death Differ. 2011; 18:1112-9.

Joosten LA, Helsen MM, Saxne T, van De Loo FA, Heinegard D, van Den Berg WB. IL-1 alpha beta blockade prevents cartilage and bone destruction in murine type II collagen-induced arthritis, whereas TNF-alpha blockade only ameliorates joint inflammation. J Immunol. 1999; 163: 5049-55.

Joosten LA, Netea MG, Fantuzzi G, Koenders MI, Helsen MM, Sparrer H, Pham CT, van der Meer JW, Dinarello CA, van den Berg WB. Inflammatory arthritis in caspase 1 gene-deficient mice: contribution of proteinase 3 to caspase 1-independent production of bioactive interleukin-1beta. Arthritis Rheum. 2009; 60:3651-62.

Kagi D, Ledermann B, Burki K, Seiler P, Odermatt B, Olsen KJ, Podack ER, Zinkernagel RM, Hengartner H. Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. Nature 1994; 369: 31-37.

Kam CM, Hudig D, Powers JC. Granzymes (lymphocyte serine proteases): characterization with natural and synthetic substrates and inhibitors. Biochim Biophys Acta. 2000; 1477:307-23.

Kanakaraj P, Ngo K, Wu Y, Angulo A, Ghazal P, Harris CA, Siekierka JJ, Peterson PA, Fung-Leung WP. Defective interleukin (IL)-18-mediated natural killer and T helper cell type 1 responses in IL-1 receptor-associated kinase (IRAK)-deficient mice. J Exp Med. 1999; 189:1129-38.

Kaplanski G, Farnarier C, Kaplanski S, Porat R, Shapiro L, Bongrand P, Dinarello CA. Interleukin-1 induces interleukin-8 secretion from endothelial cells by a juxtacrine mechanism. Blood. 1994; 84:4242-8.

Karin M, Ben-Neriah Y. Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. Annu Rev Immunol. 2000; 18:621-63.

Kawai T, Akira S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. Immunity. 2011; 34:637-50.

Kazama H, Ricci JE, Herndon JM, Hoppe G, Green DR, Ferguson TA. Induction of immunological tolerance by apoptotic cells requires caspase-dependent oxidation of high-mobility group box-1 protein. Immunity. 2008; 29:21-32.

Keller M, Rüegg A, Werner S, Beer HD. Active caspase-1 is a regulator of unconventional protein secretion. Cell 2008; 132 818-31.

Kim WJ, Kim H, Suk K and Lee WH. Macrophages express granzyme B in the lesion areas of atherosclerosis and rheumatoid arthritis. Immunol Lett. 2007; 111: 57-65.

Kluck RM, Bossy-Wetzel E, Green DR, Newmeyer DD. The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science 1997; 275: 1132-1136.

Kobayashi Y, Yamamoto K, Saido T, Kawasaki H, Oppenheim JJ, Matsushima K. Identification of calcium-activated neutral protease as a processing enzyme of human interleukin 1 alpha. Proc Natl Acad Sci U S A. 1990; 87: 5548-52.

Kono H, Karmarkar D, Iwakura Y, Rock KL. Identification of the cellular sensor that stimulates the inflammatory response to sterile cell death. J Immunol. 2010; 184:4470-8.

Kozak W, Zheng H, Conn CA, Soszynski D, van der Ploeg LH, Kluger MJ. Thermal and behavioral effects of lipopolysaccharide and influenza in interleukin-1 beta-deficient mice. Am J Physiol. 1995; 269:R969-77.

Kummer JA, Kamp AM, Citarella F, Horrevoets AJ, Hack CE. Expression of human recombinant granzyme A zymogen and its activation by the cysteine proteinase cathepsin C. J Biol Chem. 1996; 271: 9281-6.

Kuhns DB, Priel DA, Gallin JI. Induction of human monocyte interleukin (IL)-8 by fibrinogen through the toll-like receptor pathway. Inflammation. 2007;30:178-88.

Kuida K, Lippke JA, Ku G, Harding MW, Livingston DJ, Su MS, Flavell RA. Altered cytokine export and apoptosis in mice deficient in interleukin-1 beta converting enzyme. Science. 1995; 267:2000-3.

Kurt-Jones, E.A., Beller, D.I., Mizel, S.B., Unanue, E.R. Identification of a membrane-associated interleukin 1 in macrophages. Proc Natl Acad Sci U S A. 1985; 82: 1204-8.

