THE UNIVERSITY *of* EDINBURGH

Characterization of bovine granzymes and studies of the role of granzyme B in killing of *Theileria*-infected cells by CD8+ T cells

Jie Yang

PhD by Research

The University of Edinburgh

2012

Declaration

I declare that the work presented in this thesis is my own original work, except where specified, and it does not include work forming part of a thesis presented successfully for a degree in this or another university

Jie Yang

Edinburgh, 2012

Abstract

Previous studies have shown that cytotoxic CD8+ T cells are important mediators of immunity against the bovine intracellular protozoan parasite *T. parva*. The present study set out to determine the role of granule enzymes in mediating killing of parasitized cells, first by characterising the granzymes expressed by bovine lymphocytes and, second, by investigating their involvement in killing of target cells.

Experiments using the perforin inhibitor concanamycin A confirmed that CD8+ T cell killing of *T. parva*-infected cells is dependent on granule exocytosis, a process that involves release of granzymes into the target cell, resulting in activation of apoptotic pathways. Analysis of the bovine genome sequence identified orthologues of granzymes A, B, H, K and M, as well as another gene O, most closely related to granzyme A. The genes were found within 3 loci in the genome. Using specific PCR assays, all of these granzymes were shown to be expressed in *Theileria*-specific CD8+ T cells. Further studies were undertaken to study the role of granzyme B in killing. DNA constructs encoding functional and non-functional forms of bovine granzyme B were produced and the proteins expressed in COS cells were used to establish an enzymatic assay to detect and quantify expression of functional granzyme B protein. Using this assay, the levels of killing of different *T. parva*-specific CD8+ T cell clones were found to be significantly correlating with levels of granzyme B protein expression. Moreover, the granzyme B inhibitor III, Z-IETD-FMK was shown to inhibit killing by CD8+ T cell clones.

Acknowledgements

I would like to thank my supervisor, Prof. Ivan Morrison, for his patience, help, support and advice during this study, particularly at time of writing up.

Special thanks must be given to Dr Timothy Connelley for all his guidance, help and encouragement during my PhD, especially at the beginning of this study.

I wish to thank Dr Niall MacHugh for his helpful discussions and Dr Jane Hart for her technical advice and always being supportive.

Thanks also need to go to Dr Darren Shaw for his statistical help and advice.

Finally, I would like to thank family and friends for all their understanding and support.

Contents

List of figures

List of tables

Abbreviations

Bid	BH3-interaction domain death agonist
Bp	base pair
BSA	Bovine serum albumin
^{o}C	degrees Celsius
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CO_2	carbon dioxide
cpm	counts per minute
CTL	cytotoxic lymphocyte
dATP	2'- deoxyadenosine triphosphate
dCTP	2'- deoxycytidine triphosphate
dGTP	2'- deoxyguanosine triphosphate
dNTP	mixture of dATP, dCTP, dGTP and dTTP
dTTP	2'- deoxythymidine triphosphate
DDW	double distilled water
DMSO	Dimethylsulphoxide
DNA	deoxyribonucleic acid
EDTA	Ethylene-diamine-tetra-acetic acid
ER	endoplasmic reticulum
FACS	fluorescence-activated cell sorting
FITC	Fluorescein isothiocyanate
g	grams
Gzm	granzyme
h	hour
HCL	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
ICAD	inhibitor of caspase-activated DNase
IFN	interferon
Ig	immunoglobulin

IL	interleukin
^{111}In	Indium radioactive isotope 111
Kb	kilo base pairs
KCL	Potassium chloride
L	Litre
M	Molar
MAb	monoclonal antibody
Mb	mage base pairs
$MgSO_4$	Magnesium sulfate
MHC	Major histocompatibility complex
MHCI	Major histocompatibility complex class I
min	minute
ml	mili litre
mM	mili molar
mRNA	messenger ribonucleic acid
NaCl	Sodium chloride
ng	nano grams
$(NH_4)_2SO_4$	Ammonium sulphate
NK	Natural killer
nm	nano meter
PARP	poly (ADP-ribose) polymerase
PBMC	peripheral blood mono-nuclear cells
PBS	Phosphate buffered saline
PCR	polymerase chain reaction
PFN	perforin
pmol	pico mole
RNA	ribonucleic acid
RNase	ribonuclease
rmp	revolutions per minute
ROS	reactive oxygen species
RT	reverse transcriptase
RT-PCR	reverse transcriptase- polymerase chain reaction

s	second
SMAC/DIABLO	second mitochondria-derived activator of caspase
TA	*Theileria Annulata*-infected cell
TCR	T-cell receptor
TCRβ	T-cell receptor beta chain
Tp	*Theileria parva*
TPM	*Theileria parva* (Muguga) - infected cell
Tris-HCL	2-Amino-2-(hydroxymethyl)-1, 3-propanediol hydrochloride
ug	micro grams
uM	micro molar
V	volts
Vβ	T-cell receptor beta chain variable gene segment
v/v	volume to volume
w/v	weight to volume
x g	force of gravity

Standard base pair (single letter code) and amino acid (both single and three letter code) abbreviation have been used throughout this study.

Chapter 1 Introduction

1.1 *Theileria parva* and East coast fever

Theileria parasites are members of the class Sporozoa, subphylum Apicomplexa, which includes several other pathogens of great importance such as *Plasmodium*, *Babesia*, *Toxoplasma* and *Eimeria*. The most important pathogenic species are *Theileria annulata* and *Theileria parva*, which cause severe clinical diseases in cattle known as Tropical Theileriosis and East Coast Fever (ECF), respectively. *T. parva* is transmitted by the brown ear tick *Rhipicephalus appendiculatis* and is distributed in a large area of eastern, southern and central Africa.

ECF is an acute, and lymphoproliferative disease characterised by sustained fever and lymph node swelling. Infection of susceptible European breeds of cattle with *T. parva* results in death of the majority of animals within 4 weeks. Breed differences in susceptibility to the disease have been observed, although the mechanisms responsible are unknown. In particular, indigenous *Bos indicus* cattle living in ECF-endemic areas develop less severe disease and suffer lower levels of mortality than introduced European Breeds (*Bos taurus*) or breeds of *Bos indicus* cattle that have been selected for improved productivity. Therefore, ECF is considered as a serious constraint to improvement of the local livestock in affected areas. Moreover, the financial losses resulting from the disease and cost of measures taken to control it have been estimated at approximately US$168 million per year (Mukhebi et al.,

1992). Thus, ECF has a huge impact on the livestock industries and economies of countries in the affected region.

1.1.1 Life cycle of *T. parva*

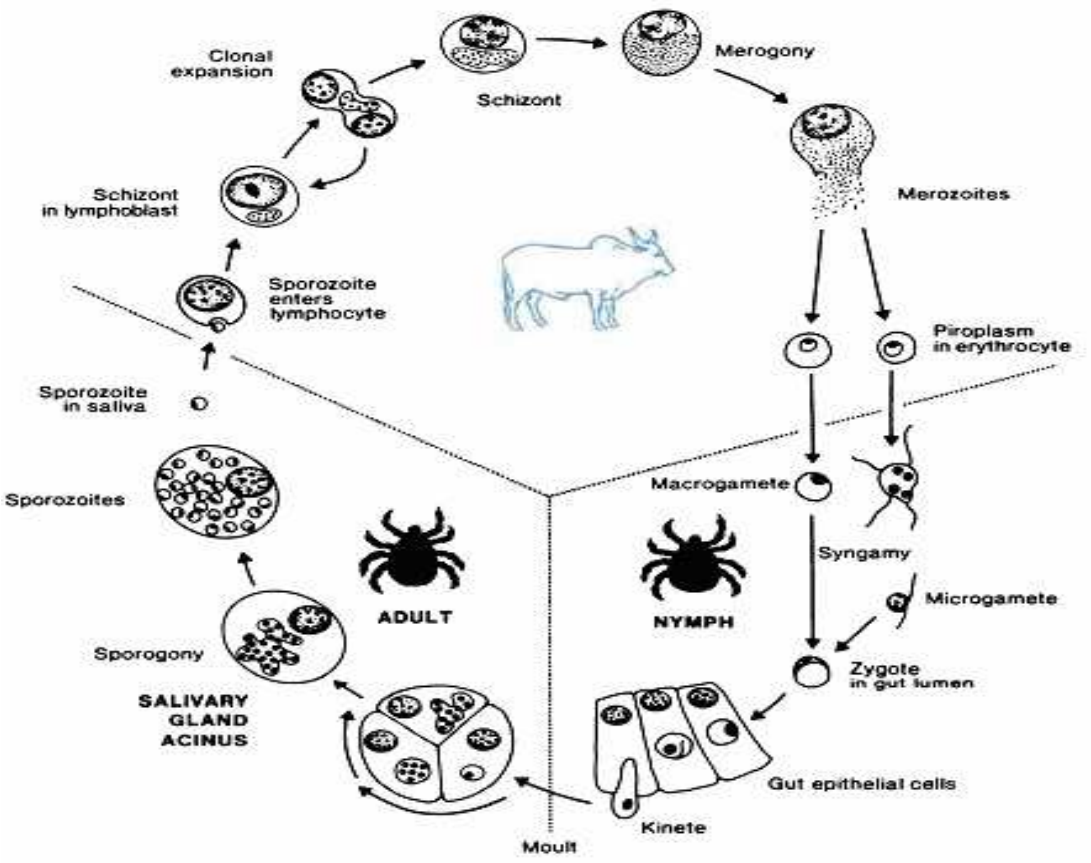


Figure 1.1 *Theileria parva* lifecycle
Reproduced from the Annual Report of the International Laboratory of Research on Animal Disease (ILRAD) 1990

The life cycle of *T. parva* is illustrated in figure 1.1. The infective stage of *T. parva* for ticks is the piroplasm form of the parasites present in erythrocytes of infected cattle. Ticks are infected during the larval or nymphal developmental stages when

they feed on infected cattle and on completion of feeding they drop onto the pasture and moult to the next developmental stage, nymphs and adults respectively, which can then transmit the infection when they feed on a fresh bovine host. Following ingestion by the tick, the parasites start to undergo a sexual cycle initially involving differentiation of the released piroplasms into micro- and macro-gametes (haploid), which fuse together to form diploid zygotes (Schein et al., 1977). The zygote forms then invade the epithelial cells of the gut wall and differentiate into motile kinetes, which migrate from the gut to the salivary glands where they infect glandular epithelial cells (Mehlhorn and Shein, 1984). Within an infected gland cell, each kinete undergoes a process of sporogony, which can give rise to 30,000 to 50,000 sporozoites (Fawcett et al., 1982), the infective stage for cattle (Shaw, 2003). The process of sporogony involves a meiotic division, during which genetic recombination can occur in zygotes arising from infection with mixed parasite genotypes. Therefore sporozoites and all subsequent developmental stages in cattle contain a haploid genome.

Infection is transmitted to cattle during tick feeding by release of sporozoites into the saliva following 2-4 days of tick feeding. The lifecycle of the parasite in cattle involves successive developmental stages in lymphocytes and erythrocytes. First, sporozoites rapidly enter lymphocytes by receptor-mediated endocytosis (Fawcett et al., 1984). In vitro studies have shown that B and T lymphocytes, including CD4+ T cells, CD8+ T cells and TCR γδ+ population, are susceptible to infection with *T. parva* at similar frequency (Baldwin et al., 1988). However, the majority of parasitized cells in cattle undergoing primary infection are CD4+ and CD8+ T

lymphocytes (Emery et al., 1988). Once inside lymphocytes, the sporozoite rapidly disrupts the surrounding host cell membrane in order to escape from the endosome and subsequently lies free in the cytoplasm within 15-30 min. Within 3-4 days, the sporozoite differentiates into a multinucleate schizont (Stagg et al., 1981), which induces activation and proliferation of the host cell. Division of parasites occurs synchronously with that of the host cell. This is achieved by close association of the parasite with the host mitotic spindle, with parasite nuclei being distributed randomly to both daughter cells during the process of division (Hulliger et al., 1964; Irvin et al., 1982). Although the mechanism of host cell transformation is not fully understood, *T. parva*-has been shown to induce constitutive activation of NF-κB (Heussler et al., 1999), which initiates host cell activation and up-regulation of a number of anti-apoptosis proteins that maintain the viability of the infected cells. Proliferation of the infected cells results in rapid clonal expansion of the cells initially infected with sporozoites. A proportion of them eventually undergo merogony involving differentiation of the schizonts to merozoites, which are released upon destruction of the host cells and infect erythrocytes giving rise to piroplasms. The piroplasms, which undergo little or no multiplication, are the infective stage for the tick vector.

1.1.2 Clinical disease

The pathogenesis of ECF in cattle is mainly attributable to the schizont-infected stage. Cattle experimentally infected with tick-derived sporozoites develop clinical signs, namely pyrexia and enlargement of the lymph nodes, 5-10 days (depending on dose) after infection, coincident with initially detectable schizonts in the lymph

nodes draining the site of infection (Emery, 1981b; Morrison et al., 1981; Shatry et al., 1981). Later, the schizont-infected cells disseminate throughout the lymphoid system (Emery, 1981b; Morrison et al., 1981; Shatry et al., 1981) and non-lymphoid tissues such as gut and lungs, causing generalized lymphadenopathy and, in the terminal stages, respiratory distress (Irvin and Mwamachi, 1983). In the later stages of disease, there is extensive lymphocytolysis and severe leucopaenia (DeMartini and Moulton, 1973; Emery, 1981b; Emery et al., 1981; Morrison et al., 1981; Shatry et al., 1981). Susceptible breeds of animals suffer high levels of mortality and death generally occurs within 4 weeks of infection. Animals that recover become asymptomatic carriers of infection. They carry small numbers of parasites (both schizonts and piroplasms), which are usually not detected microscopically, for months or years (Geysen et al., 1999; Skilton et al., 2002). This carrier state allows transmission of the parasite by tick feeding. In endemic areas, transmission from carrier animals has been estimated by mathematical modelling to account for 90% of new infections in cattle (Medley et al., 1993).

1.1.3 Control of *T. parva*

In the past, control of ECF has been largely based on spraying or dipping of cattle with acaricides to prevent tick infestation. Although this has been an effective method of controlling the disease, it has a number of shortcomings. Continuous application is required for effective prevention of disease. This is expensive and requires careful management. Contamination of the environment by the chemicals is

also a serious issue and their prolonged use has resulted in emergence of acaricide-resistant ticks.

Chemotherapy using two theilericidal compounds (parvaquone and buparvaquone) has been employed to control the disease. However, the treatment needs to be applied in the early stages of clinical disease to be effective and this is not always possible. Also, the high cost of these two drugs is not affordable for many small holder farmers.

Vaccination has been used in some areas as a control measure. An infection and treatment protocol involving infection with a defined dose of sporozoites and simultaneous treatment with a single dose of long-acting oxytetracycline, was developed for immunisation of cattle against *T. parva* (Radley et al., 1975a). This protocol generated protective immunity to challenge with the same (homologous) *T. parva* isolate for at least 3.5 years (Burridge et al., 1972). However, some animals immunised with a single parasite isolate remained susceptible to challenge with heterologous parasite isolates (Radley et al., 1975a). Nevertheless, immunisation with a cocktail of 3 different parasite isolates (Muguga, Kiambu 5 and Serengeti) resulted in immunity to a range of heterologous isolates (Radley et al., 1975b). Subsequently, immunisation with another single isolate, *T. parva* (Marikebuni), was also shown to generate immunity to challenge with a range of heterologous isolates (Morzaria et al., 1987). This isolate was subsequently found to be genetically and antigenically heterogeneous (Morzaria et al., 1987; Goddeeris et al., 1990; Toye et al., 1991). Immunisation of cattle by the infection and treatment method using the

mixture of 3 isolates referred to above (termed the Muguga cocktail) has been used for field vaccination of cattle on a local scale in some regions of Africa to control ECF (Uilenberg et al., 1976; Morzaria et al., 1987; Marcotty et al., 2002). Although it has proved to be effective, it has a number of limitations that have prevented its utilization on a large scale. The method for producing sporozoites from infected ticks requires local infrastructure and expertise and is difficult to quality control, The sporozoites require storage in liquid nitrogen and the lack of a cold chain to transport vaccine to remote areas is a serious constraint., Introduction of the strains of parasite used in the vaccine into the local tick populations as a consequence of vaccine-induced carrier infection is also considered as a disadvantage (Maritim et al., 1989; Kariuki et al., 1995; Geysen et al., 1999). Because of these constraints, research is being directed to developing alternative method of vaccination.

1.2 Effector mechanism of immunity to *T. parva*

1.2.1 The role of CD8+ T cells in protective immunity

Studies of the immune responses in cattle immunised against *T. parva* by infection and treatment have provided insight into the immune responses involved. Following challenge of immune animals there is transient emergence of schizont parasitosis (Morrison et al., 1987), indicating that infection is not prevented at the early stage of parasite development, but at the schizont-infected stage. Other studies of experimental immunization showed that animals could also be immunized against

homologous challenge by administering large numbers of schizont-infected cells (Emery et al., 1982). These findings indicated that protective immunity against *T. parva* is largely attributable to immune responses to the schizont stage rather than sporozoites. As the schizont remains within host lymphocytes during parasite multiplication, it is inaccessible to antibody. Several experiments examining the role of antibody from immune animals in immunity have failed to demonstrate a protective effect. Thus, transfer of serum and gamma-globulins from immunized to susceptible cattle (Muhammed et al., 1975) did not confer protection and attempts to demonstrate antibodies reactive with the surface of schizont-infected cells gave negative results (Wagner et al., 1974; Creemers, 1982).

In contrast, transfer of thoracic duct leukocytes from immune to naïve twin calves was shown to confer immunity, indicating an important role for cell-mediated immune response in immunity (Emery, 1981a). Other in vitro and ex vivo studies of cellular immune responses in immune animals identified strong cytotoxic activity against parasitized lymphocytes mediated by CD8+ T cells (Pearson et al., 1979; Eugui and Emery, 1981; Morrison et al., 1987). A further adoptive transfer study demonstrated that transfer of CD8+ T cells from immune to non-immune twin calves conferred strong protective immunity (McKeever et al., 1994), confirming an important role for CD8+ T cells in immunity against *T. parva*. Immunisation by infection and treatment also stimulates strong parasite-specific CD4+ T cell responses as well as CD8+ T cell responses (Baldwin et al., 1987), and Taracha et al., (1997) reported evidence that optimal activation of parasite-specific CD8+ T cells in

vitro required help from CD4+ T cells from immune animals. This supports the idea that CD4+ T cell responses may also contribute to immunity (Taracha et al., 1997).

1.2.2 Parasite stain specificity and major histocompatability class I (MHC1) restriction

Studies of cross-protection following immunisation and challenge with single parasite isolates have illustrated the parasite-stain specificity of protective immunity (Radley et al., 1975c). Further studies have demonstrated that cytotoxic CD8+ T cell responses induced by immunising with a single isolate also show parasite strain specificity (Goddeeris et al., 1986b; Morrison et al., 1987; Taracha et al., 1995a; Taracha et al., 1995b). Thus, CD8+ T cell lines derived from animals immunized with the Muguga *T. parva* isolate often recognize some, but not other parasite isolates in vitro (Goddeeris et al., 1986a; Goddeeris et al., 1986b). A cross-protection experiment with 22 animals immunized with either *T. parva* (Muguga) or a cloned population of the *T. parva* (Marikebuni) isolate (3219) and subsequently challenged with the reciprocal parasite demonstrated a close correlation between parasite strain specificity of CD8+ T cell responses and susceptibility of animals to heterologous parasite challenge. Nine of the 22 animals had cross-reactive CD8+ T cell responses following immunisation and showed solid immunity following challenge with the heterologous parasite, whereas the remaining 13 animals generated strain-specific CD8+ T cell responses and remained susceptible to heterologous challenge (Taracha et al., 1995a).

Additional experiments by Taracha et al. using the same animals provided evidence that disparity in parasite strain specificity of CD8+ T cell responses of the animals is influenced by major histocompatability class I (MHCI) phenotype (Taracha et al., 1995b). These and other studies (Morrison et al., 1987; Taracha et al., 1995b) showed that MHC restriction of the response in MHC-heterozygous animals is often biased towards one of the MHCI haplotypes and that among groups of immunised animals responses are predominantly restricted by certain haplotypes in preference to others.

1.2.3 Identification of antigens and epitopes

Studies carried out by Graham and colleagues (Graham et al., 2006; Graham et al., 2007; Graham et al., 2008) have identified a number of antigens recognized by *T. parva*-specific CD8+ T cells derived from immune animals of different MHC genotypes. Candidate antigens were identified by screening either a *T. parva* schizont cDNA expression library or expressed isolated cDNAs encoding proteins with a predicted signal peptide, selected by bioinformatic analyses of the *T. parva* (Muguga) genome. *T. parva*-specific CD8+ T cell lines were used to screen Cos cells co-transfected with parasite cDNA and cDNAs encoding the respective bovine class I MHC heavy chains. Antigen recognition by the specific CD8+ T cells was detected by measuring release of IFN-γ. Based on these immuno-screens, 6 *T. parva* antigens (Tp1, Tp2, Tp4, Tp5, Tp7 and Tp8) were identified and further epitope mapping studies identified 9 epitopes within these antigens. Tp1 and 2 are predicted as hypothetical proteins, whereas Tp4, 5, 7 and 8 show homology to the ε-subunit of T

complex protein 1, elongation translation factor 1A, heat-shock proteins 90 and cysteine protease, respectively. Analyses of the MHC restriction of T cell responses to the antigens showed that each antigen tended to be presented by different MHC genotypes and that the responses restricted by certain MHC types appear to be predominantly focused on a single dominant epitope.

1.2.4 Immunodominance

MHCI-restricted CD8+ T cell responses are important in mediating immunological control of many intracellular pathogens, including viruses, bacteria and parasites (Wong and Pamer, 2003). A well-established feature of the CD8+ T cell response to many virus infections in mice and humans is that, despite the ability of the host to respond to numerous antigenic epitopes, the response is often focused only on a few epitopes. This is known as immunodominance (Yewdell, 2006). Several studies have provided evidence that immunodominance is also a feature of CD8+ T cell responses to *Theileria parva* infection (Taracha et al., 1995b; MacHugh et al., 2009). Taracha et al. studied responses of genetically identical twin calves carrying a MHCI haplotype expressing the A10 and KN104 class I proteins, one immunized with *T. parva* (Muguga) and the other with *T. parva* (Marikebuni 3219). The Muguga immunised animal generated a KN104-restricted, strain-specific CD8+ T cell response and was susceptible to subsequent challenge with Marikebuni 3219, whereas the Marikebuni 3219-immunised animal had a KN104-restricted, cross-reactive CD8+ T cell response and was protected against challenge with Muguga. Moreover, after suffering a severe clinical reaction upon challenge with Marikebuni,

the Muguga-immunized twin recovered and subsequently had a KN104-restricted, cross-reactive CD8+ T cell response. This indicated that the initial failure of this animal to respond to the conserved epitope(s) shared by the two *T. parva* isolates was due to the immunodominance of a variable epitope in the *T. parva* (Muguga) isolate (Taracha et al., 1995b). Recent studies of CD8+ T cell responses of immune animals to defined *T. parva* antigens have confirmed the immunodominant nature of the response. CD8+ T cell responses in MHCI-homozygous animals (A18 or A10) recognised only one of 5 *T. parva* antigens tested, Tp1 in A18+ animals and Tp2 in A10+ animals. Furthermore, in each case over 60% of CD8+ T cells were specific for a single dominant epitope, $Tp1_{214\text{-}224}$ and $Tp2_{49\text{-}59}$, respectively (MacHugh et al., 2009).

1.2.5 Subunit vaccine - Immunization with *T. parva* antigens

Given the disadvantages of live vaccines for control of ECF, investigation of alternative methods of vaccination, focusing particularly on subunit vaccines, has been undertaken over the last 3 decades.

In the early 1980s, monoclonal antibodies specific for a sporozoite surface antigen, p67 were found to neutralize the infectivity of sporozoites for lymphocytes in vitro (Musoke et al., 1982; Dobbelaere et al., 1984; Musoke et al., 1984). Attempts were therefore made to use recombinant p67 protein incorporated in adjuvants for vaccination. However, although animals vaccinated with a p67 subunit vaccine generated high titres of neutralising antibodies, a field trial of the vaccine revealed

that the overall incidence of severe ECF was only reduced by approximately 50% in vaccinated animals (total number of 83 animals from 3 different sites in Kenya) (Musoke et al., 2005). These results indicated that vaccination with p67 is unable to provide sufficient protection for field use.

As experimental studies provided evidence that MHCI-restricted CD8+ T cell responses against cells infected with *T. parva* are important in mediating immunological control of the infection, investigation of subunit vaccines has recently focused on CD8+ T cell target antigens. Following identification of *T. parva* antigens recognized by specific CD8+ T cells, 5 of the antigens were used to immunise animals employing a prime-boost protocol, involving priming with antigen expressed in a DNA plasmid or a recombinant canarypox virus and boosting after 4 weeks with antigen expressed in modified vaccinia virus Ankara strain (Graham et al., 2006). Plasmid DNAs and recombinant viruses expressing individual antigens were used. Although 19 of 24 immunised animals generated detectable antigen-specific CD8+ T cell IFN- γ responses to one or more antigens, only a few of the animals showed evidence of protection against parasite challenge (Graham et al., 2006). In contrast to CD8+ T cells induced by infection and treatment, the CD8+ T cells generated by this prime boost protocol exhibited weak or no cytotoxic activity. This suggests that strong cytotoxicity in the specific CD8+ T cell response may be required for protection.

1.3 CD8+ T cell- mediated effector mechanisms against intracellular pathogens

Previous studies using T cell subset depletion and gene-knock mouse models have indicated that CD8+ T cells are important mediators of adaptive immunity against a number of viruses and intracellular protozoa and bacteria. They act as effectors upon engagement with the target cell via T cell receptor (TCR)-mediated recognition of pathogen-derived peptides complexed with self class I MHC glycoproteins (pMHCI). In general, they can act by cytokine-mediated killing of the intracellular pathogens or by directly killing the infected cells via two distinct molecular pathways: the granule exocytosis pathway, which involves release of perforin and granzymes (Henkart and Sitkovsky, 1994), or Fas/Fas ligand interaction which results in classical caspase-dependent apoptosis (Nagata and Golstein, 1995). The former frequently involves IFN- γ and a number of different intracellular mechanisms of IFN- γ-mediated killing of intracellular pathogens have been described (Boehm et al., 1997). Studies of responses to the Gram-positive intracellular bacterium *Listeria monocytogenes* in mice, in which immunity has been shown to be mediated by CD8+ T cells (Mielke et al., 1989; Roberts et al., 1993; Kaufmann and Ladel, 1994; Ladel et al., 1994), have also demonstrated that immunity can develop in mice deficient in IFN- γ, perforin or Fas or combinations of these deficiencies (Harty and Bevan, 1995; White and Harty, 1998; Badovinac and Harty, 2000). TNF-α was implicated in this immunity (Samsom et al., 1995; White and Harty, 1998; White et al., 2000), but the precise mechanisms are still poorly understood. In addition to their direct effector functions, CD8+ T cells can express chemokines that mediate recruitment of inflammatory cells towards infection sites for clearance of pathogens.

1.3.1 Cytokine-mediated mechanisms in *Theileria*

Previous in vitro studies have shown that despite the presence of large numbers of IFN-γ expressing cells in *T. parva*-specific CD8+ T cell populations (Ballingall et al., 2000), the schizont stage of *T. parva* in infected leukocytes is not susceptible to IFN-γ or TNF-α (DeMartini and Baldwin, 1991). What is more, some parasitized cell lines constitutively express IFN-γ both at the level of RNA (McKeever et al., 1997) and functionally active protein (DeMartini and Baldwin, 1991). Other studies of the in vivo response to primary infection with *T. parva* (Houston et al., 2008) have detected production of a large amount of IFN-γ by uninfected T lymphoblasts during the early stages of infection, coinciding with rapid multiplication of parasitized cells. Thus, there is no evidence of cytokine-mediated killing of *T. parva* by the specific CD8+ T cell response.

1.3.2 Mechanisms of cell-mediated cytotoxicity

1.3.2.1 Death receptor pathway (Fas-Fas Ligand)

Fas (CD95), a death receptor belonging to the tumor necrosis factor (TNF) receptor superfamily is constitutively expressed on the cell surface of many cells (Watanabe-Fukunaga et al., 1992b). In contrast, expression of Fas ligand (FasL) is mainly restricted to T and NK cells (Montel et al., 1995; Suda et al., 1995). In T cells, de novo synthesis of FasL is induced upon T cell activation via TCR/CD3 engagement with a specific peptide/MHCI complex (Vignaux et al., 1995). After binding to FasL, signalling through the Fas receptor induces apoptosis of target cells via activation of caspases, which is regulated by several intracellular inhibitors at different molecular

levels (Chang et al., 2002; Salvesen and Duckett, 2002). Although mediation of cell death by this interaction is independent of Ca^{2+} (Rouvier et al., 1993), production of FasL on the cell surface upon T cell activation requires extracellular Ca^{2+} (Lowin et al., 1996). Fas-mediated cell death is believed to primarily contribute to regulating lymphocyte homeostasis (lymphocyte proliferation and apoptosis) via an immune process known as activation-induced cell death (AICD) (Watanabe-Fukunaga et al., 1992a; Takahashi et al., 1994) and is involved in maintaining immune-privileged sites (eg. eye and testis cells constitutively express FasL, allowing them to immediately kill any invading activated inflammatory cells (Suda et al., 1993; Griffith et al., 1995). Several studies in mouse models using Fas-mutant lpr mice or FasL-mutant gld mice have revealed defective cytotoxic activity in CD4+ T cells (Henkart and Sitkovsky, 1994; Stalder et al., 1994) and, using perforin-deficient mice, demonstrated the absence of cytotoxic activity in CD8+ T cells (Kagi et al., 1994b), indicating that CD4+ T cells predominantly utilize the Fas-mediated pathway to induce apoptosis, whereas CD8+ T cells primarily mediate cell death via granule exocytosis.

A study by Kuenzi in 2003 found that *T. parva*-infected cells from cell lines maintained in vitro were less susceptible to Fas-induced cell death than the same cells from which the parasite had been removed by treatment with the theilericidal compound BW720. However, expression of both Fas and FasL was detected on the cell surface of infected cells and was unaffected by theilericidal drug treatment (Kuenzi et al., 2003). The enhanced resistance was attributed to *T. parva*-induced upregulation of several anti-apoptotic proteins including FLIP, X-chromosome-

linked inhibitor of apoptosis protein (XIAP) and c-IAP, which act as endogenous inhibitors of caspases (Kuenzi et al., 2003). Because of the apparent resistance of *Theileria*-infected cells to Fas-mediated killing, Fas-induced cell death is considered less likely to be used as a mechanism of killing of *T. parva*-infected cells by CD8+ T cells

1.3.2.2 Granule exocytosis pathway

In human and mouse, there is evidence that the granule exocytosis pathway plays a predominant role in killing of target cells by antigen-specific CD8+ T cells (Kagi et al., 1994b; Yasukawa et al., 2000). Several studies in perforin-deficient mice have demonstrated that perforin-based cytotoxic activity is required for effective control of infections with a number of intracellular pathogens (Kagi et al., 1994a; Walsh et al., 1994; Nickell and Sharma, 2000). Membrane lysis and DNA damage of target cells are achieved by polarized release of cytotoxic effector molecules, including perforin (a pore-forming protein) and granzymes, which mediate cell death by cleaving a number of substrate proteins involved in mediating cell death. These effector proteins are stored in lytic granules, referred to as secretory lysosomes, which are specialized organelles found predominantly in CD8+ T cells and NK cells (Burkhardt et al., 1990; Peters et al., 1991). Lytic granules are dual-function organelles, having the function of secretory granules (ie, storage and secretion of proteins) as well as that of conventional lysosomes (ie, degradation of proteins) found in other cell types. The acidic environment within lytic granules is important in regulating the functional activity of the effector proteins. In contrast to the lysosomal hydrolases in conventional lysosomes, which are functionally active under

acidic conditions, perforin and granzymes require neutral pH conditions to be functionally active. Within the granules, they remain functionally inactive by forming complexes with a proteoglycan, serglycin (SG) because of the acidic pH (Masson et al., 1990). This protects the CD8+ T cells/NK cells themselves from these toxic enzymes. Resting NK cells have secretory granules and constitutively express perforin and granzymes, whereas naïve CD8+ T cells do not have the secretory granules or express their enzyme contents. Upon activation of CD8+ T cells by TCR recognition of specific antigen and engagement of costimulatory molecules, they differentiate into cytotoxic CD8+ T cells after several days, concurrent with development of lytic granules and synthesis of perforin and granzymes. Degranulation and release of these effector molecules occurs rapidly when these differentiated CD8+ T cells again recognise antigen via TCR recognition alone, thus resulting in rapid apoptosis of target cells.

1.4 Perforin

Perforin is a Ca^{2+} dependent pore-forming protein, with homology to some of the complement components (Muller-Eberhard, 1986). It facilitates delivery of granzymes into the cytosol of target cells, and thus plays an essential role in lymphocyte-mediated cytotoxicity (Redelman and Hudig, 1980; Hudig et al., 1991). Several studies in perforin-deficient mice have provided direct evidence of its absolute requirement for killing target cells by CD8+ T cells (Kagi et al., 1994b; Kojima et al., 1994; Lowin et al., 1994). Purified perforin alone has been shown to induce direct lysis of target cells under some conditions; however, induction of DNA damage of the same cells only occurred in the presence of granzymes (Hayes et al.,

1989; Shi et al., 1992a; Shi et al., 1992b), indicating synergetic activity of perforin and granzymes in inducing apoptotic cell death.

Perforin expression has been found in most NK, NKT and γδ+ cells as well as CD8+ T cells and a subpopulation of CD4+ T cells (Williams and Engelhard, 1997; Kelso et al., 2002; Johnson et al., 2003; Bade et al., 2005). The nascent protein is synthesized as a 70 kDa inactive precursor, modified by addition of glycans in the Golgi apparatus and cleaved at the C-terminus in the lytic granule, thus yielding a 60 kDa active form (Uellner et al., 1997). The presence of a low concentration of Ca^{2+} in the lytic granule as well as the formation of complexes with serglycins in the acidic environment, results in the mature form of perforin remaining functionally inactive within the granules (Masson et al., 1990). Upon degranulation, perforin binds Ca^{2+} which induces conformational changes in the C2-domain that confer an ability to bind lipids (Uellner et al., 1997). This allows perforin to enter into the lipid bi-layer and by polymerisation form a 16nm membrane pore. Pores formed at the site of the T cell synapse were originally thought to allow granzymes to pass through the cell membrane (Young et al., 1986; Podack and Hengartner, 1989). However, the precise site at which granzymes enter the target cell is still under debate. Evidence has been presented that granzymes and perforin together are taken up by the target cells by endocytosis and that delivery of granzymes into the cytosol via perforin pores occurs within the endosomes (Froelich et al., 1996b; Metkar et al., 2002). Moreover, it has been proposed that mannose-6-phosphate receptor (MPR) proteins on the cell surface may bind granzymes and perforin and deliver them into endosomes (Motyka et al., 2000; Metkar et al., 2002).

1.5 Granzymes

Granzymes are a subfamily of serine proteases that make up a major proportion of the mass of lytic granules in cytotoxic CD8+ T lymphocytes and NK cells. They play an important role in killing of susceptible target cells, acting on various cellular pathways that regulate programmed cell death (Jenne and Tschopp, 1988). So far, five granzymes - A, B, K, H and M – have been identified in humans and ten granzymes - A, B, K, E, M, C, D, F, G and N - have been identified in mice (Grossman et al., 2003). Granzyme H appears to be restricted to humans, while C, D, E, F, G and N are exclusively expressed in mouse.

Granzymes are structurally related to chymotrypsin with typical features of a catalytic triad of amino acid residues (His-57, Asp-103 and Ser-195) at the active sites (Murphy et al., 1988) and a substrate-binding cleft, the shape of which determines their enzymatic specificity. In general, following specific docking into the substrate binding cleft of the protease, the substrate is cleaved after the cleavage residue (referred to as amino acid P1 position in the substrate) through the proteolytic activity of the catalytic triad. The binding site on the protease typically interacts with at least three amino acids on each side of P1 in the substrate. The nomenclature applied to amino acid positions flanking the cleavage residue in the substrate is Pn-P4-P3-P2-P1-P1'-P2'-P3'-P4'-Pn', while the nomenclature for corresponding sites in the protease is Sn-S4-S3-S2-S1-S1'-S2'-S3'-S4'-Sn' (Schechter and Berger, 1967), where S1 is the key site that interacts with the substrate P1 residue. This S1 site of the protease determines “primary substrate specificity”, which is strictly restricted to

certain amino acids, whereas other binding sites of the protease that interact with other substrate residues determine the “extended substrate specificity”, which is less specific but has substantial influence on the proteolytic efficiency. Additional shared features of granzymes have been defined including the presence of a consensus sequence at the N-terminus (Bleackley et al., 1988; Jenne and Tschopp, 1988; Murphy et al., 1988), a conserved activation peptide (Gly-Glu or Glu-Glu) (see section 1.5.1) and 3 or 4 intra-molecular disulfide bridges (Smyth et al., 1996; Trapani, 2001; Piuko et al., 2007).

Genes encoding each member of the granzyme family are classified into three distinct subgroups based on their enzymatic activity (trypsin-like, chymotrypsin-like and metase) and this corresponds to segregation into different gene loci on three chromosomes (Table1.1) (Trapani, 2001). Granzymes A and K are found together on human 5q11-q12 and mouse 13D chromosomal regions, respectively. Granzymes B and H are located on human 14q11-q12 chromosomal region and mouse granzymes B, C, E, M, D, F, G and N on 14D chromosomal region. In addition to these granzymes, the chymotrypsin-like locus contains genes encoding other serine proteases such as cathepsin G and mast cell proteases in both species. Granzyme M is located on human 19p13.3 and mouse 10q21.2 chromosomal regions, respectively; a cluster of neutrophil elastase genes including azurocidin 1(ZAU1), proteinase 3(PRTN3), neutrophil expressed (ELANE) and complement factor D (CFD) are present 200-500kb downstream of granzyme M (Pilat et al., 1994). Interspecies comparisons of granzyme sequences reveal a clearly lower level of similarity between the 3 subgroups within a species (30-40%) than between group members

across species (55-70%) (Piuko et al., 2007). Several studies have demonstrated that the substrate specificity of granzymes corresponds to their phylogenetic relatedness and their genome loci. For example, granzyme A and K are tryptases that prefer to cleave their target proteins after basic residues (arginine or lysine); Granzyme B has an unusual Aspase activity, specifically requiring an Asp residue; Granzyme H exhibits the chymase specificity and cleaves after aromatic residues (phenylalanine, tryptophan or tyrosine); Granzyme M has metase activity and prefers to cleave after long narrow hydrophobic residues (methionine or leucine). In contrast, the substrate specificities of mouse granzymes C, E, D, F, G and N were not defined (Smyth et al., 1996; Smyth et al., 2001) and are still unknown so far.

Table 1.1 Mouse and human granzymes grouped according to their chromosomal localization

Chromosomal localization	Species	Granzymes
The 'tryptase' locus		
5q11-q12	Human	A and K
13D	Mouse	A and K
The 'chymase' locus		
14q11-q12	Human	B and H
14D	Mouse	B, C, D, E, F, G and N
The 'Met-ase' locus		
19p13.3	Human	M
10q21.2	Mouse	M

Table is cited from Trapani, 2001 .

1.5.1 Synthesis and processing of granzymes

As granzymes are extremely toxic, causing cell death through various cellular pathways, expression of these molecules is regulated to avoid enzymatic activity during their post-translational transport and storage. Their function is strongly related to the structural conformation of the proteins. Nascent granzymes are synthesized as inactive zymogens and have to undergo two modification steps to become enzymatically activated. Firstly, the signal sequence is removed when it passes through the endoplasmic reticulum (ER), leaving an activation peptide attached at the amino terminus of what is destined to be the mature protein (Masson and Tschopp, 1987). This pro-protein is then delivered via the Golgi apparatus into the lytic granules. Transport occurs predominantly by the mannose-6-phosphate receptor (MPR) pathway, although approximately 30% of granzyme A and B have been shown to be delivered into the lytic granules in an MPR-independent way (Griffiths and Isaaz, 1993). Second, once inside the granules, a specific peptidase, dipeptidyl peptidase I/cathepsin C, cleaves the 2 N-terminal residues of the activation peptide, producing the active form of the enzyme (Pham and Ley, 1999) (Brown et al., 1993). This form remains enzymatically non-functional within the low pH environment of the granules and only becomes active following release upon engagement of target cells (McGuire et al., 1993). This process of granzyme production is broadly similar for the different granzymes, except for granzyme M which is converted to the active form by N-terminal cleavage of a hexapeptide instead of a dipeptide to generate the mature protein sequence (Smyth et al., 1995; Kelly et al., 1996).

1.5.2 Expression of granule enzymes

Granzymes are expressed primarily in lymphoid lineage cells such as CD4+ and CD8+ T cells and their thymic precursors, and γδ+ T cells, NK cells and NKT cells (Table 1.2) (Anthony et al., 2010; Bovenschen and Kummer., 2010). Their differential expression and discordant regulation in lymphocytes has been reported in several studies. Unstimulated thymocytes only express detectable transcripts of granzyme A. However following in vitro stimulation with PMA, ionomycin and IL-2, cultured CD4-CD8- thymocytes, both TCR γ/δ+ and TCRα/β+, were shown to contain transcripts for most granzyme genes (Garcia-Sanz et al., 1990). Lymphocyte subsets involved in innate immunity, namely NK cells γδ+ T cells, and NKT cells, were found to have constitutive expression and granule storage of granzymes (Garcia-Sanz et al., 1990), whereas CD4+ and CD8+ T cells required antigen stimulation or other types of stimuli to induce granzyme expression (Garcia-Sanz et al., 1990; Grossman et al., 2003). Initial studies of granzyme gene expression in different populations of cytotoxic lymphocytes reported that CD8+ T cells obtained from allogeneic mixed lymphocyte reactions expressed predominantly granzyme A and B, whereas levels of expression of other granzymes comparable to that of granzyme A and B were found in NK and lymphokine-activated killer cells (LAK) cells (Pham et al., 1996). Recent studies in mice indicated that, while perforin and most or all of granzymes can be expressed simultaneously by virus-specific CD8+ T cell clones and populations activated *in vitro*, the genes encoding perforin and the granzymes may be differentially regulated in activated CD8+ T cells at the single cell level (Kelso et al., 2002). This was confirmed by another single cell study of virus-specific CD8+ T cells, which showed distinct patterns of expression of perforin and

the granzyme transcripts in single cells from the same T cell culture (Johnson et al., 2003).

Additional studies have shown that the levels of expression of perforin and the various granzymes by CD8+ T cells may be influenced by several cytokines. Regulation of expression of granzymes and perforin in CD8+ T cells has been reported for IL-2, IL-4, IL-12, IL-15 and IL-21. IL-2 and IL-15 were shown capable of stimulating proliferation of CD8+ T cells and up-regulating expression of granzyme A and B and perforin genes (Ye et al., 1996). Provision of IL-12 as a third signal (ie, in addition to TCR engagement and IL-2 co-stimulation), was found to promote development of full cytolytic function of CD8+ T cells and enhanced levels of granzyme B protein expression (Curtsinger et al., 2005). IL-21 in combination with IL-15 was found to promote expression of granzyme B (Zeng et al., 2005). In contrast, IL-4 has been reported to induce a subpopulation of non-cytolytic T cells with low surface CD8+ and low levels of granzymes and perforin expression (Kienzle et al., 2002).

Table 1.2 Summary of information on cell types/tissues expressing different granzymes

Granzyme	Species	Expression
A	H, M	CD4+ T cells, CD8+ T cells, NK, NKT, γδ+ T cells and thymus
B	H, M	CD4+ T cells, CD8+ T cells, NK, NKT, γδ+ T cells and thymus
C	M	CD4+ T cells, CD8+ T cells and thymus
D	M	CD4+ T cells, CD8+ T cells, NK and thymus
E	M	CD4+ T cells, CD8+ T cells, NK and thymus
F	M	CD4+ T cells, CD8+ T cells, NK and thymus
G	M	CD4+ T cells, CD8+ T cells, NK and thymus
H	H	CD4+ T cells, CD8+ T cells, NK
K	H, M	CD4+ T cells, CD8+ T cells, NK, NKT, γδ+ T cells
L	M	Unknown
M	H, M	CD8+ T cells, NK, NKT, γδ+ T cells
N	M	Testis

H - human; M - mouse;. This table is a modified version of data reported by Anthony et al., 2010 and Bovenschen and Kummer., 2010.

1.5.3 Apoptotic function of granzymes

Granzyme A and B are the most abundant granzymes and have been studied in most detail. Current thinking regarding the molecular basis of GzmA and GzmB-induced cell death is based largely on experiments using a mast cell exocytosis model (Nakajima et al., 1995) and on *in vitro* studies using purified effector molecules delivered with sub-lytic concentrations of perforin or other pore-forming analogues. However, the relevance of these in vitro findings to the in vivo models of action of the granzymes is not always well established. Also cell death may not always be dependent on apoptosis. Despite the finding that CD8+ T cells deficient in granzyme

A and B are defective inducers of classical apoptosis, they can still mediate target cell death, suggesting that other granzymes may be operating via alternative pathways (Simon et al., 1997; Mullbacher et al., 1999; Davis et al., 2001; Waterhouse et al., 2006). Little is known about the functions of other granzymes, hence their designation as 'orphans' (Grossman et al., 2003). Although granzyme K, H and M molecules are found at low levels in human CD8+ T cell granules, it has been shown that they can induce cell death (Bovenschen and Kummer., 2010).

Table 1.3 Characteristics cell death induced by individual granzymes and phenotypes of mice with granzyme deficiencies

Granzyme	**Function in cell death**
A	Caspase-independent, depolarization of the mitochondrial membrane, generation of ROS and cleavage of SET, and DNA nicks
B	Caspase-dependent and -independent cell death. Direct processing of effector caspases such as pro-caspase-3 and cleavage activation of ICAD, cleavage of Bid leading to mitochondrial damage and release of SMAC/DIABLO and cytochrome c, formation of an apoptosome resulting in DNA fragmentation
C	Caspase-independent cell death with mitochondrial inner membrane depolarization and DNA nicks
H	Caspase-independent cell death with mitochondrial inner membrane depolarization and ROS formation, resulting in DNA nicks
K	Bid cleavage and caspase-independent cell death, with ROS production due to mitochondrial inner membrane depolarization and DNA nicks
M	Caspase-independent, with unique morphological changes, possible cleavage of ICAD and PARP
D-G,J, L and N	Not described
Deficiency	**Mouse Phenotype**
GrzA -/-	Healthy, fertile; moderate increase in susceptibility to MCMV and ectromelia.

	Unaltered apoptotic response
GrzB -/-	Healthy, fertile; moderate increase in susceptibility to ectromelia and MCMV. CD8+ T cells induce delayed nuclear changes in target cell.
GrzAB -/-	Healthy, fertile; highly susceptible to both MCMV and ectromelia. CTL induce delayed nuclear changes in target cell
GrzM -/-	Healthy, Fertile; mild susceptibility to MCMV but normal response to ectromelia. Unaltered apoptotic response

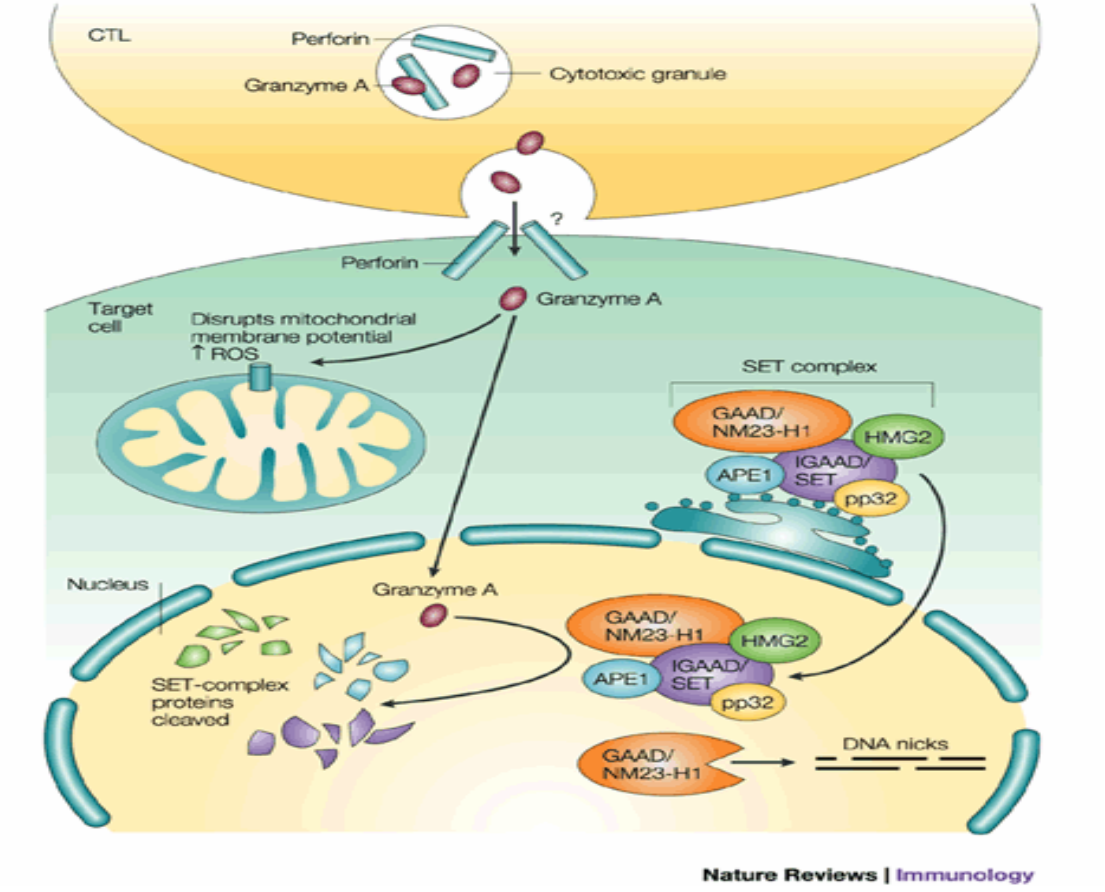
The table is cited from Anthony et al., 2010. MCMV = Murine cytomegalovirus;

1.5.3.1 Apoptotic function of Granzyme A

In vitro studies have shown that recombinant granzyme A delivered with purified perforin results in cell death by causing damage of the inner mitochondrial membrane and inducing nicks in single-stranded DNA (Figure 1.2) (Lieberman and Fan, 2003). Unlike granzyme B, granzyme A-induced cell death does not require caspase activation (Beresford et al., 1999; Fan et al., 2003a). Granzyme A has been shown to act on 3 major cellular targets: lamins, histones and the endoplasmic reticulum-associated SET complex (Beresford et al., 2001; Zhang et al., 2001a; Zhang et al., 2001b). Production of reactive oxygen species (ROS) occurs when granzyme A causes inner mitochondrial membrane damage, thus leading to translocation of the SET complex from the cytoplasmic surface of the ER to the nucleus (Beresford et al., 1999; Martinvalet et al., 2005). Although the biological function of the SET complex is not fully understood, it is probably involved in activation of transcription and transcription-related DNA repair upon oxidative stress. It contains three granzyme A substrates, the nucleosome assembly protein SET, the DNA binding protein HMG-2 and the base excision repair enzyme, Ape1 (Beresford et al., 1999; Fan et al., 2003a; Fan et al., 2003b). The SET protein acts as

an inhibitor of an endonuclease in the SET complex (NM23-H1) and cleavage of SET by granzyme A allows activation of NM23-H1, thus resulting in single-strand DNA nicks. Lamins are responsible for maintaining nuclear structure and histones are the basic building blocks of chromatin structure (Zhang et al., 2001a; Zhang et al., 2001b). Overall, the activity of granzyme A induces apoptosis of target cells through degradation of DNA and disruption of chromatin structural integrity (Pardo et al., 2004).

Figure 1.2 Granzyme A-mediated apoptotic pathway



The figure is cited from Lieberman, 2003

Although several studies have demonstrated cytotoxic activity of granzyme A *in vitro*, the physiological significance of these findings remains controversial (Metkar

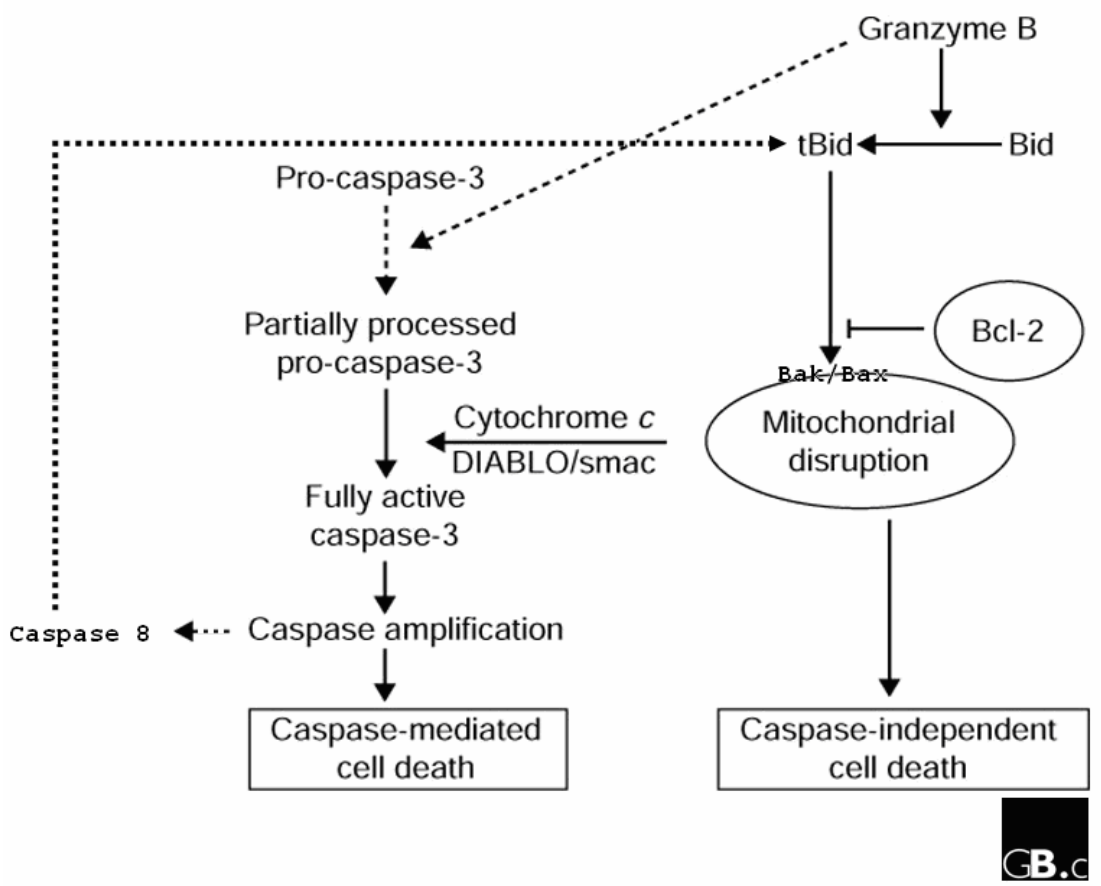
et al., 2008; Trapani and Bird, 2008; Pardo et al., 2009). A recent study of human and mouse granzyme A by Metkar et al., showed this protease does not have cytotoxic activity after delivery by human perforin or adenoviral particles and is not able to induce cell death at low concentrations (nanomolar), based on use of a short-term cell-death assay and cell survival in a long-term proliferative assay (Metkar et al., 2008). Although cell death was observed with high concentrations of granzyme A (micromolar) with sublytic concentrations of perforin, this was not associated with apoptosis but with necrosis due to virtually instantaneous membrane lysis (Metkar et al., 2008). In addition, several investigators have reported that granzyme A-deficient mice have no defect of CD8+ T cell or NK cell-mediated cytotoxicity, suggesting that granzyme A does not play an essential role in cell-mediated cytotoxicity (Ebnet et al., 1995; Mullbacher et al., 1996; Riera et al., 2000).