Kurt-Jones EA, Fiers W, Pober JS. Membrane interleukin 1 induction on human endothelial cells and dermal fibroblasts. J Immunol. 1987; 139: 2317-24.

Kuwana T, Mackey MR, Perkins G, Ellisman MH, Latterich M, Schneiter R, Green DR, Newmeyer DD. Bid, Bax, and lipids cooperate to form supramolecular openings in the outer mitochondrial membrane. Cell 2002; 111: 331-342.

Lamkanfi M, Kanneganti TD, Van Damme P, Vanden Berghe T, Vanoverberghe I, Vandekerckhove J, Vandenabeele P, Gevaert K, Núñez G. Targeted peptidecentric proteomics reveals caspase-7 as a substrate of the caspase-1 inflammasomes. Mol Cell Proteomics. 2008; 7:2350-63.

Lamkanfi M, Moreira LO, Makena P, Spierings DC, Boyd K, Murray PJ, Green DR, Kanneganti TD. Caspase-7 deficiency protects from endotoxin-induced lymphocyte apoptosis and improves survival. Blood. 2009; 113:2742-5.

Lamothe B, Besse A, Campos AD, Webster WK, Wu H, Darnay BG. Site-specific Lys-63-linked tumor necrosis factor receptor-associated factor 6 auto-ubiquitination is a critical determinant of I kappa B kinase activation. J Biol Chem. 2007; 282:4102-12.

Lauw FN, Simpson AJ, Hack CE, Prins JM, Wolbink AM, van Deventer SJ, Chaowagul W, White NJ, van Der Poll T. Soluble granzymes are released during human endotoxemia and in patients with severe infection due to gram-negative bacteria. J Infect Dis. 2000; 182: 206-13.

Li P, Allen H, Banerjee S, Franklin S, Herzog L, Johnston C, McDowell J, Paskind M, Rodman L, Salfeld J, Elizabeth Towne, Daniel Tracey, Scott Wardwell, Feng-Yi Wei, Winnie Wong, Robert Kamen, Tara Seshadri. Mice deficient in IL-1 beta-

converting enzyme are defective in production of mature IL-1 beta and resistant to endotoxic shock. Cell. 1995; 80:401-11.

Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell. 1997; 91: 479-89.

Li H, Zhu H, Xu CJ, Yuan J. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell. 1998; 94:491-501.

Lin TJ, Garduno R, Boudreau RT, Issekutz AC. Pseudomonas aeruginosa activates human mast cells to induce neutrophil transendothelial migration via mast cell-derived IL-1 alpha and beta. J Immunol. 2002; 169:4522-30.

Loeb CR, Harris JL, Craik CS. Granzyme B proteolyzes receptors important to proliferation and survival, tipping the balance toward apoptosis. J Biol Chem. 2006; 281: 28326-35.

Lu H, Hou Q, Zhao T, Zhang H, Zhang Q, Wu L, Fan Z. Granzyme M directly cleaves inhibitor of caspase-activated DNase (CAD) to unleash CAD leading to DNA fragmentation. J Immunol 2006; 177: 1171-1178.

Luo X, Budihardjo I, Zou H, Slaughter C, Wang X. Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. Cell. 1998; 94:481-90.

Lüthi AU, Martin SJ. The CASBAH: a searchable database of caspase substrates. Cell Death Differ. 2007; 14:641-50.

Lüthi AU, Cullen SP, McNeela EA, Duriez PJ, Afonina IS, Sheridan C, Brumatti G, Taylor RC, Kersse K, Vandenabeele P, Lavelle EC, Martin SJ. Suppression of interleukin-33 bioactivity through proteolysis by apoptotic caspases. Immunity. 2009; 31: 84-98.

Mahrus S, Kisiel W, Craik CS. Granzyme M is a regulatory protease that inactivates proteinase inhibitor 9, an endogenous inhibitor of granzyme B. J Biol Chem 2004; 279: 54275-54282.

Maier JA, Voulalas P, Roeder D, Maciag T. Extension of the life-span of human endothelial cells by an interleukin-1 alpha antisense oligomer. Science 1990; 249: 1570-4.

Maier JA, Statuto M, Ragnotti G. Endogenous interleukin 1 alpha must be transported to the nucleus to exert its activity in human endothelial cells. Mol Cell Biol. 1994; 14:1845-51.

Martin SJ, Reutelingsperger CP, McGahon AJ, Rader JA, van Schie RC, LaFace DM, Green DR. Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. J Exp Med. 1995; 182:1545-56.

Martin SJ, Amarante-Mendes GP, Shi L, Chuang TH, Casiano CA, O'Brien GA, Fitzgerald P, Tan EM, Bokoch GM, Greenberg AH, Green DR. The cytotoxic cell protease granzyme B initiates apoptosis in a cell-free system by proteolytic

processing and activation of the ICE/CED-3 family protease, CPP32, via a novel two-step mechanism. Embo J. 1996; 15: 2407-16.

Martinon F, Burns K, Tschopp J. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. Mol Cell. 2002; 10:417-26.

Martinon F, Pétrilli V, Mayor A, Tardivel A, Tschopp J. Gout-associated uric acid crystals activate the NALP3 inflammasome. Nature. 2006; 440:237-41.

Matsushima K, Taguchi M, Kovacs EJ, Young HA, Oppenheim JJ. Intracellular localization of human monocyte associated interleukin 1 (IL 1) activity and release of biologically active IL 1 from monocytes by trypsin and plasmin. J Immunol. 1986; 136: 2883-91.

McMahon GA, Garfinkel S, Prudovsky I, Hu X, Maciag T. Intracellular precursor interleukin (IL)-1alpha, but not mature IL-1alpha, is able to regulate human endothelial cell migration *in vitro*. J Biol Chem. 1997; 272:28202-5.

Medema JP, Toes RE, Scaffidi C, Zheng TS, Flavell RA, Melief, CJ, Peter ME, Offringa R, Krammer PH. Cleavage of FLICE (caspase-8) by granzyme B during cytotoxic T Lymphocyte-induced apoptosis. Eur J Immunol 1997; 27: 3492-3498.

Metkar SS, Froelich CJ. Human neutrophils lack granzyme A, granzyme B, and perforin. Blood. 2004; 104:905-6; author reply 907- 8.

Metkar SS, Menaa C, Pardo J, Wang B, Wallich R, Freudenberg M, Kim S, Raja SM, Shi L, Simon MM, Froelich CJ. Human and mouse granzyme A induce a proinflammatory cytokine response. Immunity. 2008; 29: 720-33.

Mizutani H, Schechter N, Lazarus G, Black RA, Kupper TS. Rapid and specific conversion of precursor interleukin 1 beta (IL-1 beta) to an active IL-1 species by human mast cell chymase. J Exp Med. 1991; 174: 821-5.

Monack DM, Navarre WW, Falkow S. Salmonella-induced macrophage death: the role of caspase-1 in death and inflammation. Microbes Infect. 2001; 3:1201-12.

Moretta L, Moretta A. Unravelling natural killer cell function: triggering and inhibitory human NK receptors. EMBO J 2004; 23: 255-259.

Moretta A, Marcenaro E, Parolini S, Ferlazzo G, Moretta L. NK cells at the interface between innate and adaptive immunity. Cell Death Differ. 2008; 15: 226-33.

Mosley B, Urdal DL, Prickett KS, Larsen A, Cosman D, Conlon PJ, Gillis S, Dower SK. The interleukin-1 receptor binds the human interleukin-1 alpha precursor but not the interleukin-1 beta precursor. J Biol Chem. 1987a; 262: 2941-4.

Mosley B, Dower SK, Gillis S, Cosman D. Determination of the minimum polypeptide lengths of the functionally active sites of human interleukins 1 alpha and 1 beta. Proc Natl Acad Sci U S A. 1987b; 84: 4572-6.

Mossman BT, Churg A. Mechanisms in the pathogenesis of asbestosis and silicosis. Am J Respir Crit Care Med. 1998; 157(5 Pt 1):1666-80.

Muzio M, Ni J, Feng P, Dixit VM. IRAK (Pelle) family member IRAK-2 and MyD88 as proximal mediators of IL-1 signaling. Science. 1997; 278:1612-5.

Nigrovic PA, Binstadt BA, Monach PA, Johnsen A, Gurish M, Iwakura Y, Benoist C, Mathis D, Lee DM. Mast cells contribute to initiation of autoantibody-mediated arthritis via IL-1. Proc Natl Acad Sci U S A. 2007; 104: 2325-30.