1.5.3.2 Apoptotic function of Granzyme B

In contrast to granzyme A, there seems to be little dispute that granzyme B is a potent inducer of apoptosis and an important mediator of cytotoxicity. Direct evidence has been obtained from studies of granzyme B-deficient mice, which show dramatically reduced efficiency in the induction of rapid cell death of allogeneic target cells by CD8+ T cells, implying its critical role in rapid granule-mediated apoptosis (Heusel et al., 1994). Granzyme B induces apoptosis by caspase-dependent and –independent pathways (Figure 1.3) (Lieberman, 2003). It can directly hydrolyse but only partially process effector caspases such as procaspase-3 and -7, as their full activation requires a further process known as autocatalysis which is regulated by the release of other pro-apoptotic mitochondrial factors (Martin et al., 1996; Goping et

al., 2003; Sutton et al., 2003). The endonuclease CAD is activated after its inhibitor, inhibitor of caspase-activated DNase (ICAD), is cleaved by the fully activated effector caspases, thus resulting in cell death via DNA fragmentation. Alternatively, granzyme B can induce cell death by triggering mitochondrial outer membrane permeabilization as a consequence of cleaving a pro-apoptotic protein of the Bcl-2 family, BH3-interaction domain death agonist (Bid). The truncated Bid protein initiates mitochondrial-dependent apoptosis by activating Bax and/or Bak (pro-apoptotic proteins of the Bcl-2 family) and promoting their oligomerization within the mitochondrial outer membrane, which leads to mitochondria permeabilization and the subsequent release of cytochrome c, SMAC/DIABLO and Htra2/Omi (Barry et al., 2000; Sutton et al., 2000; Alimonti et al., 2001; Sutton et al., 2003). Release of SMAC/DIABLO and Htra2/Omi sequesters the inhibitor of apoptosis proteins (IAP), which acts as an endogenous inhibitor of the activity of caspases, and thus facilitate full activation of caspases via autocatalysis (Martin et al., 1996; Goping et al., 2003; Sutton et al., 2003). In addition, release of cytochrome c into the cytosol leads to the formation of the apoptosome, a multimeric complex comprising cytochrome c, apoptotic protease activating factor 1 (APAF-1) and procaspase-9 (Li et al., 1997; Jiang and Wang, 2000). The assembly of apoptosomes results in activation of caspase-9, an apical caspase (ie. involved in initial stages of caspase-mediated apoptosis), followed by activation of the downstream caspase cascade (Slee et al., 1999; Hill et al., 2004).

Figure 1.3 Human granzyme B-mediated apoptotic pathway



Solid arrows refer to the major cytotoxic pathways mediated by human granzyme B; Dotted arrows refer to minor pathways, which reinforce actions of the major pathways but are not able to induce apoptosis alone; The figure is cited from Trapani, 2001.

Recent studies have shown that the precise pathways by which granzyme B mediates apoptosis are species-dependent (Kaiserman et al., 2006; Cullen et al., 2007). Human granzyme B is much more effective at mediating apoptosis than mouse granzyme B because it can cleave and activate Bid, whereas mouse granzyme B cleaves Bid poorly and does not utilise it for initiating apoptosis (Cullen et al., 2007). In addition, the failure of human granzyme B to kill cells induced to over-express the anti-

apoptotic protein Bcl-2, which blocks Bid-initiated cell death, indicates that it lacks the capacity to independently and fully activate apoptosis by direct activation of caspases (Goping et al., 2003; Sutton et al., 2003). Thus, mouse granzyme B directly activates caspases to promote apoptosis in a Bid-independent manner, whereas human granzyme B may kill by indirect activation of caspases via Bid.

Although activation of caspases was originally thought to be required for granzyme B-mediated cell death, studies by a number of groups indicate that their requirement is not absolute. An in vitro study of mouse CD8+ T cells showed that apoptotic nuclear damage induced by granule exocytosis was abrogated by a pan-caspase inhibitor Z-VAD-FMK, whereas lysis of the cells was unaffected (Sarin et al., 1997). Similar results have been obtained in studies with purified human granzyme B, caspase inhibition preventing granzyme-induced DNA damage but not cell lysis (Trapani et al., 1998). Some studies also showed that granzyme B can directly cleave and activate several downstream substrates of caspases such as the inhibitor of the caspases-activated DNase (ICAD), poly (ADP-ribose) polymerase (PARP), DNA-dependent protein kinase (DNA-PK) and lamin B (Froelich et al., 1996a; Andrade et al., 1998; Thomas et al., 2000; Sharif-Askari et al., 2001; Zhang et al., 2001a) to induce DNA fragmentation. These findings strongly indicated an important role of caspase-independent pathway(s) in granzyme B-mediated apoptosis.

1.5.3.3 Apoptotic function of orphan granzymes

Purified recombinant human granzyme H, delivered by perforin or a pore-forming analogue streptolysin O (SLO), has been shown to mediate cell death characterised

by chromosomal condensation, DNA fragmentation, mitochondrial inner membrane depolarization and ROS production (Fellows et al., 2007). The granzyme H-induced cell death in vitro is independent of Bid and caspases, implying a cell-death pathway distinct from that of granzyme B (Fellows et al., 2007). However, this mechanism of granzyme H-induced cell death has been challenged by another group, who provided evidence for caspase-dependent apoptosis involving Bid and ICAD and release of cytochrome c (Hou et al., 2008). The latter cell death was induced by purified recombinant granzyme H after delivery by adenovirus or a cationic lipid-based transfection reagent Pro-Ject into target cells.

Human granzyme K was initially thought to act in a similar way and provide an alternative to granzyme A for inducing apoptosis, since cell death was reported to occur independently of caspases and similar to granzyme A was associated with single-strand DNA nicks and ROS production (MacDonald et al., 1999; Martinvalet et al., 2005; Zhao et al., 2007b). Granzyme K has also been reported to cleave the granzyme A substrate SET to induce cell death (Zhao et al., 2007b). However, recent studies have strongly suggested that it has a distinct function, as indicated by a dependence on mitochondrial depolarisation initiated by direct processing of Bid to release cytochrome c and endonuclease G (Zhao et al., 2007a).

Purified recombinant human granzyme M delivered with perforin or streptolysin O (SLO) has been found to rapidly induce death of tumour cells in vitro, which show an unusual cell death-morphology characterised by formation of large cytoplasmic vacuoles, dilated ER and shrunken nuclei; no DNA nicking or fragmentation was

observed (Kelly et al., 2004; Bovenschen et al., 2008; Cullen et al., 2009). The cell death was independent of Bid and caspases and DNA fragmentation, indicating a potentially unique form distinct from that of granzyme A and B. However, a study of granzyme M-deficient mice has shown that killing of tumour cells by NK cells in vitro was comparable to that of wild-type mice (Pao et al., 2005).

1.5.3.4 Inhibitors of granzyme activity

Protease inhibitor 9 (serpinB9 / PI-9), a member of the clade B serpins, is an endogenous inhibitor of human granzyme B. PI-9 acts as a pseudo substrate, employing a reactive centre loop (RCL) to react with residues at the active site of granzyme B, thus blocking its function by forming an irreversible stable complex (Sun et al., 1996). PI-9 shows a very broad tissue and cellular distribution, with high and stable levels found in cytotoxic T cells, antigen-presenting cells, a number of other cell types, including endothelial and mesothelial cells, and cells at immunologically privileged sites (Sun et al., 1996; Bladergroen et al., 2001; Buzza et al., 2001; Hirst et al., 2001). PI-9 is located in both the cytoplasm and nucleus of cells (Bird et al., 2001) and is thought to protect cytotoxic T cells and bystander cells from misdirected granzyme B (Sun et al., 1996; Bladergroen et al., 2001; Buzza et al., 2001; Hirst et al., 2001; Bird et al., 1998).

Recent studies have proposed that some tumour cells up-regulate PI-9 expression in order to evade the immune system. Studies of natural levels of PI-9 expression in a number of tumour cells (including lymphoid tumours) showed that the level of expression was associated with increased resistance to perforin/ granzyme B-

mediated killing activity by CD8+ T cells and NK cells (Medema et al., 2001; Bladergroen et al., 2002; ten Berge et al., 2002; van Houdt et al., 2005; Bossard et al., 2007), although this notion is challenged by Godal et al., (2006), who presented evidence that PI-9 expression in several lymphoma cells was irrelevant to perforin-dependent killing by CD8+ T cells and NK cells (Godal et al., 2006).

Granzyme M has recently been reported to facilitate granzyme B-induced cell death by inactivating the PI-9 inhibitor. Granzyme M hydrolysed PI-9 in vitro and thus inactivated its inhibitory activity on granzyme B (Mahrus et al., 2004). Although the physiological significance of this finding remains to be examined, it suggests that granzyme M can promote the efficiency of granzyme B-induced killing by inactivating its endogenous inhibitor.

Adenovirus 5 has been shown to encode an inhibitor of granzyme B, L4-100K assembly protein, which forms a stable complex with the active sites of granzyme B, thus inhibiting its proteolytic activity (Andrade et al., 2001). However, granzyme H is able to neutralise this inhibitor, thus facilitating killing of the adenovirus-infected cells granzyme B (Andrade et al., 2007). These findings demonstrated the ability of granzymes to operate cooperatively to induce apoptosis and to bypass viral and tumour cell inhibitory strategies.

1.5.4 Other possible functions of granzymes

In addition to their role in apoptosis, evidence has emerged recently that granzymes have other biological functions, including direct inactivation of intracellular pathogens, remodelling extracellular matrix (ECM) and induction of pro-inflammatory cytokines.

Several studies in mouse models indicated an important role of granzymes for viral clearance. For example, mice deficient in granzyme A, B or M or both granzyme A and B have shown various degrees of increased susceptibility to murine cytomegalovirus (MCMV) and ectromelia infection, despite the finding that their CD8+ T cells generally retain cytotoxic activity comparable with that of wild-type mice (Table 1.3) (Anthony et al., 2010). Also as discussed above, granzyme H restricts the replication of adenovirus by directly cleaving and inactivating an adenovirus DNA binding protein DBP essential for viral replication. Overall, these findings indicate that granzymes may influence control the pathogen infections by mechanisms other than killing of infected cells.

A number of extracellular proteins have been shown to act as substrates for granzymes. For example, fibronectin, laminin, basement membrane collagen type IV and other substrates such as the thrombin receptor are processed by granzyme A (Simon et al., 1988; Simon et al., 1991; Vettel et al., 1993; Suidan et al., 1994). Granzyme B is also reported to cleave the extracellular substrates vitronectin, fibronectin and laminin (Buzza et al., 2005). As a consequence of cleavage of these three proteins involved in extracellular matrix structure and function, granzyme B

induces perforin-independent killing via a process, known as anoikis (cell death induced by detachment) and inhibits tumour cell spreading, migration, and invasion (Buzza et al., 2005). In addition to lymphoid cells, granzyme B expression has also been found in non-lymphoid cells such as mast cells (Pardo et al., 2007; Strik et al., 2007), basophils (Tschopp et al., 2006) and epidermal keratinocytes (Berthou et al., 1997) and macrophages (Kim et al., 2007). However, the function of granzyme B in these cell types is unknown.

Human and murine granzyme A in the absence of perforin have been reported to trigger expression of IL-1β, TNFα and IL-6 by human monocytes and expression of IL-1β in primary mouse macrophages, respectively (Metkar et al., 2008). The mechanisms involved were not investigated in detail, but cytokine release from human monocytes was inhibited by a caspase-1 inhibitor (Metkar et al., 2008).

1.6 Aims of the project

The above discussion indicates that the cytotoxic activity of CD8+ T cells is an important factor in mediating immunity against the bovine intracellular protozoan parasite *Theileria parva*. However, the mechanism by which the specific CD8+ T cells kill parasitized cells is not understood. In human and mouse, the predominant pathway used by CD8+ T cells to kill pathogen-infected cells is granule exocytosis, involving release of a membrane disrupting protein perforin and a number of granzymes (5 in human and 10 in mouse). Granzyme B is the best-characterized and most potent member of the granzyme family in these species and is considered to be the major initiator of granule-mediated apoptosis. However, there is no published information on the identity or biological activities of bovine granzymes.

The hypothesis addressed by this protect was that killing of *T. parva*-infected cells by CD8+ T cells occurs by release of lytic granules and that granzyme B is an important mediator of the cytotoxic activity. The overall aim was to characterise granzymes in cattle and investigate their involvement in killing of *Theileria*-infected cells by CD8+ T cells, focusing particularly on the role of granzyme B.

The specific objectives were as follows:

- Determine the role of granule exocytosis in killing of *Theileria*-infected cells by CD8+ T cells.
- Identify and characterize the functional granzyme genes in the bovine genome and examine their expression in *T. parva*-specific CD8+ T cells.

- Investigate the relationship of killing levels and the granule enzyme transcript profiles of *Theileria*-specific CD8+ T cells.
- Generate recombinant cDNA constructs of bovine granzyme B to allow production of functionally active protein for development of a biological assay to measure specific granzyme B activity.
- Using the above assay and commercially available inhibitors, determine the role of granzyme B in killing of *Theileria*-infected cells by specific CD8+ T cells.

Chapter 2 Materials and Methods

This chapter will describe methods used throughout the study, while methods peculiar to the work reported in individual results chapters will be described in the respective chapters

2.1 Experimental animals

Three animals used in the study (641, 011 and 592) were Holstein Friesians bred at the Institute for Animal Health (IAH), Compton. These animals were immunised by infection with *T. parva* and treatment The animals were bred from MHC-defined dams and sires and their MHC phenotypes were identified using a panel of MHC1-specific monoclonal antibodies (Ellis et al., 1999). Animal 641 was homozygous for the A18 MHC1 haplotype and 011 and 592 were homozygous for the A10 MHC1 haplotype.

2.2 General cell culture techniques

2.2.1 Lymphocyte preparation

Peripheral blood mononuclear cells (PBMC) were isolated as described in Goddeeris and Morrison, 1988. Blood was collected by jugular venipuncture into an equal volume of Alsever's solution (Appendix C.1). Aliquots of 30ml of blood in Alsever's solution were carefully overlaid onto 20ml of Ficoll-paque (GE Healthcare, Uppsala,

Sweden) in 50ml falcon tubes and centrifuged at 800 x g for 30 min at room temperature. PBMC were harvested from the interface and mixed with an equal volume of Alsever's solution before pelleting at 450 x g at 10 min at room temperature. The pellet was then washed 3 times in Alsever's prior to re-suspension in standard culture medium (SCM- Appendix C.2) and cells counted using a haemocytometer.

2.2.2 Concanavalin A (ConA)- activated PBMC

Uninfected lymphoblast lines were established in 24 well plates by stimulation of PBMC with ConA (Sigma-Aldrich) for 3 days and immediately harvested for the further experiment. Each well was seeded with $2x10^6$/ml PBMC in SCM together with ConA at a final concentration of 5ug/ml. Plates were incubated for 3 days in a humidified incubator in an atmosphere of 5% CO_2 at 37°C.

2.2.3 Complement-mediated cell lysis

Cells were re-suspended in SCM at a final concentration of 1 x 10^7 cells/ml and 1ml of hybridoma culture supernatant containing monoclonal antibodies ILA-12 (anti-CD4, IgG2a) and CC15 (WC1 –expressed on γδ+ T cells, IgG2a) added per 5 x 10^7 cells, and incubated on ice for 45 min. Cells were pelleted by centrifugation at 180 x g for 10 min at 4°C and washed twice in SCM. Cells were subsequently re-suspended in SCM at $5x10^6$cells/ml and rabbit serum was added, as a source of complement, to give a final dilution of one in five. The mixture was incubated at

37°C for 40 min to allow complement-mediated lysis. The cells were pelleted and washed twice in SCM, re-suspended in 9 ml SCM and overlaid on 6ml Ficoll-Paque^{TM} PLUS in 15ml polypropylene tubes and then centrifuged at 900 x g for 20 min at room temperature. Live, unlysed cells were collected from the Ficoll/SCM interface, washed in an equal volume of SCM and pelleted at 450 x g for 10 min at room temperature. The effectiveness of the lysis was assessed by immunofluorescence staining of a sample the remaining live cell population and analysis by flow cytometry (Section 2.2.7).

2.2.4 Generation of CD8+ T cell lines in vitro

T. parva-specific CD8+ T-cell lines were generated by the method described by Goddeeris and Morrison, 1988, with minor modifications:

1st-stimulation

PBMC were isolated from blood of a *T. parva* immune animal as described above. Established cell lines infected with *T. parva* (Muguga strain - TPM) derived from each of the animals from which T cell lines were generated, referred to as 641TPM, 011TPM and 592TPM were provided by Drs Niall MacHugh and Timothy Connelley in the Roslin Institute. Autologous *T .parva*-infected cells were harvested, counted and resuspended at $1x10^5$/ml SCM. They were exposed to 60Gy of gamma irradiation from a ^{137}Cesium source (Roslin Institute). Aliquots of 1 ml of PBMC at $2x10^6$/ml and irradiated autologous stimulators at $1x10^5$/ml were added to each well of 24-well plates. Plates were incubated for 7 days in a humidified incubator in an atmosphere of 5% CO_2 at 37°C.

2nd-stimulation

Cells from the first stimulation were harvested, viable cells counted and re-cultured in 24-well plates. Each well was seeded with $2x10^6$ responder cells together with $1x10^5$ irradiated autologous stimulators in a final volume of 2ml SCM. Plates were incubated for 7 days in a humidified incubator in an atmosphere of 5% CO_2 at 37°C.

3rd-stimulation

Cells from the second stimulation were harvested, counted and the CD4+ and γδ+ T-cell populations lysed as described above (section 2.2.3). The remaining viable cells were resuspended at $2x10^5$/ml in SCM and 1ml aliquots added into the wells of 24-well plates along with $1x10^6$ of irradiated autologous stimulators and recombinant human IL-2 (Chiron Corporation Emeryville, CA., USA) added to give a final concentration of 100U/ml. The plates were incubated for 7 days in a humidified incubator in an atmosphere of 5% CO_2 at 37°C.

2.2.5 Expansion of clones

Selected CD8+ T cell clones, kindly provided by Dr Tim Connelley and Ms Victoria Carroll, respectively were expanded in the wells of 24-well plates. Each well was seeded with $1x10^5$ responder cells together with $5x10^5$ irradiated autologous parasitized stimulators and recombinant human IL-2 added to give a final concentration of 100U/ml. The plates were incubated for 14 days in a humidified incubator in an atmosphere of 5% CO_2 at 37°C.

2.2.6 Cryopreservation of cellular samples

Aliquots of $2x10^6$ cells were pelleted and re-suspended in 500ul heat-inactivated Foetal bovine serum (FBS) and 20% dimethylsulphoxide (DMSO, Sigma, Poole, Dorset, UK)/500ul FBS was slowly added to each aliquot. The 1ml aliquots were transferred into polypropylene cryovials (CryoTube, Nunc, Roskilde, Denmark), places in an isopropanol jacketed container (Cryo 1°C Freezing Container, Nalgene, Neerijse, Belgium) and slowly frozen at -70°C for at least 3 hour before being transferred to liquid nitrogen storage. For recovery of the cells, cryopreserved samples were rapidly thawed by incubation in a water bath at 37 °C, made up to 10ml by addition of SCM, pelleted to remove DMSO and re-suspended in SCM. Cells were then kept in a humidified incubator in an atmosphere of 5% CO_2 at 37°C

2.2.7 Flow cytometry

Cells were stained by indirect immunofluorescence and analysed by flow cytometry using a cell analyser (FACScalibur, Beckton Dickinson, Mountain View, CA., USA). The monoclonal antibodies (mAbs) were primary antibodies used for FACS analysis. The second antibody was fluorescein isothiocyanate (FITC)-labelled, polyvalent goat anti-mouse immunogolobulin G, A, M antibody (Sigma-Aldrich, Poole, Dorset, UK).

For phenotypic analysis, aliquots of 50ul of cells suspended at $2x10^7$cells/ml in FACS medium (Appendix C.3) were distributed in wells of 96-well round-bottom well plates and 25ul of primary mAb added to each well. As a negative control, cells were incubated with FACS medium instead of primary antibody. The antibodies used are listed in table 2.1. Plates were incubated at 4°C for 30 min, washed three times in

FACS medium and re-suspended in 25ul of FITC-labelled goat polyvalent anti-mouse immunoglobulin diluted 1:100 in FACS medium. Following incubation at 4°C for 30 min, the cells were washed three times in FACS medium and then re-suspended in 200ul FACS medium for analysis using a cell analyser (FACScalibur, Beckton Dickinson, Mountain View, CA., USA).

Table 2.1 Monoclonal antibodies used for primary labelling in FACS analysis

Antibody	Isotype	Specificity	Cell distribution
MM1A	IgG1	CD3	T-cells
IL-A12	IgG2a	CD4	CD4+ T-cells
IL-A51	IgG1	CD8α chain	CD8+ T-cells, subset NK cells
IL-A105	IgG2a	CD8	CD8+ T-cells, subset NK cells
CC15	IgG2a	WC1	Peripheral γδ+ T-cells
GB21A	IgG2b	γδ TCR	All γδ+ T-cells
IL-A30	IgG1	IgM	B-cells
AKS1	IgG1	NKp46	NK cells

2.2.8 Cytotoxicity assay

Cell mediated cytotoxicity was measured using ^{111}Indium (^{111}In) 4-hour release assays as described by Goddeeris and Morrison (1988). In some experiments, longer periods of incubation of effectors with targets were used.

Preparation of target cells

Autologous TPM-infected cell lines (641TPM, 011TPM and 592TPM) were used as target cells to test cytotoxicity against infected cells. *T. annulata* (TA)-infected cell lines 641TA and 592TA from the same animals, provided by Dr Niall MacHugh, were used as to test cytotoxicity against defined MHC1 A18-restricted and A10-restricted epitopes using cells pulsed with synthetic peptides. Peptide for the A18-restricted epitope $Tp1_{214-224}$ (VGYPKVKEEML) was kindly provided by Dr. E.Taracha (ILRI, Nairobi, Kenya) and peptide for the A10-restricted epitope $Tp2_{49-59}$ (KSSHGMGKVGK) was produced by Pepscan Systems (Lelystad, Netherlands). *T. parva*-infected target cells were suspended at $2x10^7$cells/ml in cytotoxicity medium (Appendix C.4) and 5µCi of ^{111}In (GE Healthcare Ltd., Little Chalfont, Bucks, UK) was added to 50ul of this suspension (1x 10^6 cells) and incubated for 30 min at 37°C in a 15ml falcon tube. Cells were then washed six times with 10ml cytotoxicity medium by centrifugation at 180 x g for 5 min and re-suspended in 10ml of cytotoxicity medium to give a concentration of 1x 10^5 cells/ml. *T. annulata* (TA) infected cell lines, 641TA and 592TA were adjusted to $1x10^6$/ml in cytotoxicity medium and incubated with 100ng/ml $Tp1_{214-224}$ and 1000ng/ml $Tp2_{49-59}$ peptide respectively for 0.5 hour at 37°C. The peptide-pulsed target cells were then pelleted, labelled with 5µCi of ^{111}In and resuspended at a concentration of $1x10^5$ cells/ml as described above.

Preparation of effector cells

CD8+ T cells were harvested and suspended in cytotoxicity medium to a concentration of $1x10^6$cells/ml.

^{111}In 4h-release cytotoxicity assays

Duplicate aliquots of 100ul of two-fold dilutions of the effector cells were distributed into the wells of 96-well V-bottomed plates to give a range of $1x10^5$-$6.25x10^3$ cells per well; 50ul of target cells were added to each well. Spontaneous release and maximal release were measured for each of target cell in triplicate. For spontaneous release, 50ul of target cells were incubated with 100ul cytotoxicity medium, whereas, for maximal release, 50ul of target cells were incubated with 100ul 0.2% Tween 20 (Sigma, Poole, Dorset, UK). Plates were briefly centrifuged for 1 min at 120xg to sediment the effector and target cell mixtures and then incubated for 4 hour in a humidified incubator in an atmosphere of 5% CO_2 at 37°C. After 4-hour incubation, plates were centrifuged for 5 min at 180xg and 75ul of supernatants per well was transferred into individual gamma counter tubes. To capture radioactive material in solid form to avoid spillage, small drops of bromophenol blue-stained 1.5% agarose were added into each sample. Radioactivity release was measured with a Wallac Wizard 1470 Automatic Gamma Counter (PerkinElmer, Beaconsfield, Bucks., UK) and the percentage of cytotoxicity for each sample was calculated using the following formula:

$$\text{Cytotoxicity (\%)} = \frac{100 \times (\text{Test } ^{111}\text{In release} - \text{Spontaneous } ^{111}\text{In release})}{\text{Maximal } ^{111}\text{In release} - \text{Spontaneous } ^{111}\text{In release}}$$

Levels of cytotoxicity of >10% were considered to be significant positive results; these values were well in excess of 3 standard deviations above the values obtained with negative control targets.

2.3 General molecular techniques

2.3.1 Isolation of RNA

Total RNA was extracted from cell pellets using the Tri-Reagent Kit (Sigma, Sigma-Aldrich, UK) according to the manufacturer's protocol. Aliquots of 5-10 x 10^6 cells re-suspended in approximately 100ul of medium were lysed with 1ml Tri-Reagent by repeat pipetting and the mixture was incubated at room temperature for 5 min to complete dissociation of nuclear-protein complexes. Following the addition of 0.2ml of chloroform, the mixture was vigorously vortexed for 15 seconds, incubated at room temperature for 2-15 min and then centrifuged at 12,000xg for 15 min at 4°C. The aqueous phase was transferred to a new Eppendorf tube and mixed with 0.5ml isopropanol. After 5-10 min incubation at room temperature, the RNA was pelleted by centrifugation at 12,000xg for 10min at 4°C and then washed with 1ml of 75% ethanol by centrifugation at 7,500xg for 5min at 4°C. The washed pellet was air-dried and re-suspended in 25ul of nuclease-free water. RNA was stored at -70°C until further use.

2.3.2 RNA and DNA quantification

Absorbance of light at 260 and 280nm wavelengths by RNA/DNA samples was measured using a NanoDrop ND-1000 spectrophotometer (Labtech International Ltd) according to the manufacturer's protocol. RNA/DNA concentration was estimated based on the assumptions that RNA and DNA have absorbance readings of 1.0 at 260nm at concentrations of 40ug/ml and 50ug/ml, respectively. The purity of

RNA/DNA samples was determined by the ratio of readings obtained at 260nm:280nm >1.8.

2.3.3 RNA reverse transcription

cDNA was synthesized from RNA using the Reverse Transcription system (Promega, Madison, WI, USA) according to the manufacturer's protocol. Briefly, 1ug RNA was incubated at 70°C for 10 min and placed on ice until added to the reaction in the following order: 4ul $MgCl_2$ (25mM), 2ul of 10x Reverse transcription Inhibitor, 2ul dNTP mixture (10mM), 0.5ul Recombinant RNAsin Ribonuclease Inhibitor, 15U avian myeloblastoma virus reverse transcriptase (AVM-RT), 0.5ug $Oligo(dT)_{15}$ Primer or Random Primers, 1ug RNA sample and nuclease-free water added to give a final volume of 20ul. When using $Oligo(dT)_{15}$ primer, the mixture was incubated at 42°C for 1h, heated at 95°C for 5 min and then incubated at 0°C for 5 min to inactivate the AMV-RT and prevent it from binding the cDNA. When using Random Primers, the mixture was first incubated at room temperature for 10 min before incubation at 42°C for 1h, then heated at 95°C for 5 min and incubated at 0°C for 5 min. cDNA was stored at -20°C until use.

2.3.4 General RT-PCR assay

PCR primers were designed using the Primer3 programme (website http://www.bioinformatics.nl/cgi-bin/primer3/primer3_www.cgi) or manually and synthesized by MWG biotech (Ebersberg, Germany). Standard PCR reactions were

completed in a G-storm thermal cycler (Genetic Research instrumentation, Essex, UK). Standard PCR assays were composed of 10 pmol of primers, 0.5 units BIOTAQ (5 units/ul Bioline, London, UK), 2ul SM-0005 buffer (ABgene, Epsom, Surrey, UK-Appendix C.5), 1ul cDNA (0.05ug/ul) in DDW, and nuclease-free water to give a final volume of 20ul. The programme used was as follows: 94°C for 3 min, 30 cycles (94°C for 1.5 min, 55°C for 1.5 min, 72°C for 1.5 min) and a final extension period of 72°C for 10 min.

2.3.5 Agarose gel electrophoresis

The PCR products were analysed by electrophoresis in 1.5% agarose gels incorporating 10ul Gel Red (Biotium Inc, Hayward, CA, USA)/100ul 1 x TAE (Appendix C.6). Aliquots of 10ul of PCR product plus 3ul of a 1 in 6 dilution of loading buffer (Appendix C.7) were applied to each lane and run at 110 V for 50 min in the midi-gel system (PowerPac 200, BIO-RAD), using 1 x TAE as running buffer. 1ug of 1kb Plus DNA Ladder (Invitrogen Ltd., Paisley, UK) was used to enable estimation of product sizes.

2.3.6 DNA purification

PCR product purification

PCR products were purified using the Wizard Preps DNA purification system (Promega, Madison, WI, USA) according to the manufacturer's protocol. Briefly, 30-300ul of PCR products were added to 100ul of Direct Purification Buffer and

vortexed briefly. After addition of 1ml of DNA resin, the mixture was vortexed 3 times over a 1 min period and passed through a mini-column to allow the DNA/resin to bind to the membrane. The mini-column was then washed with 2ml of 80% isopropanol and centrifuged twice at 10,000x g for 2 min to dry the resin. 30ul of nuclease-free water was added to the mini-column to elute the purified DNA by centrifugation at 10,000xg for 1 min. DNA was stored at -20°C.

Gel purification

Gel purification of the desired DNA products was performed using the Wizard Preps DNA purification system (Promega, Madison, WI, USA) according to the manufacturer's protocol. The predicted sizes of DNA bands were estimated by reference to a 1kb Plus DNA Ladder. Bands were excised with a clean scalpel from the low-melting-temperature agarose and the isolated agarose slice (less than 300mg) was transferred into a 1.5ml microcentrifuge tube and incubated at 70°C until it was melted. Following the addition of 1ml of DNA resin, the mixture was briefly mixed, attached to the membrane of the mini-column, washed with 80% isopropanol and the purified DNA was eluted with 30ul of nuclease-free water as described above.

2.3.7 Sub-cloning of PCR products

Purified cDNA products of the predicted size were sub-cloned into pGEM-T Easy vector system (Promega, Madison, WI, USA) according to the manufacturer's protocol. The ligation reactions were composed of 1ul pGEM-T Easy Vector, 25ng purified PCR product, 1ul T4 DNA Ligase (3 Weiss units/ul), 5ul of 2x Rapid

ligation Buffer and DDW, to give a final volume of 10ul. The products were transferred into JM109 High Efficiency Competent Cells (Promega, Madison, WI, USA) and the cells were plated onto duplicate Luria-Bertani (LB)/ampicillin/IPTG/X-Gal plates (Appendix C.9) according to the manufacturer's protocol. The plates were incubated overnight at 37°C. White colonies containing inserts were selected from the plates and transferred into 30ml flasks with 6ml LB media (Appendix C.10) and 100ug/ml ampicillin. The cells containing clones were expanded by incubating overnight at 37°C with agitation at 220 rpm.

2.3.8 Plasmid DNA Preparation-(Mini-preps)

The plasmid DNA was extracted from bacteria and purified using the Wizard *Plus* SV Minipreps DNA Purification System (Promega, Madison, WI, USA) according to the manufacturer's protocol. 5ml of bacterial cultures were harvested, pelleted by centrifugation at 10,000xg for 5 min, re-suspended in 250ul of Cell Resuspension Solution and lysed with 250ul of Cell Lysis Solution. After addition of 10ul of Alkaline Protease Solution, the bacterial lysates were incubated for 5 min at room temperature, mixed with 350ul Neutralisation Solution and pelleted by centrifugation at 14,000xg for 10 min. The cleared lysates were transferred into a Spin Column and centrifuged at 14,000xg for 1 min to allow them to attach the membrane. The Spin Column was washed with 750ul of Column Wash Solution (ethanol added) by centrifugation at 14,000xg for 2 min and the washing was repeated with 250ul of Column Wash Solution. 50ul of nuclease-free water was added to the Spin Column

to elute the purified plasmid DNA by centrifugation at 14,000xg for 1 min. The plasmid DNA was stored at -20°C.

2.3.9 Identification the insert containing plasmid clones

To identify the presence of inserts, the plasmid DNA was digested in the reaction composed of 20units *Eco R1* (20,000 units/ml, New England Biolabs, Hitchin, Herts., UK), 1ul 10x *Eco R1* buffer(New England Biolabs, Hitchin, Herts., UK), 5ul mini-preps and 3ul DDW to give a final volume of 10ul and incubated at 37°C for 1 h. 10ul of digested products was added with 3ul loading buffer and analysed by 1.5% agarose gel electrophoresis as described above (Section 2.3.5).

2.3.10 Sequencing

Purified plasmid products with inserts (3.2pmol/ul) were sent to DBS Genomic (Durham University) for sequencing. The sequence obtained for each sample was analysed using DNAsis Max 2.0 software.

Chapter 3 Identification and characterisation of bovine granzymes

3.1 Introduction

Granzymes are a family of serine proteases that exhibit various primary substrate specificities. They are found in the lytic granules of cytotoxic T lymphocytes (CTLs) and NK cells and play a significant role in killing of susceptible target cells, acting on various cellular pathways that regulate programmed cell death (Jenne and Tschopp, 1988). Five granzymes - A, B, K, H and M - have been identified in humans. In mouse, there are ten granzymes - A, B, K, E, M, C, D, F, G and N (Grossman et al., 2003). Granzyme H appears to be restricted to human, while C, D, E, F, G and N are exclusively expressed in mouse. The granzyme family has been classified into three distinct evolutionary groups, which are clearly discriminated based on their primary substrate specificities and correspond to three different chromosomal locations (Trapani, 2001). The members of the three groups have trypsin-like (A and K on human 5q11-q12 and mouse 13D chromosomal regions, respectively), chymotrypsin-like (B and H on human 14q11-q12; B, C, E, M, D, F, G and N on mouse 14D) and metase (M on human 19p13.3 and mouse 10q21.2, respectively) enzymatic activities (Smyth et al., 1996). Genome mapping of the granzyme genes has revealed that other serine proteases such as the cathepsin G and mast cell proteases are closely linked with granzyme B and H in the chymotrypsin locus, while the metase locus holds a cluster of neutrophil elastase genes, including azurocidin 1 (ZAU1), proteinase 3 (PRTN3), neutrophil expressed (ELANE) and

complement factor D (CFD), 200 to 500kb downstream of granzyme M (Pilat Daniel, 1994). Consistent with granzymes being structurally related to chymotrypsin, studies of human and mouse granzymes by several groups have defined a number of common features, including a shared consensus sequence at the N-terminus (Bleackley et al., 1988; Jenne and Tschopp, 1988; Murphy et al., 1988), conserved activation peptides (propeptides), a three amino acid catalytic triad (His-57, Asp-103 and Ser-195) (Murphy et al., 1988) and 3-4 disulfide bridges (Smyth et al., 1996; Trapani, 2001; Piuko et al., 2007). However, sequence comparisons show a higher level of conservation within granzyme subgroups (55-70%) than between groups (30-40%) (Piuko et al., 2007). Further detailed comparison of sequence and structure also reveal that each group of granzymes has unique features. Substrate specificity is dependent on protein structure (Perona and Craik, 1995), which is determined by variation in key amino acid residues that form the substrate binding pocket (Smyth et al., 1996).

The studies described in this chapter aimed: (i). To identify and characterise bovine granzyme genes by mining existing genomic and expression sequence tag databases. (ii). To conduct inter-species comparative analysis of their nucleotide and amino acid sequences, to attempt to deduce their enzymatic specificities.

3.2 Materials and methods

3.2.1 Bovine granzyme genome analysis

The nucleotide sequences of bovine granzymes were identified in the bovine genome assembly, Btau_4.0 (http://www.ensembl.org/Bos_taurus/blastview) using the nucleotide-nucleotide basic local alignment search tool (BLASTN) with the sequences listed in table 3.1. Use of the obtained genome sequences to search the bovine expressed sequence tags (EST) database (http://www.ncbi.nlm.nih.gov/sites/entrez) identified the corresponding cDNA sequences.

Table 3.1 Accession numbers of cDNA sequences of human and mouse granzyme genes obtained from the National Center for Biotechnology Information (NCBI) RefSeq database (http://www.ncbi.nlm.nih.gov/)

Granzyme	Species	
	Human	Mouse
A	NM_006144	NM_010370
B	NM_004131	NM_013542
H	NM_033423	
K	NM_002104	NM_008196
C		NM_010371
E		NM_010373
D		NM_010372
F		NM_010374
G		NM_010375
N		NM_153052
M	NM_005317	NM_008504

3.2.2 Sequence analysis

Sequence analyses such as CLUSTAL W alignment and translations were performed by using the DNAsis Max V2.7 programme (MiraiBio, Alameda, CA, USA). Prediction of the signal sequence cleavage site was performed using an algorithm described by von Heijne et al. (1986). Residues involved in the catalytic triad and disulfide bridge formation were analysed by EBI PPsearch (http://www.ebi.ac.uk/Tools/ppsearch/) and Prosite (http://www.expasy.org/prosite/).

3.2.3 Chromosomal location analysis

In humans and mice, granzyme-related trypsin-like, chymotrypsin-like and metase loci have been found on three separate chromosomes (Smyth et al., 1996). The nucleotide sequences of bovine orthologues in three loci were identified using BLASTN searches in the bovine genome assembly, Btau_4.0 with the sequences in table 3.2. The chromosomal localization of trypsin-like, chymotrypsin-like and metase loci on bovine genome were annotated using gene mapping with identified bovine orthologues in the respective chromosome.

Table 3.2 Summary of accession numbers of human and mouse genes in trypsin-like, chymotrypsin-like and metase loci

Chromosomal Locus	Species	
	***H.sapiens* (Hs)**	***M.musculus* (Mm)**
Trypsin-like	GzmK (NM_002104)	GzmK (NM_008196)
	GzmA (NM_006144)	GzmA (NM_010370)
Chymotrypsin-like	CMA1 (NM_001836)	Cma1 / Mcpt5 (NM_010789)
	-	Mcpt1 (NM_008570)
	-	Mcpt9 (NM_010782)
	-	Mcpt2 (NM_008571)
	-	Mcpt4 (NM_010779)
	-	Mcpt8 (NM_008572)
	CTSG (NM_001911)	Ctsg (NM_007800)
	-	GzmE (NM_010373)
	-	GzmD (NM_010372)
	-	GzmG (NM_010375)
	-	GzmN (NM_153052)
	-	GzmF (NM_010374)
	-	GzmC (NM_010371)
	GzmH (NM_033423)	-
	GzmB (NM_004131)	GzmB (NM_013542)
Metase	GzmM (NM_005317)	GzmM (NM_008504)
	AZU1 (NM_001700)	-
	PRTN3 (NM_002777)	Prtn3 (NM_011178)
	ELANE (NM_001972)	Elane (NM_015779)
	CFD (NM_001928)	Cfd (NM_013459)

The cDNA sequences of these genes located on three loci were obtained from the NCBI RefSeq database (Smyth et al., 1996; Grossman et al., 2003; Gallwitz and Hellman, 2006). -, no; Gzm = granzyme; CMA /Mcpt = mast cell chymase /mast cell protease; Ctsg = cathepsin G; ZAU1 = azurocidin 1; PRTN3 = proteinase 3; ELANE = neutrophil elastase preproprotein; CFD = complement factor D.

3.2.4 Phylogenetic analysis

Phylogenetic analysis was performed on the cDNA sequence of granzyme genes of human, mouse, pig and bovine as identified in table 3.2 and 3.6. The relationships across species were established by analysing CLUSTAL W alignment with the Neighbour-joining method by using MEGA4 software (Tamura et al., 2007).

3.2.5 Amplification of granzyme and perforin transcripts from cDNA by RT-PCR

Total RNA was extracted from cell pellets of the uncloned *T.parva*-specific CD8+ T cell lines day 7 after 3rd stimulation, which were isolated from animals 641 and 011. The cDNA was synthesized from RNA as described in section 2.3.1- 2.3.3. Primers were designed to amplify full-length coding regions for bovine granzymes and perforin based on sequences identified from bovine genomic and EST databases. Primers were designed using the Primer3 programme or manually and synthesized by MWG biotech (Ebersberg, Germany). The sequences of the primers are shown in table 3.3. The PCR products were amplified, sub-cloned into the pGEM-T vector and sent to DBS Genomic (Durham University) for sequencing as described in section 2.3.4- 2.3.10.

Table 3.3 PCR primers designed for detection of bovine granzymes and perforin

Primers	Sequences (5'---3')
GzmA (For)	ATTGATTGATGTGGGGACAC
GzmA (Rev)	AAAAAGTAACAGCAAATGAAATACAA

GzmO (For)	AGTCTCCATATGTGAATAACAGGAG
GzmO (Rev)	CCCTTTCACTTGGTTACTTCG
GzmB (For)	CATCCTGGGCAGTCTTTCTA
GzmB (Rev)	CCTGCAGTGTGATTCTGGAT
GzmH (For)	CTGACCTGGGCAAATCTTCT
GzmH (Rev)	GGACAATGGTCAGTGCAGAG
GzmK (For)	TTCCTTTGCCAATACAGTCAG
GzmK (Rev)	AGCAGCTGATAGAGCCAAGA
GzmM (For)	GAGGCCCCCCAGATCCAAG
GzmM (Rev)	CCCCTTGGAACACAGAATCA
Perforin (For)	CAG GGT GGT CAA GCT AGA GG
Perforin (Rev)	AGG TGA GGC AAG CAT TTG AC

3.3 Results

3.3.1 Identification of bovine granzyme genes

Previous studies have identified five granzymes (A, B, K, H and M) in human and ten granzymes (A, B, K, C, E, D, F, G, N and M) in mouse. BLASTN searches of the bovine genome assembly, Btau_4.0 using nucleotide sequences of human and mouse granzymes identified 6 putative granzyme genes predicted to encode full-length functional proteins (Table 3.4 and accession numbers are listed in Table 3.5). These included genes orthologous to the 5 granzyme genes found in humans (A, B, K, H and M) and a further gene with no close orthologue, but most closely related to granzyme A. This novel nucleotide sequence in the bovine genome (ENSBTAG00000027865) was designated as Granzyme O. So far, a close orthologue of this gene has not been described in any other species. However, a BLASTN search of the pig genome revealed a full-length functional gene (ENSSSG00000016902) that shows 89% similarity in the coding region to the cattle gene (discussed in further detail below). To enable further cross-species comparison, we also searched the pig genome assembly, Sscrofa9 (April 2009), for other granzyme genes and found orthologues of granzymes A, B, K and H (Table 3.4 and accession numbers were listed in Table 3.5). A gene for granzyme M was not found, although assembly of the region on pig chromosome 2 predicted to contain granzyme M is incomplete.

Table 3.4 Summary of functional granzyme genes identified in different species

Species	Granzyme											
	A	**O**	**B**	**H**	**K**	**C**	**E**	**D**	**F**	**G**	**N**	**M**
Human	+	-	+	+	+	-	-	-	-	-	-	+
Murine	+	-	+	-	+	+	+	+	+	+	+	+
Cattle	+	+	+	+	+	-	-	-	-	-	-	+
Pig	+	+	+	+	+	-	-	-	-	-	-	**ND**

Genes for bovine granzymes are highlighted in red. ND – not detected (assembly of the region on pig chromosome 2 predicted to contain granzyme M is incomplete)

Sequences matching each of the bovine granzyme genes identified from the genome were found within the GenBank bovine EST database (http://www.ncbi.nlm.nih.gov/sites/entrez). Searches of this database using other murine granzyme gene sequences suggest that no additional bovine granzymes are present. The significant hits identified are listed in table 3.5.

Table 3.5 Summary of accession numbers of granzyme genes identified in bovine and pig genome and EST databases

Granzyme	Bovine		Pig
	Genome (Btau_4.0)	EST	Genome
A	ENSBTAG00000021958	1907915310	corr_ENSSSCG00000016903
O	ENSBTAG00000027865	CK830799	ENSSSG00000016902
B	corr_ENSBTAG00000010057	CK952629	corr_ENSSSCG00000001978
H	corr_ENSBTAG00000010828	CK776010	ENSSSCG00000001981
K	ENSBTAG00000005164	1382286268	ENSSSCG00000016901
M	ENSBTAG00000002100	1907810005	

Corr - Manually corrected sequence

The cattle granzyme genes range in length from 3,484 to 10,785bp, with coding regions of cDNAs ranging from 741 to 795bp (Appendix A). The nucleotide sequences of the genes all exhibit the same exon-intron gene arrangement as the human and murine genes, each composed of 5 exons, with exon 1 encoding the leader peptide. Exons 2, 3 and 5 each contain one of the three amino acids that make up the catalytic triad (Appendix A) (Trapani, 2001). Most of the exon-intron boundaries of the granzyme genes fulfil the GT-AG rule, though granzyme H has a GC-AG splice site between exon 3 and intron 3 (Appendix A-(d)) (Burset et al., 2000). The granzyme M gene displays the distinguishing feature that the intron 1 boundary falls at the codon for Gly-7 (position with respect to the mature functional protein) prior to the putative hexapeptide Asn(-6) - His(-1) (Appendix A- (f)), which is cleaved to yield the functionally active polypeptide. This distinct gene

arrangement is shared with human and mouse granzyme M and also genes in the linked neutrophil elastase gene cluster, such as azurocidin (AZU1), proteinase-3 (PRTN3) and neutrophil elastase (ELA2) (Zimmer et al., 1992; Pilat et al., 1994; Kelly et al., 1996).

3.3.2 Comparison of sequences between species

Alignments of identified granzyme sequences from different species were used to investigate the similarity between species. Alignments of the predicted amino acid sequences and levels of identity between species are illustrated in figure 3.1- 3.6. Alignments of nucleotide sequences are available in Appendix B. Analysis of sequence homology showed that each of the bovine granzymes was more closely related to the respective counterparts in human than mouse than other bovine granzyme genes, with levels of 76-83% nucleotide similarity and 70-77% amino acid identity with the respective human counterparts and 70-75% nucleotide similarity and 64-71% amino acid identity with the corresponding mouse granzymes.