Niki Y, Yamada H, Seki S, Kikuchi T, Takaishi H, Toyama Y, Fujikawa K, Tada N. Macrophage- and neutrophil-dominant arthritis in human IL-1 alpha transgenic mice. J Clin Invest. 2001; 107: 1127-35.

Niki, Y., Yamada, H., Kikuchi, T., Toyama, Y., Matsumoto, H., Fujikawa, K., Tada, N. Membrane-associated IL-1 contributes to chronic synovitis and cartilage destruction in human IL-1 alpha transgenic mice. J Immunol. 2004; 172: 577-84.

Nuki G, Simkin PA. A concise history of gout and hyperuricemia and their treatment. Arthritis Res Ther. 2006; 8 Suppl 1:S1.

Okamura Y et al. The extra domain A of fibronectin activates Toll-like receptor 4. J Biol Chem. 2001;276:10229-33.

Omoto Y, Yamanaka K, Tokime K, Kitano S, Kakeda M, Akeda T, Kurokawa I, Gabazza EC, Tsutsui H, Katayama N, Yamanishi K, Nakanishi K, Mizutani H. Granzyme B is a novel interleukin-18 converting enzyme. J Dermatol Sci. 2010; 59:129-35.

Osterloh A, Breloer M. Heat shock proteins: linking danger and pathogen recognition. Med Microbiol Immunol. 2008; 197:1-8.

Pardo J, Balkow S, Anel A, Simon MM. Granzymes are essential for natural killer cell-mediated and perf-facilitated tumor control. Eur J Immunol 2002; 32: 2881-2887.

Pardo J, Bosque A, Brehm R, Wallich R, Naval J, Müllbacher A, Anel A, Simon MM. Apoptotic pathways are selectively activated by granzyme A and/or granzyme B in CTL-mediated target cell lysis. J Cell Biol 2004; 167: 457-468.

Pardo J, Wallich R, Ebnet K, Iden S, Zentgraf H, Martin P, Ekiciler A, Prins A, Müllbacher A, Huber M, Simon MM. Granzyme B is expressed in mouse mast cells *in vivo* and *in vitro* and causes delayed cell death independent of perforin. Cell Death Differ. 2007;14:1768-79.

Petersen JL, Morris CR, Solheim JC. Virus evasion of MHC class I molecule presentation. J Immunol. 2003; 171:4473-8.

Pipkin ME, Lieberman J. Delivering the kiss of death: progress on understanding how perforin works. Curr Opin Immunol. 2007; 19: 301-8.

Pohlman TH, Harlan JM. Human endothelial cell response to lipopolysaccharide, interleukin-1, and tumor necrosis factor is regulated by protein synthesis. Cell Immunol. 1989; 119:41-52.

Prakash MD, Bird CH, Bird PI. Active and zymogen forms of granzyme B are constitutively released from cytotoxic lymphocytes in the absence of target cell engagement. Immunol Cell Biol. 2009; 87: 249-54.

Priestle JP, Schär HP, Grütter MG. Crystal structure of the cytokine interleukin-1 beta. EMBO J. 1988; 7:339-43.

Qu Y, Franchi L, Nunez G, Dubyak GR. Nonclassical IL-1 beta secretion stimulated by P2X7 receptors is dependent on inflammasome activation and correlated with exosome release in murine macrophages. J Immunol. 2007; 179:1913-25.

Quan LT, Caputo A, Bleackley RC, Pickup DJ, Salvesen GS. Granzyme B is inhibited by the cowpox virus serpin cytokine response modifier A. J Biol Chem. 1995; 270: 10377-9.

Quan LT, Tewari M, O'Rourke K, Dixit V, Snipas SJ, Poirier GG, Ray C, Pickup DJ, Salvesen GS. Proteolytic activation of the cell death protease Yama/CPP32 by granzyme B. Proc Natl Acad Sci USA 1996; 93: 1972-1976.

Reeves EP, Williamson M, Byrne B, Bergin DA, Smith SG, Greally P, O'Kennedy R, O'Neill SJ, McElvaney NG. IL-8 dictates glycosaminoglycan binding and stability of IL-18 in cystic fibrosis. J Immunol. 2010; 184:1642-52.

Reiter Z, Rubinstein M. Interleukin-1 alpha and tumor necrosis factor-alpha protect cells against natural killer cell-mediated cytotoxicity and natural killer cytotoxic factor. Cell Immunol. 1990; 125:326-36.