Figure 3.1 Granzyme A – comparison of bovine, human and murine amino acid sequences

(a). Alignment of amino acid sequences

```
             1          11         21         31         41         51
             .          .          .          .          .          .
BovineGZMA   MRNSSTFLAA TLSIVV-FLL IPEDLCE KIIGGNQVTP HSRPYMVLL- -DGGNICAGALIA
Human GZMA   ....YR...S S..V..SL.. ....V.. .....E... .........S L.RKT........
MurineGZMA   ...A.GPRGP S.ATLLFL.. ...GG.. R....DT.V. ......A..K LSSNT.......E

             61         71         81         91         101        111
             .          .          .          .          .          .
BovineGZMA   KDWVLTA AHCSLNQKSQ IILGAHSRNK EEPEKQIMFV KKEFPYPCYD PDTHEGDLKLLKL
Human GZMA   ....... ...N..KR.. V......ITR ...T....L. .......... .A.R......VR.
MurineGZMA   .N..... ...NVGKR.K F......I.. .-..Q..LT. ..A....... EY.R....Q..Q.

             121        131        141        151        161        171
             .          .          .          .          .          .
BovineGZMA   NKKATLN KNVAILQLPK EGKDVEPGTA CRVAGWGQFY NNSP-VSKIL REVNVTIIDRKIC
Human GZMA   ME..KI. .Y.T..H... K.D..K...M .Q.....RTH .SAS-W.DT. ....I......V.
MurineGZMA   K....V. R.....H... K.D..K...R .......R.G .K.A-P.ET. ....I.V......

             181        191        201        211        221        231
             .          .          .          .          .          .
BovineGZMA   NDQSHYN YNPVIGLNMI CAGSLQGGKD SCHGDSGSPL ICKDTFRGIT AFGIPGRCGDPRG
Human GZMA   ..RN... F.....M..V .....R..R. ..N....... L.EGV...V. S..LENK......
MurineGZMA   ..EK... FH........ ...D.R.... ..N....... L.DGIL.... S..GE-K...R.W

             241        251        261
             .          .          .
BovineGZMA   PGVYTLL SKKHLNWIVK TMKQAV
Human GZMA   ....I.. ........IM .I.G..
MurineGZMA   .....F. .D......K. I..GS.
```

Dot-Identical; Dash-Gap; Dark- Mature protein sequence; Yellow-Activation peptide

(b). Percentage amino acid sequence identities (and nucleotide similarities) between species

	BtGzmA	HsGzmA	MmGzmA
BtGzmA		73 (83)	67 (75)
HsGzmA			69 (77)
MmGzmA			

Figure 3.2 Granzyme O – comparison of bovine and pig amino acid sequences

(a). Alignment of amino acid sequences

```
                1          11         21          31         41         51
                .          .          .           .          .          .

BovineGZMO MNIPFPFSFP PAICLLLIPG VFPVSCEGII GGNEVAPHTR RYMALIKGLK LCAGALIKEN
PigGZMO    .E.....F.. A.M...I... ......D.. .....S.... ......E... V.........

                61         71         81          91         101        111
                .          .          .           .          .          .

BovineGZMO WVLTAAHCDL KGNPQVILGA HSTSHKEKLD QVFSIKKAIP YPCFDPQTFE GDLQLLQLEG
PigGZMO    .......... .......... ........Y. .T........ .......... ..........

                121        131        141         151        161        171
                .          .          .           .          .          .

BovineGZMO KATMTKAVGI LQLPRTEDDV KPHTKCHVAG WGSTKKDACQ MSNALREANV TVIDRKICND
PigGZMO    .......... .K..N.GK.. E......... ....R..S.K I..T...V.I ......M...

                181        191        201         211        221        231
                .          .          .           .          .          .

BovineGZMO AQHYNFNPVI DLSMICAGGR KGEDDSCEGD SGSPLICDNV FRGVTSFGKC GNPQKPGIYI
PigGZMO    .......... ..G....V.. .......... .........I .......... .......V..

                241        251                                .          .          .
BovineGZMO LLTKKHLNWI KKTIAGAI*
PigGZMO    .......... .........
```

Dot-Identical; Dash-Gap; Dark- Mature protein sequence; Yellow-Activation peptide

(b). Percentage amino acid sequence identities (and nucleotide similarities) between species

	BtGzmO	SsGzmO
BtGzmO		89 (91)
SsGzmO		

Figure 3.3 Granzyme B – comparison of bovine, human and murine amino acid sequences

(a). Alignment of amino acid sequences

```
             1          11         21         31         41         51
             .          .          .          .          .          .
BovineGZMB   MKPLLLLVAF LLTPRAKAGE IIGGHEAKPH SRPYMAYLQY WNQDVQSRCG GFLVRQDFVL
Human GZMB   .Q.I...L.. ..L...D... .......... ........MI .D.KSLK... ...I.D....
MurineGZMB   ..I....LTL S.AS.T.... ......V... ......L.SI KD.QPEAI.. ...I.E....

             61         71         81         91         101        111
             .          .          .          .          .          .
BovineGZMB   TAAHCNGSSI KVTLGAHNIK QQERTQQVIR VRRAISHPDY NPKNFSNDIM LLKLERKAKQ
Human GZMB   .....W.... N......... E..P...F.P .K.P.P..A. .......... ..Q......R
MurineGZMB   .....E..I. N......... E..K.....P MVKC.P.... ...T...... ....KS...R

             121        131        141        151        161        171
             .          .          .          .          .          .
BovineGZMB   TSAVKPLSLP RAKARVKPGQ TCSVAGWGRD S-TDTYADTL QEVKLIVQED QKCEAYLRNF
Human GZMB   .R..Q..R.. SN..Q..... ........QT APLGKHSH.. ....MT.... R...SD..HY
MurineGZMB   .R..R..N.. .RNVN....D V.Y......M APMGK.SN.. ...E.T..K. RE..S.FK.R

             181        191        201        211        221        231
             .          .          .          .          .          .
BovineGZMB   YNRAIQLCVG DPKTKKASFQ GDSGGPLVCD NVAQGIVSYG KRDGSTPRAF TKVSSFLPWI
Human GZMB   .DST.E.... ..EI..T..K .........N K......... RNN.MP...C ......VH..
MurineGZMB   ..KTN.I.A. .....R...R .........K K..A...... YK...P.... .......S..

             241
             .
BovineGZMB   KKTMKSL
Human GZMB   .....RY
MurineGZMB   ......S
```

Dot-Identical; Dash-Gap; Dark- Mature protein sequence; Yellow-Activation peptide

(b). Percentage amino acid sequence identities (and nucleotide similarities) between species

	BtGzmB	HsGzmB	MmGzmB
BtGzmB		72 (79)	69 (75)
HsGzmB			68 (76)
MmGzmB			

Figure 3.4 Granzyme H– comparison of bovine and human amino acid sequences

(a). Alignment of amino acid sequences

```
             1          11         21         31         41         51
             .          .          .          .          .          .
BovineGZMH   MQLLLLLMAF LLPPGLGEPF LSEEIIGGHE AKPHSRPYMA FVQFLGEKSW KRCGGVLIQK
Human GZMH   ..PF...L.. ..T..A.T-- --........ .......... .....Q...R .....I.VR.

             61         71         81         91         101        111
             .          .          .          .          .          .
BovineGZMH   DFVLTAAHCR GSSINVTLGA HNIKQQERTQ QVIQVKRAIH HPDYNPKTFS NDIMLLQLER
Human GZMH   .........Q .......... ....E..... .F.P...P.P ..A....N.. ..........

             121        131        141        151        161        171
             .          .          .          .          .          .
BovineGZMH   KAKQTSAVKP LSLPKAKAQV KPGEVCSLAG WGKVALGTPA TTLQEVELTV QEDRVCESLN
Human GZMH   ...W.T..R. .R..SS.... ...QL..V.. ..Y.SMS.L. ......L... .K.CQ..R.F

             181        191        201        211        221        231
             .          .          .          .          .          .
BovineGZMH   PRNYSRATQI CVGDPRKVKT GFKGDSGGPL VCKKVVHGIF SYGKTNGTPP GVFTQVSHFL
Human GZMH   HG......E. .....K.TQ. .......... ...D.AQ..L ...NKK.... ..YIK.....

             241
             .
BovineGZMH   PWIKRTMKHL
Human GZMH   ........R.
```

Dot-Identical; Dash-Gap; Dark- Mature protein sequence; Yellow-Activation peptide

(b). Percentage amino acid sequence identities (and nucleotide similarities) between species

	BtGzmH	HsGzmH
BtGzmH		77 (81)
HsGzmH		

Figure 3.5 Granzyme K – comparison of bovine, human and murine amino acid sequences

(a). Alignment of amino acid sequences

```
             1          11         21         31         41         51
             .          .          .          .          .          .
BovineGZMK   MTKFSSFFLC FLLAGTYMTP ECFNMEIIGG REVSPHSRPF MASLQYGGDH ICGGVLIHPQ
Human GZMK   .......S.F ..IV.A...H V.........  K.........  ...I....H. V......D..
MurineGZMK   .-R...WA.V S.V..V..SS ...HT.....  ...Q......  ...I..RSK. ..........

             61         71         81         91         101        111
             .          .          .          .          .          .
BovineGZMK   WVLTAAHCHL RFAKSQSSKV VLGAHSLSKN EASKQTFEIK KFIRFPGFAL APKSNDIMLV
Human GZMK   ........QY ..T.G..PT. ..........  ......L...  ...P.SRVTS D.Q.......
MurineGZMK   ........YS W.PRGH.PT. ..........  .PM.......  ...P.SRLQS GSA.H....I

             121        131        141        151        161        171
             .          .          .          .          .          .
BovineGZMK   KLHTAAILNR HVQLLHPRAK NDIKAGTKCQ VVGWGATDPE GLSLSDTLRE VTVTVISRKT
Human GZMK   ..Q...K..K ..KM..I.S. TSLRS....K .T.......D S.RP......  .....L...L
MurineGZMK   ..R...E..K N.....LGS. .YLRD.....  .T...T.K.D L.TA......  ....I....R

             181        191        201        211        221        231
             .          .          .          .          .          .
BovineGZMK   CNSRDYYNHS PVITRTMLCA GDARGQKDSC QGDSGGPLVC KGAFHALVSG GPKCGDAKKP
Human GZMK   ...QS...GD .F..KD.V..  ...K......  K.......I.  ..V...I...  .HE..V.T..
MurineGZMK   ...QS....K ....KD.I..  ..........  K.......I.  ..I......Q  .Y...I....

             241        251        261
             .          .          .
BovineGZMK   GIYILLTRKF QAWIKSNLAP SHAD
Human GZMK   ...T...K.Y .T......V. P.TN
MurineGZMK   ...T...K.Y .T....K... .R.H
```

Dot-Identical; Dash-Gap; Dark- Mature protein sequence; Yellow-Activation peptide

(b). Percentage amino acid sequence identities (and nucleotide similarities) between species

	BtGzmK	HsGzmK	MmGzmK
BtGzmK		73 (81)	71 (75)
HsGzmK			72 (78)
MmGzmK			

Figure 3.6 Granzyme M – comparison of bovine, human and murine amino acid sequences

(a). Alignment of amino acid sequences

```
             1          11         21         31         41         51
             .          .          .          .          .          .
BovineGZMM   M-----LLLL VVLEALWAGG NTFETHIIGG RDAVPHSRPY MVSLQKSSGS HQCGGVLLHQ
Human GZMM   MEACVSS..V LA.G..S-V. SS.G.Q.... .EVI...... .A...RN-.. .L.....V.P
MurineGZMM   MEVCWS.... LA.KT...A. .R...Q.... .E........ .A....A-K. .V.....V.R

             61         71         81         91         101        111
             .          .          .          .          .          .
BovineGZMM   NWVLTAAHCL TQPTQQLRLV LGLH---VLG EISPIYRIRK VVRHPEYKPV PHLENDLALL
Human GZMM   K......... A.RMA..... .......T.D SPGLTFH.KA AIQ..R.... .A........
MurineGZMM   K......... SE.L.N.K.. ....NLHD.Q DPGLTFY..E AIK..G.N-- HKY.......

             121        131        141        151        161        171
             .          .          .          .          .          .
BovineGZMM   KLDGKVKPSR TIQPLALPRG -RQMVATGTR CSLAGWGLTH QPGNLARVLQ ELDVHVLDTR
Human GZMM   Q......... ..R.....SK -..V..A... ..M....... .G.R.S...R ...LQ.....
MurineGZMM   ...RR.Q..K NVK......K P.SKP.E..W ..T....M.. .G.PR..A.. ...LR....Q

             181        191        201        211        221        231
             .          .          .          .          .          .
BovineGZMM   MCNNSRFWHG NISSHMICLA ADSKNQAPCK GDSGGPVVCK RGQVAG-ILS FSSENCTDIF
Human GZMM   ........N. SL.PS.V... ....D..... ......L..G K.R.LARV.. ...RV.....
MurineGZMM   ........N. VLIDS.L..K .G..S..... ......L..G K...D.-... ...KT.....

             241        251        261
             .          .          .
BovineGZMM   KPPVAVAVAP YMPWIKKVLR HNGSPPSP-
Human GZMM   .....T.... .VS..R..TG R-SA-----
MurineGZMM   .....T.... .SS..R..IG R-W..Q.LV
```

Dot-Identical; Dash-Gap; Dark- Mature protein sequence; Yellow-Activation peptide

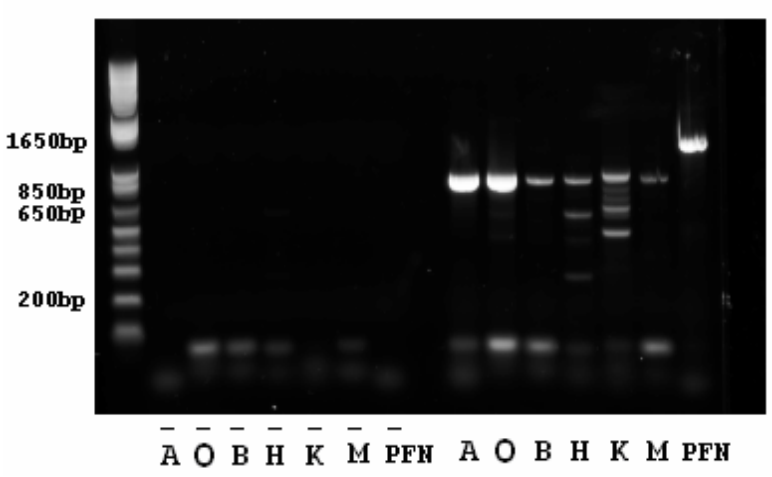
(b). Percentage amino acid sequence identities (and nucleotide similarities) between species

	BtGzmM	HsGzmM	MmGzmM
BtGzmM		70 (76)	64 (70)
HsGzmM			70 (74)

3.3.3 Validating expression of bovine granzymes and perforin by RT-PCR analyses

Based on the sequences identified above, pairs of PCR primers were designed to amplify the full-length coding region of each of the granzymes and also the perforin gene, which had been identified in the bovine genome (XM_585583). cDNA prepared from cultures of *T. parva*-specific CD8+ T cells established from animals 641 and 011, which had been immunised by infection and treatment with *T. parva*, was used to test these PCR primers. PCR assays with the designed primers detected transcripts of the expected sizes for all 6 granzyme genes and perforin in cDNA from bovine CD8+ T cells (Figure 3.7). A single band was obtained for all except granzyme H and K, which each gave three bands, one of the predicted size and two smaller bands. To confirm the identity of the PCR products, they were sub-cloned into the pGEM-T vector and clones containing inserts of the correct size were selected and sequenced. Analysis of sequences obtained confirmed that they were all identical to those originally identified from the bovine genome. Sequencing of the additional bands obtained for granzyme H showed that they represent alternatively spliced forms of granzyme H, without exon 4 (562bp) and exons 2, 3 and 4 (265bp), respectively. The alternatively spliced forms of granzyme K were identified as transcripts without exon 4 (619bp) and exons 3 and 4 (468bp), respectively. In conclusion, all of the identified bovine granzyme genes are expressed at the mRNA level in bovine CD8+ T cells.

Figure 3.7 PCR products obtained using primers designed to be specific for each of the bovine granzyme genes and perforin



Lanes on the left contain negative controls (primers with no template) for the individual granzymes. Lanes on the right contain PCR products obtained with each set of primers with cDNA template of an uncloned *T. parva*-specific CD8+ T cell line (641) 7 day after 3rd stimulation (purity, 99%). The estimated sizes are; granzyme A - 838bp; granzyme O - 849bp; granzyme B - 818bp; granzyme H - 820bp, 562bp and 265bp; granzyme K - 889bp, 619bp and 468bp; granzyme M - 833bp; Perforin (PFN) - 1275bp.

3.3.4 Chromosomal location of granzyme genes

In humans and mice, granzyme genes are separated into loci on three chromosomes termed the trypsin-like, chymotrypsin-like and metase loci (Smyth et al., 1996). Annotation of the bovine genome using the identified bovine granzyme orthologues revealed a similar organisation of granzyme genes to that described for human and mouse. Granzyme B is linked together with granzyme H and located on chromosome 21. Granzyme A and K, as well as the novel granzyme gene O, are observed on the

same cluster on bovine chromosome 20, while granzyme M is located on chromosome 7. To provide comparative data from another artiodactyl species, the genomic location of granzyme genes in the pig was also examined and found to have a similar arrangement into 3 loci. The genome accession numbers for the genes are listed in table 3.6.

Table 3.6 Summary of accession numbers of the identified genes found within the trypsin-like, chymotrypsin-like and metase loci of cattle and pig genome

Chromosomal Loci	**Species**	
	***Bos taurus* (Bt)**	***Sus scrofa* (Ss)**
Trypsin-like	GzmA(ENSBTAG00000021958)	GzmA(corr_ENSSSCG00000016903)
	GzmO(ENSBTAG00000027865)	GzmO(ENSSSG00000016902)
	GzmK(ENSBTAG00000005164)	GzmK(ENSSSCG00000016901)
Chymotrypsin-like	Cma1a(ENSBTAG00000027033)	Cma1(ENSBTAG00000039828)
	Cma1b(ENSBTAG00000037578)	
	DDN1(ENSBTAG00000039813)	DDN(ENSSSCT00000001979)
	DDN2(corr_ENSBTAG00000038080)	
	DDN3(corr_ENSBTAG00000038159)	
	DDN4(corr_ENSBTAG00000013055)	
	DDN5(corr_ENSBTAG00000039828)	
	CTSG1(ENSBTAG00000040134)	CTSG(ENSSSCG00000001980)
	CTSG2(ENSBTAG00000013234)	
	GzmH(corr_ENSBTAG00000010828)	GZMH(ENSSSCG00000001981)
	GzmB(corr_ENSBTAG00000010057)	GzmB(corr_ENSSSCG00000001978)
Metase	GzmM(ENSBTAG00000002100)	ND
	ND	AZU1(ENSSSCG00000013415)
	ND	PRTN3(ENSSSCG00000013417)
	ND	CFD(ENSSSCG00000013418)

The sequences matched in pig and cattle genome were named according to the orthologues suggested by Ensembl. Corr - Manually corrected sequence; ND - not detected. Gzm = granzyme; Cma1 = mast cell a-chymase; DDN = duodenase; CTSG = cathepsin G; ZAU1 = azurocidin 1; PRTN3 = proteinase 3; CFD = complement factor D.

3.3.4.1 Trypsin-like locus

In all 4 species examined, the trypsin-like locus contains the functional granzyme genes A and K, which are separated by between 74kb and 86kb on chromosomes 5, 13, 20 and 16 in humans, mice, cattle and pig, respectively. The genes have a conserved gene orientation and arrangement in the 4 species.

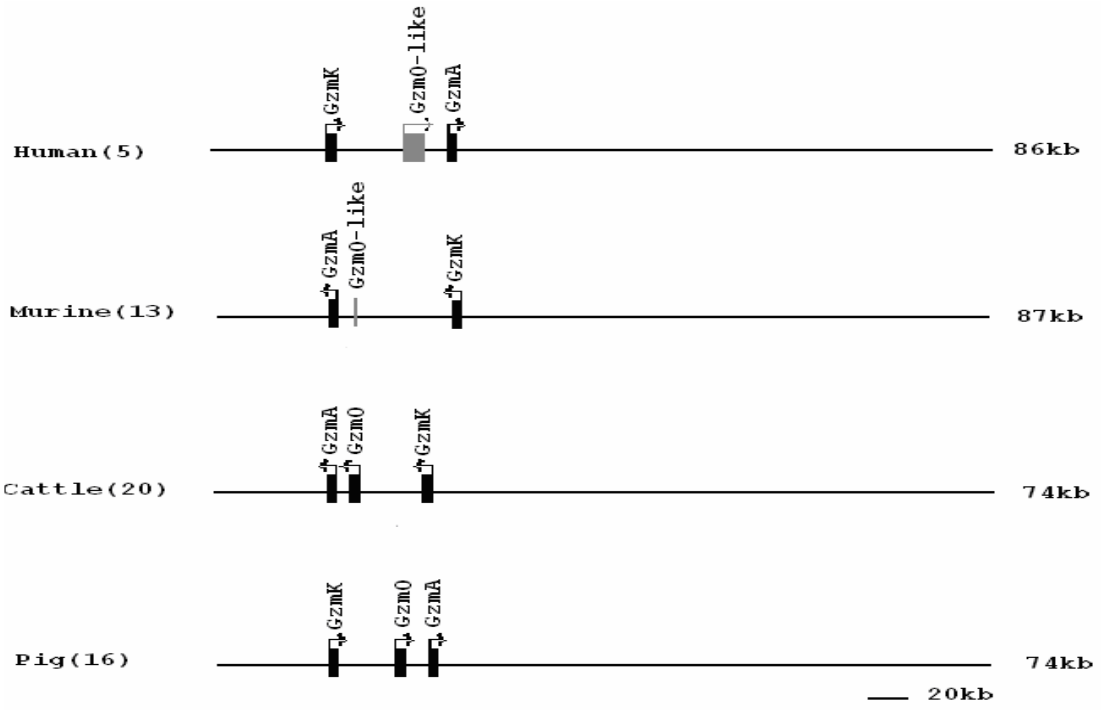


Figure 3.8 Comparison of the genomic organization of the human, murine, cattle and pig trypsin-like locus.

Bars indicate gene positions; arrows indicate transcriptional orientation; numbers indicate the length of locus. Functional genes are shown in black boxes and non-functional genes in grey. Intervals between genes are drawn to scale.

In cattle and pig, the additional granzyme O gene is situated between the A and K genes and displays the same orientation. Interspecies comparison of the novel bovine and pig granzyme gene shows a high level of similarity both in nucleotide sequence (91%) and amino acid sequence (89%). The EST sequence data in both species (pig EST database reference 2241795172) together with RT-PCR analysis of cattle T cells in this study indicate that granzyme O is functionally expressed. Analysis of genome DNA sequence in the region between the granzyme A and K genes in human and mouse revealed a pseudogene in human (ENSG00000249454) and a truncated gene in mouse (ENSMUSG00000051002), which exhibit 89% and 66% nucleotide similarity to cattle granzyme O gene, respectively (Figure 3.9- b). The human granzyme O-like pseudogene comprises 4 exons that contain several premature stop codons, whereas the mouse truncated gene contains only 269bp of DNA sequence, which based on alignments appears to correspond to exon 4 in the bovine gene (Figure 3.9- a).

Figure 3.9 Granzyme O and granzyme O-like genes – comparison of bovine, human murine and pig nucleotide sequences

(a). Alignment of nucleotide sequences

```
           1          11         21         31         41         51
           .          .          .          .          .          .
BovineGZMO ATGAATATTC CTTTTCCTTT CTCTTTTCCT CCTGCCATTT GTCTCCTTCT AATTCCTGGA
PigGzmO    ...G.A.... .......... ..T....... G.......G. ........A. ..........
HumanGZMO  .G...A...T T......C.. ......C... A..A....GC T......... ..........
MurineGZMO ---------- ---------- ---------- ---------- ---------- ----------
```

```
           61         71         81         91         101        111
           .          .          .          .          .          .
BovineGZMO GTT------- ---------- -TTTCCAGTA T--CCTGCGA GGGAAT---- ---TATAGGA
PigGzmO    ...------- ---------- .......... .--....T.. C....---- ----------
HumanGZMO  .A.AAGACAT GTTTTCATAG C.C.T..... .TG.TCAT.. .AA.G.AGAA TCC....T..
MurineGZMO ---------- ---------- ---------- ---------- ---------- ----------

           121        131        141        151        161        171
           .          .          .          .          .          .
BovineGZMO GGAAATGAAG TGGCCCCTCA CACAAGACGC TACATGGCTC TAATCAAAGG GCTGAAACTC
PigGzmO    .......... ..T.A..C.. ......G... .........T ....TG.... .......G..
HumanGZMO  .......... ..CTA..... TG.....T.. .G..G.A... ...C...... ....G..A..
MurineGZMO ---------- ---------- ---------- ---------- ---------- ----------

           181        191        201        211        221        231
           .          .          .          .          .          .
BovineGZMO TGTGCAGGGG C-TTTAATCA AAGAAAACTG GGTGTTGACA GCCGCTCATT GTGACCTGAA
PigGzmO    .......... .-...G.... .......... .......... ..T.....C. ..........
HumanGZMO  ......A... .C...G..G. ..T....... ......A.TG ..T.....C. .---------
MurineGZMO ---------- ---------- ---------- ---------- ---------- ----------

           241        251        261        271        281        291
           .          .          .          .          .          .
BovineGZMO GGGCAATCCT CAAGTTATTC TTGGGGCCCA CTCTACATCC CATAAAGAGA AACTTGACCA
PigGzmO    A.....C... .......... ....A..... .......... .......... ..TA......
HumanGZMO  -.....A.T. ...A...... .......... .......... ......C... ..........
MurineGZMO ---------- ---------- ---------- ---------- ---------- ----------

           301        311        321        331        341        351
           .          .          .          .          .          .
BovineGZMO AGTATTTTCC ATTAAAAAGG CAATTCCCTA CCCATGCTTT GATCCACAGA CATTTGAAGG
PigGzmO    GAC....... .......... .......T.. T.....T... .......... ..........
HumanGZMO  GAA.G..... .......... .G...T.... T.......A. .........C ....CAT.T.
MurineGZMO ---------- ---------- ---------- ---------- ---------- ----------

           361        371        381        391        401        411
           .          .          .          .          .          .
                                            ----------Exon4----------
BovineGZMO GGATCTTCAA CTACTTCAGC TGGAAGGTAA AGCAACTATG ACCAAAGCTG TAGGAATACT
PigGzmO    .........G ..G....... .......... .......... .......... ..........
HumanGZMO  .T.....T.. ..C....... ......T... ......C... .G........ ......-...
MurineGZMO ---------- ---------- --------.. .........A .......T.. .G..G..C..

           481        491        501        511        521        531
           .          .          .          .          .          .
------------------------------------------------------------------------------
BovineGZMO TCAGCTACCA AGAACAGAAG ACGATGTCAA ACCCCACACC AAGTGTCATG TGGCAGGATG
PigGzmO    .A.......C .AC....G.A .A......G. .......... .......... ..........
HumanGZMO  ...T...... .A.....G.C .G.....A.. ..TT...... G......... ..........
MurineGZMO ...C..C... .AG.G..G.. .G..CT.G.. ......T... ........C. G......G.C

           541        551        561        571        581        591
           .          .          .          .          .          .
------------------------------------------------------------------------------
BovineGZMO GGGAAGCACC AAAAAAGACG CATGTCAAAT GTC--TAATG CCTTGAGAGA AGCCAACG--
PigGzmO    .......... .G.......T .G..CA.... T..--A..CA .......... ..T....A--
HumanGZMO  .........A ....G....T ....CA..G. T.TGAA..CA .......... ..TG...AAA
MurineGZMO AC.GG..CTG .TG.GG..-- -GCAC..GG. T..--CC.CA T......... ..T....C--

           601        611        621        631        641        651
           .          .          .          .          .          .
------------------------------------------------------------------------------
BovineGZMO --TTACAGTG ATAGATAGGA AAATATGCAA TGATGCCCAG CACTATAATT TTAATCCAGT
PigGzmO    --....T... ......C.A. ....G..... .......... .......... ..........
HumanGZMO  CA.C..TA.. ..G..CCAA. ...C...... ......AG.. .......... .C..G.TG..
MurineGZMO --.C..T... ..T...TTA. ..C.C..... CA..C.AA.. .GT..C.... .C..A.TGT.

           661        671        681        691        701        711
           .          .          .          .          .          .
------------------------------------------------------------------------------
BovineGZMO TATTGATCTC AGTATGATCT GTGCTGGTGG TAGAAAAGGT GAAGATGATT CATGTGAAGG
PigGzmO    ......C..T G......... ....A.T... .......... .......... ..........
```

```
HumanGZMO  .G....AAA. T.C.......  .....A....  .........C  .......C-- ----------
MurineGZMO CG.G..CAA. ..C.G.C.T.  ..........  C.CG.G...G  TCTA.G.... .C...-----

           721         731         741         751         761         771
           .           .           .           .           .           .
BovineGZMO GGATTCTGGA AGTCCTCTGA TATGTGATAA TGTTTTCAGA GGTGTCACTT CCTTTGGCAA
PigGzmO    A.........  ..........  .......C..  CA........  ..G.......  .......G..
HumanGZMO  ---------- ---------- ---------- ---------- ---------- ----------
MurineGZMO ---------- ---------- ---------- ---------- ---------- ----------

           781         791         801         811         821         831
           .           .           .           .           .           .
BovineGZMO GTGTGGTAAT CCCCAGAAGC CTGGCATCTA CATCCTCCTT ACCAAAAAAC ACCTCAACTG
PigGzmO    .........C  ..........  .....G....  ...T.....C  ..........  ..........
HumanGZMO  ---------- ---------- ---------- ---------- ---------- ----------
MurineGZMO ---------- ---------- ---------- ---------- ---------- ----------

           841         851         861
           .           .           .
BovineGZMO GATAAAGAAA ACCATTGCAG GAGCCATATA A
PigGzmO    ..........  ..........  ..........  G
HumanGZMO  ---------- ---------- ---------- -
MurineGZMO ---------- ---------- ---------- -
```

Dot – Identical and Dash - Gap

(b). Percentage nucleotide sequence similarities between species

	BtGzmO	SsGzmO	HsGzmO-like	MmGzmO-like
BtGzmO		90 (91)	89 (81)	66
SsGzmO			82 (81)	67
HsGzmO-like				68
MmGzmO-like				

The nucleotide similarity values are for exon 4 and those in brackets are for the available coding region sequences

3.3.4.2 Chymotrypsin-like locus

The chymotrypsin-like locus is more complex. In human and mouse, the locus contains not only genes encoding granzymes B and H, but also a cluster of hematopoietic serine protease genes, including mast cell chymase (including 3 subgroups, a-chymase in human and mouse and β-chymase and mast cell protease 8 (Mcpt8), which are only found in mouse) and neutrophil cathepsin G, which exhibit gene sequence similarities and share similar substrate specificities with granzyme

genes (Gallwitz and Hellman, 2006). A preliminary map of the cattle chymotrypsin-like locus, based on NCBI, Build 2.1 database released on October 2005, has been reported (Gallwitz et al., 2006). However, changes in the assembly of this region have been reported in the updated cattle genome; hence, the locus has been re-drawn according to the version of Ensembl, Btau_4.0 (released on 4 October 2007). The locus is identified on chromosome 21 in cattle. Although its overall size (192kb) is similar to that in human (129kb), the number of annotated functional genes in cattle (10) is more than twice that found in human.

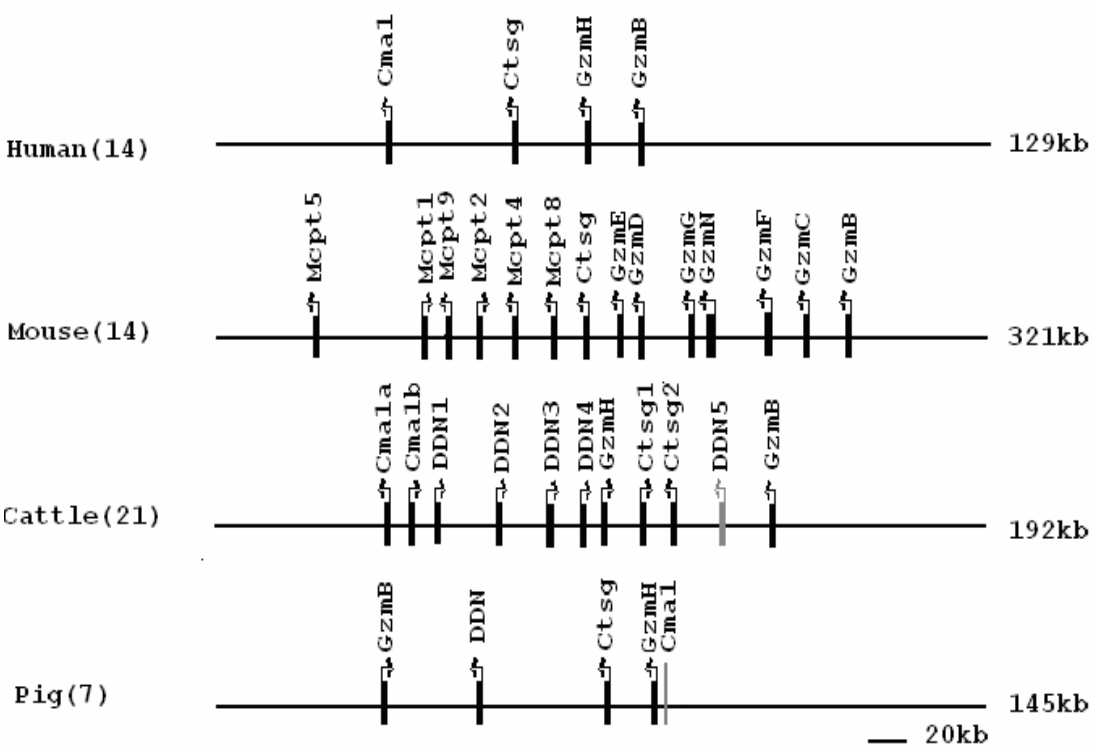


Figure 3.10 Comparison of the genomic organization of the human, murine, cattle and pig chymotrypsin-like locus.

Bars indicate gene positions; arrows indicate transcriptional orientation; numbers indicate the length of locus. Functional genes are shown in black boxes and non-functional genes in grey. Intervals between genes are drawn to scale.

Gzm = granzyme; Cma /Mcpt = mast cell chymase /mast cell protease; DDN = duodenase; Ctsg = cathepsin G.

The cattle chymotrypsin-like locus contains 2 subgroups classified as α-chymase and cathepsin G (Ctsg) based on their phylogenetic relationship (Figure 3.13). In contrast to the presence of only one gene belonging to each group in human and mouse (Gallwitz and Hellman, 2006), there are two members of each group in cattle, which show a high level of predicted amino acid identity (94% and 78%, respectively): Cma1a and Cma1b representing α-chymase and Ctsg1 and Ctsg2 representing cathepsin G (as reported in (Gallwitz et al., 2006)); however, the gene sequences and chromosomal location of Ctsg genes differ from those in the previous report (Gallwitz et al., 2006). The positions of these genes relative to granzymes B and H and their orientation differ between human and cattle. Unlike the human locus, in which the Ctsg gene lies immediately downstream of the granzyme genes and the latter are in the same orientation, the two bovine Ctsg genes lie between the granzyme B and H genes, which are in a head-to-head orientation. This suggests that there has been one or more inversions in this region.

A further 5 related genes sharing a high level of nucleotide similarity with granzyme B (64% to 66%) were found, one lying between the granzyme B and granzyme H genes and other 4 immediately downstream of granzyme H. However, none of them appear to be paralogous to granzyme B. One of these genes was identified as a duodenase by Zamolodchikova et al in 1995 (Zamolodchikova et al., 1995a) and has been named as DDN1_BOVINE in Ensembl, Btau_4.0. The other 4 genes are categorized as members of the duodenase family, based on their sequence similarity

to DDN1 and each other and their phylogenetic relationship (Figure 3.13) and have been named DDN2 – DDN5 accordingly following their order in the genome (Figure 3.10). These 5 genes were previously reported as duodenases in (Gallwitz et al., 2006) and were named as BDMD1 – BDMD5 according to their close relationship to BDMD1 (a synonym of DDN1), but the gene sequences and chromosomal location of DDN2-DDN5 differ from those in the previous report (Gallwitz et al., 2006). So far, duodenase genes have only been reported in ruminants and no counterpart has been found in human and mouse. Comparisons of cDNA sequences show a high level of similarity between the DDN members. For example, the coding sequences of DDN2 and DDN1 are identical, except for one non-synonymous substitution at position 474 (A/T), suggesting a very recent gene duplication. All duodenase genes are predicted to be functional except for DDN5, which has several premature stop codons due to a frameshift mutation. Analysis of the available genomic data on the pig chymotrypsin-like locus reveals a gene order similar to cattle, with single Ctsg and DDN genes lying between the granzyme B and H genes, and with a Cma1 fragmented gene (519bp) lying immediately downstream of granzyme H.

3.3.4.3 Metase-locus

The granzyme M gene found on chromosome 19 in human and chromosome 10 in mouse is linked downstream, in both species, with the neutrophil elastase gene cluster, which comprises azurocidin 1 (AZU1 - humans only), proteinase 3 (PRTN3), neutrophil elastase preproprotein (ELANE) and complement factor D (CFD) in human (Pilat et al., 1994). The counterpart of granzyme M in cattle is located on

chromosome 7, but no members of the neutrophil elastase gene cluster were found within the 160 kb of sequence downstream of this gene.

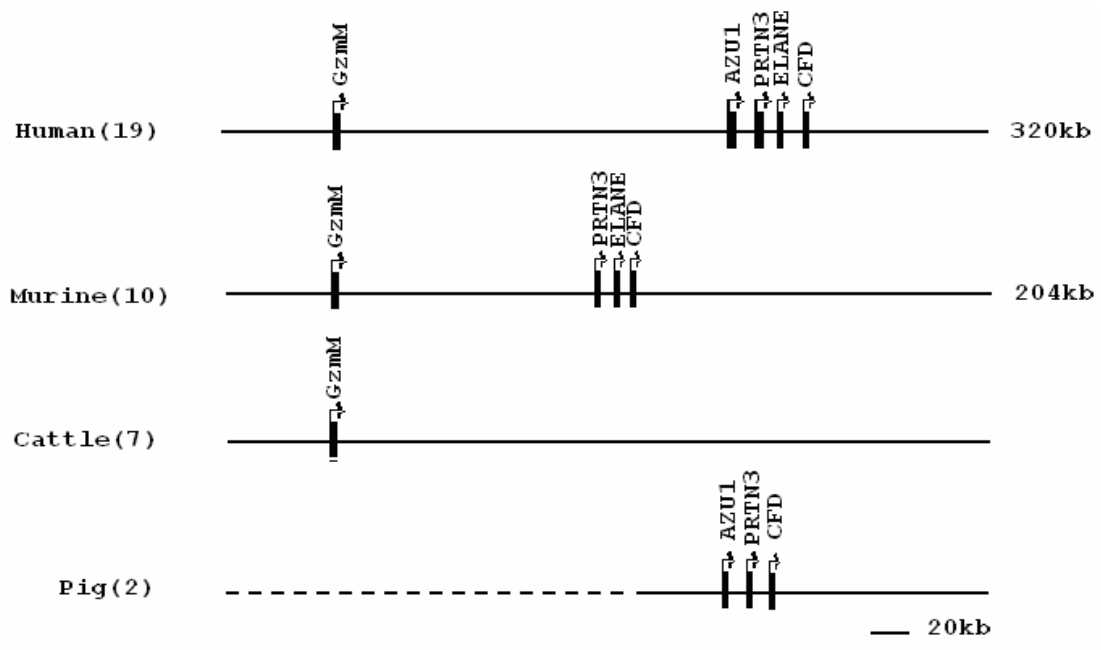


Figure 3.11 Comparison of the genomic organization of the human, murine, cattle and pig metase locus.

Bars indicate gene positions; arrows indicate transcriptional orientation; numbers indicate the length of locus. Functional genes are shown in black boxes. Dotted lines represent large regions of undermined sequence with the scaffolds. Intervals between genes are drawn to scale. Gzm = granzyme; ZAU1 = azurocidin 1; PRTN3 = proteinase 3; ELANE = neutrophil elastase preproprotein; CFD = complement factor D.

BLASTN searches of the bovine genome using the nucleotide sequences of human AZU1, PRTN3, ELANE and CFD did not identify any significant hits. In contrast searches of the EST database did identify single highly matched sequences (covering >86% gene length and exhibiting >76% identity) for each of the genes (ZAU1/1907915542, PRTN3/1907973944, ELANE/1958311623 and CFD/1909258796). In human and mice two genes flanking each side of the elastase

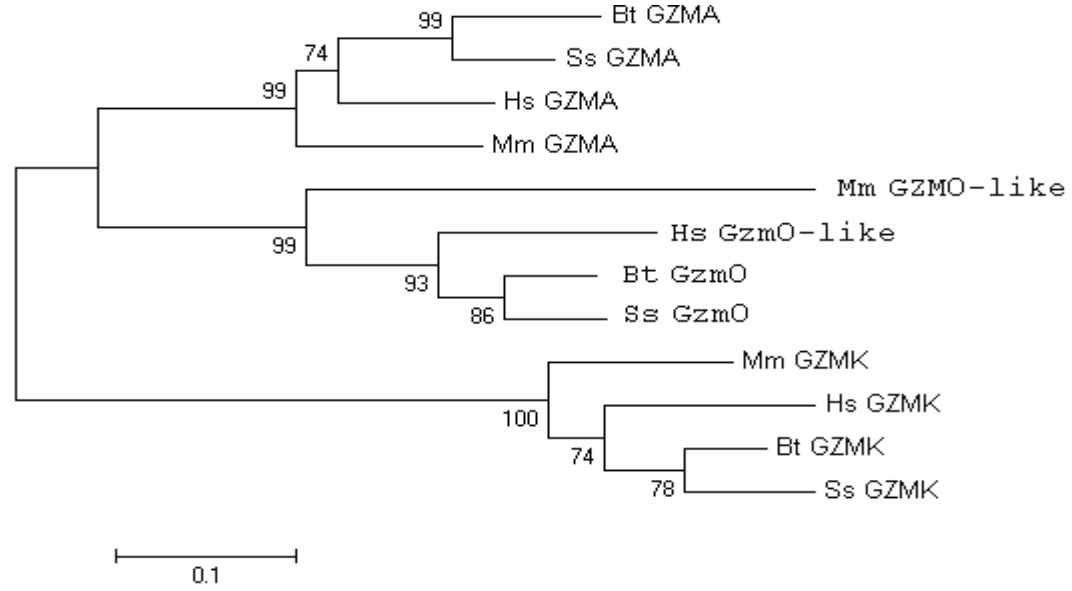
gene cluster, PTBP1 and MIR3187 on one side and MED16 and C19orf22 on the other, are highly conserved and lie close to the elastase genes. These genes also are not included in the bovine genome assembly. In contrast to these genes, other genes flanking the metase locus show syntenic locations in cattle, mice and humans. It is most likely that the absence of the neutrophil elastase genes downstream from granzyme M may be due the incomplete genomic sequencing and assembly of this region. In pig, the cluster of neutrophil elastase genes was found on chromosome 2, but it was not possible to determine its proximity to the orthologue to granzyme M, as this gene has not been identified in the genome.

3.3.5 Phylogenetic relationships between granzymes and related enzymes

3.3.5.1 Relationship between genes within each genome cluster

Phylogenetic analysis of the nucleotide sequences of granzyme A, granzyme K and granzyme O located within the trypsin-like locus in human, mouse, cattle and pig identified three independent subgroups segregated according to granzyme gene, with the O subgroup being more closely related to A than K (Figure 3.12). In each case, interspecies comparisons showed that the bovine gene was most closely related to the pig orthologue and most divergent from the murine orthologue. The results suggest that the O gene diverged prior to speciation, possibly as a result of duplication of granzyme A, and that it subsequently became non-functional in human and mouse.

Figure 3.12 Phylogenetic relationships of human, mouse, cattle and pig trypsin-like granzyme genes

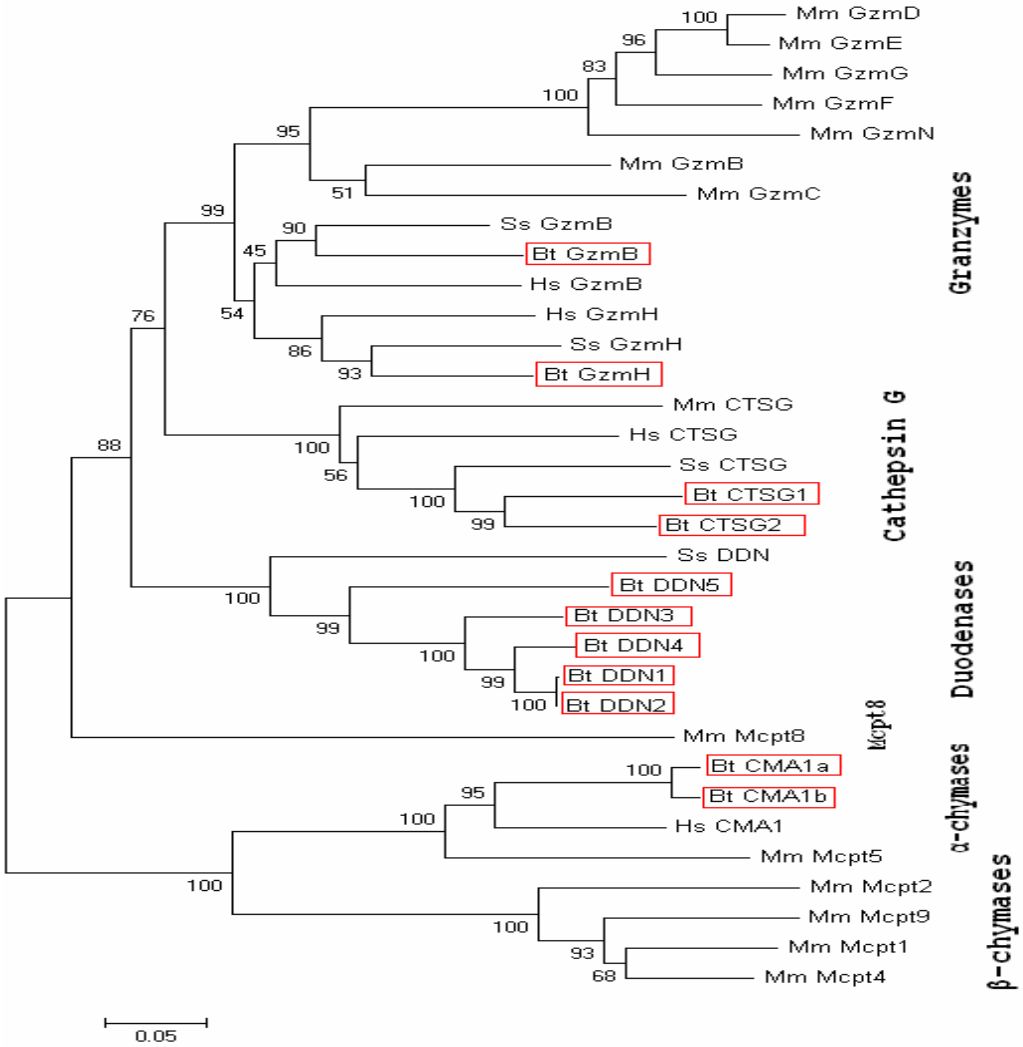


The nucleotide sequences were aligned by CLUSTAL W alignment with complete deletion of gaps for the analysis. The tree was constructed with the neighbour-joining algorithm using Mega 4.0 software. Numbers represent percentage bootstrap values out of 2,000 replications. Hs - *Homo sapiens*; Mm - *Mus musculus*; Ss - *Sus scrofa*; Bt - *Bos taurus*.

Phylogenetic analyses of genes encoded in the chymotrypsin-like locus in the different species broadly segregated the genes into four groups, namely the granzyme, cathepsin G, duodenase and chymase genes, which are expressed in mast cells and in mice are referred to as mast cell proteases (Mcpt). The chymase genes include two main subgroups, α-chymases in human, mouse and cattle and β-chymases which are only found in mouse. An additional mouse chymase, (Mcpt8), is phylogenetically distinct from both chymase subgroups. The 4 groups of genes exhibited a closer relationship across species than between the groups. Within the granzyme group,

human, bovine and pig granzyme B and H genes formed a subgroup, within which the bovine genes were most closely related to the respective pig orthologues, while the orphan mouse granzyme genes (D, E, F and G) formed a second subgroup. The murine granzyme B and C genes appeared to be in an intermediate position between these two subgroups.

Figure 3.13 Phylogenetic relationship between genes within the human, mouse, pig and cattle chymotrypsin-like locus



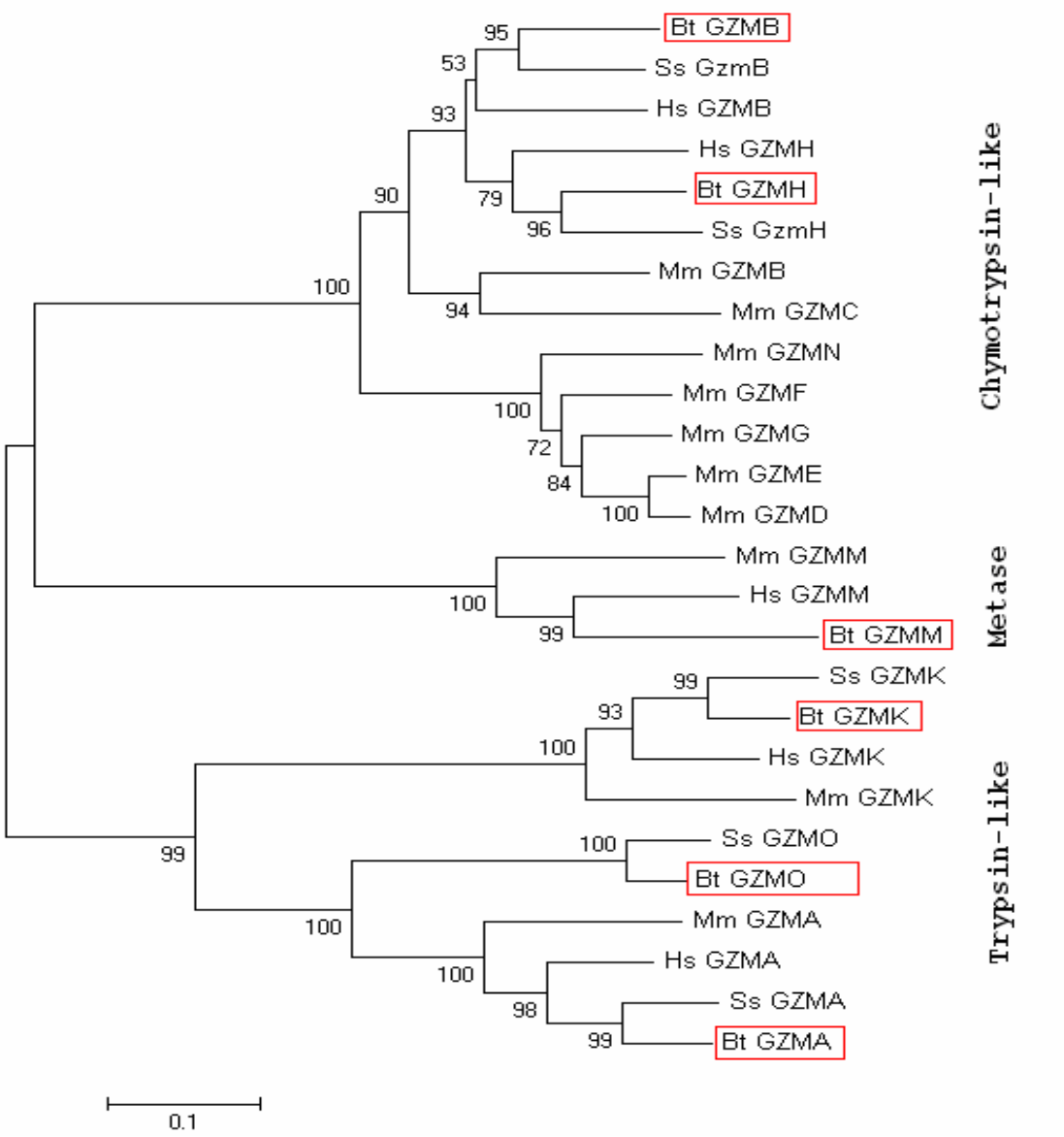
The cDNA sequences were aligned by CLUSTAL W alignment with complete deletion of gaps for the analysis. The tree was constructed with the neighbour-

joining algorithm using Mega 4.0 software. The 4 main phylogenetic groups are labelled as granzymes, cathepsin G, duodenases and chymases (α- and β-chymase). Numbers represent percentage bootstrap values out of 2,000 replications. Hs - *Homo sapiens*; Mm - *Mus musculus*; Ss - *Sus scrofa*; Bt - *Bos taurus*. Gzm = granzyme; CMA /Mcpt = mast cell chymase /mast cell protease; DDN = duodenase; CTSG = cathepsin G.

3.3.5.2 Relationship between granzyme proteins within and between species

A phylogenetic tree constructed from the translated amino acid sequences of all granzymes from human, mouse, cattle and pig is shown in figure 3.14 and the levels of amino acid identity between the granzymes are presented in table 3.7. In general, the levels of sequence identity correspond closely with the relationships observed in the phylogenetic tree. The latter demonstrates divergence into three broad groups, corresponding to the trypsin-like, chymotrypsin-like and metase proteins and to the chromosomal locations of the genes. This was reflected by much lower sequence identity between the groups (up to 44%) than within groups (45-91%).

Figure 3.14 Phylogenetic relationship of granzymes from human, mouse, pig and cattle based on analysis of amino acid sequences



The amino acid sequences translated from the cDNA sequences were aligned by CLUSTAL W alignment with complete deletion of gaps for the analysis. The tree was constructed with the neighbour-joining algorithm using Mega 4.0 software. Numbers represent percentage bootstrap values out of 2,000 replications. Hs - *Homo sapiens*; Mm - *Mus musculus*; Ss - *Sus scrofa*; Bt - *Bos taurus*.

Table 3.7 Percentage amino acid identities between mature protein sequences of bovine, human, murine and pig granzymes.

		BtGZMA	SsGZMA	HsGZMA	MmGZMA	BtGZMO	SsGZMO	BtGZMK	SsGZMK	HsGZMK	MmGZMK	BtGZMB	SsGzmB	HsGZMB	MmGZMB	BtGZMH	SsGzmH	HsGZMH	MmGZMC	MmGZMD	MmGZME	MmGZMF	MmGZMG	MmGZMN	BtGZMM	HsGZMM	MmGZMM
Trypsin-like	BtGZMA	0																									
	SsGZMA	83	0																								
	HsGZMA	73	76	0																							
	MmGZMA	70	70	71	0																						
	BtGZMO	61	61	63	59	0																					
	SsGZMO	61	61	63	60	90	0																				
	BtGZMK	46	48	49	48	48	48	0																			
	SsGZMK	46	47	47	48	46	46	83	0																		
	HsGZMK	46	46	45	45	47	48	73	77	0																	
	MmGZMK	47	47	47	47	48	48	72	75	75	0																
Chymotrypsin-like	BtGZMB	38	37	39	41	39	37	40	37	38	35	0															
	SsGzmB	39	38	40	41	39	37	41	38	40	37	77	0														
	HsGZMB	38	38	40	41	40	38	41	39	39	38	72	72	0													
	MmGZMB	41	41	43	44	42	40	42	39	39	38	69	69	69	0												
	BtGZMH	40	40	41	42	40	39	40	38	39	38	70	68	69	64	0											
	SsGzmH	41	41	40	42	42	39	40	37	39	38	69	78	67	63	76	0										
	HsGZMH	39	39	41	40	40	40	38	38	40	37	68	68	72	64	78	71	0									
	MmGZMC	39	39	39	41	39	39	41	39	40	37	63	61	62	68	60	61	61	0								
	MmGZMD	41	41	39	39	40	39	37	35	37	37	58	57	56	57	58	58	59	56	0							
	MmGZME	39	40	38	40	39	39	35	33	35	35	58	56	54	55	58	60	59	56	91	0						
	MmGZMF	40	43	43	40	42	40	36	35	37	36	56	57	52	58	55	58	58	57	72	75	0					
	MmGZMG	38	42	40	39	43	42	38	36	38	37	60	59	55	58	59	60	60	59	78	82	79	0				
	MmGZMN	38	39	38	38	38	37	37	34	36	36	55	55	52	55	57	57	58	56	68	71	72	71	0			
Metase	BtGZMM	36	35	36	37	35	34	38	36	36	34	39	39	38	38	39	40	39	37	32	33	35	35	36	0		
	HsGZMM	35	34	37	37	38	38	39	38	38	36	38	38	39	38	38	38	39	38	33	35	38	37	38	71	0	
	MmGZMM	35	34	35	36	36	36	39	36	37	36	42	42	38	38	39	42	39	39	33	34	37	37	36	65	71	0

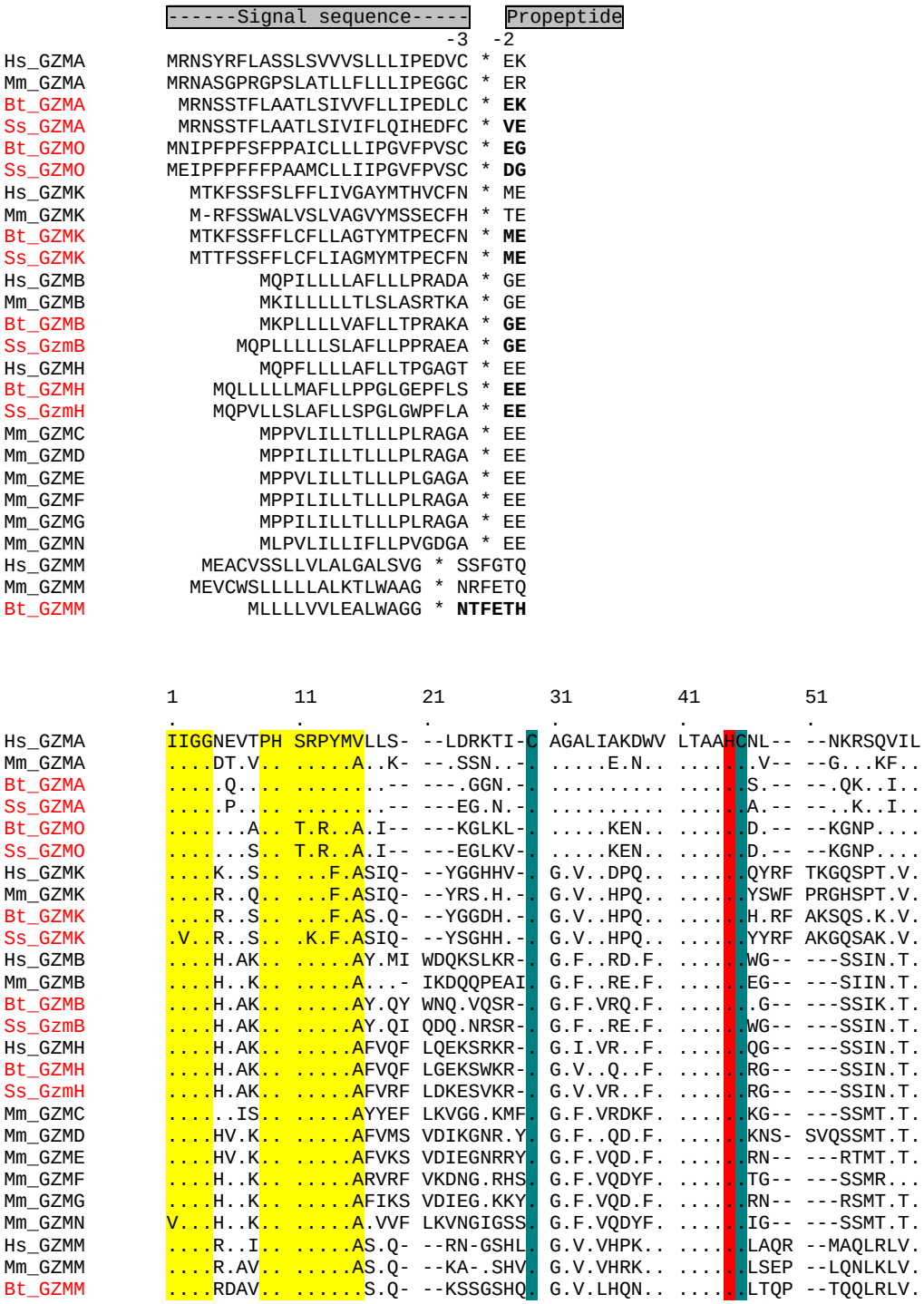
Mature protein sequences of granzyme genes were aligned by Clustal W alignment with pairwise deletion of gaps for the analysis and then manually edited for improvement. Numbers representing the amino acid identity of granzymes within each subgroup are highlighted in grey. Hs - *Homo sapiens*; Mm - *Mus musculus*; Ss - *Sus scrofa*; Bt - *Bos Taurus*.

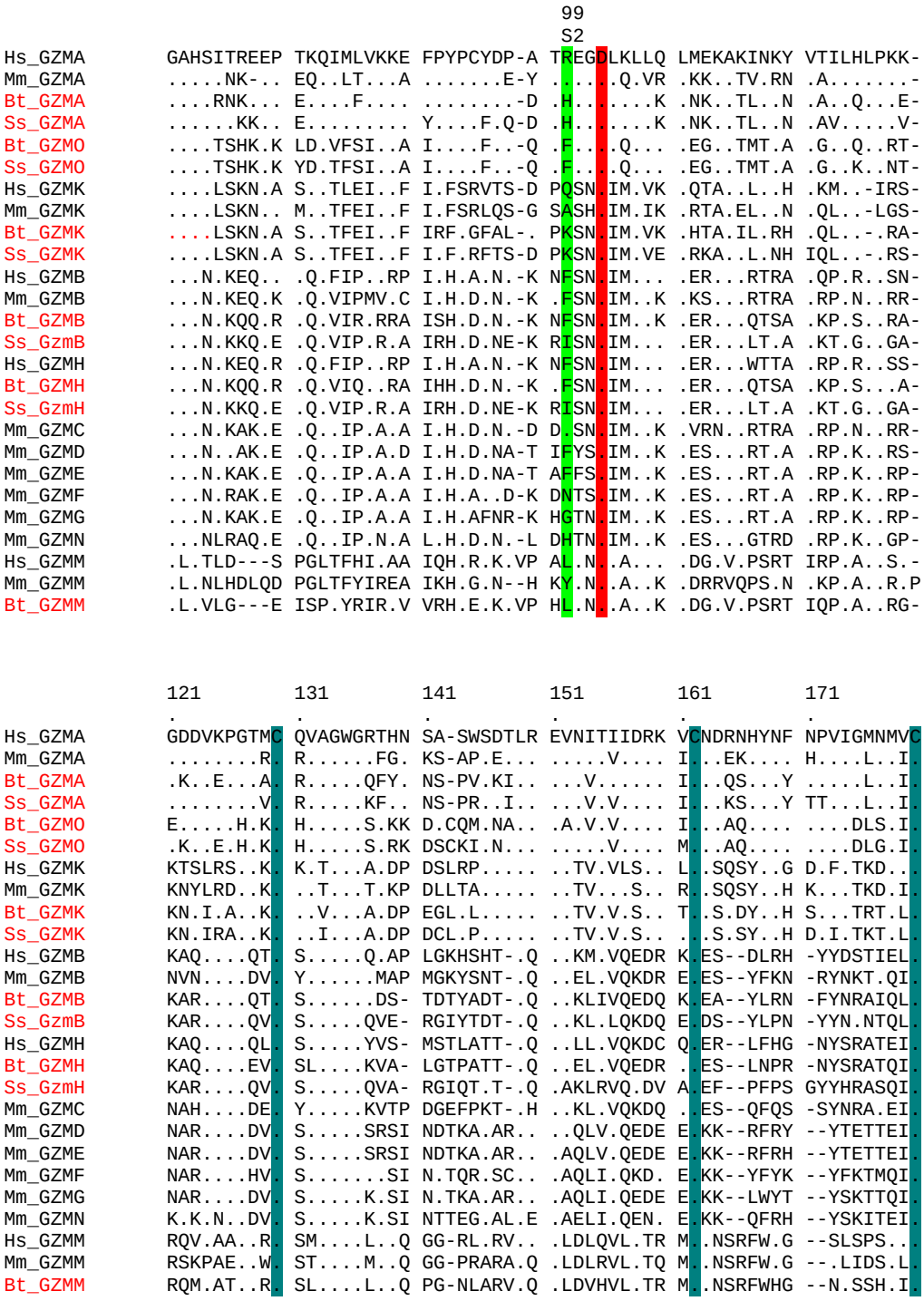
Overall, the findings support the view that the granzyme genes diverged before divergence of these mammalian species. Data on the novel Granzyme O gene indicate that it arose from early duplication of granzyme A, with subsequent loss of function in human and mouse.

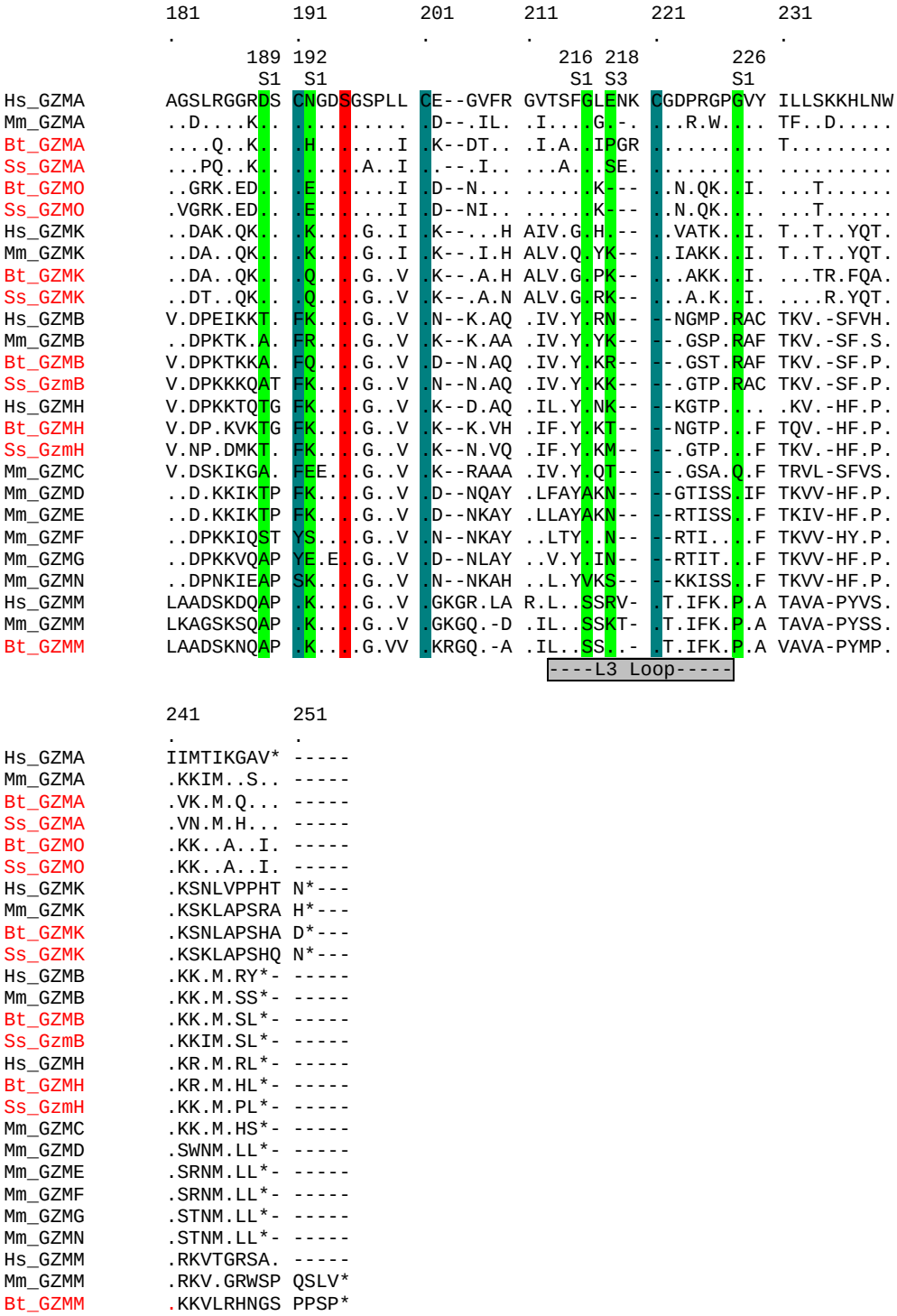
3.3.6 Characteristic structural features of granzyme proteins

To investigate their structural homologies and predicted substrate-binding specificity, multiple amino acid sequence alignments of human, mouse, cattle and pig granzymes were applied by using the Clustal W alignment and then manual manipulation for improvement (Figure 3.15). Sequence analysis revealed features of cattle and pig granzymes typical of the murine and human orthologues: highly conserved consensus sequences at positions 1-4 (IIGG) and 9-16 (PHSRPYMA); conserved signal sequence cleavage sites prior to the propeptide; conserved amino acids at the catalytic triad, ie. residues that form the active site, His-45, Asp-95 and Ser-195; 3-4 disulfide bonds, Cys30-Cys46, Cys162-Cys180, Cys191-Cys221 and Cys130-Cys201.

Figure 3.15 Amino acid sequence alignment of bovine, human, mouse and pig granzymes showing conservation of functionally relevant amino acid residues.




99
S2
Hs_GZMA GAHSITREEP TKQIMLVKKE FPYPCYDP-A TREGDLKLLQ LMEKAKINKY VTILHLPKK-
Mm_GZMA .....NK-.. EQ..LT...A ....E-Y .....Q.VR .KK..TV.RN .A.......-
Bt_GZMA ....RNK... E....F.... ....-D .H.....K .NK..TL..N .A..Q...E-
Ss_GZMA ......KK.. E..... Y....F.Q-D .H.....K .NK..TL..N .AV.....V-
Bt_GZMO ....TSHK.K LD.VFSI..A I....F..-Q .F....Q... .EG..TMT.A .G..Q..RT-
Ss_GZMO ....TSHK.K YD.TFSI..A I....F..-Q .F....Q... .EG..TMT.A .G..K..NT-
Hs_GZMK ....LSKN.A S..TLEI..F I.FSRVTS-D PQSN.IM.VK .QTA..L..H .KM..-IRS-
Mm_GZMK ....LSKN.. M..TFEI..F I.FSRLQS-G SASH.IM.IK .RTA.EL..N .QL..-LGS-
Bt_GZMK ....LSKN.A S..TFEI..F IRF.GFAL-. PKSN.IM.VK .HTA.IL.RH .QL..-.RA-
Ss_GZMK ....LSKN.A S..TFEI..F I.F.RFTS-D PKSN.IM.VE .RKA..L.NH IQL..-.RS-
Hs_GZMB ...N.KEQ.. .Q.FIP..RP I.H.A.N.-K NFSN.IM... .ER...RTRA .QP.R..SN-
Mm_GZMB ...N.KEQ.K .Q.VIPMV.C I.H.D.N.-K .FSN.IM..K .KS...RTRA .RP.N..RR-
Bt_GZMB ...N.KQQ.R .Q.VIR.RRA ISH.D.N.-K NFSN.IM..K .ER...QTSA .KP.S..RA-
Ss_GzmB ...N.KKQ.E .Q.VIP.R.A IRH.D.NE-K RISN.IM... .ER...LT.A .KT.G..GA-
Hs_GZMH ...N.KEQ.R .Q.FIP..RP I.H.A.N.-K NFSN.IM... .ER...WTTA .RP.R..SS-
Bt_GZMH ...N.KQQ.R .Q.VIQ..RA IHH.D.N.-K .FSN.IM... .ER...QTSA .KP.S...A-
Ss_GzmH ...N.KKQ.E .Q.VIP.R.A IRH.D.NE-K RISN.IM... .ER...LT.A .KT.G..GA-
Mm_GZMC ...N.KAK.E .Q..IP.A.A I.H.D.N.-D D.SN.IM..K .VRN..RTRA .RP.N..RR-
Mm_GZMD ...N..AK.E .Q..IP.A.D I.H.D.NA-T IFYS.IM..K .ES...RT.A .RP.K..RS-
Mm_GZME ...N.KAK.E .Q..IP.A.A I.H.D.NA-T AFFS.IM..K .ES...RT.A .RP.K..RP-
Mm_GZMF ...N.RAK.E .Q..IP.A.A I.H.A..D-K DNTS.IM..K .ES...RT.A .RP.K..RP-
Mm_GZMG ...N.KAK.E .Q..IP.A.A I.H.AFNR-K HGTN.IM..K .ES...RT.A .RP.K..RP-
Mm_GZMN ...NLRAQ.E .Q..IP.N.A L.H.D.N.-L DHTN.IM..K .ES...GTRD .RP.K..GP-
Hs_GZMM .L.TLD---S PGLTFHI.AA IQH.R.K.VP AL.N..A... .DG.V.PSRT IRP.A..S.-
Mm_GZMM .L.NLHDLQD PGLTFYIREA IKH.G.N--H KY.N..A..K .DRRVQPS.N .KP.A..R.P
Bt_GZMM .L.VLG---E ISP.YRIR.V VRH.E.K.VP HL.N..A..K .DG.V.PSRT IQP.A..RG-
121 131 141 151 161 171
Hs_GZMA GDDVKPGTMC QVAGWGRTHN SA-SWSDTLR EVNITIIDRK VCNDRNHYNF NPVIGMNMVC
Mm_GZMA ....R. R......FG. KS-AP.E... .....V.... I...EK.... H....L..I.
Bt_GZMA .K..E...A. R.....QFY. NS-PV.KI.. ...V...... I...QS...Y .....L..I.
Ss_GZMA ....V. R.....KF.. NS-PR..I.. ...V.V.... I...KS...Y TT...L..I.
Bt_GZMO E.....H.K. H.....S.KK D.CQM.NA.. .A.V.V.... I...AQ.... ....DLS.I.
Ss_GZMO .K..E.H.K. H.....S.RK DSCKI.N... .....V.... M...AQ.... ....DLG.I.
Hs_GZMK KTSLRS..K. K.T...A.DP DSLRP..... ..TV.VLS.. L..SQSY..G D.F.TKD...
Mm_GZMK KNYLRD..K. ..T...T.KP DLLTA..... ..TV...S.. R..SQSY..H K...TKD.I.
Bt_GZMK KN.I.A..K. ..V...A.DP EGL.L..... ..TV.V.S.. T..S.DY..H S...TRT.L.
Ss_GZMK KN.IRA..K. ..I...A.DP DCL.P..... ..TV.V.S.. ...S.SY..H D.I.TKT.L.
Hs_GZMB KAQ....QT. S.....Q.AP LGKHSHT-.Q ..KM.VQEDR K.ES--DLRH -YYDSTIEL.
Mm_GZMB NVN....DV. Y......MAP MGKYSNT-.Q ..EL.VQKDR E.ES--YFKN -RYNKT.QI.
Bt_GZMB KAR....QT. S......DS- TDTYADT-.Q ..KLIVQEDQ K.EA--YLRN -FYNRAIQL.
Ss_GzmB KAR....QV. S.....QVE- RGIYTDT-.Q ..KL.LQKDQ E.DS--YLPN -YYN.NTQL.
Hs_GZMH KAQ....QL. S.....YVS- MSTLATT-.Q ..LL.VQKDC Q.ER--LFHG -NYSRATEI.
Bt_GZMH KAQ....EV. SL....KVA- LGTPATT-.Q ..EL.VQEDR ..ES--LNPR -NYSRATQI.
Ss_GzmH KAR....QV. S.....QVA- RGIQT.T-.Q .AKLRVQ.DV A.EF--PFPS GYYHRASQI.
Mm_GZMC NAH....DE. Y.....KVTP DGEFPKT-.H ..KL.VQKDQ ..ES--QFQS -SYNRA.EI.
Mm_GZMD NAR....DV. S.....SRSI NDTKA.AR.. ..QLV.QEDE E.KK--RFRY --YTETTEI.
Mm_GZME NAR....DV. S.....SRSI NDTKA.AR.. .AQLV.QEDE E.KK--RFRH --YTETTEI.
Mm_GZMF NAR....HV. S.......SI N.TQR.SC.. .AQLI.QKD. E.KK--YFYK --YFKTMQI.
Mm_GZMG NAR....DV. S.....K.SI N.TKA.AR.. .AQLI.QEDE E.KK--LWYT --YSKTTQI.
Mm_GZMN K.K.N..DV. S.....K.SI NTTEG.AL.E .AELI.QEN. E.KK--QFRH --YSKITEI.
Hs_GZMM RQV.AA..R. SM....L..Q GG-RL.RV.. .LDLQVL.TR M..NSRFW.G --SLSPS...
Mm_GZMM RSKPAE..W. ST....M..Q GG-PRARA.Q .LDLRVL.TQ M..NSRFW.G --.LIDS.L.
Bt_GZMM RQM.AT..R. SL....L..Q PG-NLARV.Q .LDVHVL.TR M..NSRFWHG --N.SSH.I.



Hs - *Homo sapiens*; Mm - *Mus musculus*; Ss - *Sus scrofa*; Bt - *Bos taurus*. Multiple signal sequence cleavage sites are shown by an asterisk; conserved consensus sequences at the N-terminus at positions 1-4 (IIGG) and 9-16 (PHSRPYMA) are shown in yellow; the three amino acids of the catalytic triad are shown in red; cysteine residues that form disulfide bonds are shown in blue; Substrate-determining residues in S1, S2 and S3 are shown in green.

Numbering of residues based on chymotrypsinogen amino acid sequence is used to refer to specific amino acids.

Application of an algorithm based on a weight-matrix approach (von Heijne, 1986) indicates that the signal sequence cleavage site occurs between positions -3 and -2 in cattle and pig granzyme A, O, B and H. The algorithm failed to predict a cleavage site in granzyme K, but since Asn^{-3}–Met^{-2} has been identified experimentally as the cleavage site in human granzyme K (Sayers et al., 1996) and is conserved in the bovine orthologue, this is likely to represent the cleavage site in the bovine protein. The residues forming the potential peptidase cleavage site, Gly(-7)-Asn(-6), deduced from the cattle granzyme M sequence, are identical to those in the mouse and rat proteins, which are cleaved at this site (Pilat et al., 1994; Smyth et al., 1995; Kelly et al., 1996), suggesting that a hexapeptide is cleaved from cattle granzyme M to generate the mature protein. Eight cysteine residues are conserved in the same respective positions in cattle and pig granzyme A, O, K and M and are predicted to form four internal disulfide bonds Cys30-Cys46, Cys162-Cys180, Cys191-Cys221 and Cys130-Cys201. Six of these residues are also conserved in the corresponding positions in granzyme B and H; the lack of cysteine residues at positions 191 and 221 in these granzymes, resulting in the absence of the disulfide bond in the vicinity of the enzyme active site, is a characteristic feature of the chymotrypsin family which distinguishes them from other serine proteases (Zamolodchikova et al., 2003).

Like other proteases, the function of granzymes is determined by which substrate they cleave. In general, following specific docking into the substrate binding cleft of

the protease, the substrate is cleaved after the cleavage residue (the amino acid at P1 position in the substrate) through the proteolytic activity of the catalytic triad in the protease. The protease binding site typically interacts with at least three amino acids on each side of P1 in the substrate (nomenclature for amino acid positions in the substrate is Pn-P4-P3-P2-P1-P1'-P2'-P3'-P4'-Pn' (Schechter and Berger, 1967)). It is known that interaction of the substrate P1 residue with the S1 site of the protease (nomenclature for corresponding sites in the protease is Sn-S4-S3-S2-S1-S1'-S2'-S3'-S4'-Sn' (Schechter and Berger, 1967)) determines “primary substrate specificity”, which is rigorously restricted to certain amino acids, whereas interaction of other substrate residues with the binding site of the protease determines “extended substrate specificity” that is less specific but has substantial influence on the proteolytic efficiency.

Substrate specificity is determined by the 3-dimensional structure of the enzymes. Previously obtained data on the structure of murine and human granzymes (Perona and Craik, 1995) and the use of homology molecular models provide the opportunity to predict the substrate specificity of granzymes from other species by analysis of the corresponding specificity-determining residues. Residues 189, 192, 216 and 226 (based on chymotrypsinogen numbering) important for primary substrate specificity (Odake S 1991 and Kam C M 2000), deduced in cattle and pig granzymes in this model, are shown in table 3.8.

Table 3.8 Comparison of key amino acid residues in the substrate binding site and predicted enzymatic specificities of granzymes from different species

	S3 (218)	S2 (99)	(S1) 192	S1 (189)	S1 (216)	S1 (226)	Chr.	Activity	P1 specificity
HsGZMA	E	R	N	D	G	G	Trypsin locus	Tryptase	R/K
MmGZMA	E	R	N	D	G	G	Trypsin locus	Tryptase	R/K
BtGZMA	P	H	H	D	G	G	Trypsin locus	*Tryptase	*R/K
SsGZMA	S	H	N	D	G	G	Trypsin locus	*Tryptase	*R/K
BtGZMO	-	F	E	D	G	G	Trypsin locus	*Tryptase	*R/K
SsGZMO	-	F	E	D	G	G	Trypsin locus	*Tryptase	*R/K
HsGZMK	E	Q	K	D	G	G	Trypsin locus	Tryptase	R/K
MmGZMK	K	A	K	D	G	G	Trypsin locus	Tryptase	R/K
BtGZMK	K	K	Q	D	G	G	Trypsin locus	*Tryptase	*R/K
SsGZMK	K	K	Q	D	G	G	Trypsin locus	*Tryptase	*R/K
HsGZMB	N	F	K	T	G	R	Chymase locus	Aspase	D
MmGZMB	K	F	R	A	G	R	Chymase locus	Aspase	D
BtGZMB	R	F	Q	A	G	R	Chymase locus	*Aspase	*D
SsGzmB	K	I	K	A	G	R	Chymase locus	*Aspase	*D
HsGZMH	K	F	K	T	G	G	Chymase locus	Chymase	F/Y
BtGZMH	T	F	K	T	G	G	Chymase locus	*Chymase	*F/Y
SsGzmH	M	I	K	T	G	G	Chymase locus	*Chymase	*F/Y
MmGZMC	T	R	E	A	G	Q	Chymase locus	Unknown	Unknown
MmGZMD	N	F	K	T	A	G	Chymase locus	Unknown	Unknown
MmGZME	N	F	K	T	A	G	Chymase locus	Unknown	Unknown
MmGZMF	N	N	S	S	G	G	Chymase locus	Unknown	Unknown
MmGZMG	N	G	E	A	G	G	Chymase locus	Unknown	Unknown
MmGZMN	S	H	K	A	V	G	Chymase locus	Unknown	Unknown
HsGZMM	R	L	K	A	S	P	Metase locus	Metase	M/L
MmGZMM	K	Y	K	A	S	P	Metase locus	Metase	M/L
BtGZMM	E	L	K	A	S	P	Metase locus	*Metase	*M/L

Based on data from (Odake et al., 1991; Kam et al., 2000), with additional data on bovine and pig in red from the present study. Models from which data derived in original analysis: The molecular model of GzmA was constructed based on the crystal structure of bovine trypsin and RMCPII (Murphy et al., 1988). The model for GzmB was based on structure of RMCPII (Murphy et al., 1988). GzmM was modelled based on the crystal structure of α-chymotrypsin (Smyth et al., 1996). P1 specificity from human granzymes is derived from (Mahrus and Craik, 2005). The potential enzymatic function and P1 specificity of cattle and pig granzymes are highlighted by an asterisk. The sequence residue numbering is based on the chymotrypsinogen numbering. Hs - *Homo sapiens*; Mm - *Mus musculus*; Ss - *Sus scrofa*; Bt - *Bos taurus*.

Generally, three residues at positions 189, 216 and 226 are responsible for the primary substrate specificity in serine proteases (Perona and Craik, 1995). The presence of a negatively charged residue Asp189 is of great importance in determining the primary substrate specificity to trypsin-like enzymes (Ruhlmann et al., 1973). This amino acid is positioned at the bottom of S1 pocket and electrostatically interacts with the basic residue at P1 in the substrate. Gly residues at positions 216 and 226 help to provide access of the bulky lysine or arginine in the substrate to Asp189. As cattle and pig granzyme A, O and K share the identical specificity-determining triplet (Asp189-Gly216-Gly226) with their human and mouse counterparts, these novel enzymes most likely have the trypsin-like specificity and prefer to cleave after Arg or Lys residues.

In chymotrypsin-like enzymes, the crucial role is shifted to the amino acid on position 226 that is situated at the back of the specificity pocket (Caputo et al., 1994; Edwards et al., 1999). This is associated with the absence of a disulfide bond in the vicinity of the enzyme active site, compared with other granzyme groups (Zamolodchikova et al., 2003). An uncharged amino acid is present at position 189 in all of chymotrypsin-like enzymes and it is observed in granzyme B and H in both cattle and pig. The small size of the uncharged residue Gly226, present in all chymotrypsins, is thought to permit bulky hydrophobic side chains to be accommodated and cleaved (Edwards et al., 1999). Granzyme H has no direct rodent counterpart but is reported to have the typical chymotrypsin-like activity to cleave after Phe or Tyr at P1. The presence of conserved amino acids at Thr189, Gly216 and Gly226 (S1 subsite) in cattle and pig granzyme H are predictive of chymotrypsin-like

activity. However, the existence of a positively charged residue, Arg226, in bovine granzyme B suggests a unique specificity for the negatively charged acidic side-chain of Asp in the substrate (Poe et al., 1991; Caputo et al., 1994; Waugh et al., 2000). Experiments in which point mutations were introduced into mouse granzyme B have shown that replacement of Asp226 with Gly226 causes loss of Aspase specificity but facilitates hydrolysis of hydrophobic substrates (Caputo et al., 1994). As the Asn189-Gly216-Arg226 amino acid triplet within the S1 pocket of cattle and pig granzyme B are highly conserved with those in human and mouse, these enzymes are predicted to act on substrates containing the acidic residue Asp in P1.

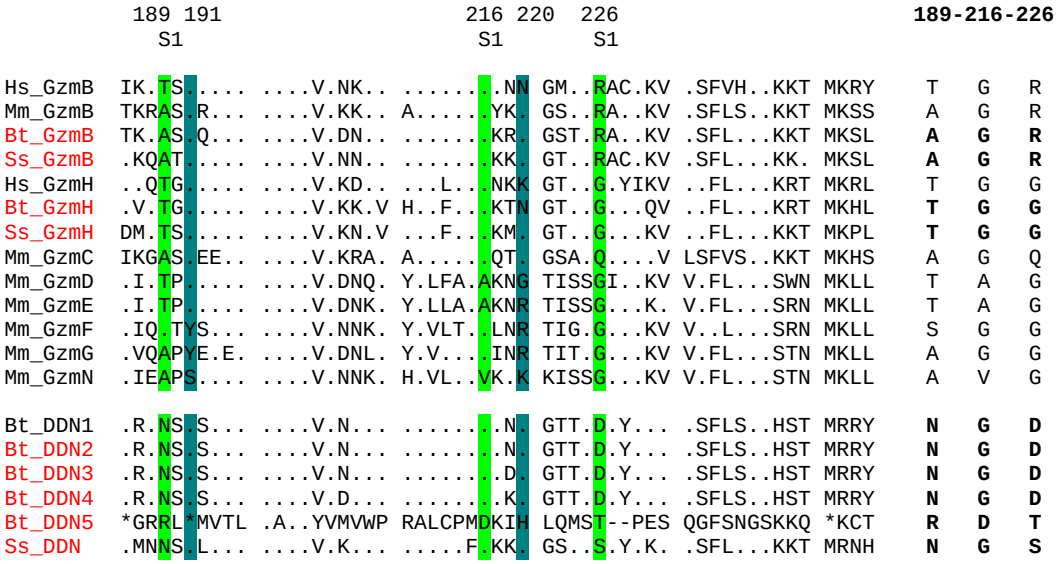
Structural studies of chymotrypsin A have indicated that the substrate specificity of granzyme M for the carboxyl terminal of long narrow hydrophobic amino acids, Met and Leu, is dependent on two key residues, Lys192 and Ser216. Replacement of Lys192 with Met and Ser216 with Gly in human granzyme M has been shown to result in a marked reduction in hydrolysis of substrates containing Met at P1, but the acquisition of chymase activity on Phe at P1 (Smyth et al., 1996). The presence of conserved amino acids Lys192 and Ser216 in cattle granzyme M indicates that it should have metase specificity similar to the human and murine counterparts.

Comparison of predicted functions of related genes in the chymotrypsin-like locus

The bovine chymotrypsin-like locus contains a number of genes in addition to granzymes. It includes the duplication of non-granzyme genes (a-chymase and cathepsin G) and also a series of closely related genes, encoding enzymes termed duodenases, not detected in humans or mice. They show high sequence similarity to

granzyme B (64-66%) and in early studies one of these genes (DDN1, identified by Zamolodchikova et al in 1995) was erroneously reported as granzyme B (NM_174296). Therefore, the primary structure of duodenases was examined to investigate their potential enzymatic specificities in comparison with those of the chymotrypsin-like granzymes. The amino acids found in the three key specificity-determining residues (189-216-226) in the S1 pocket of cattle and pig chymotrypsin-like genes are shown in figure 3.16.

Figure 3.16 Alignment of specificity-determining residues in the S1 pocket of chymotrypsin-like genes



Bovine and pig sequences are labelled in red. The triplet of S1 pocket residues at positions 189, 216 and 226 are highlighted in green and listed adjacent to the sequences to the right of the figure. Chymotrypsinogen numbering is used to refer to residue positions. Hs - *Homo sapiens*; Mm - *Mus musculus*; Ss - *Sus scrofa*; Bt - *Bos taurus*.

DDN1 was found to have unique amino acids Asn189 and Asp226 at two of the primary specificity determining residues in the S1 pocket. The presence of these amino acids suggests that DDN1 has both trypsin-like and chymotrypsin-like activities (Zamolodchikova et al., 1995a; Zamolodchikova et al., 1995b); the uncharged residue Asn at position 189 indicates chymotrypsin-like specificity while the negatively charged Asp at position 226 indicates acquisition of trypsin-like activity (Zamolodchikova et al., 1995b). The same structure-function relationship of duodenase has been reported for sheep mast cell protease (Smcp-1) (McAleese et al., 1998). As the other functional duodenases DDN2-4 have the identical triplet (N-G-D) with DDN1 and sheep Smcp-1, these novel enzymes should exhibit similar specificity. In contrast, pig DDN has an uncharged residue Ser at position 226, suggesting that it does not have trypsin-like specificity. Overall, the duodenase genes in cattle, sheep and pig display similar key residues in the S1 pocket and are likely to have similar enzymatic activity. On the other hand, their functional sites show clear differences from granzyme proteins, indicating that these proteins have distinct biological functions.

3.4 Discussion

The studies reported in this chapter have identified six classes of granzymes in cattle, A, O, B, K, H and M, based on analysis of genome and EST databases. Specific PCR protocols have been developed and optimised for all of these granzymes. Analysis of cDNA from un-cloned *T. parva*-specific CD8+ T cell lines using these PCR assays showed that all of the enzymes are expressed at the RNA level in activated CD8+ T cells. Consistent with findings in humans and mice, the granzyme-encoding genes were located on 3 different loci within the genome, which correspond to different proteolytic enzymatic activities, namely trypsin-like, chymotrypsin-like and metase. Analysis of primary amino acid sequences suggested that the granzyme proteins have enzymatic specificities similar to their human and murine counterparts.

The identified granzymes included a novel functional granzyme, termed granzyme O, not previously reported among the granzymes described in any species. A functional orthologue was also identified in the pig and non-functional/truncated genes were present in human and mouse, indicating that this novel gene evolved by gene duplication before the human-mouse-cattle-pig species divergence and subsequently became non-functional in humans and mice.

Annotation of the genome loci containing the bovine granzyme genes has demonstrated that the gene organisation is essentially identical to that in human, mouse and pig. In each species, the genes are found in three distinct chromosomal locations, each of which has similar gene organisation across the species (Table 3.9).

Analysis of phylogenetic trees has revealed the homologous relationship between each subgroup of cattle and pig granzyme genes and their human and mouse counterparts. Comparison of mature protein sequences of all granzymes from human, mouse, cattle and pig has shown obviously higher levels of identity within each group between species (45%-91%) than between the groups (Up to 44%). All of these findings suggest that the granzyme family of genes in human, mouse, cattle and pig have evolved from common ancestors, and that differences in gene complement have arisen mainly due to differential deletion or loss of function. Thus, unlike cattle and pigs, humans and mice have lost granzyme O and, in contrast to the other species mice have lost granzyme H. In place of the latter, mice have an additional set of genes encoding granzyme C, D, E, F, G and N, which although no remnants are found in other species may also have been lost in a common ancestor of these species by a gene deletion event.

The gene content and organisation of the trypsin-like and metase loci were similar to those in humans and mice, although a cluster of elastase genes and their flanking genes in the human and mouse metase locus were not identified in cattle. The latter may be due to incomplete or incorrect genome assembly. However, the findings indicate conservation of these loci following species divergence. In contrast, the chymotrypsin-like locus has expanded in gene content due to duplication of non-granzyme genes (a-chymase and cathepsin G) and the presence of an additional set of closely related genes (duodenases), not detected either in the chymotrypsin locus or elsewhere in the human or mice genomes. Identification of a duodenase orthologue in pig has shown that the duodenase is not exclusively expressed in ruminant,

suggesting either that these may have been lost in human and mice or that they evolved later through gene duplication. These findings together with the expanded chymotrypsin-like locus of mice including the presence of Mcpt8 family and multiple β-chymases and granzymes in mouse (Gallwitz and Hellman, 2006), illustrate a massive change undertaken in this locus after human, mouse, cattle and pig divergence.

Table 3.9 Summary of granzyme genes found in different mammalian species and their genomic locations

Chromosomal localization	Species	Granzymes
The 'tryptase' locus		
5q11-q12	Human	A and K
13D	Mouse	A and K
20	Cattle	A, O and K
16	Pig	A, O and K
The 'chymase' locus		
14q11-q12	Human	B and H
14D	Mouse	B, C, D, E, F, G and N
21	Cattle	B and H
7	Pig	B and H
The 'Met-ase' locus		
19p13.3	Human	M
10q21.2	Mouse	M
7	Cattle	M
2 ?	Pig	M

Data on human and mouse granzymes was extracted from (Trapani, 2001). Pig granzyme M has not been detected but assembly of the region on chromosome 2 predicted to contain the gene is incomplete.

Analysis of primary amino acid sequences of bovine granzymes in relation to known crystal structures of human or murine orthologues by homology molecular models allows us to investigate the corresponding substrate specificity of granzymes within each group. These initial analyses of amino acid sequences of cattle granzymes

indicate that they have primary specificities comparable with their human and mouse counterparts, but their extended substrate specificities appear to show some differences.

To date, crystal structures of human granzyme A, B, Pro-K and M have been reported by several groups but structures for K and H have not been described so far (Estebanez-Perpina et al., 2000; Hink-Schauer et al., 2002; Bell et al., 2003; Hink-Schauer et al., 2003; Wu et al., 2009). The crystal structures, coupled with results of biological experiments and structural modelling, provide information on residues that are critical for interacting with the substrates. For example, a crystallography study of granzyme A by Bell (2003) has shown that the specificity for a basic residue of the S1 pocket is primarily due to the side chain of Asp189 at the bottom of the pocket (Bell et al., 2003), whereas the primary specificity of granzyme B for Asp occurs through a salt bridge with Arg226, anchored at the back of the S1-specificity pocket of granzyme B (Estebanez-Perpina et al., 2000; Waugh et al., 2000). Crystal structure comparison of the active form of granzyme M with the inactive Asp86Asn-GzmM mutant has revealed that a peptide loop (L3 - residues 214-226) is most important in determining substrate specificity M (Wu et al., 2009). Mahrus and Craik (2005) provided experimental evidence to support the respective substrate specificities of human granzyme A, B, K, H and M, using positional scanning of synthetic combinatorial libraries of 4-amino acid peptides (Mahrus and Craik, 2005). The results of these studies suggest that homology molecular modelling is a reliable and productive method for identifying differences in fine substrate specificity of related granzymes and novel orthologues of the same granzyme. Comparison of the

substrate-determining amino acid residues in bovine granzymes with those in orthologues in other species, predict that cattle granzymes have primary substrate specificities identical to their human and mouse counterparts. The findings indicate that the novel granzyme O is most likely to have trypsin-like activity, consistent with the specificity of the other granzymes (A and K) in the same gene locus. The trypsin-like specificity of granzyme A, O and K is also consistent with the idea that these genes arose by duplication before primate/rodent/cattle/pig species divergence. Although phylogenetic analysis shows that granzyme B and C are most closely related in mouse, as are granzymes B and H in human, cattle and pig, experiments have demonstrated that the mechanism of cell death induced by granzyme C is quite different from the classical apoptosis induced by granzyme B (Johnson et al., 2003). Although the enzymatic function of granzyme C is still unknown, these findings indicate that the substrate specificity of granzyme C is distinct from that of granzyme B.

Although both human granzyme A and K have the trypsin-like specificity characterised by cleavage after basic residues Arg or Lys (Mahrus and Craik, 2005), they display highly restricted substrate specificities that only partially overlap (Bovenschen et al., 2009). This was demonstrated by screening a library comprising 1,000 fully randomized 15-amino acid peptides; no clear consensus sequence was found near the cleavage site, although both granzymes were shown to prefer P1Arg (Bovenschen et al., 2009). Cattle and pig granzyme A, O and K are predicted to have the identical primary substrate specificity (trypsin-like activity). However, comparison of extended specificity-determining residues at S2 and S3 of granzyme A,

O and K in both cattle and pig (shown in table 3.8) revealed different amino acids both between granzymes and between species orthologues, indicating differences between species in the extended substrate specificities of these granzymes. Although human and mouse granzyme B exhibit high sequence similarity, including conserved primary substrate-determining residues, and both are aspases, they exhibit divergent substrate preferences. This is a consequence of differences in the residues that determine extended substrate specificity. Using a phage display substrate assay, human and mouse granzyme B were shown to have distinct substrate preferences at the key P4, P2 and P2' substrate residues because of the presence of divergent residues at the corresponding granzyme binding sites (Kaiserman et al., 2006). The detection of divergent amino acids in the S2 and S3 extended specificity-determining residues in cattle and pig granzyme B suggests that they may also have different substrate preferences than those of the human and mouse orthologues.

The bovine chymotrypsin-like locus contains a number genes closely related to granzymes, including genes encoding duodenases, which located adjacent to granzyme B and one of which (DDN1) was previously erroneously reported as granzyme B. However, comparison of their amino acid sequences revealed different primary specificity-determining residues in the S1 pocket predicted to result in enzymatic functions distinct from granzyme B. Compared to the aspase activity of granzyme B, comparison with other chymotrypsin-like proteins indicates duodenase genes are likely to have dual specificity with trypsin-like as well as chymotrypsin-like activity. DDN1 was originally isolated from duodenal mucosa (Antonov et al., 1992; Zamolodchikova et al., 1995a). It is found in epithelial cells and the ducts of

Brunner's gland and is a potential activator of enteropeptidase, as indicated by its ability to hydrolyse recombinant bovine pro-enteropeptidase, which is an important enzyme in the digestive protease cascade (Zamolodchikova et al., 1997).

In conclusion, work described in this chapter has identified the complement of functional granzyme genes in cattle, developed PCR assays to examine their expression and demonstrated that they are all expressed in activated antigen-specific CD8+ T cells. Comparisons of their amino acid sequences with orthologues in other species have identified differences in residues that determine extended substrate specificities, suggesting that they differ in their protein substrate specificities. These findings provide the basis for further work to examine their role in killing of target cells by CD8+ T cells.

Chapter 4 Expression and characterization of bovine granzyme B

4.1 Introduction

Cell-mediated killing by cytotoxic T-lymphocytes (CTL) is an important immune response against viral infection, intra-cellular bacteria and neoplastic cells. One important mechanism used by CTL is induction of apoptosis of target cells by granule exocytosis, involving release of several toxic granzymes and a membrane disrupting protein perforin. Granzyme B is the best-characterized and most potent member of the granzyme family and is considered to be the major initiator of granule-mediated apoptosis. This protease induces target cell apoptosis by several pathways, including mitochondrial-mediated mechanisms, via direct cleavage of 'BH3-only' members of the Bcl-2 family, by enzymes such as BH3-interaction domain death agonist (Bid), which result in mitochondrial permeabilization (Heibein et al., 2000; Alimonti et al., 2001; Pinkoski et al., 2001; Sutton et al., 2003), activation of caspases (Darmon et al., 1995; Martin et al., 1996; Quan et al., 1996) and direct cleavage of downstream substrates (Andrade et al., 1998; Casciola-Rosen et al., 1999; Browne et al., 2000; Thomas et al., 2000; Sharif-Askari et al., 2001; Adrain et al., 2006). As granzyme B is highly active, expression of this molecule is regulated to avoid enzymatic activity during the post-translational modification process. Typically, granzyme B is translated as an inactive zymogen containing a leader peptide of 18 amino acids, followed by a conserved dipeptide/GE. Activation

of the zymogen requires cleavage of the dipeptide/GE by dipeptidyl peptidase I/cathepsin C within the granules (McGuire et al., 1993).

Granzyme B is a 32-kDa serine protease with a structure closely resembling that of chymotrypsin and characterized as an Aspase because of its preference for substrate cleavage after aspartic acid residues (Poe et al., 1991). Granzyme B is the only known serine protease with this specificity in mammals (Atkinson and Bleackley, 1995), although cleavage after aspartic acid residue also occurs in cysteine proteases of the ICE family (caspases) (Duan et al., 1996), which are inactive zymogens under normal cellular conditions but can be activated during apoptosis. The preferred substrate sequence at the P4-P3-P2-P1 (amino-terminal to the proteolytic cleavage site) for human granzyme B cleavage is I-E-P-D defined by a combinatorial chemistry approach (Thornberry et al., 1997), but optimal substrate recognition also involves features extending carboxyl terminal to the proteolytic cleavage, P1'-P2'-P3'-P4'(Waugh et al., 2000; Rotonda et al., 2001; Sun et al., 2001).

Although native granzyme B from NK cell granules has been purified to homogeneity and characterized as an Aspase based on it activity on synthetic substrates, the purification procedure, involving biochemical and immunological methods, is time-consuming and has yielded limited amounts of active granzyme B (Odake et al., 1991; Poe et al., 1991; Trapani et al., 1993). As other members of the chymotrypsin-like subfamily (granzyme H and other hematopoietic serine proteases such as mast cell chymase and neutrophil cathepsin G) are of similar size and exhibit similarity in amino acid sequence (50-70%), they may contaminate preparations of

native granzyme B. However, studies by Caputo et al in 1993 and Smyth et al in 1995 have enabled a system to produce this enzymatically active granzyme B by preparation of recombinant DNA constructs that can be expressed in mammalian COS-7 cells (Caputo et al., 1993; Smyth et al., 1995). In this eukaryotic expression system, deletion of nucleotide sequences encoding an amino-terminal dipeptide is required because of the absence of cathepsin C in COS-7 cells (Caputo et al., 1993; Smyth et al., 1995).

This chapter describes the preparation of recombinant cDNA constructs of cattle granzyme B for production of functionally active protein, and testing of the enzymatic activity and substrate specificity of the protein. The reactivity of six anti-monoclonal antibodies raised against equine granzyme B (eqGzmB) was also examined.

4.2 Materials and Methods

4.2.1 Preparation of cDNA

Total RNA was extracted from an un-cloned *T.parva*-specific CD8+ T cell line from the cow 011 and cDNA was synthesized from RNA using the Reverse Transcription system as described in section 2.3.1- 2.3.3.

4.2.2 Site-directed mutagenesis strategies

4.2.2.1 Wild-type cattle granzyme B cDNA

Wild-type cattle granzyme B cDNA was amplified by High fidelity PCR, using primers flanking the full-length cattle granzyme B coding sequence as follows:

5'- CATCCTGGGCAGTCTTTCTA-3' (Forward);

5'- CCTGCAGTGTGATTCTGGAT-3' (Reverse).

The High fidelity PCR protocol was composed of 10pmol of primers, 1.2 unit *Pfu* DNA polymerase (3units/ul, Promega), 5ul of 10x Buffer with $MgSO_4$ (200nM Tris-HCl, 100mM KCl, 100mM $(NH_4)_2SO_4$, 20mM $MgSO_4$, 1mg/ml nuclease-free BSA and 1% Triton X-100, Promega), 10mM dNTP, 0.5ug of cDNA template and nuclease-free water to give a final volume of 50ul. The programme used was as follows: 95°C for 2 min, 30 cycles of 95°C for 1min followed by 55°C for 0.5 min and 72°C for 2.5 min, and a final extension period of 72°C for 5 min. The PCR product was analysed by electrophoresis as described in section 2.3.5.

4.2.2.2 Cloning of the purified cDNA

Blunt-ended PCR products of the predicted size were purified (section 2.3.6) and then a single adenosine was added to the 3'-ends of a double stranded DNA molecule (Zhou and Gomez-Sanchez, 2000). The TA cloning reaction was composed of 0.5unit *BIOTAQ* (5 units/ul Bioline, London, UK), 1ul SM-0005 buffer (Appendix C.5), 4ul of purified DNA (30ng/ul), and nuclease-free water to give a final volume of 10ul. The reaction was incubated at 72°C for 30 min and then transferred to ice.

The A-tailed granzyme B cDNAs were subcloned into a linearized T-vector which has single 3'-T overhangs on both ends (section 2.3.7). The plasmid DNA was extracted from selected colonies and purified using the Wizard Mini-preps DNA Purification System (section 2.3.8). To identify the presence of inserts, the plasmid DNA was digested with *EcoR1* (section 2.3.9). Plasmid products purified from clones containing inserts were submitted to DBS Genomic (Durham University) for nucleotide sequencing (2.3.10).

4.2.2.3 Dipeptide/GE knockout of cattle granzyme B cDNA

The deletion of six nucleotides encoding the dipeptide of cattle granzyme B cDNA was performed by PCR splice overlap extension (PCR-SOE) based on procedures described for human granzyme B (Smyth et al., 1995), as discussed by Ling and Robinson in an overview of DNA mutagenesis (Ling and Robinson, 1997).

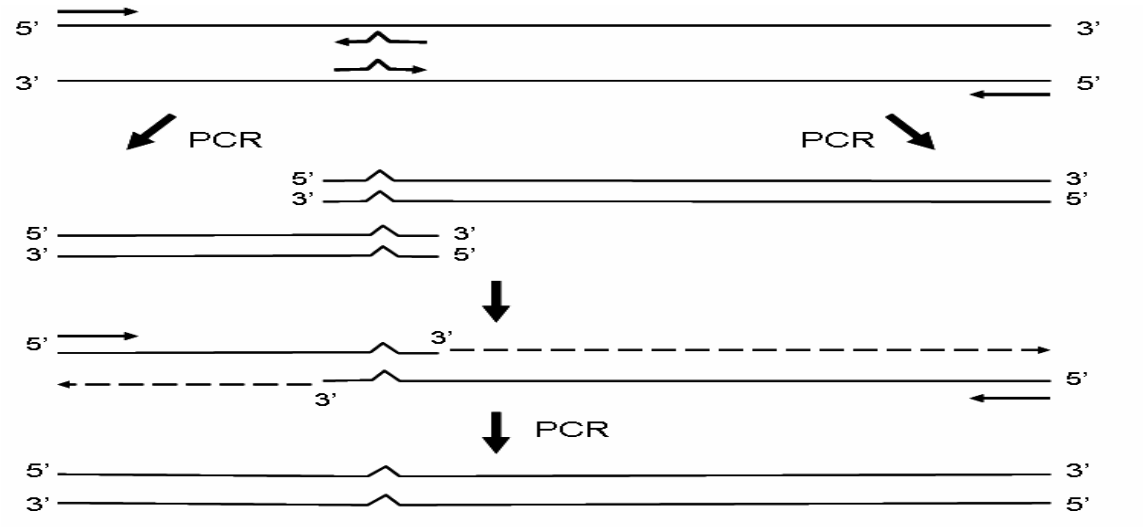


Figure 4.1 Schematic illustration of the "overlap extension PCR" for introducing a deletion into granzyme B cDNA.

Two PCR assays were initially performed to generate two overlapping fragments that carry the 6 nucleotide deletion in the overlapping segment. These two fragments were mixed, denatured and annealed to produce mutant DNA templates and amplification of the extended mutant DNAs was performed with flanking primers in a further PCR.

Principle:

The scheme for generating the dipeptide-deleted mutant is summarised in figure 4.1. Firstly, two PCRs were performed to generate two DNA fragments with overlapping sequence containing the target site for mutagenesis. Forward and reverse mutagenic internal primers extending across the site and from which the 6 nucleotides had been omitted, were used. The sequences of these internal primers for cattle granzyme B were as follow: 5'- CAAAGGCAATCATCGGGGGCCATG-3' (Forward); 5'-CCCGATGATTGCCTTTGCCCTGGG-3' (Reverse). External primers flanking the full-length coding region of cattle granzyme B were as follow: 5'-CATCCTGGGCAGTCTTTCTA - 3' (Forward); 5'-

CCTGCAGTGTGATTCTGGAT - 3' (Reverse). The PCR reaction was composed of 10 pmol of primers, 1.2 unit *Pfu* DNA Polymerase (3units/ul, Promega), 5ul of 10x Buffer with $MgSO_4$, 10mM dNTP, 0.5ug of cDNA template and nuclease-free water to give a final volume of 50ul. The programme used was as follows: 95°C for 2 min, 30 cycles (95°C for 1min, 45°C for 0.5 min, 72°C for 2.5 min) and a final extension period of 72°C for 5 min. The PCR products were analysed by electrophoresis as described in section 2.3.5.

Secondly, PCR products of the two overlapping DNA fragments were purified, mixed, denatured and annealed in a PCR-ready buffer to produce mutant DNA templates. Amplification of the extended mutant DNAs was achieved by using the pair of external primers in a further PCR. The PCR reaction was composed of 10 pmol of primers, 1.2unit *Pfu* DNA Polymerase (3units/ul, Promega), 5ul of 10x Buffer with $MgSO_4$, 10mM dNTP, 5ng of each purified DNA fragments and nuclease-free water, to give a final volume of 50ul. The programme used was as follows: 95°C for 2 min, 30 cycles (95°C for 1min, 45°C for 0.5 min, 72°C for 2.5 min) and a final extension period of 72°C for 5 min. The PCR product was analysed by electrophoresis as described in section 2.3.5. PCR products of the predicted size were purified, TA cloned and sub-cloned into the T-vector as described above in section 4.2.2.2.

4.2.2.4 Introduction of a Ser195Ala substitution at the active site in the Dipeptide/GE knockout granzyme B cDNA

Substitution of Ser with Ala at the active site of dipeptide-knockout cDNA was performed by 'megaprimer' PCR mutagenesis (Picard et al., 1994; Tyagi et al., 2004), as summarised in figure 4.2.

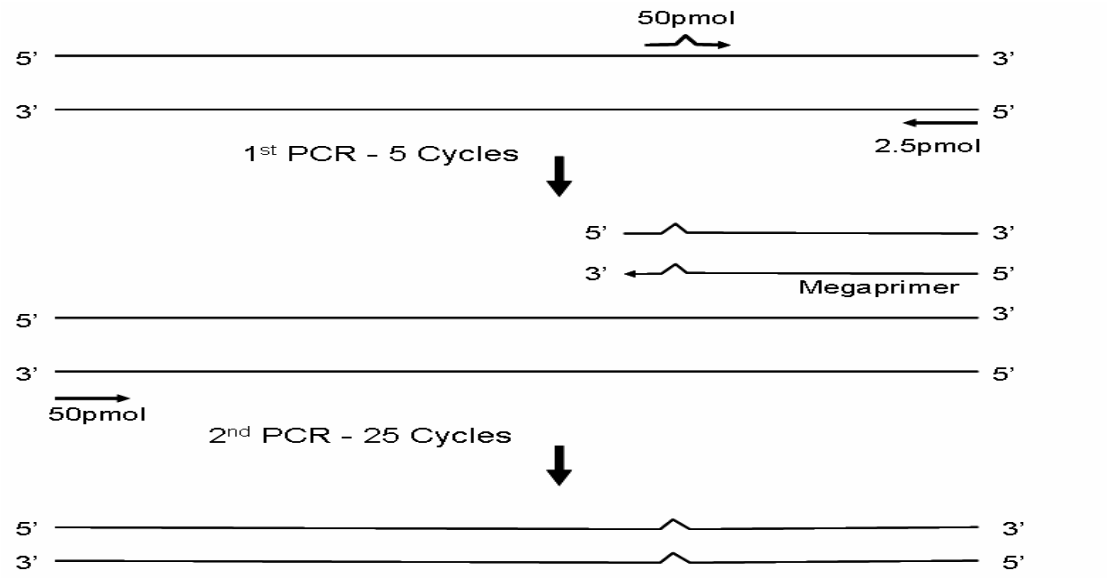


Figure 4.2 Schematic representation of the "megaprimer PCR" for introducing a mutation into granzyme B DNA.

An initial 5 cycles of a PCR reaction containing a standard concentration of mutagenic internal primer and a limiting concentration of a flanking primer was followed by a prolonged extension step to generate mutant mega fragments. The other flanking primer was added to the mutant templates and the PCR reaction subjected to a further 25 cycles to generate full-length product containing the mutation.

Principle:

This procedure is based on first introducing the desired mutation into a terminal fragment of the cDNA (in this case the 5' end) in an initial PCR reaction and then using this fragment as a megaprimer in a second PCR reaction to obtain the mutated

full-length cDNA. The first round of this ‘Megaprimer’ PCR protocol uses a limiting concentration of the standard unmodified reverse primer in combination with a standard concentration of an internal mutagenic forward primer incorporating substitutions to achieve the amino acid change. The PCR protocol incorporates a prolonged additional extension period.

Two different internal mutagenic forward primers incorporating the mutation were designed and tested in the first step due to the GC rich nature of the primer binding site; their sequences were:

Primer1, 5’-AGAAAGCTTCCTTTCAGGGGGACGCGG-3’; or Primer2, 5’-ACGCGGGGGGCCCTCTCGTGTGTGACAAT-3’; these were used with the reverse primer 5’- CCTGCAGTGTGATTCTGGAT-3’; The PCR reaction was composed of 50pmol of internal mutagenic forward primer, 2.5pmol of external reverse primer, 1.2 unit *Pfu* DNA Polymerase (3units/ul, Promega), 5ul of 10x Buffer with $MgSO_4$, 10mM dNTP, 1ul of dipeptide-knockout cattle granzyme B cDNA containing plasmid (60ng/ul) and nuclease-free water to give a final volume of 50ul. The first round PCR programme used was as follows: 95°C for 2 min, 5 cycles (95°C for 1min, 55°C for 0.5 min, 72°C for 2.5 min) and an additional extension period of 72°C for 35 min.

In the second round of PCR, 50pmol of the external forward primer, 5’-CATCCTGGGCAGTCTTTCTA - 3’ was added into the same tube and the programme was resumed under the following conditions: 95°C for 2 min, 25 cycles (95°C for 1min, 55°C for 0.5 min, 72°C for 2.5 min) and a final extension period of 72°C for 10 min. The PCR product was analysed by electrophoresis as described in

section 2.3.5. PCR products of the predicted size were purified, TA cloned and sub-cloned into the T-vector as described above in section 4.2.2.2.

4.2.3 Mammalian expression vector construction

4.2.3.1 PCR amplifications of Granzyme B cDNAs lacking a stop codon (TGA)

Forward and reverse PCR primers were designed to incorporate unique *SalI* and *BamH1* sites on the ends of the functional granzyme B cDNA and the corresponding controls and to eliminate the 3' stop codon, in order to express the protein with a c-terminal peptide tag. The forward primer containing six nucleotides encoding the SalI restriction site and the reverse primer containing six nucleotides encoding a BamH1 site with a stop codon (TGA) deletion were as follows:

5'- CCAGGTCGACCATCCTGGGCAGTCTTTCTA-3' (*SalI*, Forward); 5'-CCTGGGATCCGAGGCTTTTCATGGTTTTCTTTATCCAGGGC-3' (*BamH1*, Reverse). These primers were used in a high fidelity PCR protocol composed of 10pmol of primers, 1.2 unit *Pfu* DNA Polymerase (3units/ul, Promega), 5ul of 10x Buffer with $MgSO_4$, 10mM dNTP, 1ng of plasmid templates and nuclease-free water to give a final volume of 50ul. The programme used was as follows: 95°C for 2 min, 30 cycles (95°C for 1min, 55°C for 0.5 min, 72°C for 2.5 min) and a final extension period of 72°C for 5 min. The PCR product was analysed by electrophoresis as described in section 2.3.5.

4.2.3.2 Incorporation of granzyme B cDNAs into the pFLAG-CMV^{tm}-5a expression vector

PCR products of the predicted size were purified and double digested by using restriction endonuclease reactions (BioLabs Inc, New England) to generate the *SalI* and *BamH1* sticky-end cDNAs. The reaction was composed of 1ug DNA, 5ul of 10x NEBuffer 3, 0.5ul of 100x BSA, 2ul *SalI* (20,000 units/ml), 2ul *BamH1* (20,000 units/ml) and nuclease-free water to give a final volume of 50ul. The reaction was incubated at 37°C for 3h and stored at -20°C. The pFLAG-CMV^{tm}-5a expression vector (Sigma, Cat.E7523) was double digested with *SalI* and *BamH1* and the restriction endonuclease reaction was performed at 37°C for 3h.