Rhim, J.H., Kim, S.A., Lee, J.E., Kim, D.J., Chung, H.K., Shin, K.J., Chung, J. Cancer cell-derived IL-1αlpha induces IL-8 release in endothelial cells. J Cancer Res Clin Oncol. 2008; 134: 45-50.

Rissoan MC, Duhen T, Bridon JM, Bendriss-Vermare N, Péronne C, de Saint Vis B, Brière F, Bates EE. Subtractive hybridization reveals the expression of immunoglobulin-like transcript 7, Eph-B1, granzyme B, and 3 novel transcripts in human plasmacytoid dendritic cells. Blood. 2002; 100: 3295-303.

Rock KL, Latz E, Ontiveros F, Kono H. The sterile inflammatory response. Annu Rev Immunol. 2010; 28:321-42.

Romero V, Andrade F. Non-apoptotic functions of granzymes. Tissue Antigens. 2008; 71: 409-16.

Ronday HK, van der Laan WH, Tak PP, de Roos JA, Bank RA, TeKoppele JM, Froelich CJ, Hack CE, Hogendoorn PC, Breedveld FC, Verheijen JH. Human granzyme B mediates cartilage proteoglycan degradation and is expressed at the invasive front of the synovium in rheumatoid arthritis. Rheumatology (Oxford). 2001;40:55-61.

Russell JH, Ley TJ. Lymphocyte-mediated cytotoxicity. Annu Rev Immunol 2002; 20: 323-370.

Sarin A, Williams MS, Alexander-Miller MA, Berzofsky JA, Zacharchuk CM Henkart PA. Target cell lysis by CTL granule exocytosis is independent of ICE/Ced-3 family proteases. Immunity 1997; 6: 209-215.

Sasson R, Dantes A, Tajima K, Amsterdam A. Novel genes modulated by FSH in normal and immortalized FSH-responsive cells: new insights into the mechanism of FSH action. FASEB J. 2003; 17: 1256-66.

Sasu S, Cooper AL, Beasley D. Juxtacrine effects of IL-1 alpha precursor promote iNOS expression in vascular smooth muscle cells. Am J Physiol Heart Circ Physiol. 2001; 280: H1615-23.

Sayers TJ, Wiltrout TA, Sowder R, Munger WL, Smyth MJ, Henderson LE. Purification of a factor from the granules of a rat natural killer cell line (RNK) that reduces tumor cell growth and changes tumor morphology. Molecular identity with a granule serine protease (RNKP-1). J Immunol. 1992; 148: 292-300.

Sayers TJ, Brooks AD, Ward JM, Hoshino T, Bere WE, Wiegand GW, Kelly JM, Smyth MJ. The restricted expression of granzyme M in human lymphocytes. J Immunol. 2001; 166: 765-71.

Schmitz J, Owyang A, Oldham E, Song Y, Murphy E, McClanahan TK, Zurawski G, Moshrefi M, Qin J, Li X, Gorman DM, Bazan JF, Kastelein RA. IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. Immunity. 2005; 23:479-90.

Schroder K, Tschopp J. The inflammasomes. Cell. 2010; 140:821-32.

Shao W, Yeretssian G, Doiron K, Hussain SN, Saleh M. The caspase-1 digestome identifies the glycolysis pathway as a target during infection and septic shock. J Biol Chem. 2007; 282:36321-9.

Silverman GA, Bird PI, Carrell RW, Church FC, Coughlin PB, Gettins PG, Irving JA, Lomas DA, Luke CJ, Moyer RW, Pemberton PA, Remold-O'Donnell E, Salvesen GS, Travis J, Whisstock JC. The serpins are an expanding superfamily of structurally similar but functionally diverse proteins. Evolution, mechanism of inhibition, novel functions, and a revised nomenclature. J Biol Chem. 2001; 276: 33293-6.

Simon MM, Hausmann M, Tran T, Ebnet K, Tschopp J, ThaHla R, Müllbacher A. *In vitro* - and *ex vivo*-derived cytolytic leukocytes from granzyme A x B double knockout mice are defective in granule-mediated apoptosis but not lysis of target cells. J Exp Med. 1997; 186:1781-6.

Slee EA, Adrain C, Martin SJ. Serial killers: ordering caspase activation events in apoptosis. Cell Death Differ 1999; 6: 1067-1074.