Double digested cDNAs and pFLAG-CMV^{tm}-5a expression vector of the expected size were gel purified and ligated together by using T4 DNA ligase. The reactions were composed of 50ng modified pFLAG-CMV^{tm}-5a expression vector, 26ng sticky-end granzyme B cDNA, 1ul T4 DNA ligase (3 weiss units/ul), 5ul 2x Rapid ligation Buffer and DDW to give a final volume of 10ul. The reaction was performed at 4°C overnight. The products were then transferred into JM109 High Efficiency Competent Cells and the cells were plated onto duplicate LB/ampicillin plates. The plates were incubated overnight at 37°C.

Colonies containing appropriate inserts were identified by PCR amplification with primers specific for flanking vector sites. Selected colonies were picked and streaked onto new LB agar plates with 100ug/ml ampicillin. The plates were incubated for 4h or overnight at 37°C. The expanded colonies were harvested, added into 96-well V

bottom plates with 50ul of nuclease-free water and denatured by heating at 95°C for 5 min. The cellular debris were sedimented by centrifugation at 13,000 x g for 5 min and the aqueous phase containing plasmid DNAs was used as template in further PCR amplification. The primers flanking the multiple cloning region of the pFLAG-CMV^{tm}-5a expression vector were as follows:

5'-AATGTCGTAATAACCCCGCCCCGTTGACGC- 3' (N-CMV-30, Forward);

5'-TATTAGGACAAGGCTGGTGGGCAC-3'(C-CMV-24, Reverse);

Annealing of the primers was performed at 55°C. The reaction and programme were performed as described in section 2.3.4 and PCR products were analysed by electrophoresis as described in section 2.3.5.

The colonies containing inserts were selected from the streaked plates and each transferred into a 30 ml flask with 6ml LB and 100ug/ml ampicillin. The clones were expanded overnight (section 2.3.7) and the plasmid DNA was purified (section 2.3.8) and sent to DBS Genomic (Durham University) for sequencing (section 2.3.10)

4.2.3.3 Plasmid DNA Preparation-(Maxi-preps)

The EndoFree Plasmid Maxi Kit (QIAGEN, Cat.12362) was used to prepare the purified Maxi-preps. Colonies containing inserts of the correct sequence and orientation were picked from the streaked plate, added into a starter culture of 5ml LB with 100 ug/ml ampicillin and incubated for 8h at 37°C with vigorous shaking at 300rpm. The starter cultures were then added into 150ml fresh LB with 100 ug/ml ampicillin and incubated overnight for 37°C with vigorous shaking at 300 rpm. The bacterial cells were pelleted by centrifugation at 6000 x g for 15 min at 4°C,

suspended in 10ml buffer P1 with 100ug/ml RNase A, lysed by the addition of 10ml Buffer P2 at room temperature for 5 min and precipitated by the addition of 10ml chilled Buffer P3. The lysates were immediately poured into the barrel of a QIAfilter Cartridge at room temperature for 10min, filtered into a 50ml tube by gently inserting the plunger into the QIAfilter Maxi Cartridge and then mixed with 2.4ml Buffer ER for 30min on ice. Following equilibration with 10ml Buffer QBT and addition of filtered lysates by gravity flow, a QIAGEN-tip 500 was washed twice in 30ml Buffer QC. DNA was eluted in 15ml Buffer QN and precipitated in 10.5ml isopropanol at room-temperature by centrifugation at 15,000 x g for 30min at 4°C. The DNA pellet was washed with 5ml of endotoxin-free 70% ethanol at room temperature, centrifuged at 15,000 x g for 10min and air-dried for 20min, re-dissolved in 500ul of endotoxin-free Buffer TE and stored at -20°C until use.

4.2.4 Cell culture and transfection

4.2.4.1 Growth of COS cells and 293T cells

COS cells and 293T cells were maintained in the culture medium containing DMEM (Invitrogen) supplemented with 10% FCS, $5x10^{-5}$M 2-Mercaptoethanol, 4mM glutamine, 100U/ml penicillin and 100ug/ml streptomycin, passaged every 3-4 days by diluting 1:5 in the culture medium and cultured at 37°C under 5% CO_2.

To remove COS cells or 293T cells adherent to the bottom of 75cm^2 flasks, the cells were gently washed with 10ml 1x PBS/EDTA (Appendix C.11) and the washing medium was discarded. Following addition of 9ml 1x PBS/EDTA and 1ml of 0.25%

Trypsin-EDTA (Invitrogen) at 37°C for 5 min, the COS cells or 293T cells in suspension were harvested into 15ml Falcon tubes, centrifuged at 1200 x g for 5min and suspended in 5ml culture medium. One ml of cell suspension was added to 15ml fresh culture medium in a 75mm flask and maintained at 37°C under 5% CO_2.

4.2.4.2 Transient transfection in COS cells

COS cells ($3x10^6$-$4x10^6$) in 20ml culture medium without antibiotics were seeded in a $75cm^2$ flask one day before transfection. The transient transfection was performed by using the Lipofectamine^{tm} 2000 reagent (Invitrogen, Cat.11668-027) according to the manufacturer's protocol. The pFLAG-CMV^{tm}-5a vector only(60ug) and vectors containing three cattle granzyme B recombinant cDNAs containing the wild type (60ug), the dipeptide knockout (60ug) and the knockout with an additional Ser195Ala substitution (40ug) were firstly diluted in 2ml Opti-MEN I Reduced Serum Medium without serum (Invitrogen) and mixed gently. Then 80ul Lipofectamine^{tm} 2000 was diluted in 2ml Opti-MEN I Reduced Serum Medium without serum, mixed gently and incubated for 5min at room temperature. The diluted DNA and Lipofectamine^{tm} 2000 were combined together and incubated for 20min at room temperature to form the DNA- Lipofectamine^{tm} 2000 complexes. 4ml of complexes were then added to a $75cm^2$ flask containing cells at 90-95% confluency and the complexes removed by replacing the culture medium without antibiotics 4-6h after transfection. Cells were cultured at 37°C under 5% CO_2 for 48h to allow expression of the transgene. Transfected cells were harvested after 48h, washed and suspended in cold PBS (Appendix C.12) and analysed in further experiments.

4.2.4.3 Detection of transfection efficiency

Cytospin smears of cells were examined to test for expression of transfected DNA products. Aliquots of 150-200ul of PBS-washed cells ($8x10^4$) in PBS were added into a cytospin cuvette with cleaned glass slides, spun for 5 min at 800rpm in a Shandon Cytospin 3 Centrifuge (Block Scientific, Inc) and the resultant smears air-dried for 5-10 min. Cells were then fixed with acetone for 5-10 min, air-dried for 5-10 min and the location of the cytocentrifuged cells marked by a Liquid Blocker Super Pap Pen. Following addition of 50ul anti-FLAG M2 antibody (1:500 dilution; IgG1; Sigma) cells were incubated for 20 min at room temperature in a moist atmosphere and washed three times with PBS. 50ul FITC-conjugated anti-mouse polyvalent immunoglobulins (G,A,M) antibody diluted 1:100 (Sigma) was added and cells were incubated for 20 min at room temperature in a moist atmosphere and then washed three times with PBS. Following removal of excess PBS, slides were mounted with the DakoCytomation Fluorescent Mounting Medium (DakoCytomation). Duplicate slides were examined on a Leitz Laborlux S microscope (Leica Mikroskopie und Systeme GmbH, Germany) and images recorded using a DXC-390P 3CCD Color Video camera (Sony Corporation, Japan) and analysed using the Scion Image 1.62c software (Wayne Rasband, National Institutes of Healthe, USA).

4.2.5 Production of cell lysates

The cell lysis protocol was performed as described by Piuko et al. (2007) for equine granzyme B. Aliquots of 1ml of PBS-washed cells adjusted to $2x10^6$cells/ml in PBS were centrifuged at 180 x g for 10 min and the pellet was lysed by using 0.2ml lysis buffer (1%Triton X-100, 50mM Tris, pH8.0 and 2ul of Benzonase Nuclease 25U/ml, Purity>99%, Merck). Following incubation on ice for 20min, lysed cells were centrifuged for 10min at 0°C at 21,000 x g to pellet cell nuclei and other cell debris. Supernatants were harvested and either stored at -70°C or immediately subjected to activity assay.

4.2.6 Assay of protease activity

The protease activity protocol was performed as described by Piuko et al. (2007) for equine granzyme B. In duplicate, aliquots of 25ul of lysis supernatant, granzyme B substrate VIII, Ac-IEPD-pNA, (Calbiochem, Cat.368067) in a final concentration of 300uM and the reaction buffer (0.1M Hepes, pH 7.0; 0.3M NaCl; 1mM EDTA) in a total volume of 250ul/well were added into the wells of Falcon™ 96-Well Flat bottomed Microplates (BD, Cat. 353072). The chymotrypsin substrate I, Suc-GGF-*p*NA (Calbiochem, Cat.230912), was used as a negative control for the substrate specificity. The reaction was composed of 25ul of lysis supernatant, 1mM Suc-GGF-*p*NA in the final concentration and the reaction buffer (50mM Tris, 100mM NaCl, pH8.0) in a total volume of 125ul. Mixtures were incubated at 37°C for 4h and the colour reaction generated by cleavage of the pNA substrate measured at a wavelength of 405nm by using a Synergy™ HT Multi-Mode Microplate Reader (BioTek)

4.2.7 Inhibition of protease activity in lysates

The specific granzyme B inhibitor IV, Ac-IEPD-CHO (Calbiochem, Cat.368056) was tested for its ability to inhibit cattle granzyme B activity as described by Piuko et al. (2007) for equine granzyme B. In duplicate, aliquots of 25ul of lysis supernatant containing released bovine granzyme B were pre-incubated with 10uM Ac-IEPD-CHO at 37°C for 0.5h. Following the addition of 300uM granzyme B substrate VIII, Ac-IEPD-*p*NA and the reaction buffer (0.1M Hepes, pH7.0; 0.3M NaCl; 1mM EDTA), mixtures in a total volume of 250ul were incubated at 37°C for 4h and the colour reaction generated by cleavage of the pNA substrate measured at a wavelength of 405nm by using a Synergy™ HT Multi-Mode Microplate Reader (BioTek).

4.2.8 Cross-reactivity of antibodies specific for equine granzyme B

An expression construct containing human granzyme B cDNA, pcDNA3.1-hsGzmB, and six anti-eqGzmB monoclonal antibodies produced in mice were kindly provided by Dr. Martin Muller (Deutsches Krebsforschungszentrum, Heidelberg, Germany). First, 293T cells were transiently transfected with either cDNA3.1-hsGzmB (80ug) or Bt-pFLAG-SOE (60ug) using the Lipofectaminetm 2000 reagent as described in section 4.2.4.2 and following incubation for 48 hours, cytospin staining was

performed to determine the transfection efficiency and to test reactivity of the monoclonal antibodies as described above in section 4.2.4.3.

Following incubation of smears with the monoclonal antibodies (mAbs) (Table 4.1) they were washed and incubated FITC-conjugated anti-mouse polyvalent immunoglobulins (G, A, M) antibody diluted 1:100 (Sigma).

Table 4.1 Monoclonal antibodies used for staining cytospin preparations of 293T cells transfected with granzyme B constructs

Monoclonal Antibody	Specificity	Final Dilution	Undiluted concentration
XIXG7	EqGzmB, HsGzmB	1:50	0.93ug/ul
SZ	EqGzmB	1:10	ND[a]
8-5B	EqGzmB	1:10	0.57ug/ul
8D8	EqGzmB	1:10	0.56ug/ul
7-7D	EqGzmB	1:10	1.0ug/ul
108	EqGzmB	1:10	0.63ug/ul
Anti-pFLAG M2	pFLAG tag epitope	1:500	1mg/ml

[a] The concentration of monoclonal antibody SZ was not determined.

4.3 Results

4.3.1 Site-directed PCR mutagenesis

To obtain the functional recombinant granzyme B, deletion of an amino-terminal dipeptide is required (Caputo et al., 1993; Smyth et al., 1995). The splice overlap extension (SOE) PCR mutagenesis was developed to delete 6 nucleotides encoding the GE dipeptide to allow expression of the activated cattle granzyme B. Control constructs without the dipeptide deletion and with the deletion and an additional Ser195 to Ala substitution in the active site, generated by 'megaprimer' PCR mutagenesis, were also produced (Table 4.2). cDNAs prepared from a culture of *T. parva*-specific CD8+ T cells established from an animal that had been immunised by infection and treatment with *T. parva*, was used as the template.

Table 4.2 Bovine granzyme B cDNA constructs and predicted functional activities

cDNA type	**Structure**	**Predicted Function**
Wild-type (WT)	Not activated	No
Dipeptide/GE-deleted	activated	Yes
Dipeptide/GE-deleted with additional Ser195Ala substitution	Loss of activity	No

Wild-type granzyme B

The use of High fidelity PCR with flanking primers successfully generated a PCR product of the expected size (818bp) for the coding region of the wild-type granzyme B gene (Figure 4.3- A). To confirm the identity of the PCR product, it was sub-cloned into the pGEM-T vector and 3 clones containing inserts of the correct size, WT-1, 2 and 5, were selected for sequencing (Figure 4.3- B). Analysis of sequences obtained from WT-1 and 2 confirmed that they are identical to those originally identified from the bovine genome in section 3.3.1 of this study (Corr_ENSBTAG00000010057).

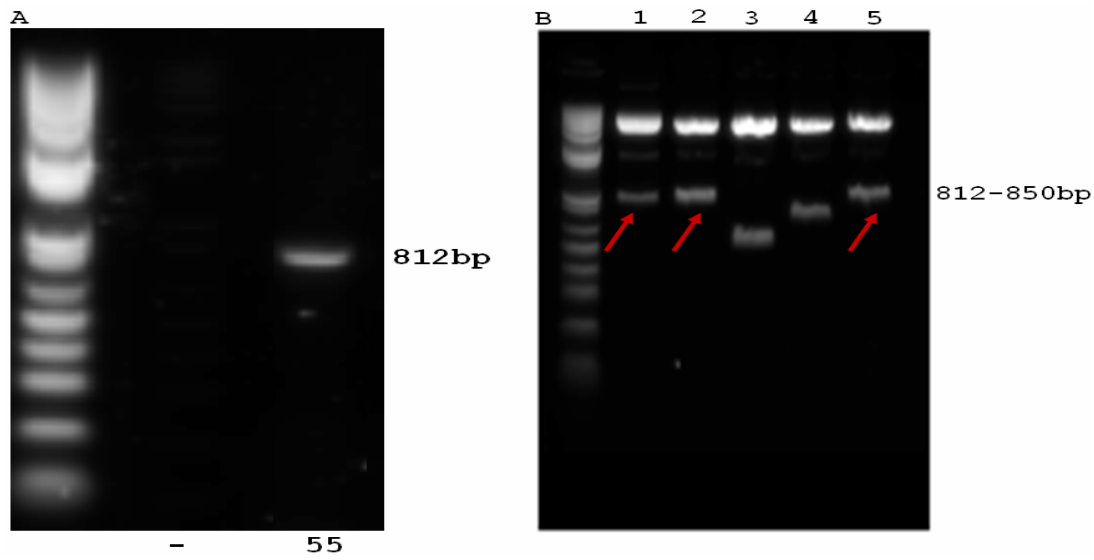


Figure 4.3 PCR amplification of wild-type bovine granzyme B cDNA.
(A). High fidelity PCR performed using proof reading taq (*Pfu*) with cDNA template of an uncloned *T. parva*-specific CD8+ T cell line (011) 7 day after 3rd stimulation (purity, 99%), yielded a product of the expected size (818bp) at an annealing temperature of 55°C. (B). Analysis of clones of the PCR product (1-5) revealing products of the expected size in 3 of the clones (1, 2 and 5).

Dipeptide/GE-deleted granzyme B

To complete the splice overlap extension (SOE) PCR mutagenesis, three individual high fidelity PCR assays were required. Two partially overlapping PCR fragments carrying the 6 nucleotide deletion in the overlapping segment were successfully produced using complementary internal primers each containing one end of flanking primer and the other end of internal mutagenic primer (Figure 4.4- A and B). Generation of the full-length mutated sequence required two further steps. First, the selected overlapping fragments were mixed, denatured and annealed to produce mutant DNA templates. In the second step, amplification of the extended mutant DNAs with flanking primers successfully generated a PCR product of the expected size (812bp) (Figure 4.4- C). To confirm the authenticity of the PCR products, purified PCR product was sub-cloned and 3 clones containing inserts of the correct size, SOE-2, 4 and 5, were selected for sequencing (Figure 4.4- D). Analysis of sequences obtained from SOE-4 and 5 confirmed that the 6 nucleotides encoding the dipeptide/GE were deleted and that the remainder of the sequence was identical to the wild-type granzyme B sequence.

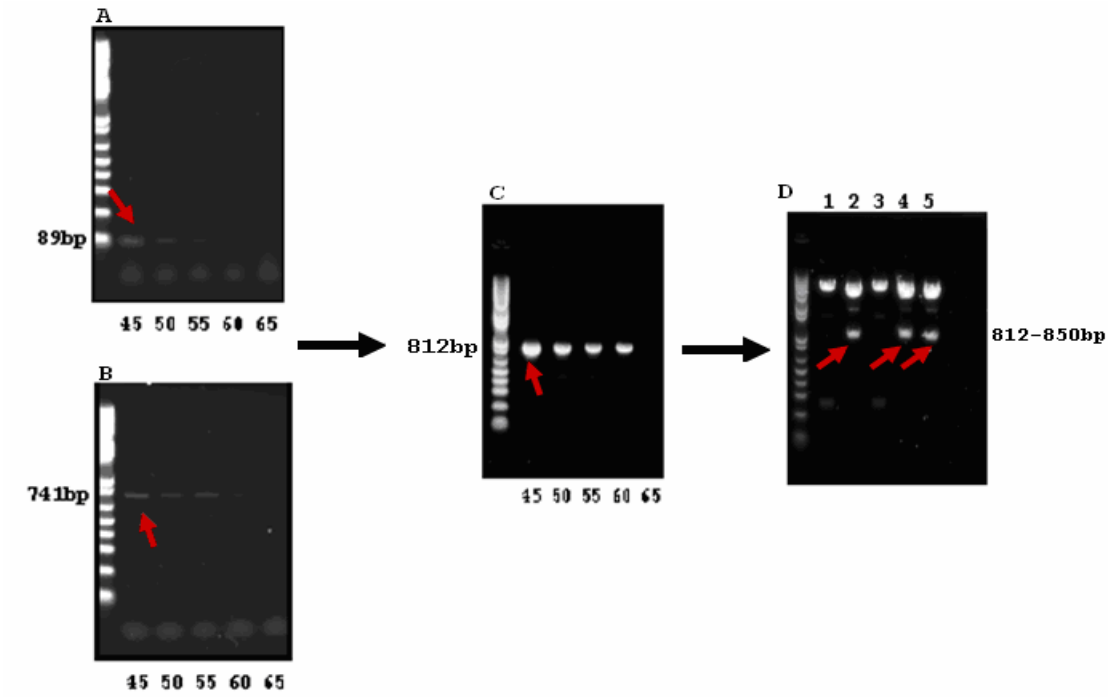


Figure 4.4 Amplification of dipeptide/GE deleted mutant of granzyme B cDNA by splice overlap extension PCR.

Results are presented for high fidelity PCR assays performed at a range of annealing temperatures (45-65°C) by using: (A). A forward flanking primer and the reverse internal mutagenic primer, giving a fragment of the expected size (89bp); (B). A forward internal mutagenic primer and the reverse flanking primer, giving a fragment of the expected size (741bp); (C). Use of flanking primers to amplify full length mutated cDNA, using a mixture of the products from (A) and (B) as template (expected size 812bp); (D). Analysis of subclones of the product obtained in step (C), revealing three clones containing inserts of the correct size (arrows).

Granzyme B with dipeptide/GE deletion and additional Ser195 to Ala substitution

Production of the dipeptide/GE knockout cDNA plasmid allowed further manipulation to generate a substitution of Serine with Alanine at the active site.

Using the SOE-4 clone as template, ‘Megaprimer’ PCR assays were performed by 2-round high fidelity PCR reactions in the single PCR tube. Because of the high GC content of the targeted sequence, two different internal mutagenic primers were tested (Primer1 and Primer2). Mega fragments of the expected size (213bp for primer1 and 194bp for primer2) were amplified with each of the forward internal mutagenic primers when used with a limiting concentration of reverse flanking primer during the first 5 cycles and completed by a prolonged additional extension time. Use of the mega fragment as the reverse primer and addition of the forward flanking primer in a second round PCR, produced a mutated product of the expected size (812bp) (Figure 4.5- A, Lane 2 and 4 Top). As only the 5’- 3’ chain of the mega fragments can be used as the reverse primer, the complementary mega fragments still remained in the mixture (Figure 4.5- A, Lane 2 and 4 Bottom). To confirm the identity of the PCR product obtained with both primer1 and primer2, they were sub-cloned and 8 clones containing inserts of the correct size, $SMA_{Primer1}$ -1, 2, 3 and 4 and $SMA_{Primer2}$ -2, 5, 12 and 8, were selected for sequencing (Figure 4.5- B). Analysis of sequences obtained from $SMA_{Primer1}$-2 and $SMA_{Primer2}$-12 confirmed the success of the Ser195 to Ala substitution and in each case the remainder of the sequence was identical to the parent functional granzyme B cDNA.

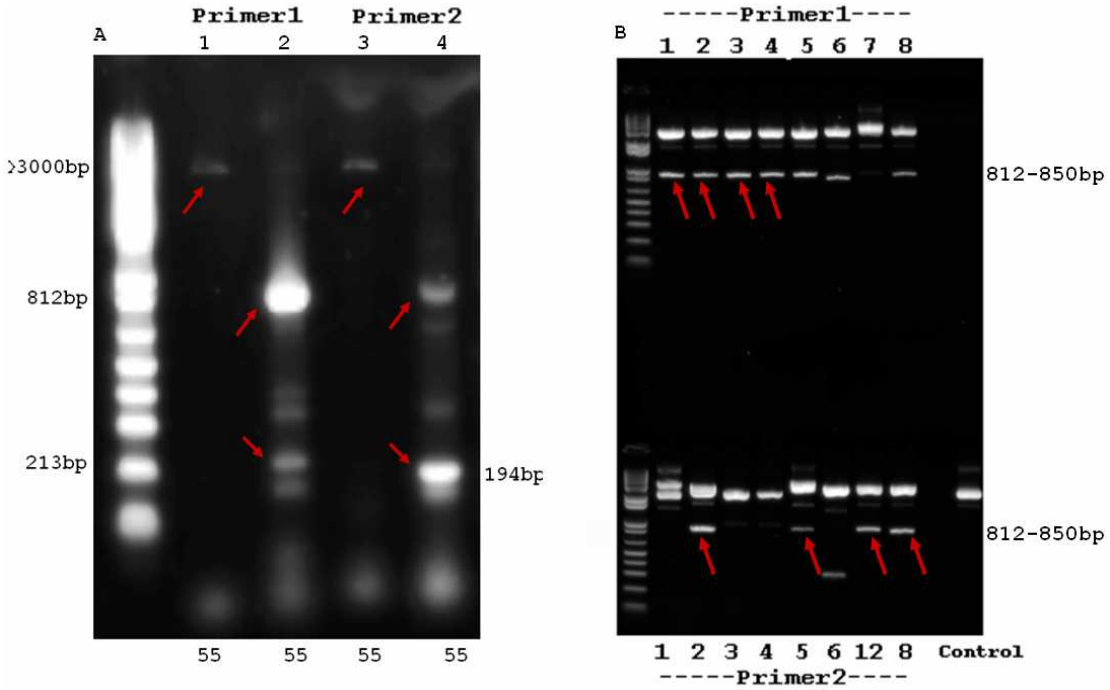


Figure 4.5 Amplification of dipeptide/GE deletion granzyme B cDNA containing an additional Ser195 to Ala substitution using 'Megaprimer' PCR.

(A). Results of first round PCR amplification using two different forward internal mutagenic primers (Primer1 and Primer2) and the same reverse flanking primer at the limiting concentration are shown in lanes 1 and 3. Mega fragment products of the expected size (213 and 194bp, respectively) were not detectable. Residual plasmids with DNA inserts (expected size over 3000bp) are detectable (arrows). PCR products obtained following addition of the forward flanking primer to amplify full-length mutated cDNA (using the 5'- 3' chain of mega mutants from 1st round PCR reaction as the reverse primer) are shown in lanes 2 and 4. A product of the expected size (812bp) together with amplified remaining mega fragments (213bp and 194bp, lanes 2 and 4 respectively) were obtained (arrows). (B). Analysis of clones of the full-length product revealing eight clones containing inserts of the correct size (arrows).

4.3.2 Construction of recombinant cDNAs

To construct recombinant cDNAs for expression in mammalian cells, three DNA clones representing the wild-type GzmB (WT-1), the dipeptide deletion mutant (SOE-4) and the dipeptide mutant with the point mutation in the active site ($SMA_{Primer1}$-2), were first sub-cloned from the pGEM-T vector using primers designed to add *SalI BamH1* 5' and 3' restriction sites and to eliminate their stop codon (TGA). High fidelity PCR assays with these primers yielded products of the expected sizes (Figure 4.6- A). The resultant DNAs were sub-cloned and 2 clones of each containing inserts of the correct size, pFLAG-SOE-1 and 3, pFLAG-SMA-1 and 3 and pFLAG-WT-1 and 2, were selected for sequencing (Figure 4.6- B). Analysis of sequences obtained from pFLAG-SOE-1 and 3, pFLAG-SMA-1 and 3 and pFLAG-WT-2 confirmed that they were all successfully sub-cloned into the pFLAG-CMVtm-5a vector and retained the correct nucleotide sequences (Figure 4.7).

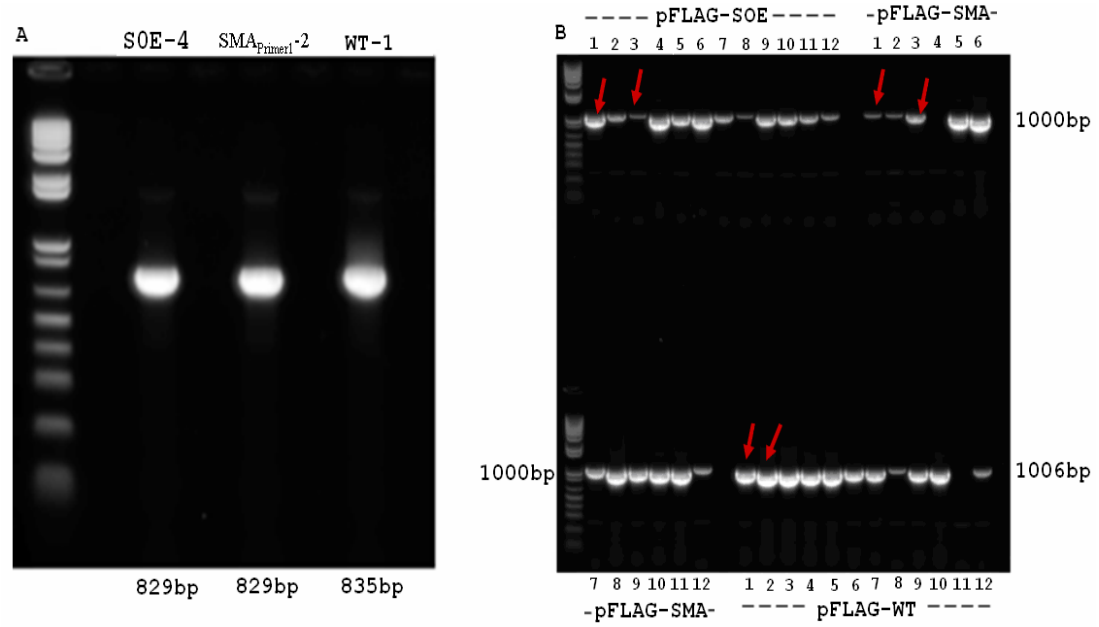


Figure 4.6 Production of cDNA clones of each of the 3 granzyme B cDNA constructs suitable for expression in the pFLAG expression vector.

(A). High fidelity PCR assays were performed on each of the 3 granzyme B cDNA constructs using primers designed to create a *SalI* restriction site at the 5' end and to create a *BamH1* site and remove the stop codon (by deleting 3 nucleotides) at the 3' end. Products of the expected sizes - 829bp for SOE-4 and $SMA_{primer1}$-2 and 835bp for WT-1 – were obtained. (B). Inserts were amplified from cloned plasmid DNAs following insertion into the pFLAG vector by conventional PCR using a pair of pFLAG-CMVtm-5a vector sequencing primers, CMV30 and CMV24. Two clones of each construct containing inserts of the correct size (1000bp for SOE-4, $SMA_{primer1}$-2 and 1006bp for WT-1) were selected for nucleotide sequencing (indicated by arrows)

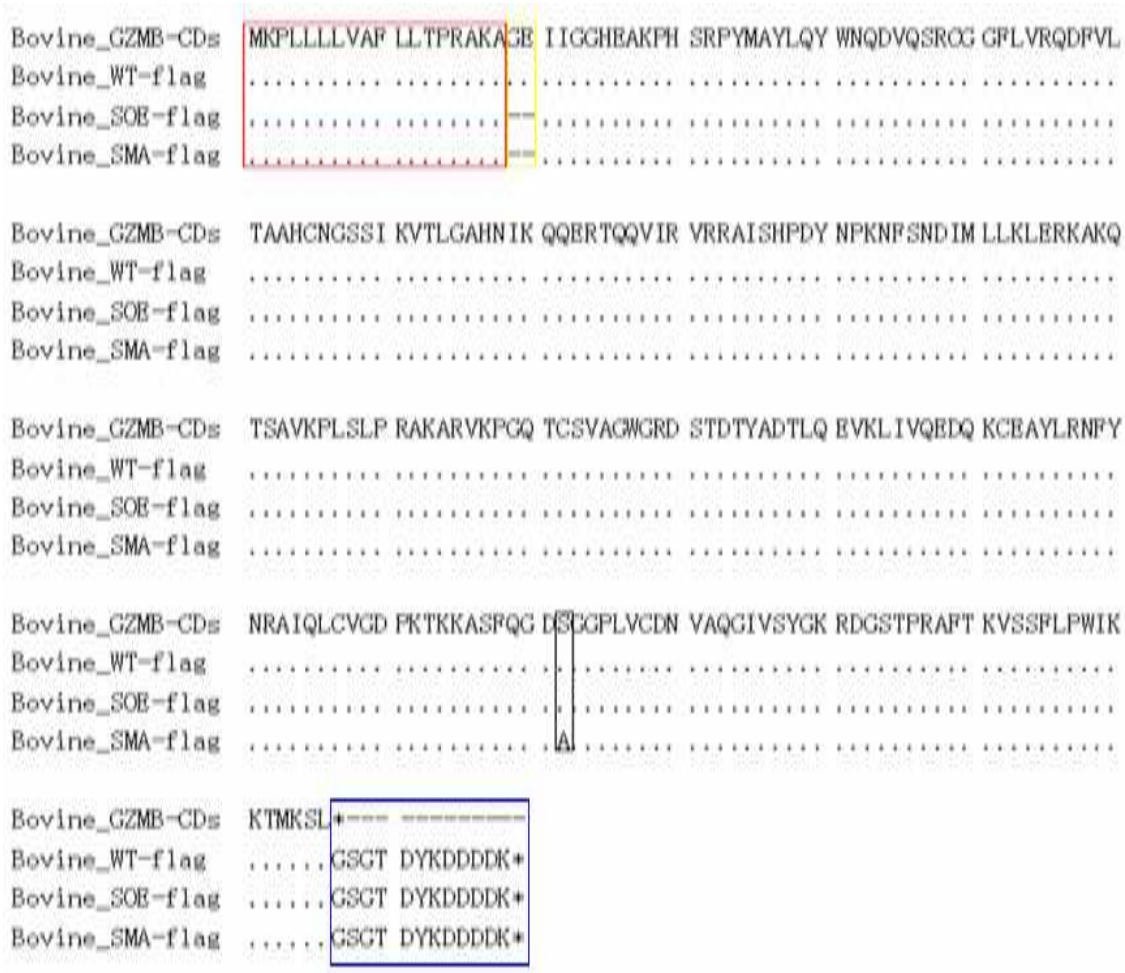


Figure 4.7 Predicted amino acid sequences from nucleotide sequences of three recombinant forms of bovine granzyme B cDNA, aligned with the reference sequence from the genome database.

Bovine_GZMB-CDs – the full length cDNA from bovine genome (corr_ENSBTAG00000010057); Bovine_WT-flag - pFLAG-CMVtm-5a vector containing wide type granzyme B; Bovine_SOE-flag - pFLAG-CMVtm-5a vector containing functional granzyme B; Bovine_SMA-flag - pFLAG-CMVtm-5a vector containing functional granzyme B with Ser195 to Ala mutation. Dot-Identical; Dash-Gap; Leader peptide is highlighted in red; Dipeptide/GE is in yellow; Ser195Ala is in black; FLAG epitope-tag sequence of the pFLAG-CMVtm-5a vector is in blue

4.3.3 Expression in COS cells

As a large quantity of DNA was needed for the transient transfection, pFLAG-CMVtm-5a vector without inserts (mock) and with inserts (pFLAG-WT-2, pFLAG-SOE-1 and pFLAG-SMA-1) were first prepared in Maxi-preps and the resultant DNAs transfected into COS cells. The efficiency of transfection was determined by staining cells in cytospin preparations with a monoclonal anti-FLAG M2 antibody that detects the FLAG epitope-tag sequence, GSGTDYKDDDDK. Figure 4.8 shows staining of transfected cells, with negative controls including an isotype-control antibody and mock-transfected cells. Transfection efficiency was very similar, 33-35%, in all preparations. Clear expression of pFLAG-WT-2, pFLAG-SOE-1 and pFLAG-SMA-1 was observed in the respective transfected COS cells, but not in the controls.

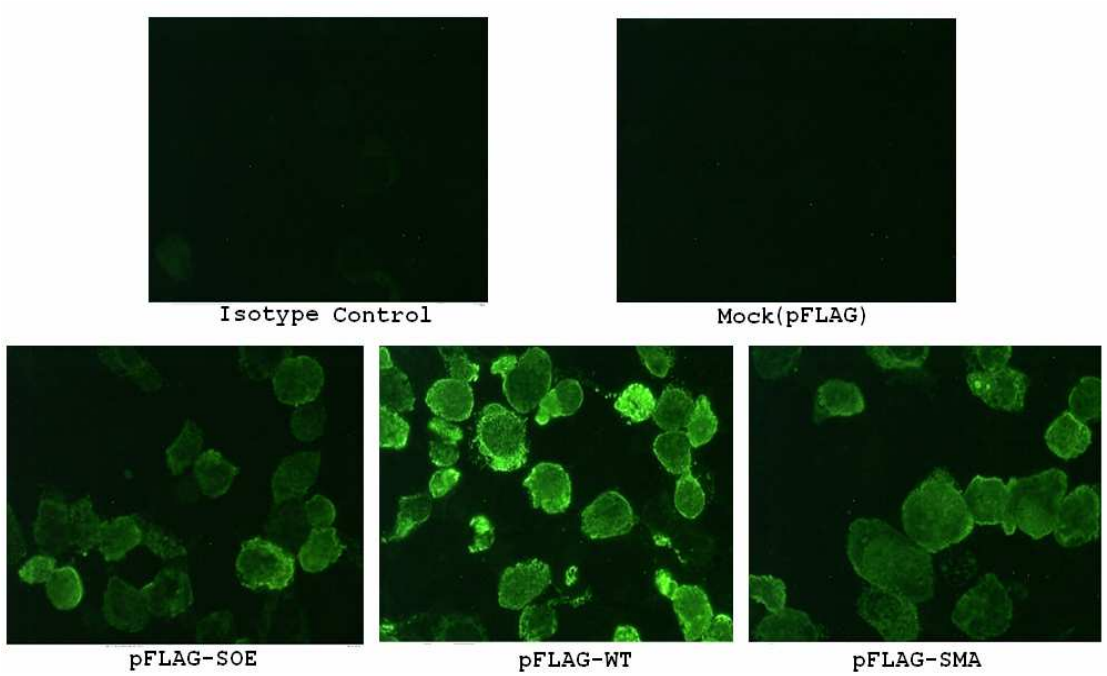


Figure 4.8 Immunofluorescence staining of COS cells transiently transfected with different recombinant granzyme B cDNAs in the pFLAG expression plasmid.

Cells transfected with unmodified cDNA (pFLAG-WT), cDNA containing the GE dipeptide deletion (FLAG-SOE) and cDNA containing both the GE dipeptide deletion plus an alanine substitution at position 195 (pFLAG-SMA), as well as mock-transfected cells, were stained with the anti-FLAG M2 antibody (IgG1, 1:500). In addition cells transfected with FLAG-SOE stained with an irrelevant isotype control primary antibody were shown (Isotype control). The transfection efficiency of pFLAG-SOE, pFLAG-WT and pFLAG-SMA was 33%, 35% and 33%, respectively.

4.3.4 Functional characterization and inhibition of cattle granzyme B

To examine the functional activities of the recombinant versions of cattle granzyme B, substrate assays were performed using a substrate, AC-IEPD-pNA containing a tetrapeptide, which has been shown to be recognized specifically by human, rat, mouse and equine granzyme B (Caputo et al., 1993; Smyth et al., 1995; Xia et al., 1998; Piuko et al., 2007). The enzymatic activities of COS cell lysates prepared 48 hours following transfection with mock, pFLAG-SOE, pFLAG-WT and pFLAG-SMA are shown in figure 4.9. Transfection with the unmodified cattle granzyme B yielded a signal close to the background level produced by mock transfection. In contrast, lysates from cells transfected with the dipeptide/GE knockout granzyme B exhibited dramatically increased enzymatic activity, whereas the additional substitution of Ser with Ala at the active site resulted in a complete loss of activity. As a substrate-specific control, the chymotrypsin substrate Suc-GGF-pNA was used in the assay and no signal was detected with any of cattle granzyme B constructs. Given this specificity and the similar transfection efficiency (33-35%) achieved with the different constructs, it is concluded that the observed high level of enzymatic activity generated by cells transfected with the dipeptide deletion mutant is attributable to granzyme B. The results also confirm that cattle granzyme B has a similar structure-function relationship to the human, mouse, rat and equine orthologues (Caputo et al., 1993; Smyth et al., 1995; Xia et al., 1998; Piuko et al., 2007).

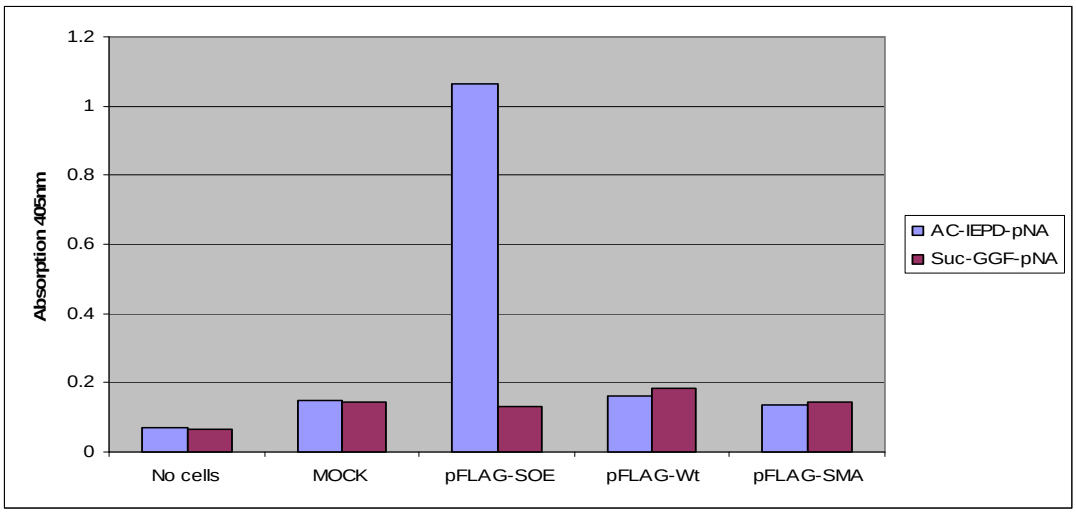


Figure 4.9 Enzymatic activity of different recombinant forms of bovine granzyme B tested on a granzyme B-specific substrate AC-IEPD-pNA and a control substrate Suc-GGF-pNA.

COS cells were transiently transfected with unmodified granzyme B cDNA (pFLAG-Wt), cDNA with the GE dipeptide deleted (pFLAG-SOE) or cDNA containing a deletion of the dipeptide and an alanine substitution at position 195 (pFLAG-SMA) and lysates of the transfected cells collected after 48 hours were incubated with the substrates for 4 hours. Controls consisted of lysates of cells transfected with pFLAG without an insert (MOCK) and buffer (No cells) added to the substrate. Colour reaction generated after 4 hours by cleavage of the pNA substrate were measured at a wavelength of 405nm using a Synergy™ HT Multi-Mode Microplate Reader (BioTek)

To test whether a granzyme B-specific inhibitor is able to inhibit the enzymatic activity of the functional bovine granzyme B, enzymatic activity of the cell lysate used above was measured in the presence or absence of AC-IEPD-CHO (Thornberry et al., 1997; Harris et al., 1998). The presence of this specific inhibitor dramatically reduced the activity of the cattle granzyme B preparation by about 4 fold, close to the

background level (Figure 4.10). The substantial loss of enzymatic function indicates an effective inhibitory capacity of AC-IEPD-CHO for cattle granzyme B.

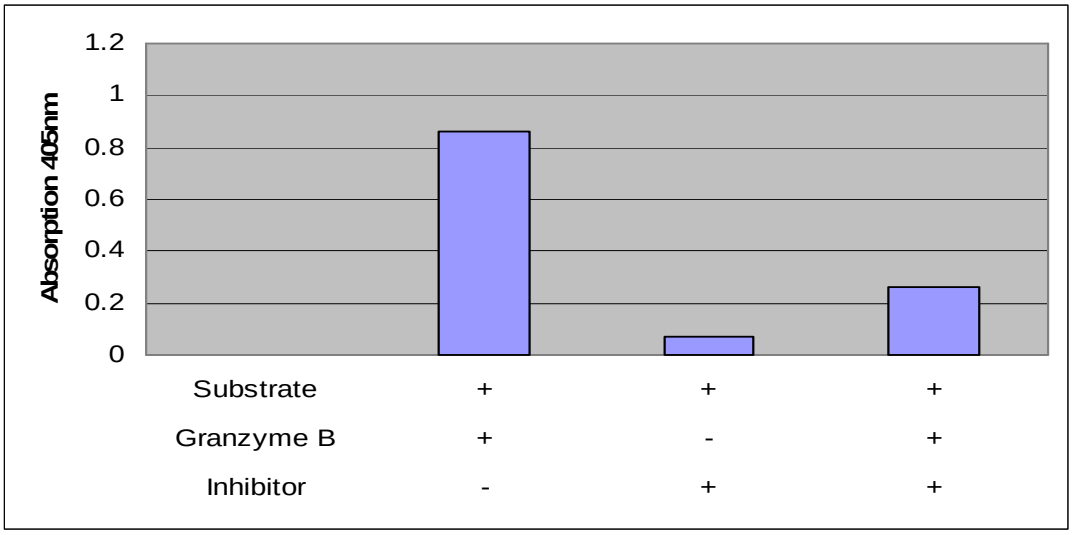


Figure 4.10 Inhibition of the activated recombinant cattle granzyme B by the specific inhibitor AC-IEPD-CHO.

Functional cattle granzyme B (expressed in pFLAG-SOE) was produced by transient transfection of COS cells and cell lysates prepared after 48 hours used to test the enzymatic activity of lysates on the substrate AC-IEPD-pNA, in the presence or absence of the specific inhibitor AC-IEPD-CHO (10uM). Colour reaction generated after 4 hours by cleavage of the pNA substrate were measured at a wavelength of 405nm by using a Synergy™ HT Multi-Mode Microplate Reader (BioTek).

4.3.5 Equine antibodies cross-react

One of the six murine anti-eqGzmB monoclonal antibodies (XIXG7) kindly provided by Dr. Martin Muller has been demonstrated previously to cross-react with human granzyme B by a western-blot assay (Piuko et al., 2007). To test whether any of the six antibodies cross-react with the functional form of cattle granzyme B used in the enzyme activity assay, they were tested for recognition of transfected cells by immunofluorescence staining of cytospin preparations (Figure 4.11). Positive controls confirmed expression of cattle pFLAG-SOE in approximately 20% of cells following transfection, using M2 antibody to the pFLAG epitope tag, and expression of human granzyme B in a small percentage of cells (<10%) transfected with pcDNA3.1-1 (kindly provided by Dr. Martin Muller), using the anti-eqGzmB XIXG7 antibody. However, none of six anti-eqGzmB monoclonal antibodies stained cells expressing the functional cattle granzyme B, even when used at twice the concentration that resulted in staining of human granzyme B by antibody XIXG7.

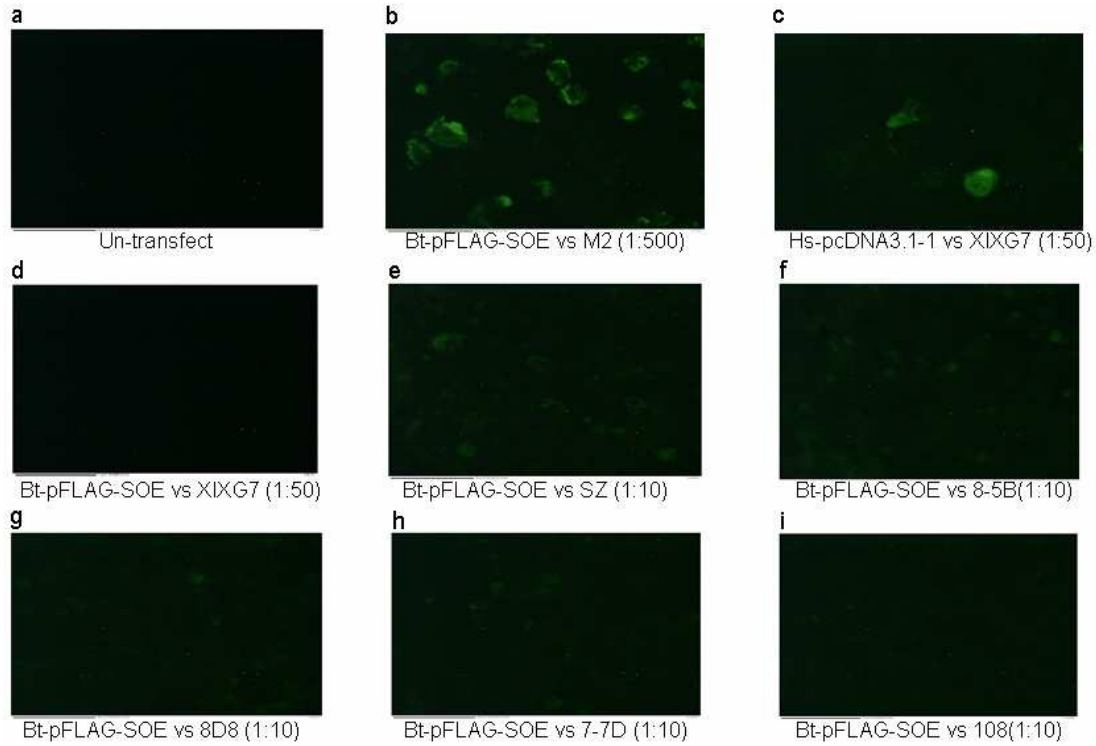


Figure 4.11 Reactivity of 6 anti-eqGzmB monoclonal antibodies with 293T cells transfected with a vector (Bt-pFLAG-SOE) expressing the active form of bovine granzyme B.

Cytospin preparations of the cells were stained by indirect immunofluorescence with the 6 anti-eqGzmB monoclonal antibodies, X1XG7(1:50), SZ(1:10), 8-5B(1:10), 8D8(1:10), 7-7D(1:10) and 108(1:10) shown in panels (d) - (i), or with the anti-FLAG M2 antibody (panel b). Staining of cells transfected with human granzyme B (Hs-pc-DNA3, kindly provided by Dr. Martin Muller) with the monoclonal antibody XIXG7 is shown in panel (c) and staining of untransfected cells with the anti-FLAG M2 antibody as a negative control is shown in panel (a). The experiments were repeated twice with similar results.

4.4 Discussion

In human and mouse, granzyme B is thought to be the most potent effector molecule used by CTL to induce apoptosis of virally infected and tumour cells. The substrate specificities and biological activities of granzyme B in these species are well characterized (Heusel et al., 1994; Shresta et al., 1995; Thornberry et al., 1997; Kaiserman et al., 2006; Casciola-Rosen et al., 2007; Cullen et al., 2007). However, at the beginning of my study, no information on the biological activity of bovine granzyme B or its specificity was available. Therefore, it was difficult to investigate its role in killing mediated by CTL in cattle. As a first step to examining the activity of bovine granzyme B, this study set out to obtain expressed recombinant protein, first to determine whether processing of the translated polypeptide to generate functional protein is similar to that observed with the human and mouse orthologues and, second, to obtain initial information on the enzymatic activity of the mature protein. Several attempts to produce biologically active human granzyme B in baculovirus, bacteria and yeast had failed (cited by Smyth et al., 1995). However, the successful generation of recombinant human and mouse granzyme B by expression in mammalian COS cells (Caputo et al., 1993; Smyth et al., 1995) provided a potential system for investigating production of active bovine granzyme B. The success of the eukaryotic expression system is likely to relate to its ability to generate protein with appropriate post-translational modifications such as protein folding, disulfide bond formation and glycosylation, although direct evidence for this is not available. No specific antibodies were available to detect bovine granzyme B protein. Hence, to enable us to detect the recombinant bovine granzyme B expressed in COS

cells, a vector containing an antibody epitope tag was chosen to express the protein. The study incorporated production of a version of the protein with a point mutation in the predicted active site to further test the specificity of the activity.

The results of these studies demonstrated successful expression of biologically active granzyme B from cattle. This work is the first description of the biological activity of a member of the granzyme family in cattle. The demonstration that deletion of the dipeptide/GE is a prerequisite for activation of cattle granzyme B in COS cells indicates that processing of the translated polypeptide is similar to that described for humans and mice (Caputo et al., 1993; Smyth et al., 1995). Moreover, the finding that mutation of Ser195, one of the triad at the catalytic site (His, Asp and Ser), causes the loss of enzyme activity of activated cattle granzyme B confirms the involvement of this residue in the active site of the bovine enzyme (Caputo et al., 1994).

The activity of the recombinant mature granzyme B protein was also examined employing a substrate assay using a peptide substrate, AC-IEPD-pNA containing an aspartic acid residue, which in other species acts as a specific substrate for granzyme B. The demonstration of strong activity against this substrate confirms that cattle granzyme B displays Aspase activity, which is a characteristic feature of granzyme B, as no other known serine protease in mammals has the cleavage preference for Asp-containing substrates (Poe et al., 1991). The GzmB inhibitor, AC-IEPD-CHO, is a known non-cell-permeable compound that inhibits human and rat granzyme B (Thornberry et al., 1997; Harris et al., 1998) by binding to residues at P4-P3-P2-P1

(amino-terminus with the proteolytic cleavage site) of active granzyme B, thus competitively blocking its enzymatic function. The effective inhibitory capacity of AC-IEPD-CHO for cattle granzyme B provides further evidence that the functional activity is attributable to granzyme B. Overall, these results indicate that the expressed product of the isolated granzyme B gene has functional properties characteristic of the granzyme B subfamily.

Although one of six anti-eqGzmB monoclonal antibodies tested in these experiments had previously been reported to cross-react with human granzyme B, none of these antibodies was found to react with the bovine protein. As there was no available information on the epitope specificity of these antibodies (Piuko et al., 2007), it is possible that only two different epitopes may have been recognized. However, the development of a system for expressing granzyme B provides a means of producing protein for generating specific antibodies in future studies.

In the absence of specific antibodies, the granzyme B substrate assay established in these studies provides a potentially valuable tool to determine and quantify the expression of functional cattle granzyme B in CD8+ T cells. Moreover, the demonstration that AC-IEPD-CHO is an effective inhibitor of cattle granzyme B in vitro also allows investigation of the enzyme specificity. However, this inhibitor is not cell-permeable and therefore cannot be used to examine granzyme B activity in intact cells. Therefore, other cell-permeable inhibitors of granzyme B need to be tested. These aspects are further investigated in chapters 5 and 6 of this thesis.

Chapter 5 Expression of perforin and granzymes by *Theileria*-specific CD8+ T cells in relation to their killing activity

5.1 Introduction

Class I MHC (MHCI)-restricted CD8+ T cell responses are important in mediating immunological control of many intracellular pathogens, including viruses, bacteria and parasites (Wong and Pamer, 2003). The importance of CD8+ T cells in immunity to *T. parva* has been confirmed by demonstrating that immunity could be transferred from immune to naïve twin calves with CD8+ T cells (McKeever et al., 1994). Recently, a number of antigens recognised by *T. parva*-specific CD8+ T cells have been identified (Graham et al., 2006; Graham et al., 2007; Graham et al., 2008) and responses in individual animals have been shown to be focused on a few highly dominant antigens (Taracha et al., 1995; MacHugh et al., 2009). However, experiments attempting to immunise cattle with these antigens using prime-boost strategies have given poor protection against parasite challenge (Graham et al., 2006). In contrast to the strong cytotoxic activity of CD8+ T cells induced by infection and treatment, immunisation of cattle with 5 defined *T. parva* antigens induced CD8+ T cells with weak cytotoxic activity (Graham et al., 2006). This suggests that strong cytotoxicity in the specific CD8+ T cell response may be required for protection.

The predominant pathway used by CD8+ T cells to kill pathogen-infected cells is granule exocytosis (Kagi et al., 1994; Walsh et al., 1994; Nickell and Sharma, 2000), involving release of a membrane disrupting protein perforin and a number of granzymes (5 in human and 10 in mouse), which are co-localized in the lytic granules. Perforin and granzymes induce apoptosis of target cells in a synergetic manner, perforin generating membrane pores that allow delivery of the granzymes into the cytosol of target cells where they initiate cell death by cleaving a number of protein substrates (Redelman and Hudig, 1980; Hudig et al., 1991). Granzyme expression in other species has shown to be primarily restricted to lymphoid lineage cells such as T lymphocytes and natural killer cells, but not B cells (Anthony et al., 2010; Bovenschen and Kummer 2010). Studies of mouse and human CD8+ T cells indicate that granzyme B is the most potent effector granzyme in T cell-mediated killing of virally infected cells and tumour cells. It is reported to be predominantly expressed in CD8+ T cells with effector function (Lieberman, 2003; Takata and Takiguchi, 2006; Chattopadhyay et al., 2009; Harari et al., 2009). Serine protease inhibitor 9 (serpinB9 / PI-9), an endogenous inhibitor of human granzyme B has been reported to be expressed broadly in many tissues, with high and stable levels in cytotoxic T cells, antigen-presenting cells, a number of endothelial and mesothelial cells and at immune privileged sites (Sun et al., 1996; Bladergroen et al., 2001; Buzza et al., 2001; Hirst et al., 2001). It is localized in the cytoplasm and nucleus of cells (Bird et al., 2001) and protects cytotoxic T cells and bystander cells from misdirected granzyme B (Bird et al., 1998).