Slee EA, Keogh SA, Martin SJ. Cleavage of BID during cytotoxic drug and UV radiation-induced apoptosis occurs downstream of the point of Bcl-2 action and is catalysed by caspase-3: a potential feedback loop for amplification of apoptosis-associated mitochondrial cytochrome c release. Cell Death Differ. 2000; 7:556-65.

Smeets TJ, Dolhain RJ, Breedveld FC, Tak PP. Analysis of the cellular infiltrates and expression of cytokines in synovial tissue from patients with rheumatoid arthritis and reactive arthritis. J Pathol. 1998; 186:75-81.

Smyth MJ, O'Connor MD, Trapani JA, Kershaw MH, Brinkworth RI. A novel substrate-binding pocket interaction restricts the specificity of the human NK cell-specific serine protease, Met-ase-1. J Immunol. 1996; 156: 4174-81.

Smyth MJ, Thia KY, Street SE, MacGregor D, Godfrey DI, Trapani, JA. Perforin-mediated cytotoxicity is critical for surveillance of spontaneous lymphoma. J Exp Med 2000; 192: 755-760.

So A, De Smedt T, Revaz S, Tschopp J. A pilot study of IL-1 inhibition by anakinra in acute gout. Arthritis Res Ther. 2007; 9:R28.

Sower LE, Froelich CJ, Carney DH, Fenton JW 2nd, Klimpel GR. Thrombin induces IL-6 production in fibroblasts and epithelial cells. Evidence for the involvement of the seven-transmembrane domain (STD) receptor for alpha-thrombin. J Immunol. 1995; 155: 895-901.

Sower LE, Klimpel GR, Hanna W, Froelich CJ. Extracellular activities of human granzymes. I. Granzyme A induces IL6 and IL8 production in fibroblast and epithelial cell lines. Cell Immunol. 1996a; 171: 159-63.

Sower LE, Froelich CJ, Allegretto N, Rose PM, Hanna WD, Klimpel GR. Extracellular activities of human granzyme A. Monocyte activation by granzyme A versus alpha-thrombin. J Immunol. 1996b; 156: 2585-90.

Spaeny-Dekking EH, Hanna WL, Wolbink AM, Wever PC, Kummer JA, Swaak AJ, Middeldorp JM, Huisman HG, Froelich CJ, Hack CE. Extracellular granzymes A and B in humans: detection of native species during CTL responses *in vitro* and *in vivo*. J Immunol. 1998; 160: 3610-6.

Strik MC, de Koning PJ, Kleijmeer MJ, Bladergroen BA, Wolbink AM, Griffith JM, Wouters D, Fukuoka Y, Schwartz LB, Hack CE, van Ham SM, Kummer JA. Human mast cells produce and release the cytotoxic lymphocyte associated protease granzyme B upon activation. Mol Immunol. 2007; 44: 3462-72.

Street SE, Hayakawa Y, Zhan Y, Lew AM, MacGregor D, Jamieson AM, Diefenbach A, Yagita H, Godfrey DI, Smyth MJ. Innate immune surveillance of spontaneous B cell lymphomas by natural killer cells and gammadelta T cells. J Exp Med 2004; 199: 879–884.

Sun J, Bird CH, Sutton V, McDonald L, Coughlin PB, De Jong TA, Trapani JA, Bird PI. A cytosolic granzyme B inhibitor related to the viral apoptotic regulator cytokine response modifier A is present in cytotoxic lymphocytes. J Biol Chem 271 1996; 27802–27809.

Sun Q, Zheng Y, Liu Q, Cao X. Rapamycin reverses TLR4 signaling-triggered tumor apoptosis resistance by disrupting Akt-mediated Bcl-xL upregulation. Int Immunopharmacol. 2008; 8:1854-8.

Sutton VR, Vaux DL, Trapani JA. Bcl-2 prevents apoptosis induced by perforin and granzyme B, but not that mediated by whole cytotoxic lymphocytes. J Immunol 1997; 158: 5783-5790.

Tak PP, Kummer JA, Hack CE, Daha MR, Smeets TJ, Erkelens GW, Meinders AE, Kluin PM, Breedveld FC. Granzyme-positive cytotoxic cells are specifically increased in early rheumatoid synovial tissue. Arthritis Rheum. 1994; 37:1735-43.