Studies in mice have indicated that the perforin and granzyme genes can be differentially regulated in activated CD8+ T cells (Kelso et al., 2002). Thus, differences in cytolytic function of *T. parva*-specific CD8+ T cell responses might be related to differential expression of these granule enzymes (especially granzyme B). Recent work in the Roslin laboratory has revealed that CD8+ T cell clones that share epitope specificity have markedly different levels of cytotoxic activity against the same target epitope (Dr. Tim Connelley, personal communication).

The aim of this chapter is to investigate whether heterogeneity in cytotoxic function is related to differential expression of granzymes and/or perforin by *T. parva*-specific CD8+ T cell clones.

5.2 Materials and Methods

5.2.1 Animals and cell lines

Freshly isolated PBMC established from naïve animals 1683 and 1693 were generated as described in section 2.2.1. Uninfected lymphoblasts were established from the same animals by stimulation of PBMC with ConA for 3 days (section 2.2.2). Seven *Theileria parva*-infected cell lines of different cell surface phenotypes - 641, 468, 109, 011, F44-951, F31-951 and 592 TPM - were provided by Drs Niall MacHugh and Timothy Connelley.

CD8+ T cell clones established from animals 641 and 633, homozygous for the A18 class I MHC haplotype, were kindly provided by Ms Victoria Carroll. The autologous *T. parva*-infected cell line, 641TPM (MHC A18/A18), was used as infected target cells in cytotoxicity assays with these clones. A *T. annulata*-infected cell line from the same animals incubated with synthetic peptide representing the target epitope, (641TAA-pulsed-$Tp1_{214-224}$,100ng/ml) was used to investigate killing of peptide-pulsed target cells, as described in section 2.2.8.

An uncloned *T. parva*-specific CD8+ T cell line established from animal 592, homozygous for the A10 class I MHC haplotype, was generated as described in section 2.2.4 and used with the autologous *T. parva*-infected cell line (592TPM-MHC A10/A10), as an antigen-specific stimulator to examine the kinetics of expression of granule enzymes.

5.2.2 Standard RT-PCR

The expression of genes encoding bovine granzymes, perforin and serine protease inhibitor 9 (serpinB9 / PI-9) was investigated. PI-9 is an intracellular inhibitor of granzyme B, which protects cytotoxic T cells and bystander cells from misdirected granzyme B. cDNA prepared from *T. parva*-infected cell lines of different phenotypes and resting PBMC and ConA-stimulated PBMC from two animals (1683 and 1693) was prepared and analysed by RT-PCR as described in section 2.3.1- 2.3.5. A further two cDNAs prepared from ConA-stimulated cell lines (1706 and 1707) that had subsequently been maintained by passage in culture with IL-2 were kindly provided by Dr Timothy Connelley and also used in these assays. PCR primers for the full-length coding region of PI-9 gene, identified in the bovine genome (NM_001075859), were designed using the Primer3 programme. The sequences of these primers were as follows: forward primer- 5'- ACCTGGTTTCCAATGTCAGG -3' and reverse primer - 5'- CTGGGGACACAGGGAAGG -3'. The primers were tested with cDNA prepared from a culture of *T. parva*-specific CD8+ T cells. The identity of the resultant PCR product (1270bp) was confirmed by sequencing as described in section 2.3 (data not shown). Primers used for granzymes and perforin were listed in table 3.3.

5.2.3 Semi-quantitative PCR

A semi-quantitative reverse transcriptase PCR was used to investigate the relative levels of expression of bovine granzymes and perforin in selected CD8+ T cell lines. A PCR for the house-keeping gene GAPDH was used as a control employing the

following primers: forward primer - 5'-ACC CCT TCA TTG ACC TTC AC-3' and reverse primer - 5'-TTC ACG CCC ATC ACA AAC ATG-3'. Primers for granzymes were redesigned using DNAsis Max 2.0 software to obtain a single-band product under the same annealing temperature for each gene. This was achieved for all except granzyme K, which gave three bands, one of the expected size and two smaller bands. The sequences of primers are listed in table 5.1. The primers for granzyme genes and GAPDH were tested with cDNA prepared from a culture of *T. parva*-specific CD8+ T cells. The identity of the PCR products were confirmed by sequencing as described in section 2.3 (data not shown). Primers used for perforin were listed in table 3.3. The PCR reactions were composed of 20pmol of granzymes/perforin primers and 10pmol of GAPDH primers, 2.5 units BIOTAQ (5 units/ul Bioline, London, UK), 2.5ul SM-0005 buffer, 2ul cDNA (derived by Reverse Transcription of RNA samples as described in section 2.3.3) and nuclease-free water to give a final volume of 25ul. The reaction programme was as following: 94°C for 3 min, 30 cycles (94°C for 1.5 min, 55°C for 1.5 min, 72°C for 1.5 min) and a final extension period of 72°C for 10 min. The Semi-quantified PCR products were analysed by 1.5% agarose gel electrophoresis and the density of the specific bands was measured by computer software (KODAK 1D 3.6 version).

Table 5.1 PCR primers designed to amplify bovine granzyme sequences from cDNA

Primers	Sequences (5'---3')
GzmA (For)	TGA CTC CTC ATT CAA GAC CCT A
GzmA (Rev)	CCA GTTGAG GTG TTT CTT TGA G
GzmO (For)	TTC CTC CTG CCA TTT GTC TC

GzmO (Rev)	AGG CTT CTG GGG ATT ACC AC
GzmB (For)	ACT GGA ATC AGG ATG TCC AGA G
GzmB (Rev)	TTT GGG TCC CCC ACA CAC AG
GzmH (For)	GGC TTT CGT TCA GTT TCT GG
GzmH (Rev)	GAG TCA CCC TTG AAG CCG
GzmK (For)	AGC CGG GAC TTA CAT GAC TC
GzmK (Rev)	AGG TTT CTT GGC ATC ACC AC
GzmM (For)	AGA TCC AAG ATG CTG CTC CT
GzmM (Rev)	GAT GTC GGT GCA ATT CTC AG

5.2.4 Cytotoxicity Assay

A standard 4h ^{111}In-release assay was performed as described in section 2.2.8.

5.2.5 CD8+ T cell line lysate preparation

Cell lysates for analysis of granzyme B content were prepared as described by Ewen et al., (2003) and Piuko et al., (2007). Aliquots of 1ml of PBS-washed CD8+ T cells adjusted to 1x10^{6}cells/ml in PBS were centrifuged at 180 x g for 10 min and the pellet was lysed by addition of 50ul of a lysis buffer composed of 1%Triton X-100 in 50mM Tris, pH8.0 and 0.5ul of Benzonase Nuclease (25U/ml, Purity>99%, Merck). Following incubation on ice for 20min, lysed cells were centrifuged for 10min at 0°C at 21,000 x g to pellet cell nuclei. Supernatants were harvested and either stored at -70°C or immediately used in activity assays.

5.2.6 Granzyme B release by CD8+ T cells

Samples containing released granzyme B were prepared as described by Ewen et al., (2003). Aliquots of $1x10^6$ effector cells were distributed into the wells of 96-well V-bottomed plates together with $5x10^5$ target cells in a total volume of 200ul phenol-red-free complete media (Invitrogen, RPMI1640, Cat:11835-063). Control wells containing effector cells and medium were also included. Plates were briefly centrifuged for 1 min at 120xg and then incubated in a humidified incubator in an atmosphere of 5% CO_2 at 37°C. After incubation for 4h, supernatants were obtained by centrifuging for 10 min at 400xg.

5.2.7 Granzyme B enzymatic assay

Measurement of granzyme B activity in samples was performed as described by Ewen et al. (2003). Aliquots of 10ul of cell lysates or 40ul of culture supernatants were added in duplicate to wells of Falcon™ 96-well flat-bottomed Microplates (BD, Cat. 353072) together with 200uM granzyme B substrate VIII, Ac-IEPD-pNA, (Calbiochem, Cat.368067) and reaction buffer (0.1M HEPES, pH7.0; 0.3M NaCl; 1mM EDTA) in a total volume of 100ul/well. Wells containing reaction buffer and substrate control were also included as controls. Mixtures were incubated at 37°C for 4h and the colour reaction generated by cleavage of the pNA (p-nitroaniline) substrate measured at a wavelength of 405nm using a Synergy™ HT Multi-Mode Microplate Reader (BioTek)

5.2.8 Statistical analysis

The statistical analysis was performed using Minitab software (Minitab® 15.1.20.0, Minitab Inc.). The correlation between variables was analysed by Pearson's correlation test. A P-value of less than 0.05 was considered significant.

5.3 Results

5.3.1 Expression of granule enzymes and protease inhibitor 9 by resting and activated lymphocytes

To investigate the transcript expression of bovine granule enzymes and PI-9, resting PBMC and ConA-stimulated PBMC from two animals (1683 and 1693) were examined by PCR (Figure 5.1- 5.2 and Figure 5.4). Transcripts of granzymes A, K H and M were detected in both resting PBMC, but granzymes O and B and PI-9 were not expressed. By contrast, all granzyme transcripts except granzyme K were observed in the corresponding ConA-stimulated PBMC; PI-9 transcripts were also detected in these cells. A similar profile of expression was obtained for another two ConA-stimulated PBMC (1706 and 1707) maintained by further stimulation with IL-2 (Figure 5.3 and 5.4).

5.3.2 Expression of granule enzymes and PI-9 in *T. parva*-infected cell lines

Previous studies have shown *T. parva* can infect and transform CD4+ T cells, CD8+ T cells, γδ+ T cells and B cells at similar frequencies in vitro (Baldwin et al., 1988). Six *T. parva*-infected cell lines of different phenotypes were examined by PCR for expression of transcripts of granule enzymes and PI-9 (Figure 5.3 and 5.4). Infected cell lines of different T cell subtypes (CD4+, CD8+ and γδ+) all expressed transcripts for perforin and granzyme A and displayed variable expression of the other granzymes. In contrast, there is no detectable expression of perforin or any of the granzymes in a B cell line infected with *T. parva.* The granzyme inhibitor PI-9 was found to be consistently expressed in all of the *T. parva*-infected cells. An additional weaker band of slightly smaller size was obtained in four infected cell lines (641, 109, 011 and 592 TPM) which had the most abundant PCR product. The identity of this PCR product was not determined, but it may be an alternatively spliced form of PI-9.

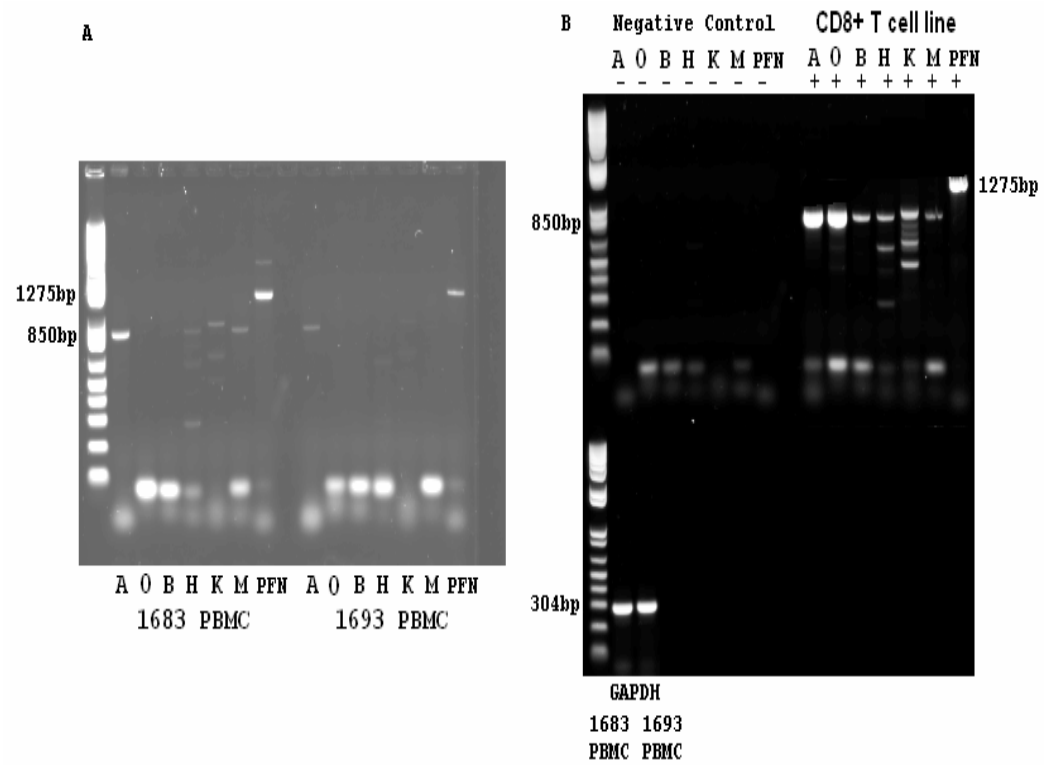
Table 5.2 Summary of relative levels of expression of genes encoding granule enzymes and PI-9 in resting and activated PBMC and in *T. parva*-infected cell lines of different phenotypes, determined by RT-PCR

Cells	Animal	A	O	B	H	K	M	PFN	PI-9
PBMC	1683	++	-	-	+	-/+	+	++	-
	1693	+	-	-	-/+	-/+	-/+	++	-
ConA Blast	1683	+++	++	++	++	-	+	++	+
	1693	+++	++	++	+	-	-/+	++	+
ConA line	1706	++	+	-	-	-	-/+	++	+
	1707	+	+	-	-	-	-/+	+	+
CD8+ TPM	641	+++	-/+	+++	+++	-/+	+	++	+++
	109	++	-/+	++	+	-/+	+	++	+++
CD4+ TPM	011	++	-/+	++	+	NT	+	+	++
	F44-951	++	-	-	-	-/+	-	++	++
γδ+ TPM	F31-951	+++	+++	-	-	-/+	-	+++	++
B+ TPM	592	-	-	-	-	-	-	-	++

PBMC = freshly harvested blood mononuclear cells; ConA blasts = PBMC harvested 3 days after stimulation with ConA; ConA line = PBMC stimulated with ConA and subsequently passaged in medium containing recombinant IL-2; TPM = cell lines infected with *T. parva*. NT = not tested. The intensity of PCR products in agarose gels was assessed visually: ranging from very weak (-/+) to very strong (+++).

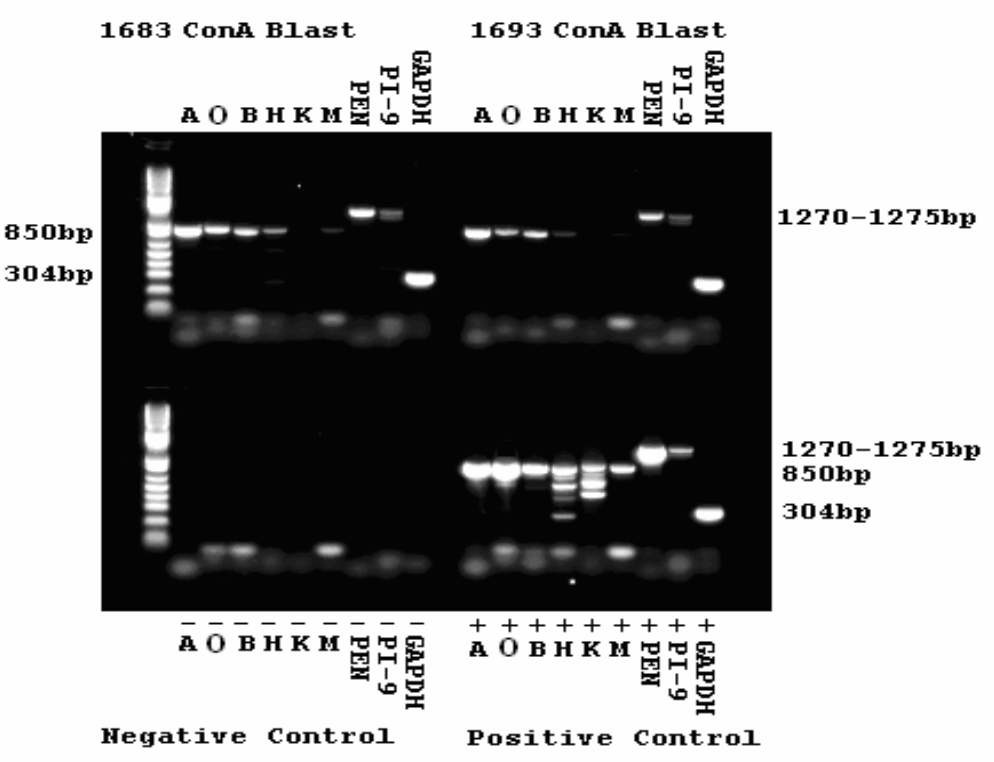
Figure 5.1 PCR products obtained for each of the bovine granule enzymes from (A). resting PBMC from two animals and (B). an uncloned *T. parva*-specific CD8+ T cell line (641).

A. B.



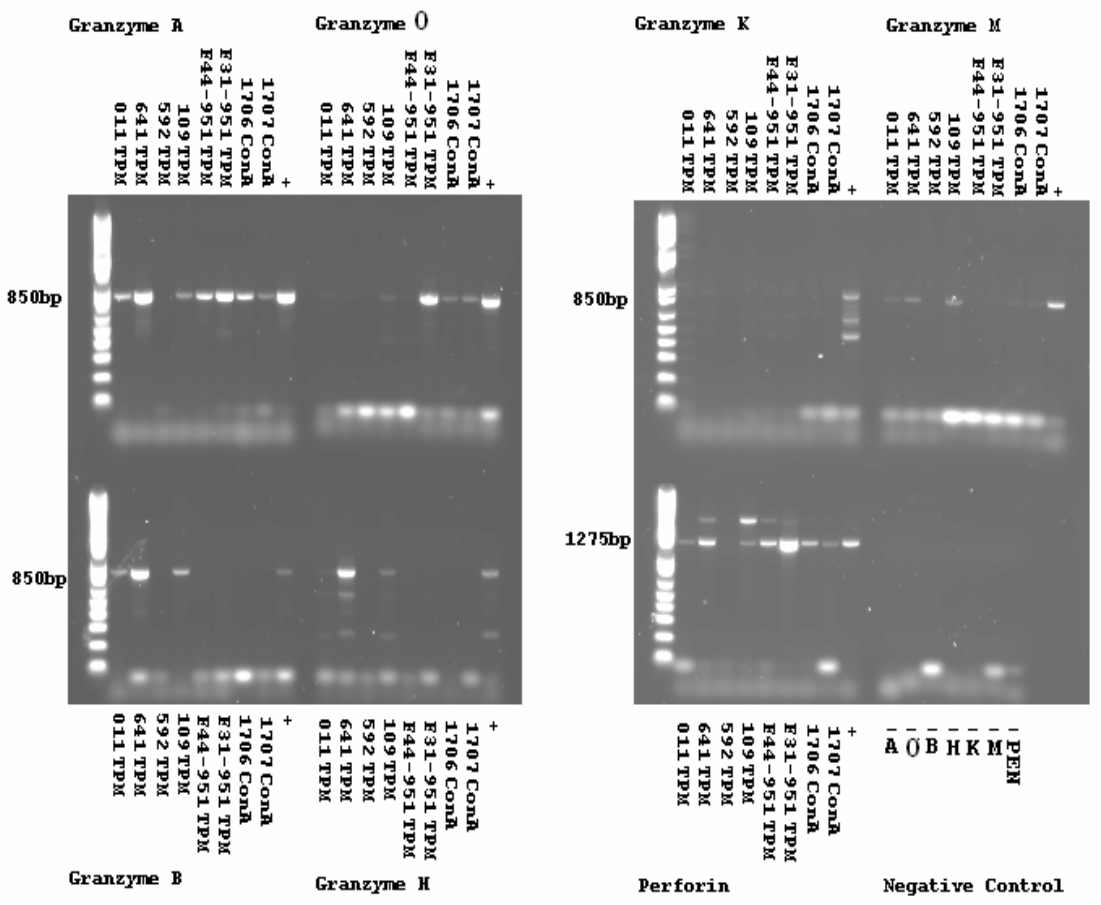
The sizes of the PCR products obtained were: granzyme A - 838bp; granzyme O - 849bp; granzyme B - 818bp; granzyme H - 820bp; granzyme K - 889bp; granzyme M - 833bp; Perforin (PFN) - 1275bp; (B). Negative controls (primers with no added cDNA template) are included in the upper left of panel B and positive controls (GAPDH) in the lower part of the same panel.

Figure 5.2 PCR products obtained for each of bovine granule enzymes and PI-9 from ConA-stimulated PBMC from two animals



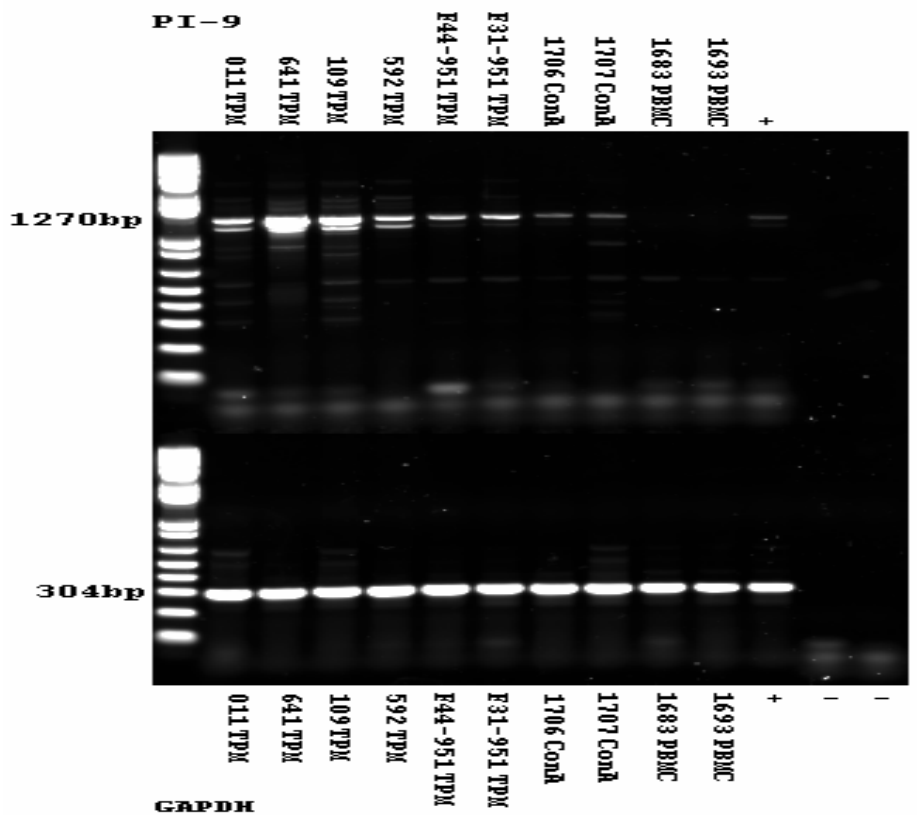
PCR products for each of granule enzymes and PI-9 (sizes as described in figure 5.1) are shown in the upper part of the panel. The negative and positive controls shown in the bottom part of the panel represent PCR products obtained without and with cDNA template respectively from cDNA of an uncloned *T. parva*-specific CD8+ T cell line (641) 7 days after stimulation with irradiated autologous parasitized cells.

Figure 5.3 PCR products obtained for each of the bovine granule enzymes from six *T. parva*-infected cell lines of different surface phenotypes and from ConA-stimulated PBMC from two animals



The sizes of the PCR products are given in the legend to figure 5.1. Positive controls, labelled + in the set of PCRs for each enzyme, consisted of primers with cDNA template of an uncloned *T. parva*-specific CD8+ T cell line (641). The negative controls, consisting of PCR assays with no added template, are shown together in the bottom right of the figure.

Figure 5.4 PCR products obtained for PI-9 from cDNA of *T. parva*-infected lines of different phenotypes and ConA-stimulated PBMC



Results are shown for 6 *T. parva*-infected cell lines, IL-2-maintained ConA lines from two animals (1706 and 1707), PBMC from two animals stimulated for 3 days with ConA (1683 and 1693) and an uncloned *T. parva*-specific CD8+ T cell line (641) (labelled +). Results obtained with the PI-9 PCR are show in the upper part of the panel and those for the GAPDH control in the lower part. Negative controls for PI-9 and GAPDH (labelled -), without added template, are also included.

5.3.3 Kinetics of expression of granule enzyme transcripts in un-cloned CD8+ T cells

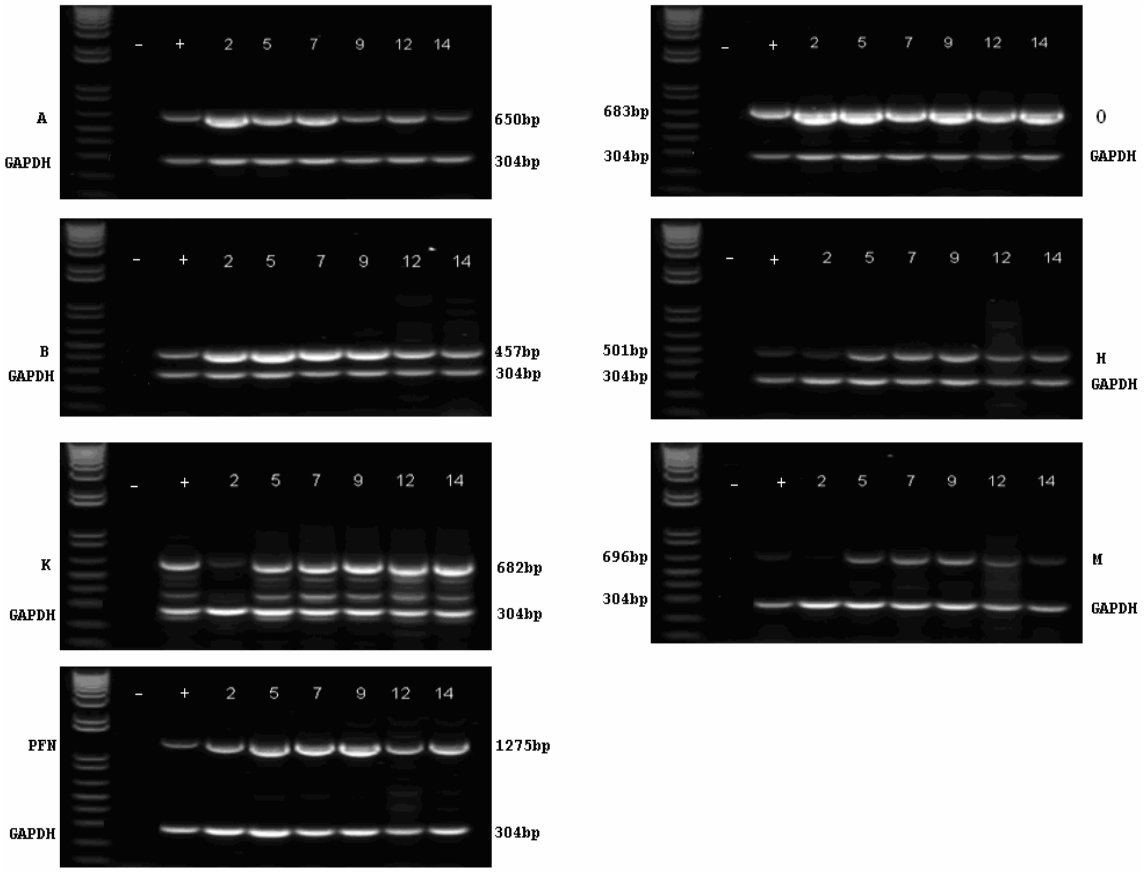
The kinetics of expression of mRNA for bovine granzymes and perforin by an uncloned CD8+ T cell line were examined following stimulation with autologous *T. parva*-infected cells, using semi-quantitative PCR assays. A CD8+ T cell culture established from animal 592; these T cells were stimulated with a B cell line infected with *T. parva* (Muguga) to avoid detection of granzyme transcripts derived from stimulator cells. A sample of cDNA prepared from this parasitized line prior to the assay was confirmed as negative for perforin and granzyme transcripts (data not shown). Analysis of the *T. parva*-specific T cell line by flow cytometry confirmed that it was predominantly CD3+, CD8+, CD4- and TCRγδ-. Following antigenic stimulation, T cell proliferation developed between days 2 and 7, during which the numbers of CD8+ cells in the culture increased at least 10-fold and their proportion in the whole population increased from 54% to 99.5% (the negative cells representing surviving stimulator cells). Cells collected from the stimulated cultures were examined by semi-quantitative PCR on days 2, 5, 7, 9, 12, 14 after stimulation.

The results revealed some differences in the kinetics of expression of the different mRNA species (Figure 5.5). Granzyme O showed high levels of expression at all time points, granzymes A and B showed high levels between days 2 and 7, while granzymes H, K and M showed low or undetectable expression on day 2 and gradually increased thereafter. The expression of perforin showed high levels between days 7 and 9.

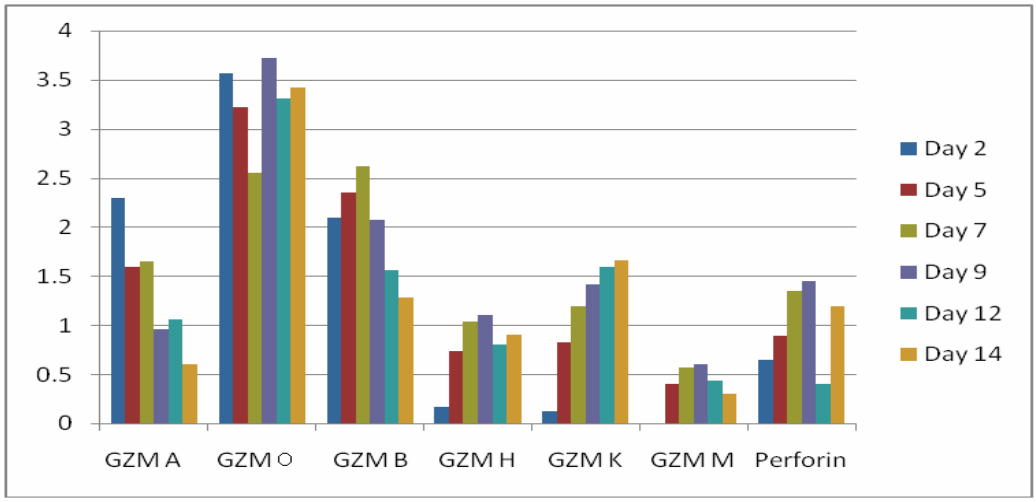
Figure 5.5 Time course of granzymes and perforin mRNA expression in an uncloned *T. parva*-specific CD8+ T cell line (592) following stimulation with autologous irradiated parasitized cells.

A. Agarose gels showing the PCR products for individual enzymes and the GAPDH control. Days after antigenic stimulation are shown at the top of each panel. A negative control (-), without added template, and a positive control (+), consisting of primers with cDNA template of an uncloned *T. parva*-specific CD8+ T cell line (641) day 7 after 3rd stimulation are included for each enzyme; B. Changes in quantity of PCR product (vertical axis) at different times following antigenic stimulation, normalised in relation to that of the GAPDH product obtained from the same sample.

A.



B.



The density of the bands was measured by Kodak 1D software (version 3.6) and results (vertical axis) are expressed as ratios of the density of the specific bands to that of the corresponding GAPDH bands.

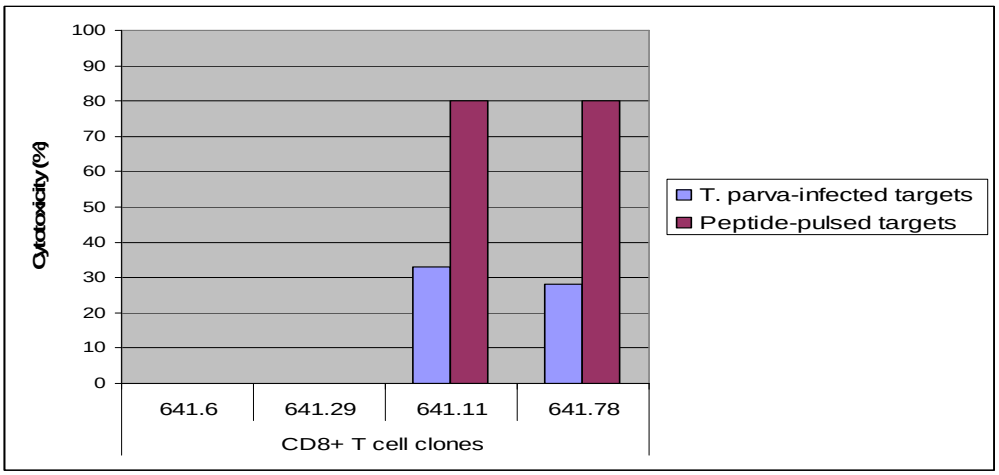
5.3.4 Relationship of killing levels and granule enzyme transcript profiles

To determine whether the levels of killing by CD8+ T cells are related to expression of particular granule enzymes, CD8+ T cell clones derived from the same animals but exhibiting different levels of killing were analysed using a semi-quantitative PCR. Dr Tim Connelley provided the cDNA and cytotoxicity data for these T cell clones. A standard time point of 7 days was chosen for analysis of cDNA, based on the results of the kinetics study as described above. The T cell clones had also been assayed for cytotoxicity on day 7. Two sets of cloned CD8+ T cell lines derived from different animals (641 and 1011) were examined; each set of lines expressed

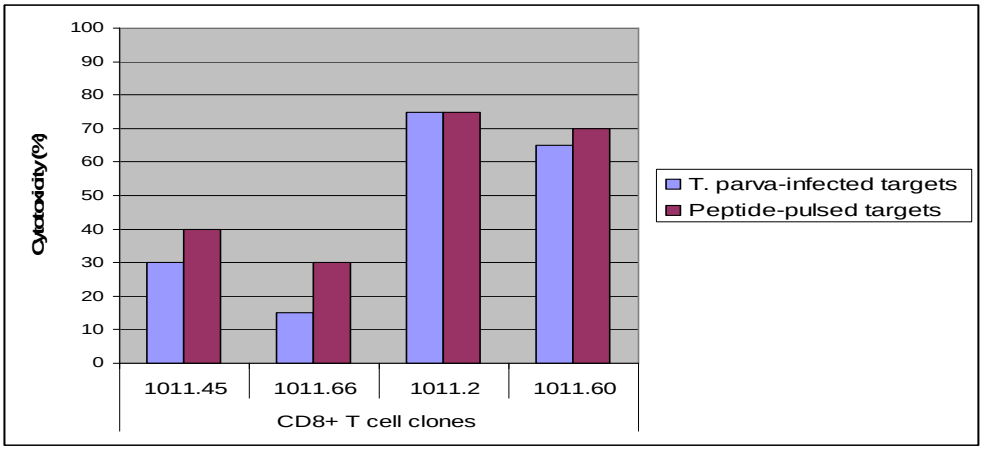
identical TCRβ chains and recognised the same epitope but exhibited different levels of cytotoxic activity (data provided by Dr. Timothy Connelley). The levels of cytotoxicity on autologous parasitized cells and on non-parasitised cells pulsed with peptide epitope are shown in figure 5.6. Of the 4 clones examined from animal 641, two showed no cytotoxic activity whereas the other 2 gave 19-32% killing of parasitized cells and 80% killing of peptide-pulsed targets. Two of the T cell clones from animal 1011 gave high levels of killing (64-75%) of both parasitized and peptide-pulsed target cells while the other two clones gave low levels of killing (<40%). Transcripts for perforin and all of the granzymes were detected in all 8 T cell clones (Figure 5.7 and 5.8). Amongst the 641 clones, perforin expression was higher in the clones with killing activity compared to the non-killing clones. In the 1011 clones, those displaying high levels of cytotoxicity had higher granzyme A expression than clones with low cytotoxicity, but there was no obvious difference in the level of perforin expression. Overall, there was no consistent pattern of Gzm/PFN expression that correlated with killing activity.

Figure 5.6 Cytotoxic activity of *T. parva*-specific CD8+ T cell clones, 4 from animal 641 (A) and 4 from animal 1011 (B), assayed on autologous *T. parva*-infected targets and *T. annulata*-infected targets pulsed with the target epitope peptide.

A.



B.

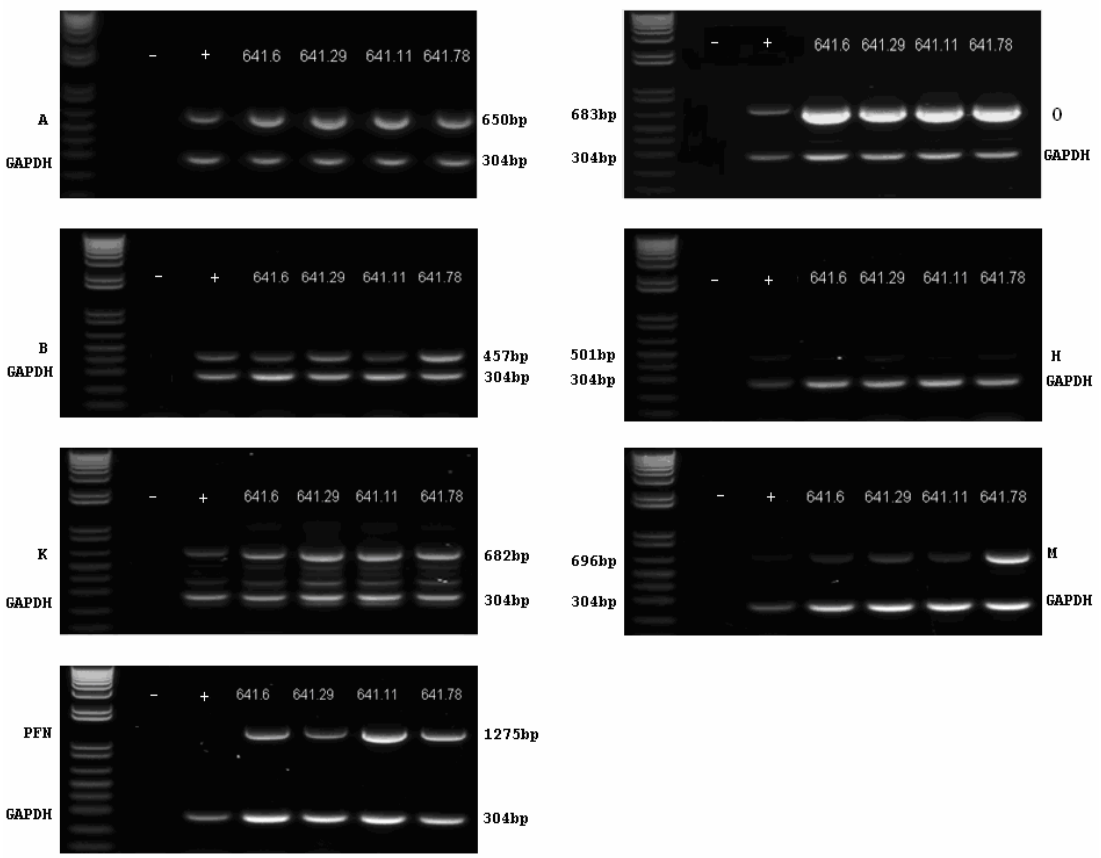


The clones were also tested on MHC-mismatched *T. parva*-infected targets and all were negative.

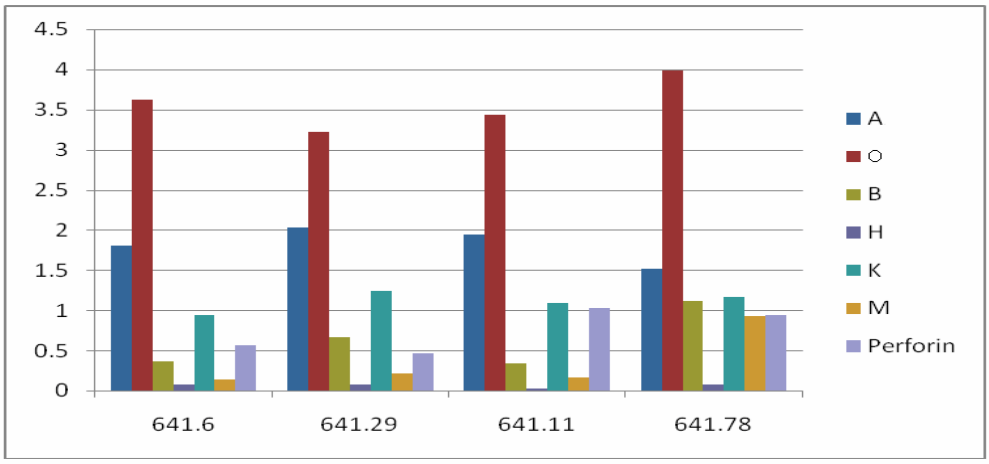
Figure 5.7 Relationship of killing levels and granule enzyme transcript profiles in CD8+ T cell clones (641).

A. Agarose gels showing the PCR products for individual enzymes and the GAPDH control. A negative control (-), without added template, and a positive control (+), consisting of primers with cDNA template of an uncloned *T. parva*-specific CD8+ T cell line (641) are included for each enzyme; B. Changes in quantity of PCR product (vertical axis) in different T cell clones, normalised in relation to that of the GAPDH product obtained from the same sample.

A.



B.

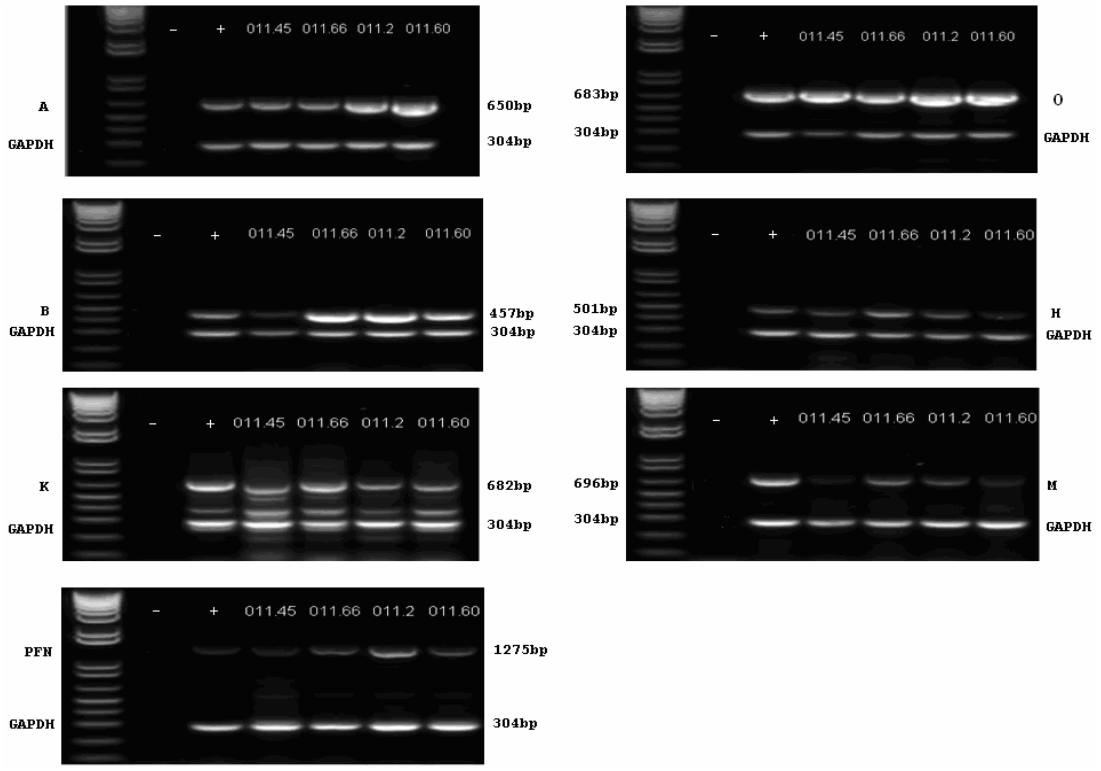


The density of the bands was measured by Kodak 1D software (version 3.6) and results (vertical axis) are expressed as ratios of the density of the specific bands to that of the corresponding GAPDH bands.

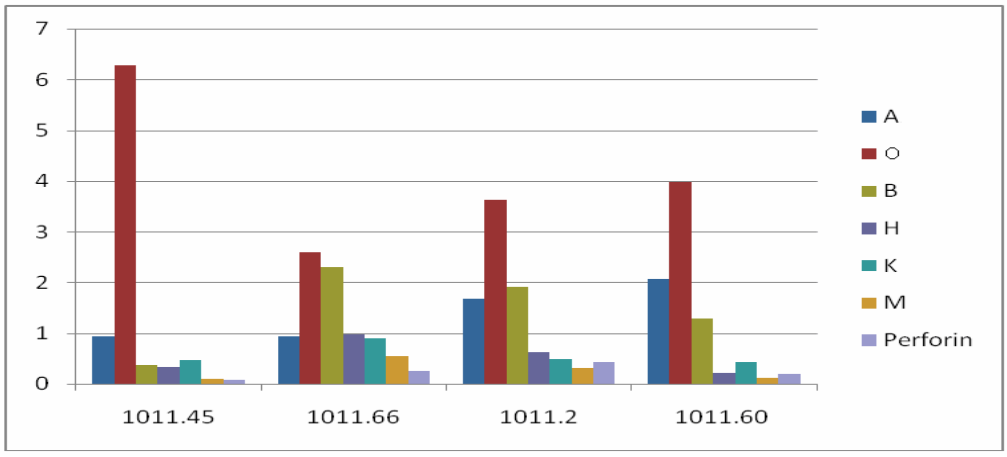
Figure 5.8 Relationship of killing levels and granule enzyme transcript profiles in CD8+ T cell clones (1011).

A. Agarose gels showing the PCR products for individual enzymes and the GAPDH control. A negative control (-), without added template, and a positive control (+), consisting of primers with cDNA template of an uncloned *T. parva*-specific CD8+ T cell line (641) are included for each enzyme; B. Changes in quantity of PCR product (vertical axis) in different T cell clones, normalised in relation to that of the GAPDH product obtained from the same sample.

A.



B.



The density of the bands was measured by Kodak 1D software (version 3.6) and results (vertical axis) are expressed as ratios of the density of the specific bands to that of the corresponding GAPDH bands.

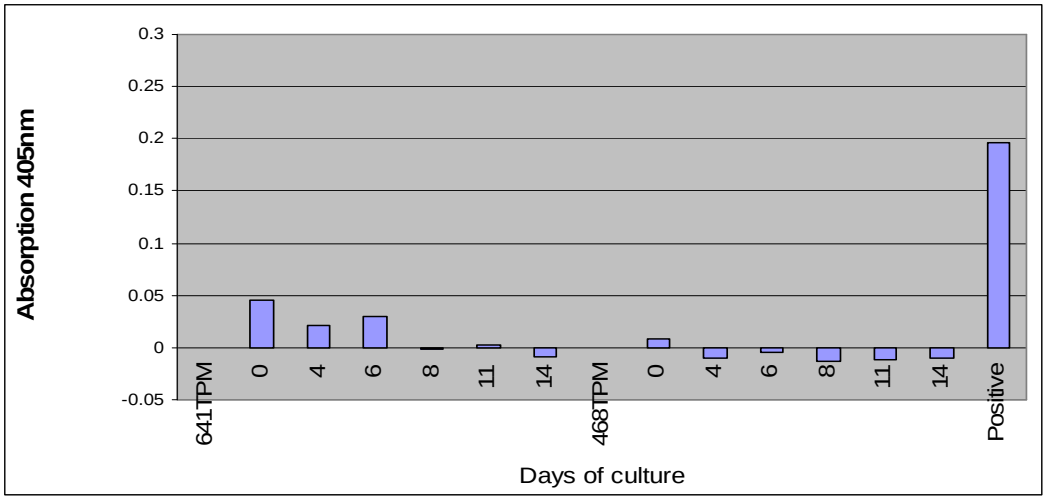
5.3.5 Relationship of killing levels and granzyme B protein expression

To further investigate whether the different levels of killing by CD8+ T cells is related to expression of granzyme B or degranulation, twelve $Tp1_{214-224}$–specific CD8+ T cell clones derived from two *T. parva*-immune animals (641 and 633), kindly provided by Ms Victoria Carroll were examined for levels of granzyme B enzymatic activity (Ewen et al., 2003). In a separate assay, these T cells gave variable levels of killing of autologous parasitized target cells and $Tp1_{214-224}$ peptide-pulsed target cells but did not kill MHC-mismatched parasitized target cells, indicating that they are antigen-specific and MHC restricted (data not shown).

MHC-homozygous *T. parva*-infected cells, 641TPM and 468TPM (MHC A18/18) were used as a source of stimulator cells for the 641 and 633 CD8+ T cell clones, respectively. In earlier experiments, RT-PCR assays had demonstrated expression of granzyme B transcripts in the 641TPM line. To determine the possible contribution of granzyme release from these stimulator cells to the T cell assays, lysates prepared from the 641TPM and 468TPM cell lines at different intervals up to 14 days after gamma-irradiation were analysed for granzyme B activity. No significant granzyme B activity was detected in the 468TPM line at any time point, while the 641TPM contained very low levels on days 0, 4 and 6 but was negative thereafter (Figure 5.9). A further experiment was undertaken to examine granzyme B release from the *T. annulata*-infected cell line (641TAA) used to present peptides to the CD8+ T cell lines. Consistent with the RNA analyses, no granzyme B was detected in lysates

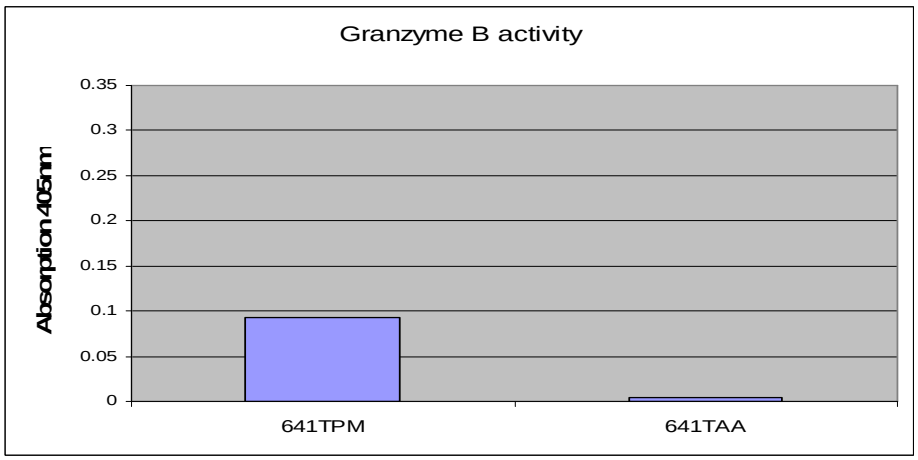
from this line, whereas 641TPM again exhibited low levels of granzyme B (Figure 10).

Figure 5.9 Granzyme B enzymatic activity in lysates of two *T. parva*-infected cell lines cultured for different intervals following gamma-irradiation.



Aliquots of 5x10^5 of gamma-irradiated *T. parva*-infected cells were cultured in 2ml SCM for the indicated times Granzyme B enzymatic activity in cell lysates was assayed by measuring the colour reaction generated after 4 hours incubation with the pNA substrate using a Synergy™ HT Multi-Mode Microplate Reader (BioTek).

Figure 5.10 Granzyme B activity in lysates prepared from *T. parva*-infected (TPM) and *T. annulata*-infected (TAA) cell lines from animal 641

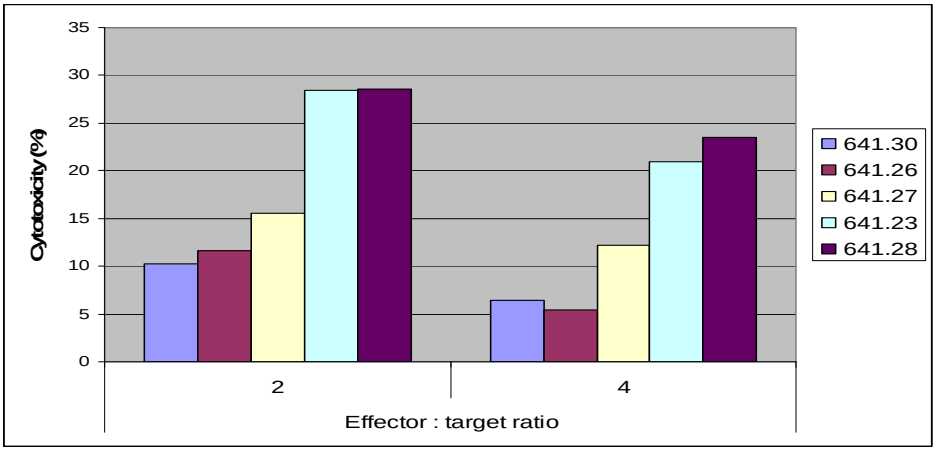


Aliquots of 1x10^6 of *Theileria*-infected cells in 1 ml were lysed and assayed for granzyme B enzymatic activity by measuring the colour reaction generated after 4 hours incubation with the pNA substrate using a Synergy™ HT Multi-Mode Microplate Reader (BioTek).

A pilot experiment was performed with five 641 CD8+ T cell clones to examine whether the levels of killing activity on [^{111}In]-labelled autologous 641TPM were associated with the granzyme B content of the cells. Since the T cell clones were assayed for cytotoxicity on day 7 after stimulation, this time point was also chosen for analysis of granzyme B activity in CD8+ T cell lysates. Figure 11- A shows the levels of killing obtained with the clones when tested at effector to target ratios of 2:1 and 4:1. Although the levels of killing observed at the two effector to target ratios differed slightly, the relative ranking of cytotoxic activity between the clones was similar. Interestingly, when the granzyme B content of lysates from these lines was examined they displayed a clear trend, namely that those CD8+ T cell clones with the highest levels of granzyme B showed the highest killing of autologous *T. parva*-infected cells (Figure 5.11- A and B).

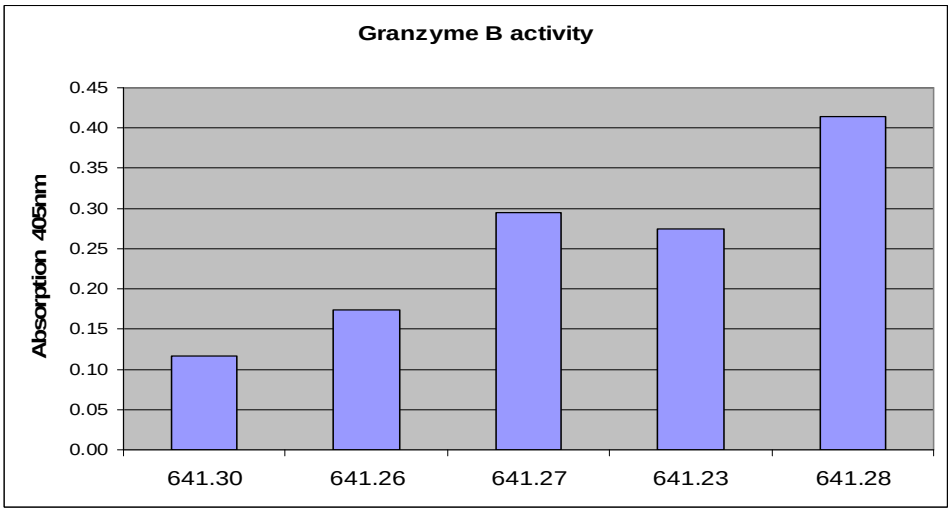
Figure 5.11 Cytotoxicity activity (A) and granzyme B activity (B) of five *T. parva*-specific CD8+ T cells clones from animal 641

A.



Five cloned 641 CD8+ T cell lines (1x10^{4}cells/well) were tested in a 4-hour cytotoxicity assay with [^{111} In]-labelled autologous *T. parva*-infected target cells at effector to target ratios of 2:1 and 4:1.

B.

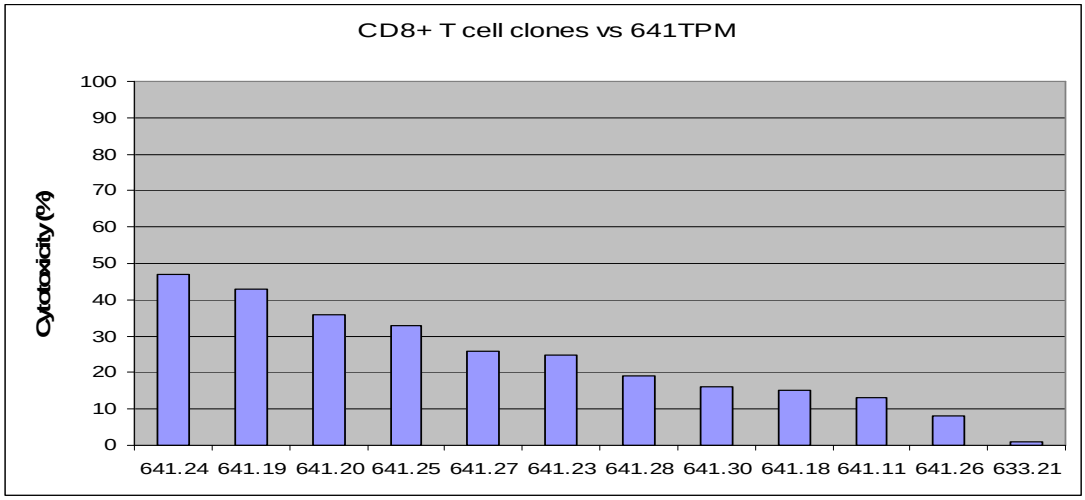


Aliquots of 1x10^6 CD8+ T cells were lysed and granzyme B enzymatic activity assayed by measuring the colour reaction generated after 4 hours incubation with pNA substrate, using a Synergy™ HT Multi-Mode Microplate Reader (BioTek).

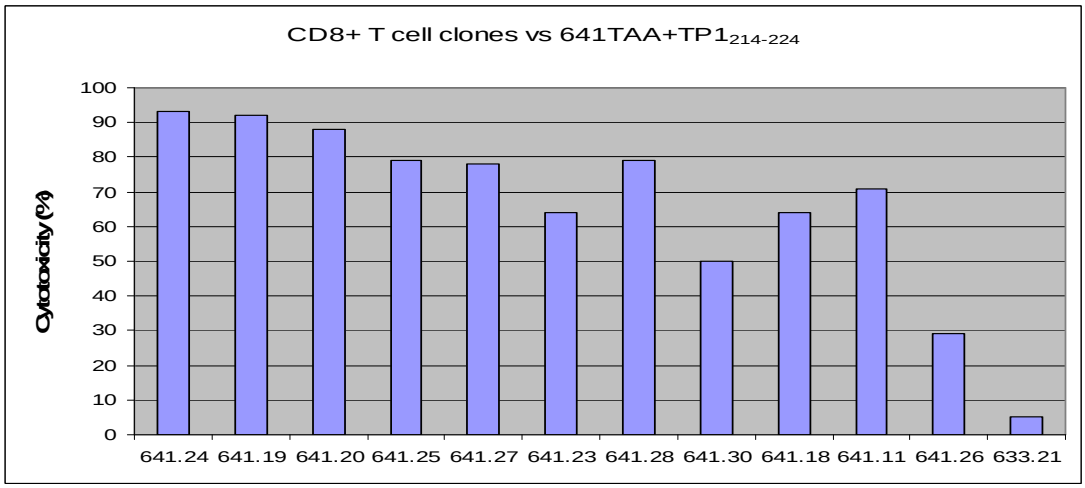
To further investigate this observation, experiments were undertaken using a larger set of 12 $Tp1_{214-224}$–specific CD8+ T cell clones (including the 5 clones used in the previous experiment). Degranulation was also assessed by measuring granzyme B released into the supernatant following incubation with 641TAA pulsed with $Tp1_{214-224}$ peptide. A standard effector to target ratio of 2:1 was used. Since the T cell clones were assayed for cytotoxicity on day 7 after stimulation, this time point was also chosen for analysis of granzyme B activity in CD8+ T cell lysates. The levels of cytotoxicity on autologous parasitized cells and on cells pulsed with epitope peptide ranged from 1% to 47% and from 5% to 93%, respectively (Figure 5.12). The levels of granzyme B in cell lysates of these CD8+ T cell clones and in supernatants following incubation with target cells are shown in figure 5.13.

Figure 5.12 Cytotoxic activity of *T. parva*-specific CD8+ T cell clones assayed with (A) autologous *T. parva*-infected cell target cells and (B) autologous *T. annulata*-infected target cells pulsed with Tp1214-224 peptide

A.



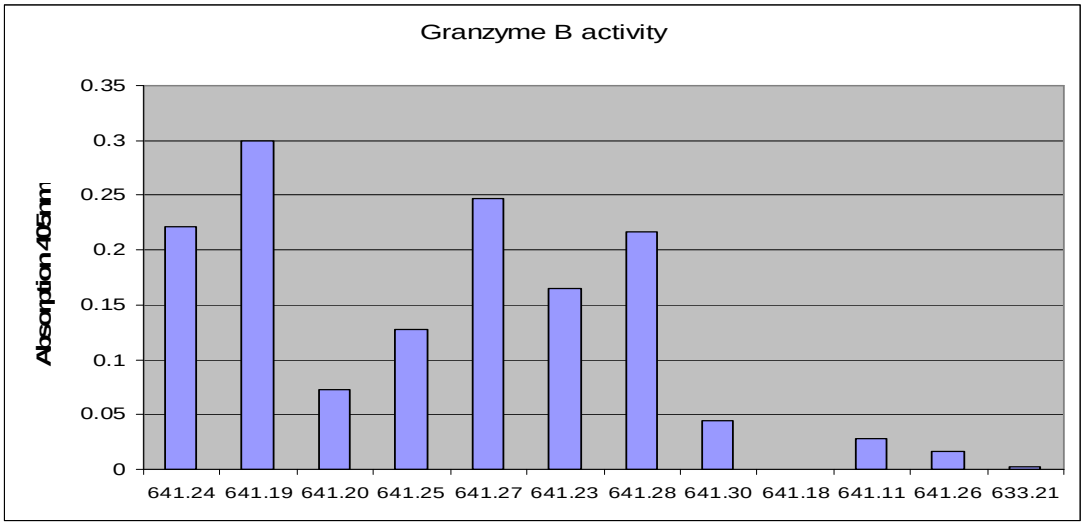
B.



CD8+ T cell lines ($1x10^4$cells) were tested in a 4-hour cytotoxicity assay with the respective [^{111} In]-labelled target cells. *T. annulata*-infected targets were incubated with 100ng/ml of the $Tp1_{214-224}$ peptide. A standard effector to target ratio of 2:1 was used.

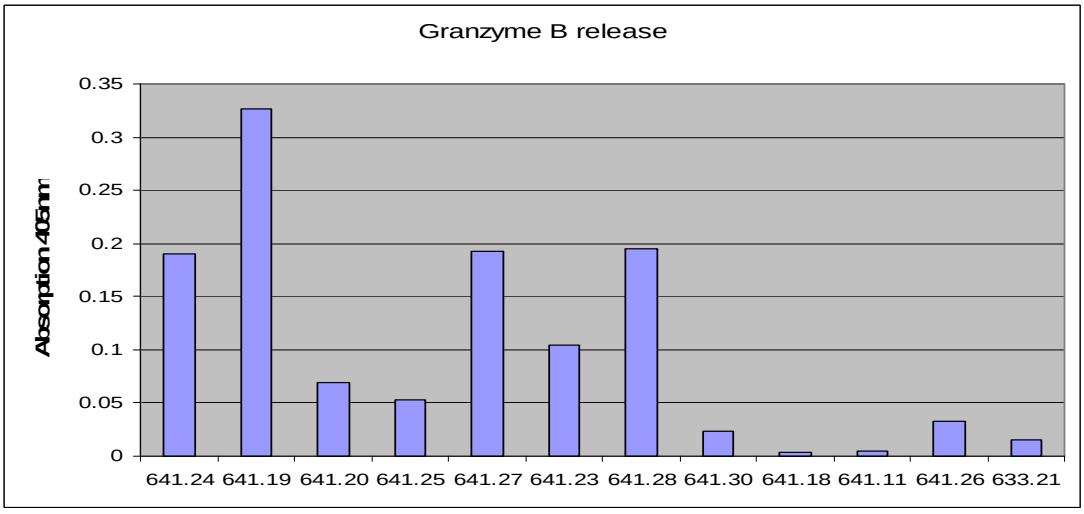
Figure 5.13 Granzyme B activity in, (A) cell lysates from 12 *T. parva*-specific CD8+ T cell clones and, (B) in supernatants of the same 12 clones following incubation with peptide-pulsed target cells.

A.



Aliquots of 1x10^6 CD8+ T cells were lysed and granzyme B enzymatic activity in lysates assayed by measuring the colour reaction generated after 4 hours incubation with pNA substrate using a Synergy™ HT Multi-Mode Microplate Reader (BioTek).

B.



Aliquots of 1x10^6 CD8+ T cells were incubated for 4 hours with autologous *T. annulata*-infected cells with Tp1$_{214-224}$ peptide added at 100ng/ml. Supernatants were harvested and granzyme B activity measured as described for (A) above.

The values for all of the data are presented in table 5.3 and plots of cytotoxicity versus levels of granzyme B in cell lysates or supernatants and their statistical associations are presented in figure 5.14- 5.16. When the levels of granzyme B activity in lysates are plotted against killing of parasitized cells, there is a statistically significant positive correlation ($r=0.732$, $p=0.007$) (Figure 5.14- A). The levels of granzyme B activity in lysates or in supernatants also showed a statistically significant positive correlation with killing of cells pulsed with epitope peptide (the former, $r=0.679$, $p=0.015$; the latter, $r=0.599$, $p=0.039$) (Figure 5.14- B and 5.15), although these associations were weaker than those observed with killing of parasitized cells. When the levels of granzyme B activity in lysates are plotted against its release, a strong positive correlation was observed ($r=0.953$, $p=0.000$) (Figure 5.16).

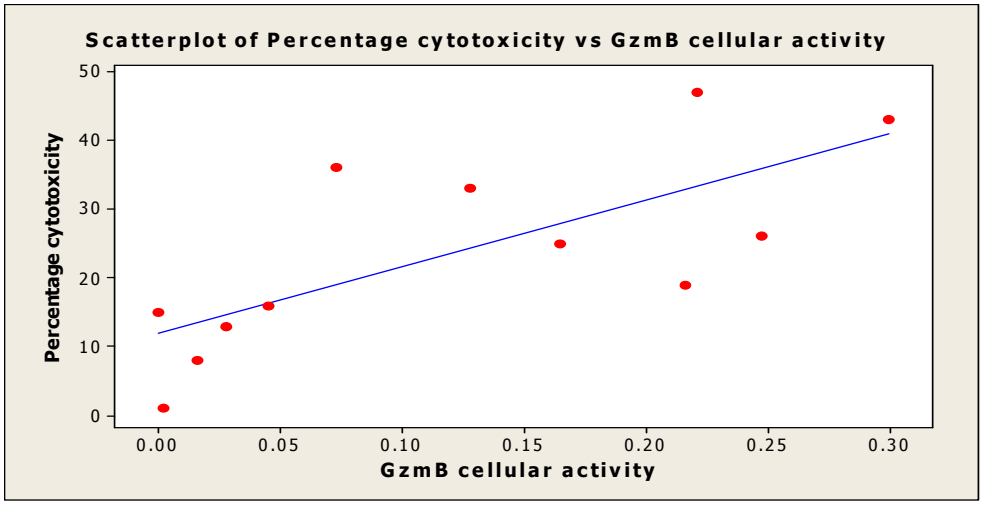
Table 5.3 Summary of cytotoxicity and granzyme B data on 12 *T. parva*-specific CD8+ T cell clones

T cell clone	TCR Vβ	Killing of TPM (%)	Killing of TAA+ $Tp1_{214-224}$ (100ng/ml) (%)	GzmB activity (405nm)	GzmB release (405nm)
641.24	3	47	93	0.22	0.19
641.19	3	43	92	0.30	0.33
641.20	3	36	88	0.07	0.07
641.25	3	33	79	0.13	0.05
641.27	NT	26	78	0.25	0.19
631.23	NT	25	64	0.16	0.10
641.28	NT	19	79	0.22	0.20
641.30	NT	16	50	0.05	0.02
641.18	1	15	64	0.00	0.00
641.11	1	13	71	0.03	0.01
641.26	NT	8	29	0.02	0.03
633.21	NT	1	5	0.00	0.02

Data on the TCRVβ genes expressed by individual T cell clones are cited from (Macdonald et al.); NT=not tested.

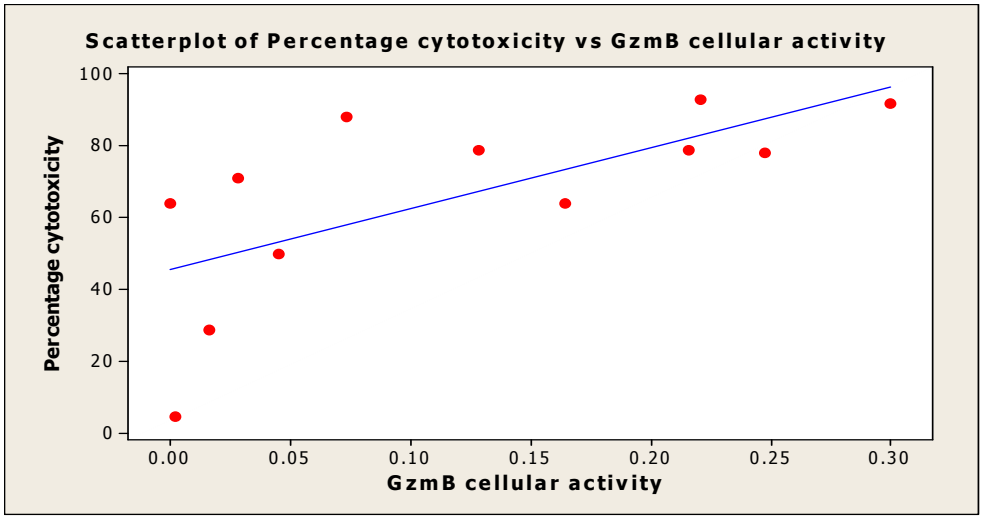
Figure 5.14 Correlation of granzyme B cellular activity with (A) levels of killing of *Theileria-infected* target cells by CD8+ T cell clones and, (B) levels of killing of peptide-pulsed target cells by the same CD8+ T cell clones

A.



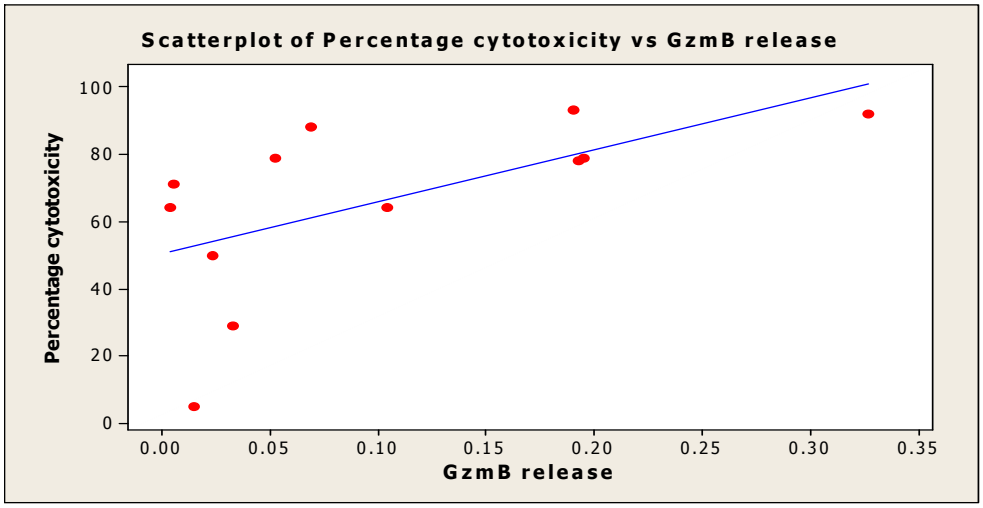
Correlation coefficient=0.732; P value=0.007

B.



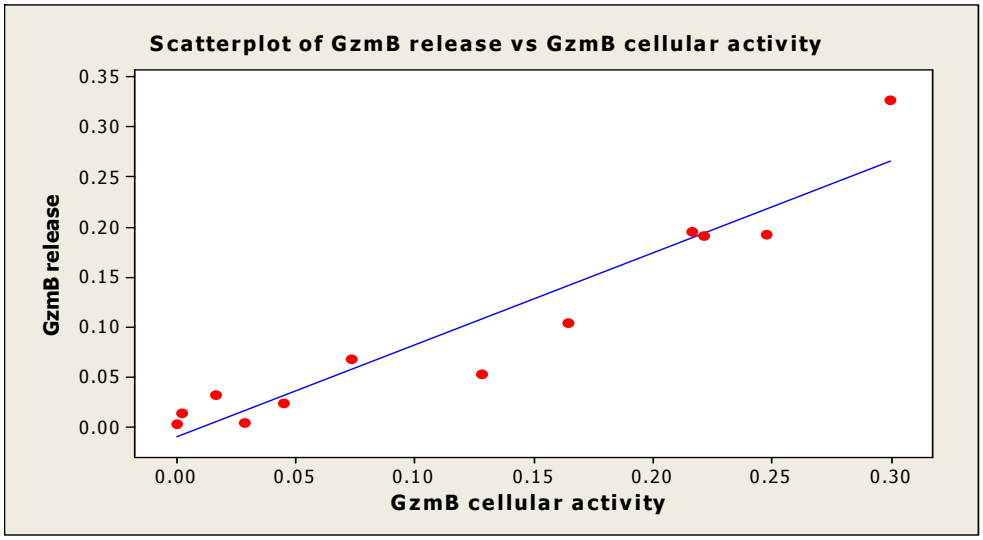
Correlation coefficient=0.679; P value=0.015

Figure 5.15 Correlation of granzyme B release by CD8+ T cell clones following stimulation with irradiated parasitized cells and their levels of killing of peptide-pulsed target cells.



Correlation coefficient=0.599, P value=0.039.

Figure 5.16 Association of levels of granzyme B cellular activity of CD8+ T cell clones and levels released following antigenic stimulation.



Correlation coefficient=0.953, P value =0.000

Since cytotoxicity activity and granzyme B release by CD8+ T cells had been measured using target cells loaded with a relatively high concentration of epitope peptide (100ng/ml), further experiments were undertaken to test the minimal

concentrations of peptide required to mediate cytotoxicity. Based on preliminary experiments, 100-fold dilutions of $Tp1_{214-224}$ peptide, ranging from 0.0001ng/ml to 1ng/ml, were tested (Figure 5.17). Three of the 641 CD8+ T cell clones, including one (641.20) that tended to have low levels of granzyme B content but relatively strong killing, were used. The granzyme B content in these three T cell clones and their cytotoxic activity against autologous *T. parva*-infected cells (641) were examined at the same day (7 day after the last stimulation) (Table 5.4). All 3 clones showed a similar cytotoxicity profile, with killing of between 52% and 63% of targets pulsed with 1ng/ml of peptide decreasing to undetectable levels at 0.0001ng/ml of peptide. However, the levels of granzyme B release at the highest peptide concentration (1ng/ml) differed for the 3 clones, and the values were similar to those obtained with 100ng/ml in the previous experiments. Nevertheless, the decrease in levels of killing observed with lower peptide concentrations was associated with a progressive decrease in granzyme B release by all 3 clones. As in the previous experiments, clone 641.20 showed low levels of granzyme B release but relatively strong killing.

Table 5.4 Supporting data of three CD8+ T cell clones

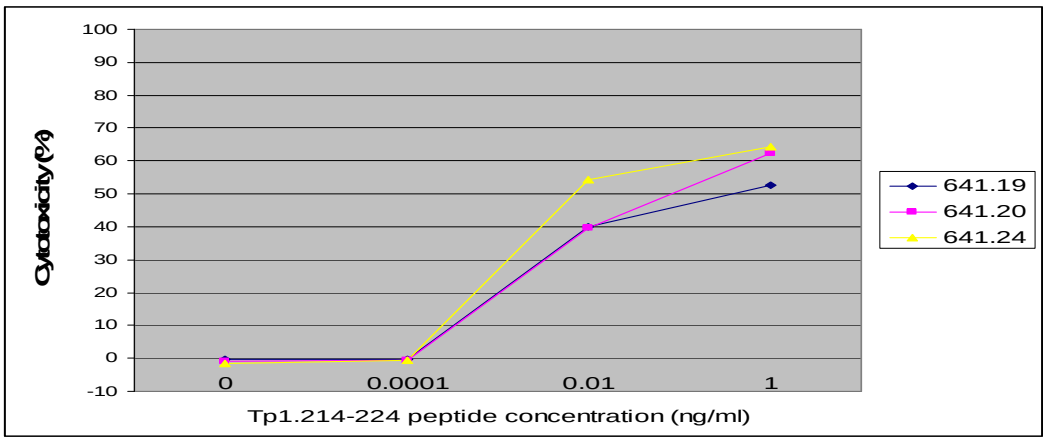
T cell clone	TCR Vβ	Granzyme B activity (405nm)	Cytotoxicity (%) against autologous *T. parva*-infected cells
641.19	3	0.17	28
641.20	3	0.09	33
641.24	3	0.14	35

The assays were carried out on the same day as the assays described in figure 5.17. The data on expressed TCRVβ genes are cited from the reference (Macdonald et al.)

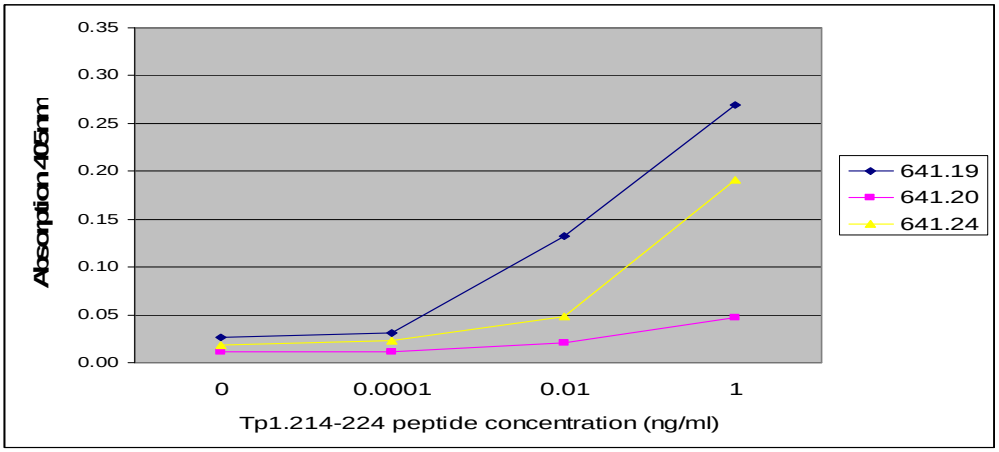
Figure 5.17 Influence on target peptide density on levels of cytotoxicity and granzyme B release by 3 *T. parva*-specific T cell clones.

(A). cytotoxicity assayed on *T. annulata*-infected target cells incubated with different concentrations of $Tp1_{214-224}$ peptide. (B). granzyme B concentrations in supernatants of the same T cell clones following incubation with peptide pulsed targets for 4 hours.

A.



B.



A standard effector to target ratio of 2:1 was used in for both assays.

These results indicate that although there is a statistically significant association between the levels of killing activity of different *T. parva*-specific CD8+ T cell clones and granzyme B protein cell content and release, this correlation is not absolute. It suggests that other factors in addition to granzyme B, which vary between T cell clones, contribute to the cytotoxic activity of the CD8+ T cells.

5.4 Discussion

Several studies in other species have shown differential expression and discordant regulation of granule enzymes in different subpopulation of lymphocytes, both at the transcription level (Grossman et al., 2003) and protein expression (Sedelies et al., 2004; Bade et al., 2005; Bratke et al., 2005). Transcripts of perforin and all except granzymes B and O were detected in resting PBMC. Granzymes B and O were expressed in ConA-activated PBMC, indicating that the expression of these two granzyme is activation-dependent. In contrast, while granzyme K was expressed in resting PBMC, there was no detectable transcript in ConA-activated cells. This might relate to a more restricted cellular expression of this granzyme K, although in humans expression of granzyme K at the protein level has been reported in T cells, $CD56^{bright}$ NK cells and a subpopulation of NKT cells (Bade et al., 2005; Bratke et al., 2005). Granzyme expression was also detected in *T. parva*-infected cell lines of different T cell subtypes, but not in infected lines of B cell origin, reflecting their lineage-restricted expression to T lymphoid lineage and NK cells (Anthony et al., ; Bovenschen and Kummer). Therefore, although infection with *T. parva* results in altered expression of some host proteins, the presence of granzyme expression appears to be consistent with the origin of cells.

PI-9 acts as an endogenous inhibitor of human granzyme B. It protects CTL and bystanders against the actions of granzyme B (Sun et al., 1996; Bladergroen et al., 2001; Buzza et al., 2001; Hirst et al., 2001; Bird et al., 1998). Several studies have reported that expression of PI-9 in tumour cell lines gives rise to resistance to

perforin/ granzyme B-mediated killing activity by CTL and NK cells (Medema et al., 2001; Bladergroen et al., 2002; Bots et al., 2006; Jiang et al., 2006), although this conclusion remains controversial (Godal et al., 2006). Expression of PI-9 in cattle is activation-dependent, as it detected in ConA-activated but not resting PBMC. Of particular interest was the observation that all of *T. parva*-infected cell lines examined consistently expressed higher levels of PI-9 transcripts than ConA-activated lines and uncloned CD8+ T cell lines (used as a positive control). This conclusion is based on visual comparison of the intensity of PCR products in agarose gels and needs to be examined by fully quantitative PCR to accurately determine the relative differences in expression. *Theileria parva* has been shown to protect infected cells from Fas/Fas ligand-induced apoptosis by upregulating a number of anti-apoptotic proteins (Guergnon et al., 2003; Kuenzi et al., 2003). Further studies are required to determine whether enhanced expression of PI-9 in *T. parva*-infected cells reduces their sensitivity to perforin/granzyme-mediated killing in comparison to uninfected cells.

Levels of transcripts for perforin and granzymes detected in 8 cloned CD8+ T cell lines with a semi-quantitative assay showed no consistent pattern of expression that correlated with the levels of killing of *T. parva*-infected target cells. One possible reason for this finding is that the PCR method used to measure Gzm/Prf expression may not be sufficiently quantitative to detect significant differences. This could be overcome by using a quantitative PCR. Alternatively, proteins of perforin and the granzymes are synthesized and stored in lytic granule after the initial activation when CTL precursor recognizes the target (Griffiths, 1995). It is therefore possible that

several CD8+ T cells which do not have active GzmB RNA transcriptions may still have proteins of perforin and the granzymes and are capable to mediate the cytotoxicity by immediately releasing them upon the recognition of the same target. Hence there may be no directly relevant expression pattern of perforin/ granzymes between the gene and protein. It is also possible that the cells within the cloned populations are heterogeneous in their patterns of Gzm/PFN expression and that only certain combinations of expression give effective killing. This would not be detected by examining the whole population. A study of mouse CD8+ T cells has reported that granzyme and perforin genes are differentially expressed in single CD8+ cells within cloned populations (Kelso et al., 2002). The ability of the different clones to preload and/or degranulate their effector molecules following recognition of the target cells might also vary.

Granzyme B has been shown to be the most potent effector molecule utilized by CD8+ T cells to kill infected cells in human and mouse. However, the lack of specific antibodies for bovine granzyme B as described in chapter 4 makes it difficult to investigate its expression at the protein level to examine its role in CD8+ T cell killing in cattle. An alternative approach is to measure the specific enzymatic activity of granzyme B. Ewen et al (2003) established a sensitive and reliable method to measure murine and human granzyme B activity in cell lysates and supernatants employing a peptide substrate, AC-IEPD-pNA. Work described in chapter 4 of this thesis demonstrated that AC-IEPD-pNA acts as a substrate for bovine granzyme B, thus providing an assay to detect and quantify the expression of functional bovine granzyme B in CD8+ T cells in relation to their cytotoxic activity.

The levels of granzyme B enzymatic activity in cell lysates of 12 cloned CD8+ T cell lines, as well as levels released into supernatants during target cell killing, showed statistically significant correlations with the levels of killing of *T. parva*-infected cells and peptide-pulsed cells by the CD8+ T cell lines. The results indicate that the amount of granzyme B released during killing reflects the level of granzyme B content of the T cells and the statistical associations of both parameters with levels of cell killing suggest that granzyme B is likely to be involved in killing of the target cells. However, this correlation was not absolute. For example, clone 641.20, which had low levels of granzyme B content and release, actually displayed relatively strong killing. This suggests that other factors, in addition to granzyme B, that vary between T cells clones may contribute to the cytotoxic activity of CD8+ T cells. Co-expression of more than one granzyme has been shown to enhance cytotoxic activity in vitro. For example, co-transfection of rat basophilic leukemia (RBL) cells with granzyme A and granzyme B in the presence of perforin resulted in enhanced killing of tumour targets in a synergistic manner (Nakajima et al., 1995). Also, human granzyme H, which itself can mediate cytotoxicity (Fellows et al., 2007), has been reported to augment granzyme B-mediated killing of adenovirus-infected cells (Andrade et al., 2001; Andrade et al., 2007; Waterhouse and Trapani, 2007). It was shown to directly restrict the replication of virus by cleaving and inactivating an essential viral protein (DNA binding protein/ DBP) and also enhanced granzyme B-induced death by neutralizing the viral inhibitor of granzyme B (L4-100K assembly protein) (Andrade et al., 2001; Andrade et al., 2007). Recent studies in vitro have demonstrated the ability of human granzyme M to rapidly induce cell death of tumor

cells, which displayed a unique cell death-morphology (Kelly et al., 2004; Bovenschen et al., 2008; Cullen et al., 2009). In addition to directly killing target cells, granzyme M has been reported to hydrolyse PI-9 and inactivate its inhibitory function for granzyme B (Mahrus et al., 2004). These findings have shown the potential for other granzymes to cooperate with granzyme B in achieving CD8+ T cell-mediating cell death. Further investigation of these interactions in cattle is hampered by the current lack of specific antibodies and biological assays to measure other bovine granzyme proteins.

It has been observed that T cell clones that give poor killing of *T. parva*-infected cells show substantially increased levels of killing against peptide-pulsed cells, particularly at high peptide concentration. The strong correlation between granzyme B content and its release detected in 12 cloned T cell lines indicated that there was no difference in degranulation ability among these clones. However, the higher levels of cytotoxicity detected against peptide-pulsed compared to infected targets indicates that the peptide ligand concentration can influence the extent of degranulation by the T cells. This was confirmed by showing increased levels of granzyme B release by *T. parva*-specific T cells exposed to targets incubated with increasing concentrations of peptide. Recent studies in mice have revealed that the strength of T cell receptor signal regulates the polarization of lytic granules to the immunological synapse and that a high threshold of signalling is required for full recruitment of granules to the synapse, implying a fine level of control of granule-mediated cell death by CD8+ T cells (Jenkins et al., 2009). Thus, in addition to effector molecules, the strength of T cell signalling also influences the efficiency of killing of target cells.

Chapter 6 Granzyme B is an important mediator involved in killing of *Theileria*-infected cells by specific CD8+ T cells

6.1 Introduction

It has been well established that the CD8+ T cell response against cells infected with *T. parva* schizonts is important in mediating immunological control of the infection (Morrison et al., 1987; Goddeeris et al., 1990; McKeever et al., 1994). Despite the evidence that CD8+ T cells are involved in mediating immunity, the mechanism by which they act against the parasitized cells is not well understood. CD8+ T cells in other species have been shown to act as effectors by releasing soluble mediators, such as IFN-γ (Boehm et al., 1997), TNF-α (Vassalli, 1992) and interleukins (Biron, 1994), which induce killing of the intracellular pathogens. However, previous experiments have provided evidence that the schizont stage of *Theileria parva* in infected leukocytes is not susceptible to IFN-γ or TNF-α (DeMartini and Baldwin, 1991). CD8+ T cells can also directly kill pathogen-infected cells by inducing rapid apoptosis, utilising either Fas/Fas ligand interaction which results in classical caspase-dependent apoptosis (Nagata and Golstein, 1995) or the granule exocytosis pathway, which involves release of perforin and granzymes (Henkart and Sitkovsky, 1994). Studies in mice have shown that the perforin-based cytotoxicity of CD8+ T cells is the predominant effector mechanism employed by CD8+ T cells against a number of intracellular pathogens (Kagi et al., 1994a; Walsh et al., 1994; Nickell and

Sharma, 2000). Perforin is a pore-forming protein, which facilitates delivery of granzymes into the cytosol of target cells, and thus plays an essential role in lymphocyte-mediated cytotoxicity. Although the precise delivery mechanism is still under debate, expression of perforin is absolutely required for killing by CTL granule exocytosis, as indicated by absence of killing activity in T cells from perforin-deficient mice (Kagi et al., 1994b; Kojima et al., 1994; Lowin et al., 1994). Purified perforin alone has been shown to induce direct lysis of target cells under some conditions; however, induction of DNA damage of the same cells only occurred in the presence of granzymes (Hayes et al., 1989; Shi et al., 1992a; Shi et al., 1992b). Granzymes, a subfamily of serine proteases, comprise a small group of enzymes (5 in human and 10 in mouse), with various primary substrate specificities. Granzyme A and B are the most abundant granzymes and have been studied in most detail. In vitro studies by the Lieberman group showed that purified human granzyme A induces target cell death via damage of the inner mitochondrial membrane and DNA nicking (Lieberman and Fan, 2003). However, the physiological significance of these findings remains controversial (Metkar et al., 2008; Trapani and Bird, 2008; Pardo et al., 2009), and several investigators have reported that granzyme A-deficient mice have no defect of CTL/NK-mediated cytotoxicity, suggesting that granzyme A does not play an essential role in cell mediated cytotoxicity (Ebnet et al., 1995; Mullbacher et al., 1996; Riera et al., 2000). In contrast, there seems to be little dispute that granzyme B is a potent inducer of apoptosis and an important mediator of cytotoxicity. In vitro studies have shown that granzyme B is critical for the rapid induction of target cell lysis and DNA fragmentation by CD8+ T cells generated from granzyme B-deficient mice (Heusel et al., 1994). Granzyme B induces

apoptosis by two main pathways: one involving direct activation of caspases and the other by triggering mitochondrial outer membrane permeabilization via cleaving the pro-apoptotic protein, BH3-interaction domain death agonist (Bid). The truncated Bid protein initiates mitochondrial-dependent apoptosis via activating Bax and/or Bak and promotes their oligomerization within the mitochondrial outer membrane, which results in permeabilization of mitochondria and release of cytochrome c (Kuwana et al., 2002). Recent studies have shown that the pathways utilised by granzyme B to mediate killing are species-dependent. Mouse granzyme B directly activates caspases to promote apoptosis, whereas human granzyme B acts by a Bid-dependent pathway (Kaiserman et al., 2006; Cullen et al., 2007). Despite the finding that GzmA/B-deficient CD8+ T cells are defective inducers of classical apoptosis, they can still mediate target cell death in some systems, suggesting that other granzymes may be operating via alternative pathways (Simon et al., 1997; Mullbacher et al., 1999; Davis et al., 2001; Waterhouse et al., 2006).

The studies described in this chapter, investigated the role granule exocytosis and specifically granzyme B in killing of *T. parva*-infected target cells by testing a series of commercially available specific inhibitors.

6.2 Materials and Methods

6.2.1 Animals and cell lines

Selected CD8+ T cell clones established from two cattle (592 and 641) homozygous for the A10 or A18 class I MHC haplotypes were kindly provided by Dr Tim Connelley and Ms Victoria Carroll, respectively. An un-cloned CD8 T cell culture established from animal 011 was generated as described in section 2.2.4. Three autologous *T. parva*-infected cell lines, 641TPM (MHC A18/A18), 011TPM and 592TPM (both MHC A10/A10), were used as infected target cells in cytotoxicity assays. *T. annulata*-infected cell lines from the same animals loading with a specific peptide epitope, 641TAA-pulsed-Tp1$_{212\text{-}224}$ and 592TAA-pulsed-Tp2$_{49\text{-}59}$, were used to investigate killing of peptide-pulsed-target cells, as described in section 2.2.8.

6.2.2 Inhibitors of proteins involved in the exocytosis pathway

Ten inhibitors specific for four molecules involved in the exocytosis pathway were used in this chapter. The properties of these inhibitors are shown in Table 6.1. Concanamycin A, CMA (SIGMA, Cat. C9705) is an inhibitor of perforin (PFN) activity. Four inhibitors of granzyme B were tested: GzmB inhibitor I (Calbiochem, Cat.368050), GzmB inhibitor II (Calbiochem, Cat.218773), GzmB inhibitor III (Calbiochem, Cat.218840) and GzmB inhibitor IV (Calbiochem, Cat.368056). They all contain short peptide sequences that bind to amino acids at the active sites of granzyme B and thus act as competitive inhibitors of enzymatic function. Of these,

GzmB inhibitor IV, used in previous experiments described in section 4.3.4, is a non-cell-permeable competitive inhibitor containing a 4-amino acid sequence (IEPD). The other three granzyme B inhibitors tested are cell-permeable and also act as competitive inhibitors. GzmB inhibitor I contains a three-amino acid peptide (AAD) (Odake et al., 1991; Shi et al., 1992a), and GzmB inhibitor II and III have a same 4-amino acid peptide (IETD). The IETD sequence in the latter two inhibitors also binds to caspase 8 and has caspase 8 inhibitory activity (Thornberry et al., 1997; Harris et al., 1998; Martin et al., 1998; Sweeney et al., 1998). Caspase inhibitor I (Calbiochem, Cat.627610), containing a 3 amino acid peptide (AAD), is a broad competitive inhibitor of caspases, acting on several caspases involved in apoptotic pathways (Garcia-Calvo et al., 1998). It reacts with active sites on most caspases and irreversibly inactivates their activity; the negative control of caspase inhibitor I, cathepsin B inhibitor I (Calbiochem, Cat.342000), was also included (Sarin et al., 1997). Bax, a pro-apoptotic member of the Bcl-2 family, plays an important role in mitochondrial-dependent apoptosis (Korsmeyer et al., 1999). It is activated via cleaving by truncated Bid protein and involved in inducing permeabilization of mitochondria and release of cytochrome c (Kuwana et al., 2002). Two inhibitors Bax Channel Blocker (Calbiochem, Cat.196805) and Bax-inhibiting Peptide, V5 (Calbiochem, Cat.196810) along with its negative control (Calbiochem, Cat.196811) were used.

Table 6.1 Inhibitors tested in the current study and their properties

Inhibitor Target	Name	Inhibitor Sequence	Other Target	Cell-permeable	Reversible
PFN	CMA	NT	-	+	-
GzmB	Inhibitor I	Z-AAD-CMK	-	+	-
	Inhibitor II	AC-AAVALLPAVLLALLAP-IETD-CHO	Caspase 8	+	+
	Inhibitor III	Z-IE(OMe)TD(OMe)-FMK	Caspase 8	+	-
	Inhibitor IV	AC-IEPD-CHO	-	-	+
Caspase	Inhibitor I	Z-VAD(OMe)-FMK	Pan Caspases	+	-
Caspase, NC	Cathepsin B Inhibitor 1	Z-FA-FMK	-	+	-
Bax Channel Blocker	Bis TFA	3,6-Dibromocarbazole Piperazine Derivatives of 2-Propanol	-	+	NT
Bax-inhibiting Peptide, V5	BIP-V5	H-VPMLK-OH	-	+	NT
Bax-inhibiting Peptide, NC	BIP-NC	H-IPMIK-OH	-	+	NT

+, yes, -, no; NT, not determined; NC, negative control; Amino acid sequence of the inhibitor peptide is highlighted in red. Z = benzyloxy-carbonyl group; AC = acetyl group; OMe = O-methyl ester group; CHO = aldehyde group; CMK = chloromethyl ketone; FMK = fluoromethyl ketone.

6.2.3 Inhibition of enzymatic granzyme B activity in CD8+ T cell lines

The cell-permeable granzyme B inhibitors, GzmB inhibitor I, GzmB inhibitor II and GzmB inhibitor III (described above), were tested for their abilities to inhibit cattle

granzyme B activity in CD8+ T cell lines. Z-VAD-FMK, a pan-caspase inhibitor was used as negative control for the inhibition specificity, whereas a non-cell-permeable GzmB inhibitor IV, AC-IEPD-CHO, which was found to substantially inhibit cattle granzyme B activity in lysates in section 4.3.4, was used as a positive control.

6.2.3.1 Inhibition of enzymatic granzyme B activity in CD8+ T cell lines

Aliquots of 1ml of CD8+ T cells adjusted to $1x10^6$cells/ml were centrifuged at 180 x g for 10 min and re-suspended in 150ul SCM. Following addition of 50ul of each inhibitor to the CD8+ T cells (GzmB inhibitor I, 80uM; GzmB inhibitor II, 80uM; GzmB inhibitor III, 40uM; caspase inhibitor I, 80uM), the cells were incubated for 1 hour in a humidified incubator in an atmosphere of 5% CO_2 at 37°C. Cells were then washed, pelleted and lysed as described in section 5.2.5. In duplicate, aliquots of 10ul of lysate supernatant, granzyme B substrate VIII, Ac-IEPD-pNA (Calbiochem, Cat.368067) at a final concentration of 200uM and the reaction buffer (0.1M Hepes, pH7.0; 0.3M NaCl; 1mM EDTA) in a total volume of 100ul/well were added into the wells of Falcon™ 96-Well Flat bottomed Microplates (BD, Cat. 353072). Mixtures were incubated at 37°C for 4h and the colour reaction generated by cleavage of the pNA (p-nitroaniline) substrate measured at a wavelength of 405nm by using a Synergy™ HT Multi-Mode Microplate Reader (BioTek).

6.2.3.2 Positive control - Inhibition of enzymatic granzyme B activity in lysates

The inhibition of the enzyme in lysates (section 4.2.7) was used as a positive control for experiments with CD8+ T cell lines. The protocol was modified slightly as follows: CD8+ T cells ($1x10^6$cells) were washed, pelleted and lysed as described in section 5.2.5. In duplicate, aliquots of 10ul of lysates were pre-incubated with 10uM GzmB inhibitor IV (Ac-IEPD-CHO) at 37°C for 30min. Granzyme B substrate VIII (Ac-IEPD-*p*NA) at a final concentration of 200uM and the reaction buffer were added to give a total volume of 100ul, which was incubated at 37°C for 4h. The colour reaction generated by cleavage of the pNA substrate was measured at a wavelength of 405nm by using a Synergy™ HT Multi-Mode Microplate Reader (BioTek).

6.2.4 Cytotoxicity assay with inhibitors

An ^{111}In release cytotoxicity assay was used as described in section 2.2.8, but with some adaptations:

6.2.4.1 Cisplatin-induced apoptosis

Cisplatin, cis-diamminedichloroplatinum II, an important chemotherapeutic agents, has been shown to induce cancer cell death through apoptosis (Dive and Hickman, 1991). The drug was kindly provided by Dr Kyoko Hayashida (Hokkaido University, Japan) and was used to induce cell lysis of *Theileria*-infected cells. Aliquots of 50ul of two-fold dilutions of cisplatin in SCM were distributed in duplicate into wells of

96-well V-bottomed plates to give concentrations ranging from 75uM to 2.4mM and 100ul of ^{111}In labelled target cells ($5x10^5$) was added to each well. Hence, the final concentration of cisplatin ranged from 25uM to 800uM. Spontaneous release and maximal release of isotope were determined by adding 100ul cytotoxicity medium and 100ul 0.2%Tween respectively to the 50ul aliquots of cells. After 4, 20, 24 and 48 hour incubation, 75ul supernatant were harvested from each well and measured for radioactivity.

6.2.4.2 CD8+ T cell-induced apoptosis

Aliquots of 50ul of each inhibitor were pre-incubated in duplicate with 100ul effector cells ($1x10^5$-$6.25x10^3$ cells) or 50ul ^{111}In labelled target cells ($5x10^3$ cells) for various times and at different concentrations as specified below for the individual inhibitors. Following pre-incubation, aliquots of 100ul of effector cells or 50ul of ^{111}In labelled target cells were added to wells containing pre-incubated target cells and effector cells respectively, to give a total volume of 200ul/well. Spontaneous release and maximal release from untreated target cells were determined by adding 150ul of cytotoxicity medium and 150ul 0.2% Tween 20 respectively to 50ul aliquots of target cells. After 4-hour incubation, 100ul supernatant was harvested from each well and measured for radioactivity.

For the inhibition of perforin activity, effector cells were pre-incubated with ten fold dilutions of CMA at final concentrations ranging from 0.1ug/ml to 1000ug/ml for 2 hour at 37 °C. For the inhibition of granzyme B activity, effector cells were pre-incubated for 1 hour at 37°C with the following inhibitors: GzmB inhibitor I (80uM);

GzmB inhibitor II (80uM), GzmB inhibitor III (40uM) and the negative control, caspase inhibitor I (80uM). For inhibition of caspase activity, ^{111}In labelled target cells were pre-incubated with caspase inhibitor I (80uM) and the negative control, cathepsin B Inhibitor I (80uM) for 1 hour at 37°C. For inhibition of Bax activity, ^{111}In labelled target cells were pre-incubated for 1h at 37°C with 2 fold dilutions of Bax Channel Blocker at final concentrations ranging from 2.5uM to 20uM or Bax-inhibiting Peptide, V5 and its negative control at final concentrations ranging from 25uM to 200uM.

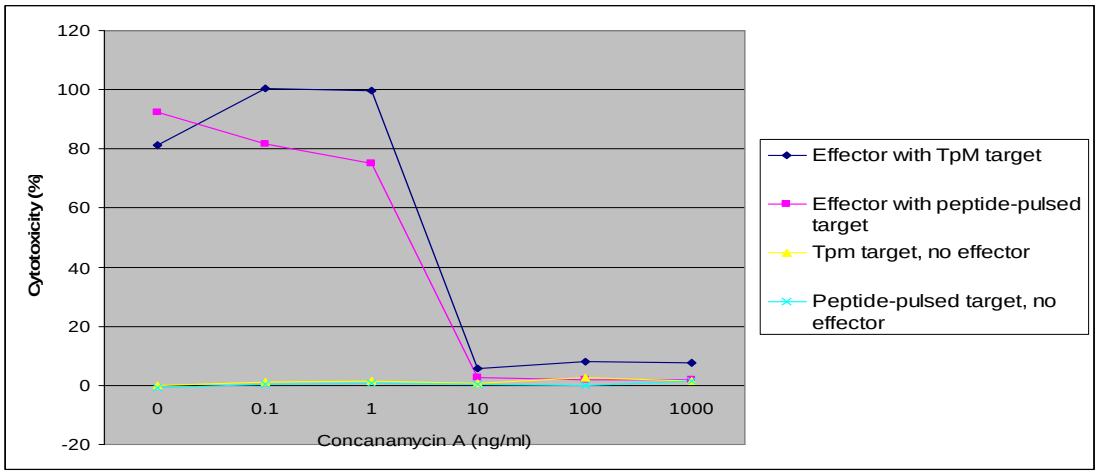
6.3 Results

6.3.1 Theileria-infected cell death induced by CD8+ T cells is mediated by granule exocytosis pathway

To investigate whether killing of *Theileria parva*-infected cells by CD8+ T cell is mediated by granule enzymes, concanamycin A (CMA), an inhibitor of vacuolar type H^+-ATPase was used (Kataoka et al., 1996). CMA induces degradation of perforin by raising the pH of the lytic granule in which acidification is essential for maintaining its structure and function (Kataoka et al., 1994). An uncloned CD8+ T cell line derived from animals 011 and 3 cloned CD8+ T cell lines from animal 592, harvested 7 days after the last antigen stimulation, were pre-incubated with various concentrations of CMA for 2 h and tested in a 4-hour cytotoxicity assay with [^{111}In]-labelled autologous *Theileria*-infected target cells. In preliminary experiments, these T cell lines were shown to kill autologous parasitized target cells but not MHC-mismatched parasitized target cells and also killed $Tp2_{49-59}$ peptide-pulsed target cells, indicating that they are antigen-specific and MHC restricted (data not shown). CMA completely inhibited the cytolytic activity of all cell lines tested when added at concentrations of 10ng/ml or greater and had no effect on background release from the target cells indicating that it did not adversely affect their viability (Figure 6.1- A and B). The results indicate that the killing of bovine CD8+ T cells specific for *T. parva*-infected cells is dependent on perforin, implying that it is mediated by release of granule enzymes.

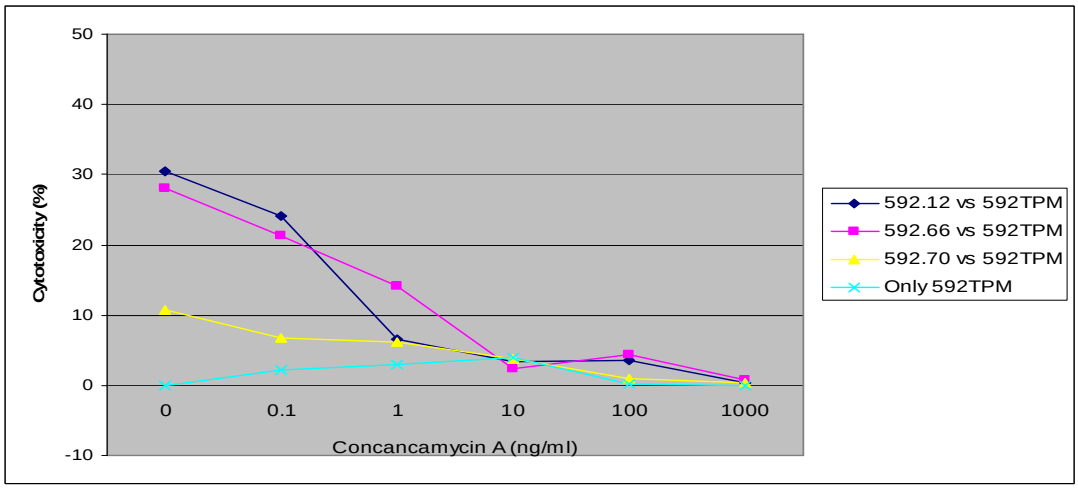
Figure 6.1 Inhibition of the cytotoxic activity of (A). an un-cloned CD8+ T cell line and (B). three CD8+ T cell clones by incubation with the perforin inhibitor CMA

A.



An un-cloned CD8+ T cell line (1x10^{5}cells) from animal 011 was pre-incubated with various concentrations of CMA for 2h and tested in a 4-hour cytotoxicity assay with [^{111}In]-labelled autologous 011TPM and MHC-matched (592) TAA-infected target cells pulsed with TP2$_{49\text{-}59}$ peptide (1000ng/ml). Labelled target cells alone were also incubated with CMA in the assay. A standard effector to target ratio of 20:1 was used.

B.



Three cloned CD8+ T cell lines (2.5x10^{4}cells) from animal 592 were pre-

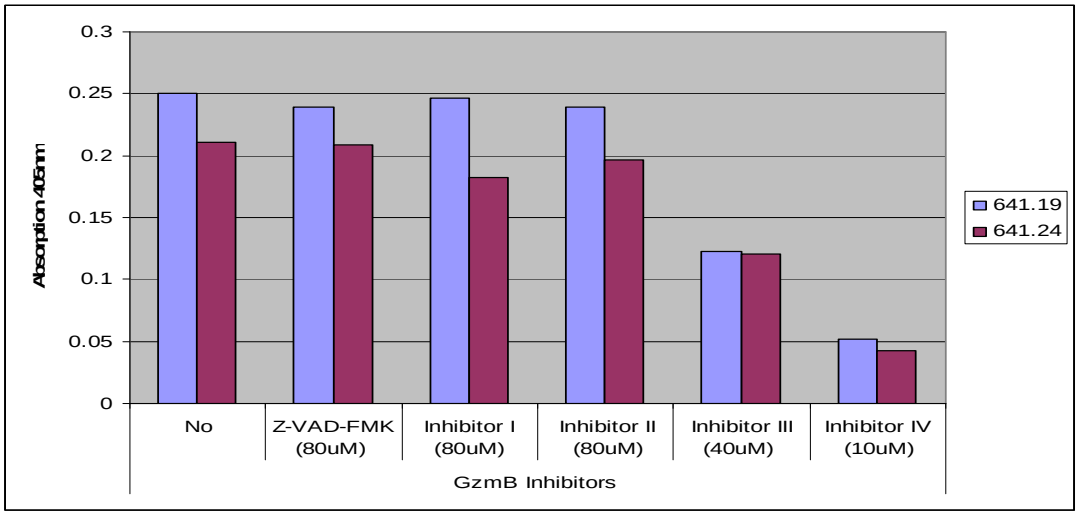
incubated with various concentrations of CMA for 2h and tested in a 4-hour cytotoxicity assay with [^{111}In]-labelled autologous 592TPM. The labelled target cells alone were also incubated with CMA in the assay. A standard effector to target ratio of 5:1 was used.

6.3.2 *Theileria*-infected cell death induced by CTL granule exocytosis is granzyme B-dependent

Although the capacity of the GzmB inhibitor IV, AC-IEPD-CHO to inhibit bovine granzyme B in cell lysates was successfully demonstrated in section 4.3.4, this inhibitor is inactive when added to viable cells due to its non-cell-permeable property. To address the problem, three additional cell-permeable granzyme B inhibitors (GzmB inhibitor I, GzmB inhibitor II and GzmB inhibitor III) were tested for their abilities to inhibit cattle granzyme B activity in two 641 CD8+ T cell clones (used in the previous experiments in section of 5.3.5). Z-VAD-FMK, a pan-caspase inhibitor, which has no effect on granzyme B activity and does not inhibit cytotoxic activity of human/mouse CD8+ T cells, was used as a negative control (Sarin et al., 1996). GzmB inhibitor IV was included as a positive control in assays of CD8+ T cell lysates. Figure 6.2 shows the results of an experiment in which lysates of CD8+ T cells in the presence or absence of inhibitors were assayed for GzmB activity using a specific substrate assay. As demonstrated in section 4.3.4, the presence of inhibitor IV (AC-IEPD-CHO) dramatically reduced the granzyme B activity of the lysates of both clones to levels that were close to the background level obtained by addition of assay buffer alone to the substrate. The presence of GzmB inhibitors I and II had no

effect on the granzyme B activity in either of the clones. In contrast, GzmB inhibitor III reduced the activity of granzyme B activity in lysates from both CD8+ T cell lines by approximately 50%, indicating a capacity to inhibit cattle granzyme B enzymatic activity. The negative control inhibitor Z-VAD-FMK has no effect on cattle granzyme B activity, as observed in other species.