Tak PP, Spaeny-Dekking L, Kraan MC, Breedveld FC, Froelich CJ, Hack CE. The levels of soluble granzyme A and B are elevated in plasma and synovial fluid of patients with rheumatoid arthritis (RA). Clin Exp Immunol. 1999; 116: 366-70.

Takaesu G, Kishida S, Hiyama A, Yamaguchi K, Shibuya H, Irie K, Ninomiya-Tsuji J, Matsumoto K. TAB2, a novel adaptor protein, mediates activation of TAK1 MAPKKK by linking TAK1 to TRAF6 in the IL-1 signal transduction pathway. Mol Cell. 2000; 5:649-58.

Taylor RC, Cullen SP, Martin SJ. Apoptosis: controlled demolition at the cellular level. Nat Rev Mol Cell Biol. 2008; 9:231-41.

Thiery J, Keefe D, Saffarian S, Martinvalet D, Walch M, Boucrot E, Kirchhausen T, Lieberman J. Perforin activates clathrin- and dynamin-dependent endocytosis, which is required for plasma membrane repair and delivery of granzyme B for granzyme-mediated apoptosis. Blood. 2010; 115:1582-93.

Thiery J, Keefe D, Boulant S, Boucrot E, Walch M, Martinvalet D, Goping IS, Bleackley RC, Kirchhausen T, Lieberman J. Perforin pores in the endosomal membrane trigger the release of endocytosed granzyme B into the cytosol of target cells. Nat Immunol. 2011; 12:770-7.

Thomas DA, Du C, Xu M, Wang X, Ley TJ. DFF45/ICAD can be directly processed by granzyme B during the induction of apoptosis. Immunity 2000; 12: 621-632.

Thornberry, N.A., Bull, H.G., Calaycay, J.R., Chapman, K.T., Howard, A.D., Kostura, M.J., Miller, D.K., Molineaux, S.M., Weidner, J.R., Aunins, J., et al. A novel heterodimeric cysteine protease is required for interleukin-1 beta processing in monocytes. Nature 1992; 356: 768-74.

Thornberry NA, Rano TA, Peterson EP, Rasper DM, Timkey T, Garcia-calvo M, Houtzager VM, Nordstrom PA, Roy S, Vaillancourt JP, Chapman KT, Nicholson DW. A combinatorial approach defines specificities of members of the caspase family and granzyme B. J Biol Chem 1997; 272: 17907-17911.

Trapani JA. Granzymes: a family of lymphocyte granule serine proteases. Genome Biol. 2001; 2:REVIEWS3014.

Tschopp CM, Spiegl N, Didichenko S, Lutmann W, Julius P, Virchow JC, Hack CE, Dahinden CE. Granzyme B, a novel mediator of allergic inflammation: its induction and release in blood basophils and human asthma. Blood. 2006; 108: 2290-9.

van den Broek ME, Kagi D, Ossendorp F, Toes R, Vamvakas S, Lutz WK, Melief CJ, Zinkernagel RM, Hengartner H. Decreased tumor surveillance in perforin-deficient mice. J Exp Med 1996; 184: 1781–1790.

van Tetering G, Bovenschen N, Meeldijk J, van Diest PJ, Vooijs M. Cleavage of Notch1 by granzyme B disables its transcriptional activity. Biochem J. 2011; 437:313-22.

Vega-Carrascal I, Reeves EP, Niki T, Arikawa T, McNally P, O'Neill SJ, Hirashima M, McElvaney NG. Dysregulation of TIM-3-galectin-9 pathway in the cystic fibrosis airways. J Immunol. 2011; 186:2897-909.

Wagner C, Iking-Konert C, Denefleh B, Stegmaier S, Hug F, Hänsch GM. Granzyme B and perforin: constitutive expression in human polymorphonuclear neutrophils. Blood. 2004; 103:1099-104.

Wagner C, Stegmaier S, Hänsch GM. Expression of granzyme B in peripheral blood polymorphonuclear neutrophils (PMN), myeloid cell lines and in PMN derived from haemotopoietic stem cells *in vitro*. Mol Immunol. 2008; 45:1761-6.

Walsh JG, Cullen SP, Sheridan C, Lüthi AU, Gerner C, Martin SJ. Executioner caspase-3 and caspase-7 are functionally distinct proteases. Proc Natl Acad Sci U S A. 2008; 105:12815-9.

Walsh JG, Logue SE, Lüthi AU, Martin SJ. Caspase-1 promiscuity is counterbalanced by rapid inactivation of the processed enzyme. J Biol Chem. 2011; 286:32513-24.

Wang K, Yin XM, Chao DT, Milliman CL, Korsmeyer SJ. BID: a novel BH3 domain-only death agonist. Genes Dev. 1996; 10:2859-69.

Wang S, Miura M, Jung YK, Zhu H, Li E, Yuan J .Murine caspase-11, an ICE-interacting protease, is essential for the activation of ICE. Cell. 1998; 92:501-9.

Wang C, Deng L, Hong M, Akkaraju GR, Inoue J, Chen ZJ. TAK1 is a ubiquitin-dependent kinase of MKK and IKK. Nature. 2001; 412:346-51.

Wang HL, Akinci IO, Baker CM, Urich D, Bellmeyer A, Jain M, Chandel NS, Mutlu GM, Budinger GR. The intrinsic apoptotic pathway is required for lipopolysaccharide-induced lung endothelial cell death. J Immunol. 2007; 179:1834-41.

Watanabe, N., Kobayashi, Y. Selective release of a processed form of interleukin 1 alpha. Cytokine. 1994; 6: 597-601.

Waterfield M, Jin W, Reiley W, Zhang M, Sun SC. IkappaB kinase is an essential component of the Tpl2 signaling pathway. Mol Cell Biol. 2004; 24:6040-8.

Werman A, Werman-Venkert R, White R, Lee JK, Werman B, Krelin Y, Voronov E, Dinarello CA, Apte RN. The precursor form of IL-1 alpha is an intracrine proinflammatory activator of transcription. Proc Natl Acad Sci U S A. 2004; 101: 2434-9.

Wessendorf JH, Garfinkel S, Zhan X, Brown S, Maciag T. Identification of a nuclear localization sequence within the structure of the human interleukin-1 alpha precursor. J Biol Chem. 1993; 268:22100-4.

White E, Sabbatini P, Debbas M, Wold WS, Kusher DI, Gooding LR. The 19-kilodalton adenovirus E1B transforming protein inhibits programmed cell death and prevents cytolysis by tumor necrosis factor alpha. Mol Cell Biol. 1992; 12:2570-80.

Yeretssian G, Correa RG, Doiron K, Fitzgerald P, Dillon CP, Green DR, Reed JC, Saleh M. Non-apoptotic role of BID in inflammation and innate immunity. Nature. 2011; 474:96-9.

Yoshifuji H, Umehara H, Maruyama H, Itoh M, Tanaka M, Kawabata D, Fujii T, Mimori T. Amelioration of experimental arthritis by a calpain-inhibitory compound: regulation of cytokine production by E-64-d *in vivo* and *in vitro*. Int Immunol. 2005; 17: 1327-36.

Zhang D, Beresford PJ, Greenberg AH, Lieberman J. Granzymes A and B directly cleave lamins and disrupt the nuclear lamina during granule-mediated cytolysis. Proc Natl Acad Sci USA 2001; 98: 5746-5751.

Zhang WH, Wang X, Narayanan M, Zhang Y, Huo C, Reed JC, Friedlander RM. Fundamental role of the Rip2/caspase-1 pathway in hypoxia and ischemia-induced neuronal cell death. Proc Natl Acad Sci U S A. 2003; 100:16012-7.

Zhao Y, Li S, Childs EE, Kuharsky DK, Yin XM. Activation of pro-death Bcl-2 family proteins and mitochondria apoptosis pathway in tumor necrosis factor-alpha-induced liver injury. J Biol Chem. 2001; 276:27432-40.

Zhao T, Zhang H, Guo Y, Zhang Q, Hua G, Lu H, Hou Q, Liu H, Fan Z. Granzyme K cleaves the nucleosome assembly protein SET to induce single-stranded DNA nicks of target cells. Cell Death Differ. 2007; 14: 489-99.

Zhao T, Zhang H, Guo Y, Fan Z. Granzyme K directly processes bid to release cytochrome c and endonuclease G leading to mitochondria-dependent cell death. J Biol Chem. 2007b; 282:12104-11.

Zheng H, Fletcher D, Kozak W, Jiang M, Hofmann KJ, Conn CA, Soszynski D, Grabiec C, Trumbauer ME, Shaw A, et al. Resistance to fever induction and impaired acute-phase response in interleukin-1 beta-deficient mice. Immunity. 1995; 3:9-19.