Figure 6.2 Effect of pre-incubation with granzyme B inhibitors on the granzyme B enzymatic activity in lysates of two *T. parva*-specific CD8+ T cell clones

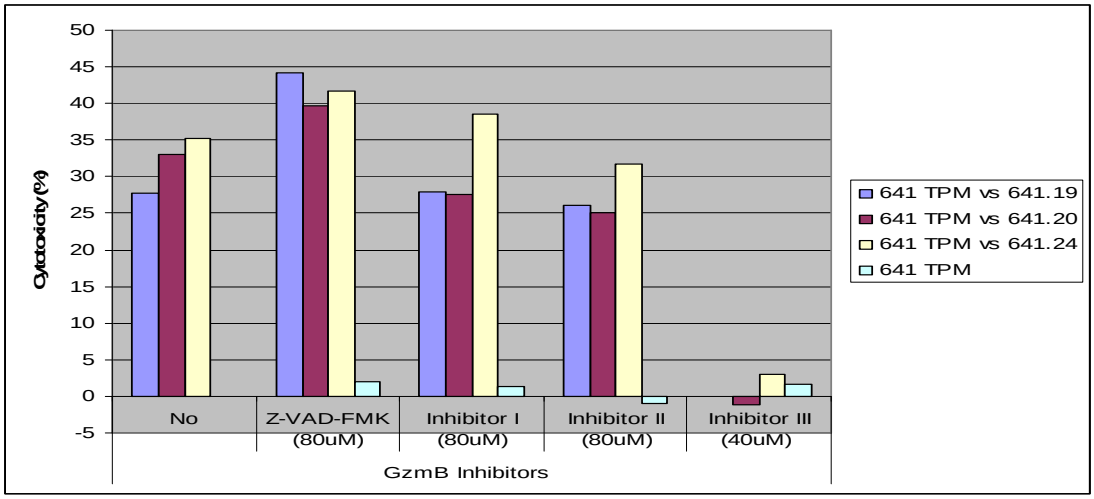


Two cloned CD8+ T cell lines (1x10^{6}cells) derived from animal 641 were pre-incubated for 1 hour with indicated concentrations of GzmB inhibitors I, II and III and a negative control, Z-VAD-FMK, and tested in a 4-hour substrate assay. As a positive control, lysates of CD8+ T cell lines (1x10^{6}cells) were also pre-incubated with GzmB inhibitor IV for 30min and tested in the same substrate assay.

To further investigate the role of granzyme B in killing of target cells by *T. parva*-specific CD8+ T cells, the GzmB inhibitors used above were pre-incubated for one hour with three 641 CD8+ T cell clones (including the two clones from which lysates were tested and one (641.20) that tended to have low levels of granzyme B content

but relatively strong killing as described in section 5.3.5) and tested in a 4-hour cytotoxicity assay with [^{111}In]-labelled target cells. The 3 clones exhibited levels of killing between 28% and 35% when assayed on autologous parasitized target cells at an effector to target ratio of 2:1). Preincubation with GzmB inhibitor III (40uM) completely inhibited the cytolytic activity of all CD8+ T cell lines and had no effect on the viability of the target cells, as indicated by low levels of spontaneous isotope release (Figure 6.3). GzmB inhibitors I and II and the negative control inhibitor (Z-VAD-FMK) did not have any effect on killing by any of the CD8+ T cell lines. Hence, the inhibitory effects on viable CD8+ T cells are consistent with those obtained by testing CD8+ T cell lysates. Although GzmB inhibitor III is also an inhibitor of caspase 8, which is involved in an early stage of apoptosis in target cells, the lack of inhibition by the pan-caspase inhibitor Z-VAD-FMK, indicates that the GzmB inhibitor III is not acting via caspase 8.

Figure 6.3 Effect incubation with GzmB inhibitors on the cytotoxic activity of 3 *T. parva*-specific cloned 641 CD8+ T cell lines



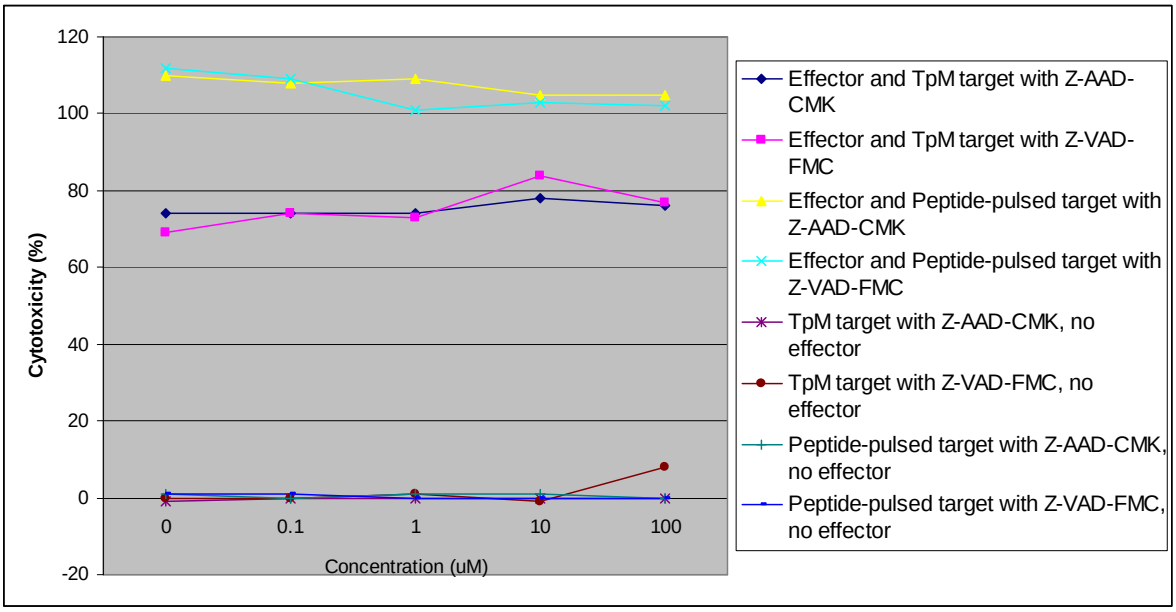
Three cloned CD8+ T cell lines (1x10^{4}cells) from animal 641 were pre-incubated for 1 hour with indicated concentrations of GzmB inhibitors and a negative

control, Z-VAD-FMK and tested in a 4-hour cytotoxicity assay with [^{111}In]-labelled autologous 641TPM target cells. The labelled target cells alone were also incubated with these inhibitors in the assay. A standard effector to target ratio of 2:1 was used

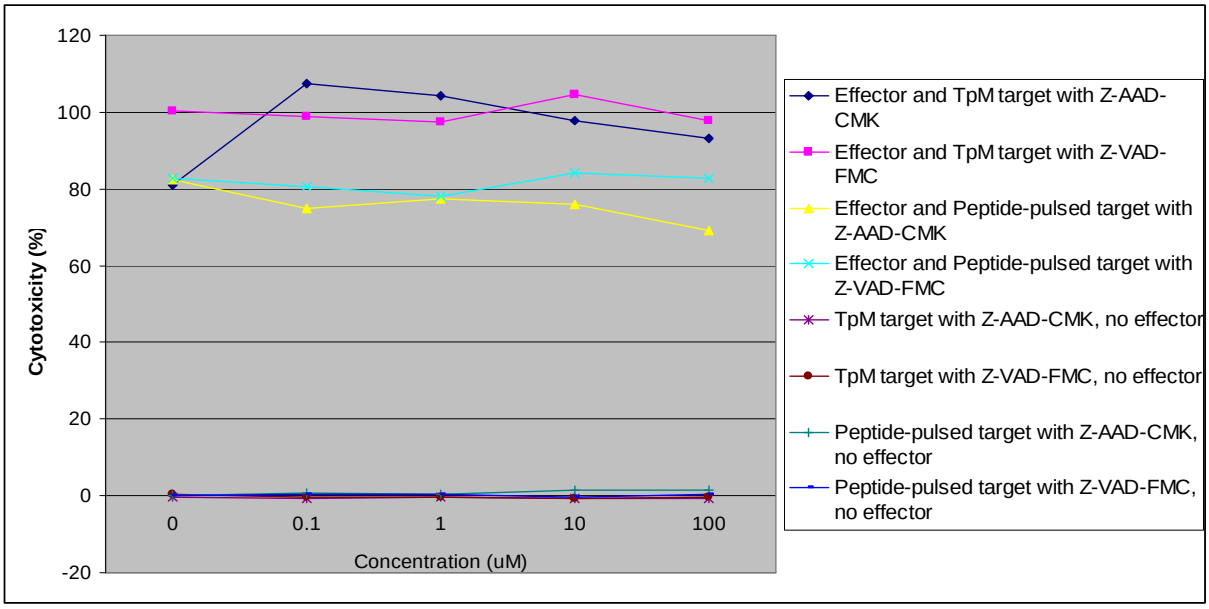
Since GzmB inhibitor I (Z-AAD-CMK) had been shown to be effective at inhibiting human and murine granzyme B activity in lysates (Odake et al., 1991) and blocking cell apoptosis induced by granzyme B (Gorak-Stolinska et al., 2001; Saito et al., 2008), further experiments were undertaken to test different concentrations of the inhibitor and different incubation times. An un-cloned CD8+ T cell line established from animal 011 and both autologous infected target cells (011TPM) and peptide-pulsed target cells (592TAA-pulsed-Tp2$_{49\text{-}59}$) were used. Pre-incubation of the CD8+ T cells with various concentrations (ranging from 0.1uM to 100uM) of Z-AAD-CMK for 0.5 hour had no inhibitory affect on the killing activity with either of the target cells (Figure 6.4- A). Pre-incubation times ranging from 0.5h to 4h also had no detectable effect on killing at any of the time points (Figure 6.4- B). The results show that GzmB inhibitor I, a known inhibitor for both human, mouse and rat granzyme B, has no effect on either cattle granzyme B activity or killing activity of bovine CD8+ T cells.

Figure 6.4 Effect of GzmB inhibitor I, Z-AAD-CMK on cytolytic activity of an un-cloned *T. parva*-specific CD8+ T cell line (A). after 0.5-h incubation and (B). after 4-h incubation

A



B



An un-cloned CD8+ T cells (1x10^{5}cells) from animal 011 was pre-incubated for 0.5 hour or 4 hours with various concentrations of GzmB inhibitor I, Z-AAD-CMK, and tested in a 4-hour cytotoxicity assay with [^{111}In]-labelled autologous

011TPM and MHC-matched (592) TAA-infected target pulsed with $TP2_{49-59}$ peptide (1000ng/ml). Z-VAD-FMK, the pan-caspase inhibitor, served as negative control. Labelled target cells alone were also incubated with Z-AAD and Z-VAD. A standard effector to target ratio of 20:1 was used.

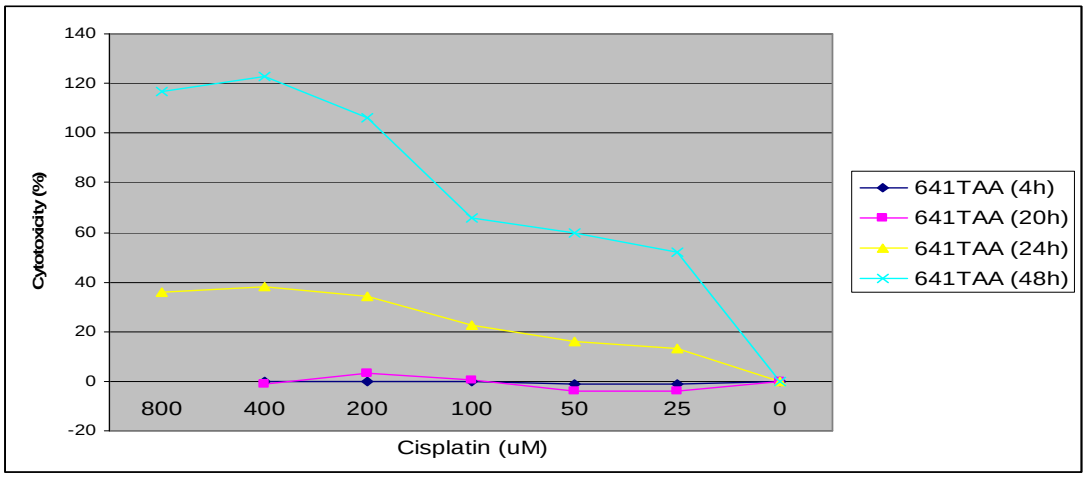
In conclusion, these findings reveal that the GzmB inhibitor III, Z-IETD-FMK specifically and effectively blocks the activity of cattle granzyme B and also inhibits killing of target cells by bovine CD8+ T cells, indicating that granzyme B is an important mediator of killing of *T. parva* infected cells by specific CD8+ T cell.

6.3.3 *Theileria*-infected cell death mediated by granzyme B is independent of caspases

Granzyme B, implicated above in the killing of *Theileria*-infected cells by CD8+ T cells, has been shown in other species to act by caspase-dependent and/or caspase-independent pathways (Lieberman, 2003). To examine the role of caspases in cell killing, experiments were undertaken to test the ability of the pan-caspase inhibitor Z-VAD-FMK and its control Z-FA-FMK to block killing. In contrast to previous experiments in which this inhibitor was pre-incubated with effector cells (as a negative control), these experiments involved pre-incubation with the target cells. Firstly, the ability of Z-VAD-FMK to block apoptosis of *Theileria*-infected cells induced by drug cisplatin, which causes apoptotic cell death in tumor cells (Sarin et al., 1997; Lau, 1999), was examined. Two parasitised cell lines, one infected with *T. parva* (468TPM) and one with *T. annulata* (641TAA) and two uninfected CD8+ T

cell clones (641.18 and 641.25) were used as target cells following incubation with various concentrations of cisplatin for a range of times (4h, 20h, 24h and 48h). Only one of the 4 lines (641TAA) showed significant cell lysis when tested in a ^{111}In release assay, and cell death was not observed before 24 hour (Figure 6.5). Figure 6.6- A and B show that cisplatin (100uM and 200uM) induced 20-30% cell death of 641TAA after incubation of 24 hour and this was blocked specifically by Z-VAD-FMK. A shortcoming of these experiments is that the longer incubation periods resulted in high levels of spontaneous isotope release from the cell lines reducing the ability to detect specific lysis. Notably, this spontaneous release was partially inhibited by Z-VAD-FMK, suggesting that this spontaneous cell death was also caspase-dependent.

Figure 6.5 Kinetic studies of the ability of the pro-apoptotic drug cisplatin to induce lysis of *Theileria annulata*-infected cells

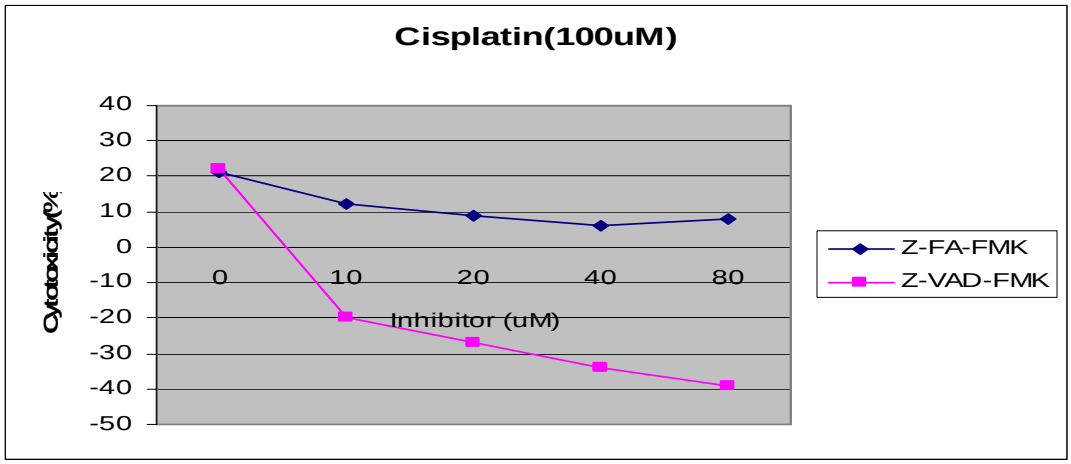


^{111}In-labelled *T. annulata*-infected cells (641TAA) (5x10^5/well) were incubated for indicated times (4h, 20h, 24h and 48h) with various concentrations of cisplatin and radioactivity released was measured. A further 3 cell lines, a *T. parva*-infected line 468TPM and two CD8+ T cell clones 641.18 and 641.25, were

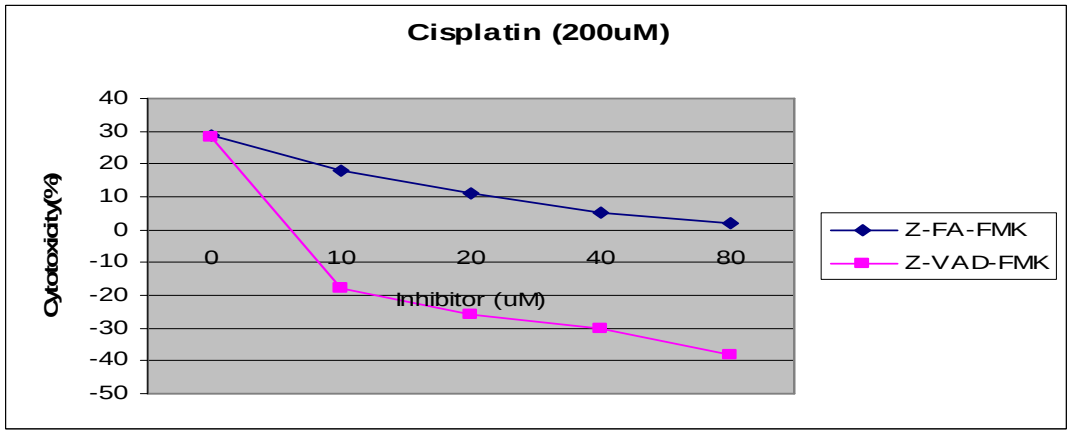
also used as target cells in this experiment, but the cell viability of these lines beyond 4 hours was too low to give interpretable results.

Figure 6.6 Inhibition of cytolysis of *Theileria annulata*-infected cells induced by cisplatin, by incubation with Z-VAD-FMK for 24 hour: (A). Cisplatin added at 100uM and (B). Cisplatin added at 200uM.

A.



B.

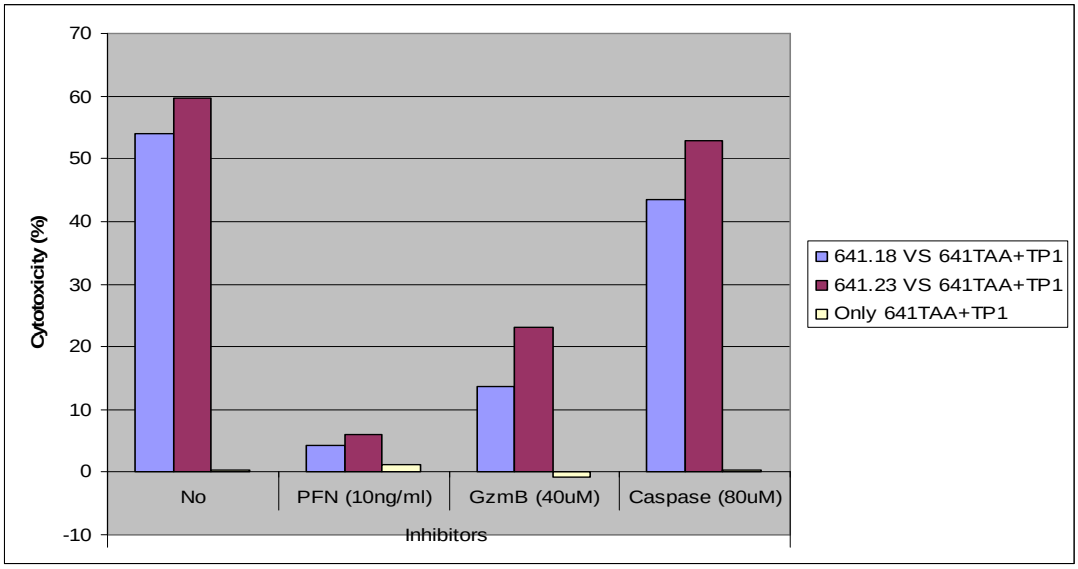


^{111}In-labelled *T. annulata*-infected cells (641TAA, 5x10^5/wells) were incubated for 24 hour with cisplatin (100um or 200uM) in the presence or absence of Z-VAD-FMK or Z-FA-FMK and radioactivity released was measured. Labelled target cells alone were also incubated with Z-VAD-FMK (80uM) or Z-FA-FMK

(80uM) for 24 hour and the values of cytotoxicity were -43% and -15% respectively.

To examine the role of caspases in CD8+ T cell-mediated killing, the same ^{111}In-labelled 641TAA target cell loaded with a specific peptide epitope was pre-incubated with Z-VAD-FMK (80uM) for 1 hour and tested in a 4-hour cytotoxicity assay with two epitope-specific CD8+ T cell clones (used in the previous experiment in section 5.3.5). Inclusion of the perforin and granzyme B inhibitors (CMA and Z-IETD-FMK) confirmed that killing by these cell lines is mediated by granule exocytosis; figure 6.7 shows that CMA completely blocked the cell death of both cell lines at 10ng/ml and that Z-IETD-FMK dramatically reduced killing by about 70%. However, Z-VAD-FMK did not affect the killing activity of either CD8+ T cell clone. The inhibitors had no effect on the viability of the target cells in these assays. Thus, although Z-VAD-FMK specifically blocked lysis of *Theileria*-infected cells induced by the pro-apoptotic agent cisplatin, it had no inhibitory effect on the granzyme B-dependent killing of *T. parva*-specific CD8+ T cell lines. In conclusion, these results suggest that granzyme B-mediated killing of *Theileria*-infected cells by specific CD8+ T cells is largely independent of caspases.

Figure 6.7 Effect of a pan-capase inhibitor, Z-VAD-CMK, on the susceptibility of peptide-pulsed *Theileria*-infected targets cells to killing by two CD8+ T cell clones



^{111}In-labelled Tp1 peptide-pulsed target cells (641TAA + $TP1_{214-224}$, 100ng/ml) was pre-incubated with Z-VAD-FMK (80uM) for 1h and tested in a 4-hour cytotoxicity assay with two Tp1-specific cloned CD8+ T cell lines ($1x10^4$cells) from animal 641. As controls, effector cells pre-incubated with the perforin inhibitor CMA (10ng/ml) for 2h or the granzyme B inhibitor Z-IETD-FMK (40uM) for 1h were tested in the same experiment. Labelled target cell alone were also incubated with these inhibitors in the assay. A standard effector to target ratio of 2:1 was used

6.4 Discussion

The results of work described in this chapter provide evidence that killing of *Theileria*-infected cells by cattle CD8+ T cells is mediated by the granule exocytosis pathway and that cattle granzyme B plays a pivotal role for induction of cell death. Cytotoxicity of CD8+ T cell lines was completely abolished by concanamycin A, an inhibitor of perforin, which is membrane pore-forming protein released from cytolytic granules. An inhibitor (GzmB inhibitor IV, AC-IEPD-CHO) shown to inhibit granzyme B activity in T cell lysates was not tested on intact T cells, as it is known to be membrane-impermeable. However, one of 3 additional membrane-permeable granzyme B inhibitors (GzmB inhibitor III, Z-IETD-FMK) was shown to inhibit the cytotoxic activity of CD8+ T cell lines by 70-100%. This compound functions as a competitive inhibitor that inhibits human and rat granzyme B (Thornberry et al., 1997; Harris et al., 1998) by binding to residues at P4-P3-P2-P1 (amino-terminal to the proteolytic cleavage site) of active granzyme B, thus competitively blocking its enzymatic function. In contrast, another two inhibitors (Inhibitor I and II) had no inhibitory effect on bovine granzyme B. GzmB inhibitors II (AC-AAVALLPAVLLALLAPIETD-CHO) and III (Z-IE(OMe)TD(OMe)-FMK) contain the identical 4-amino acid sequence (IETD) to bind the active sites of granzyme B but Inhibitor III contains the ester groups (OMe) which contribute to cell permeability. Hence, poorer permeability of bovine cells might account for the lack of inhibitory activity of Inhibitor II. GzmB inhibitor I (Z-AAD-CMK) contains distinct amino acid sequence (AAD), which might be unable to bind effectively to bovine granzyme B.

Previous studies of mechanisms of killing by mouse CD8+ T cells showed that cells derived from granzyme B-deficient mice induced tumor cell lysis with reduced efficiency in a 4 hour ^{51}Cr release assay but that levels of killing comparable to those obtained with wild-type CD8+ T cells could be achieved by incubation for 18 hours (Heusel et al., 1994). It revealed that granzyme B is required for the rapid induction of cell death and also suggested that other granzymes (eg. granzyme A) might be involved in the late onset killing. In the absence of gene knockout animals, determining the role of granzyme B in killing at different times is difficult. Although CD8+ T cells utilize pre-formed granule enzymes to kill target cells, de novo synthesis of granzyme B proteins occurs 4-6 hours after antigen stimulation (Isaaz et al., 1995; Wolint et al., 2004) and therefore late onset killing initiated by newly synthesised granzyme B cannot be distinguished from that mediated by other granzymes. Moreover, the ^{111}In release cytotoxicity assay used in our lab gave high spontaneous isotope release from labelled target cells with longer incubation, increasing from under 10% at 4 h to 30-40% during 18- 24 hour, resulting in reduced sensitivity to detect specific lysis.

Although activation of caspases was originally thought to be important in granzyme B-mediated cell death, studies by many groups revealed that their requirement was not absolute. An in vitro study of mouse CD8+ T cells showed that apoptotic nuclear damage induced by granule exocytosis was abrogated by the caspase inhibitor Z-VAD-FMK, whereas lysis of the cells was unaffected. In contrast, target cell lysis induced by the pro-apoptotic drug cisplatin was specifically blocked by this inhibitor

(Sarin et al., 1997). Similar results have been obtained in studies with purified human granzyme B, caspase inhibition preventing granzyme-induced DNA damage but not cell lysis (Trapani et al., 1998). These observations are consistent with the results of the present study, which showed that Z-VAD-FMK inhibited cisplatin-induced apoptosis of *Theileria*-infected cells, but did not inhibit granzyme B-mediated cytolytic activity of cattle CD8+ T cells, although DNA damage of the cells has not been examined. *T. parva* has been shown to protect infected cells from apoptosis by utilizing NF-κB activation to induce the expression of anti-apoptotic proteins such as FLIP, which functions as a catalytically inactive form of caspase-8, and X-chromosome-linked inhibitor of apoptosis protein (XIAP) as well as c-IAP, which block caspase-9 and also downstream executioner caspases (caspase 3 and 7) (Kuenzi et al., 2003). Studies by Guergnon et al. 2003 showed that drug-induced parasite death in *Theileria*-infected cells resulted in apoptosis involving activation of caspases 9 and 3 and was inhibited by Z-VAD-FMK (Guergnon et al., 2003). These findings demonstrated the requirement of the parasite for retaining cell viability and confirmed that the Z-VAD-FMK inhibitor is active in bovine cells. Inhibition of killing by *T. parva*-specific CD8+ T cell clones by GzmB inhibitor III in the current study demonstrates that T cell-mediated cell death (detected in a 4-hour assay) is dependent on granzyme B. As activation of caspases in the parasitized cells is tightly regulated by intracellular inhibitors induced by the NF-κB pathway, granzyme B is unlikely to break this regulation to fully activate caspases. The results strongly suggest that granzyme B bypasses the blockage and induces cell death by caspase-independent mechanisms.

Further experiments were undertaken to test two inhibitors of Bax, which plays an important role in mitochondrial-dependent apoptosis. Unfortunately, these experiments did not provide informative data on the role for Bax in cell killing. One of the inhibitors (a Bax Channel Blocker) was found to be very toxic to *T. parva*-infected cells, killing target cells in the absence of effector cells in a 4 hour ^{111}In release cytotoxicity assay. The other inhibitor (BIP-V5) and its negative control (BIP-NC) did not affect the killing activity of two 641 CD8+ T cell clones used in the previous study in section 5.3.5 (data not shown). Although BIP had been shown to inhibit Bax-mediated apoptosis induced by anti-cancer drugs such as cisplatin in several types of human cells (Sawada et al., 2003), it had no effect on cisplatin-induced cell death after incubation with parasitized cells (641TAA) at concentrations of 100-200uM for 24 hour (data not shown). In addition to absence of lysis in a 4-hour ^{111}In release cytotoxicity assay, no evidence of apoptotic nuclear changes were detected by immunofluorescence staining with Hoechst dye (Sawada et al., 2003). There are several possible explanations for these findings. First, BIP which is described as an inhibitor for human, mouse, rat and porcine Bax, may be ineffective as an inhibitor in cattle. Secondly, Bax may not be essential for killing of *Theileria*-infected cells by CD8+ T cells, other pro-apoptotic proteins possibly mediating mitochondrial-dependent apoptosis. A previous experiment has demonstrated that murine granzyme B induces mitochondrial depolarization and cell death in the absence of Bid, Bax and Bak in vitro (Thomas et al., 2001). As no inhibitors for other pro-apoptotic proteins are commercially available now, further work is required to determine whether the apparent resistance of *T. parva*-infected cells to apoptosis

induced by granzyme B is due to over-expression of anti-apoptotic proteins such as Bcl-2 (Davis et al., 2000).

Recent studies have revealed that human and mouse granzyme B are functionally divergent with respect to their substrate preferences (Kaiserman et al., 2006; Cullen et al., 2007). GzmB inhibitor I (Z-AAD-CMK), an effective inhibitor for human and murine granzyme B in both cell lysates and live cells (Odake et al., 1991; Gorak-Stolinska et al., 2001; Saito et al., 2008), has been shown to be ineffective at inhibiting bovine granzyme B. Sequence analyses described in section 3.3.6 revealed that key residues in granzyme B protein predicted to influence substrate recognition differed between the bovine, human and mouse proteins. Thus, although cattle granzyme B shares a high level of identity with human and mouse counterparts (72% and 69%, respectively) and is able to cleave a synthetic substrate AC-IEPD-pNA that has an optimal peptide recognition sequence (IEPD) for human granzyme B (section 4.3.4), it may have a distinct profile of substrate preferences.

Chapter 7 General discussion

It is well established that cytotoxic CD8+ T cells are important mediators of immunity against the bovine intracellular protozoan parasite *T. parva*. However, the mechanism by which the specific CD8+ T cells kill parasitized cells is not understood. Dissection of the mechanisms and host proteins involved in killing by specific CD8+ T cells and how they may be regulated could be of value in designing vaccination protocols for inducing protective CD8+ T cell responses. In human and mouse, the predominant pathway used by CD8+ T cells to kill pathogen-infected cells is granule exocytosis, involving release of perforin and granzymes. Although bovine perforin has been described, there is to date a lack of published information on the identity and biological activities of bovine granzymes. The aim of this study was to determine the role of granule enzymes in mediating killing of *T. parva*-infected cells, first by characterising the granzymes expressed by bovine lymphocytes and then by investigating their involvement in killing of target cells, focusing particularly on the role of granzyme B. The work had three major outputs:

1. Identification and genome annotation of granzyme genes expressed in cattle, including a novel granzyme not found in humans or mice.

Six bovine granzyme genes (A, O, B, K, H and M) were identified by mining existing genomic and expression sequence tag databases. Specific PCR assays designed for amplification of the full length coding region of these granzyme genes were validated and used to demonstrate expression of transcripts for all of the genes in activated antigen-specific CD8+ T cells. Genome annotation revealed that the

genes were organised within 3 gene loci on separate chromosomes in a similar manner to the human and murine genes. Inter-species comparative analysis of their nucleotide and amino acid sequences was undertaken to infer the likely enzymatic specificities of the proteins. The 6 identified genes included one encoding a novel granzyme, termed granzyme O, found within the trypsin-like locus. This granzyme is most closely related to granzyme A. The detection of gene remnants in humans and mice showing similarity to the granzyme O gene indicates that this gene was present prior to species divergence but became non-functional in humans and mice.

2. Successful development of molecular and biochemical methods to define the functional activities of bovine granzyme B.

To investigate the role of granzyme B in killing by *T. parva*-specific CD8+ T cells it was necessary to establish methods to detect and quantify expression of bovine granzyme B protein. First, site-directed mutagenesis strategies, including PCR splice overlap extension and 'megaprimer' PCR, were used to produce DNA constructs encoding functional and non-functional forms of bovine granzyme B. Recombinant forms of these proteins were expressed in mammalian COS-7 cells and used with defined substrates to establish a biological assay to measure specific granzyme B activity. Initial experiments with known granzyme B inhibitors indentified one compound (AC-IEPD-CHO) that blocked the biological activity detected in this assay, confirming that it was attributable to granzyme B. Results obtained from these experiments confirmed that bovine granzyme B has the primary substrate specificity similar to that of its human and murine orthologues. This work is the first description of the biological activity of a member of the granzyme family in cattle and the

approach taken provides a system that could readily be applied to study other active bovine granzymes.

3. Demonstration that granzyme B is an important mediator of the cytotoxic activity of CD8+ T cells specific for *T. parva*

Initial experiments with the perforin inhibitor, concanamycin A, showed that cytotoxic activity of *T. parva*-specific CD8+ T cells is dependent on perforin, indicating that killing is mediated predominantly by the granule exocytosis pathway. Moreover one of the granzyme B inhibitors tested, Z-IETD-FMK, which was shown to be an effective and specific inhibitor of bovine granzyme B in the substrate assay, dramatically inhibited killing by *T. parva*-specific CD8+ T cell clones, thus indicating a predominant role of granzyme B in cytotoxic activity of these T cells. Experiments in which granzyme B activity was measured in lysates and supernatants of *T. parva*-specific CD8+ T cell clones, using the specific substrate assay, revealed a significant correlation of granzyme B protein expression and its release upon incubation with target cells with the levels of killing of different T cell clones. However, this correlation was not absolute, suggesting that other factors that vary between T cells clones (possibly other granzymes) contribute to the cytotoxic activity of CD8+ T cells. Further studies showed that granzyme B-mediated death of *T. parva*-infected cells is independent of caspases. This is consistent with previous findings with some human and mouse CD8+ T cell models (Sarin et al., 1997; Trapani et al., 1998) and provides direct evidence for the important role of caspase-independent pathway(s) in a non-human/non-murine species. These findings indicate that other pro-apoptotic proteins, most likely involved in mitochondrial-dependent

apoptosis, are required to be activated by bovine granzyme B for induction of cell death.

This study represents the first dissection of the effector mechanisms employed in killing of target cells by bovine CD8+ T cells and specifically provides the first evidence that granzyme B plays a key role in killing of *T. parva*-infected cells by specific CD8+ T cells. The findings are consistent with previous evidence that granzyme B is a potent inducer of apoptosis and an important mediator of cytotoxicity in human and mouse.

Despite the evidence that granzyme B plays a central role in the killing of *T. parva*-infected cells by CD8+ T cells, a number of questions remain concerning the precise mechanisms of killing and how the level of expression of granzyme B is regulated:

(i) Which features of apoptosis are induced in *T. parva*-infected cells by bovine granzyme B?

CD8+ T cell-induced cell death has classically been measured as cytolysis using ^{51}Cr release assays (Henkart et al., 1997), which have been considered to represent an apoptotic phenotype. The role of granzyme B in killing has been determined by testing specific inhibitors and/or by examining CD8+ T cells from gene knockout mice. The current study of CD8+ T cell-mediated killing of *T. parva*-infected T cells and the role of granzyme B relied on an ^{111}In release assay, similar to the ^{51}Cr release assay. By contrast, many of the experiments done to analyse the activities of granzyme B have studied the detailed cell biology of cells incubated with purified

granzyme protein along with sublytic concentrations of perforin. Using these systems, granzyme B has been shown to induce many cellular changes that can contribute to cell death, including rapid DNA fragmentation, chromatin condensation, membrane blebbing, phosphatidylserine (PS) exposure, mitochondrial membrane depolarisation and subsequent cytochrome c release, loss of inner mitochondrial membrane potential ($\Delta\Psi m$), generation of reactive oxygen species (ROS) and membrane lysis (Shi et al., 1992a; Shi et al., 1992b; Nakajima et al., 1995; Heibein et al., 1999; MacDonald et al., 1999). However, not all of these are required to result in cell death and different features may be induced in different target cells. Further more detailed studies are required to characterise which of these changes are induced in *T. parva*-infected cells by specific CD8+ T cells and the pathways activated by bovine granzyme B.

(ii) Does bovine granzyme B-mediated cytotoxicity require Bid?

The pathways utilised by granzyme B to mediate killing are species-dependent. Mouse granzyme B directly activates caspases to promote apoptosis, whereas human granzyme B acts by a Bid-dependent pathway (Kaiserman et al., 2006; Cullen et al., 2007). The evidence that granzyme B-mediated cell death of *T. parva*-infected cells is caspase-independent needs to be confirmed and, if so, places the pro-apoptotic protein Bid as a candidate for future work to investigate mitochondrial-dependent pathways of apoptosis.

(iii) Do other granzymes cooperate with bovine granzyme B in achieving CD8+ T cell-mediating cell death?

Previous studies of human CD8+ T cells provided evidence that other granzymes, such as granzyme A, H and M, cooperate with granzyme B in achieving CD8+ T cell-mediating cell death (Nakajima et al., 1995; Mahrus et al., 2004; Andrade et al., 2007). Specific antibodies and/or biological assays for measuring other bovine granzyme proteins, as well as reagents to specifically inhibit their activities, would be required to investigate these interactions in cattle. The system used for eukaryotic expression of recombinant proteins in this study could be used for development of further biological assays or generation of specific antibodies. In the case of those granzymes where specific inhibitors are not commercially available, use of biochemical methods to synthesize the substrate specificity-based inhibitors would be desirable. Knock-down of gene expression could be considered as an alternative way, although the storage of granzyme proteins in pre-formed granules means that prolonged knockdown would be required. The function of Granzyme O, a novel functional granzyme in cattle, might be of interest in this regard, although further studies are required to determine the cell types in which this granzyme is expressed.

(iv) Investigate cytokines involved in regulation of expression of granule enzymes in CD8+ T cells specific for *T.parva*

The lack of cytotoxic activity of *T. parva* antigen-specific CD8+ T cells induced following prime-boost vaccination with defined antigens was reported by Graham et al (2006). One possible reason for this finding is that CD4+ T cells may be required for efficient induction and/or recall of the CD8 T cell response and that parasite-specific CD4+ T cell responses were not induced by the vaccination protocol. A previous study involving co-culture of different combinations of purified CD4 T cells

and CD8 T cells from naïve and *T. parva*-immune calves suggested that CD4 T cells are required for efficient induction of CD8 T cell responses in vitro (Taracha et al., 1997). Alternatively, the CD8+ T cell responses induced by vaccination may not have undergone appropriate functional differentiation and therefore were defective in expression of granule enzymes including granzyme B. Further work is required to investigate the functional properties of these responses. Regulation of expression of granzymes and perforin in murine CD8+ T cells has been reported for IL-2, IL-4, IL-12, IL-15 and IL-21 (Ye et al., 1996; Kienzle et al., 2002; Curtsinger et al., 2005; Zeng et al., 2005). Of these, provision of IL-12 as a third signal to the T cells (ie, in addition to TCR engagement and IL-2 co-stimulation), was found to promote development of full cytolytic function of CD8+ T cells and enhanced levels of granzyme B protein expression (Curtsinger et al., 2005). The current study has provided some of the tools to examine the levels of expression of granzyme B and other granzymes in different populations of CD8+ T cells and to investigate whether their expression is influenced by one or more cytokines.

(v) Does *T. parva* enhance resistance to granzyme B-mediated cell death by upregulating of PI-9 in host cells?

Previous studies have provided evidence that *T. parva* protects infected cells from Fas/Fas ligand-induced apoptosis by upregulating a number of anti-apoptotic proteins (Guergnon et al., 2003; Kuenzi et al., 2003). Recent studies have proposed that some tumour cells up-regulate PI-9 expression in order to evade the immune system (Medema et al., 2001; Bladergroen et al., 2002; ten Berge et al., 2002; van Houdt et al., 2005; Bossard et al., 2007). PI-9 acts as an endogenous inhibitor of human

granzyme B and protects CTL itself against the actions of misdirected granzyme B (Sun et al., 1996; Bladergroen et al., 2001; Buzza et al., 2001; Hirst et al., 2001; Bird et al., 1998). The present study has demonstrated that *T. parva*-infected cells contain abundant transcripts for PI-9. Future work is required to examine the relative susceptibility of *T. parva*-infected and comparable uninfected target cells to CD8+ T cell-mediated killing (using defined peptide epitopes) and to determine whether expression of PI-9 in *T. parva*-infected cells enhances their resistance to granzyme B-mediated killing in comparison to uninfected target cells.

In summary, the results presented in this study have established a system to further characterise the granzyme family in cattle and have demonstrated that cytotoxic activity of *T. parva*-specific CD8+ T cells is mediated by the granule exocytosis pathway and that bovine granzyme B plays a pivotal role for induction of cell death.

Chapter 8 Appendices

8.1 Appendix A - Nucleotide sequences and intron-exon structures of individual bovine granzyme genes

(a). Granzyme A: Full length nucleotide sequence:

ACATTTCACATTGATTGATGTGGGGACACCAGCCACAATGAGGAACTCCTCTACCTTTCTGGCAGCCACTCTCTCAATTGTCGTTTTTCTCCTAATTCCTGAAG[3505]A
TCTCTGTGAAAAAATTATTGGAGGAAATCAAGTGACTCCTCATTCAAGACCCTACATGGTGCTACTTGATGGGGGAAACATCTGTGCCGGAGCTTTGATTGCCAAAGACTG
GGTATTGACTGCAGCTCACTGTTCCCT[1773]GAACCAGAAATCCCAGATCATTCTTGGGGCCCACTCAAGAAACAAGGAAGAGCCTGAAAAACAGATTATGTTTGTTAA
GAAAGAGTTTCCCTATCCATGCTATGACCCGGACACACATGAAGGCGATCTTAAACTTCTAAAG[198]CTGAACAAAAAAGCAACACTTAATAAAAACGTGGCTATCCTT
CAGCTCCCAAAAGAGGGCAAAGATGTGGAACCAGGAACTGCATGTCGAGTTGCAGGGTGGGGACAGTTTTACAATAATTCCCCTGTGTCCAAGATTTTGAGAGAAGTCAAT
GTCACCATCATAGACAGAAAAATCTGCAATGATCAATCACACTATAATTATAATCCTGTGATTGGACTGAATATGATTTGTGCTGGAAGCCTCCAAGGTGGAAAAGACTCC
TGCCAT[1987]GGAGATTCTGGAAGCCCTCTGATATGTAAAGACACTTTCAGAGGCATCACTGCCTTTGGCATTCCGGGGAGATGTGGAGACCCTCGAGGGCCCGGCGTC
TATACACTTCTCTCAAAGAAACACCTCAACTGGATAGTTAAGACTATGAAGCAAGCAGTTTAAATACTTGTATTTCATTTGCTGTTACTTTTTAAAAAAATTGTCTTGAAG
TATAGTTGATTTACAATGTTGTATTAGTTTCAGGTGTACAGCAAAGTGAATCAGAGTTACACATATATCCACTCTTTTTTTAGATTCTTTTCCTATATAAGCCATTGCAGA
CTATTAAATAGAGTTCCCTGTGCTATATAGCAGGTTCTTATTAGTTACCCATTTTAAATAAATTGATAGTAGTATGTATATGTCAGTCCCAATCTCTCAATATATCACTCC
CCCCATCCCTGATAACCTTAAGTTTGTTTTCTACATCTGTGACTCTACTTTTGTTTCATCAATAAGTTCATTTTACCCTTTTTTTTCGATTCCACATACATGTGATATCAT
ATGATATTTGACCTTCTGTGTCTGACTTACTTCACTCAGTATGACAATCTACATCCATATTGCTGCAAATGACATTACTTTGTTCTTTTTTATGGCTGAGTAATATTCCAT

TGTATATATGTACCACATCTTTATTATCCATTCCTCTGATGGACATTTAGGTTGCTTCCACGTCCTGGTTATTGTTAAGCGGTGCTGCAATGAACATTGGGATGCATATAT
CTTTTTGAATTATGGTTTTCTTCAGATATACACCCAGTGCTGTCGTTTCTTAATTTCATTATAAATAAAATCAACTTCTAT

Granzyme A					Start	Stop	Length	Splice sites		Coding region		UTR		
	Ori			Chromosome								Length	Start	Stop
Exon 1	Rev	1	104	20	25672789	25672723	67			1	67	37	25672826	25672790
Intron1								GTAAG	AATAG					
Exon 2	Rev	105	243	20	25669217	25669079	139			68	206			
Intron 2								GTAAG	TTCAG					
Exon 3	Rev	244	385	20	25667305	25667164	142			207	348			
Intron 3								GTACA	AACAG					
Exon 4	Rev	386	655	20	25666965	25666696	270			349	618			
Intron 4								GTAAG	CCAAG					
Exon 5	Rev	656	1501	20	25664708	25664547	162			619	780	684	25664546	25663863

Gene A is reverse strand and locates in Chromosome 20: 25, 672,826-25,663,863. One transcript in this gene which consists of 5 exons has been identified and the length of cDNA and amino acid is 1501bp and 259residues, respectively.

(b). Granzyme O: Full length nucleotide sequence:

GAAACTAGTCTCCATATGTGAATAACAGGAGCCATGAATATTCCTTTTCCTTTCTCTTTTCCTCCTGCCATTTGTCTCCTTCTAATTCCTGGAG[2100]TTTTTCCAGTA
TCCTGCGAGGGAATTATAGGAGGAAATGAAGTGGCCCCTCACACAAGACGCTACATGGCTCTAATCAAAGGGCTGAAACTCTGTGCAGGGGCTTTAATCAAAGAAAACTGG
GTGTTGACAGCCGCTCATTGTGACCT[2968]GAAGGGCAATCCTCAAGTTATTCTTGGGGCCCACTCTACATCCCATAAAGAGAAACTTGACCAAGTATTTTCCATTAAA
AAGGCAATTCCCTACCCATGCTTTGATCCACAGACATTTGAAGGGGATCTTCAACTACTTCAG[3337]CTGGAAGGTAAAGCAACTATGACCAAAGCTGTAGGAATACTT
CAGCTACCAAGAACAGAAGACGATGTCAAACCCCACACCAAGTGTCATGTGGCAGGATGGGGAAGCACCAAAAAAGACGCATGTCAAATGTCTAATGCCTTGAGAGAAGCC

AACGTTACAGTGATAGATAGGAAAATATGCAATGATGCCCAGCACTATAATTTTAATCCAGTTATTGATCTCAGTATGATCTGTGCTGGTGGTAGAAAAGGTGAAGATGAT
TCATGTGAA[1429]GGGGATTCTGGAAGTCCTCTGATATGTGATAATGTTTTCAGAGGTGTCACTTCCTTTGGCAAGTGTGGTAATCCCCAGAAGCCTGGCATCTACATC
CTCCTTACCAAAAAACACCTCAACTGGATAAAGAAAACCATTGCAGGAGCCATATAACATTTCTACTTCAAAGTAGAAAATCGAAGTAACCAAGTGAAAGGGTCTTAAACT
TGACCTGTCCGAAGAAGCATCTTGCAGCCCTCTGAACTTCACATACAAAATGCCGCCTGTCTAAAATAAATTCAGCAATGAAAGAAA

Granzyme O					Start	Stop	Length	Splice sites		Coding region		UTR		
	Ori			Chromosome								Length	Start	Stop
Exon 1	Rev	1	94	20	25689314	25689254	61			1	61	33	25689347	25689315
Intron1								GTAAG	TACAG					
Exon 2	Rev	95	242	20	25687153	25687006	148			62	209			
Intron 2								GTAAG	TCAG					
Exon 3	Rev	243	384	20	25684037	25683896	142			210	351			
Intron 3								GTAAA	TCTAG					
Exon 4	Rev	385	657	20	25680558	25680286	273			352	624			
Intron 4								GTAAG	TTCAG					
Exon 5	Rev	658	951	20	25678856	25678704	153			625	777	141	25678703	25678563

Gene J is reverse strand and locates in Chromosome 20: 25,689,347-25,678,563. One transcript in this gene which consists of 5 exons has been identified and the length of cDNA and amino acid is 951bp and 258residues, respectively

(c). Granzyme B: Full length nucleotide sequence:

GACACTGTGTTTCTCTCCAAGAGGCTGGGGGATCTGAAAGAGAGCCAGGAGGTAGCACCAGCATCTCCATCCTGGGCAGTCTTTCTAGGAAGATGAAGCCTCTCCTGCTCC
TGGTGGCCTTTCTCCTGACCCCCAGGGCAAAGGCAG[1243]GGGAGATCATCGGGGGCCATGAAGCCAAGCCCCACTCCCGCCCCTACATGGCATATCTTCAGTACTGGA
ATCAGGATGTCCAGAGTAGGTGCGGTGGGTTCCTGGTTCGACAGGACTTCGTGCTGACAGCCGCTCACTGCAACGGAAG[486]CTCAATCAAAGTCACCCTGGGGGCCCA

CAACATCAAACAGCAGGAGAGGACCCAGCAGGTCATCAGGGTGAGAAGAGCCATCAGCCACCCTGACTATAATCCTAAGAACTTCTCCAACGACATCATGTTATTAAAG[197]CTGGAGAGAAAGGCCAAGCAGACATCAGCTGTGAAGCCCCTTAGTCTGCCCAGGGCCAAGGCCCGGGTGAAGCCAGGACAGACGTGCAGCGTGGCCGGCTGGGGGAGGGACTCCACGGACACCTACGCTGACACACTACAGGAGGTAAAGCTGATCGTGCAGGAGGATCAAAAGTGTGAGGCCTACTTACGCAACTTTTATAACCGCGCCATCCAGCTGTGTGTGGGGGACCCAAAGACAAAGAAAGCTTCCTTTCAG[629]GGGGACTCGGGGGGCCCTCTCGTGTGTGACAATGTGGCCCAGGGCATTGTCTCTTATGGAAAAAGAGATGGATCAACTCCACGGGCCTTCACCAAAGTCTCAAGTTTCCTGCCCTGGATAAAGAAAACCATGAAAAGCCTCTGACTGCAGGAACCAGACCCTCTTCCCTGGGGCTGATCCAGAATCACACTGCAGGGTTGGGGGTGCCCACAGCTCAATAAATGTCTCTCAGCAGAGCTC

Granzyme B					Start	Stop	Length	Splice sites		Coding region		UTR		
	Ori			Chromosome								Length	Start	Stop
Exon 1	Rev	1	147	21	34986649	34986595	55			1	55	92	34986741	34986650
Intron1								GTGAG	TTCAG					
Exon 2	Rev	148	295	21	34985351	34985204	148			56	203			
Intron 2								GTGAG	CACAG					
Exon 3	Rev	296	431	21	34984717	34984582	136			204	339			
Intron 3								GTAAA	CACAG					
Exon 4	Rev	432	689	21	34984384	34984127	258			340	597			
Intron 4								GTGAG	CTCAG					
Exon 5	Rev	690	929	21	34983497	34983354	144			598	741	96	34983353	34983258

Gene B is reverse strand and locates in Chromosome 21: 34,986,741-34,983,258. One transcript in this gene which consists of 5 exons has been identified and the length of cDNA and amino acid is 929bp and 246residues, respectively.

(d). Granzyme H: Full length nucleotide sequence:

CTGGAAGGAAGAAACCCCAGCAGCTCTGACCTGGGCAAATCTTCTGGAAGATGCAGCTACTCCTGCTCCTGATGGCCTTTCTTCTGCCTCCTGGGCTGGGAGAG[1022]C
CTTTTCTTTCAGAGGAGATCATTGGGGGCCATGAGGCCAAGCCCCACTCCCGCCCCTACATGGCTTTCGTTCAGTTTCTGGGTGAGAAGAGTTGGAAGAGGTGTGGCGGTG
TTCTCATACAAAAAGACTTTGTTCTGACAGCTGCTCACTGCAGAGGAAG[529]CTCAATCAATGTCACCCTGGGGGCCCACAACATCAAACAGCAGGAGAGGACCCAACA
GGTCATCCAGGTGAAAAGAGCCATCCACCACCCAGACTATAATCCTAAGACCTTCTCCAACGACATCATGTTACTGCAG[201]CTGGAGAGAAAGGCCAAGCAGACATCA
GCTGTGAAGCCCCTTAGTCTGCCCAAGGCCAAGGCCCAGGTGAAGCCAGGAGAAGTGTGCAGTCTGGCCGGCTGGGGGAAGGTGGCCCTGGGCACTCCAGCCACCACCCTG
CAGGAGGTAGAGCTGACGGTTCAGGAGGATCGGGTGTGTGAATCACTCAACCCCAGGAACTACAGTCGGGCCACCCAGATTTGTGTCGGGGACCCAAGGAAGGTGAAAACC
GGCTTCAAG[379]GGTGACTCCGGTGGACCCCTCGTGTGTAAAAAAGTGGTCCATGGTATTTTCTCCTATGGAAAGACGAATGGGACACCTCCAGGAGTCTTCACCCAGG
TCTCACACTTCCTACCCTGGATAAAGAGAACAATGAAGCACCTCTAACAGCAGCTTGAGACTGATCTTTCTCTGCACTGACCATTGTCCTGGGGCAGCAGCAAGAATCCCA
CAGGGATTGGCAGTGGGATCACAGGGCCATAATAAATGGGATCTCCAAAGC

Granzyme H					Start	Stop	Length	Splice sites		Coding region		UTR		
	Ori			Chromosome								Length	Start	Stop
Exon 1	For	1	104	21	34903703	34903756	54			1	54	50	34903653	34903703
Intron1					1022			GTGAG	TTCAG					
Exon 2	For	105	265	21	34904779	34904939	161			55	215			
Intron 2					529			GTGAG	TGCAG					
Exon 3	For	266	401	21	34905469	34905604	136			216	351			
Intron 3					201			GCAAG	ATCAG					
Exon 4	For	402	659	21	34905806	34906063	258			352	609			
Intron 4					379			GTCAG	CACAG					
Exon 5	For	660	918	21	34906443	34906586	144			610	753	115	34906587	34906701

Gene H is forward strand and locates in Chromosome 21: 34,903,653-34,906,701. One transcript in this gene which consists of 5 exons has been identified and the length of cDNA and amino acid is 918bp and 250residues.

(e). Granzyme K: Full length nucleotide sequence:

CTTCCTTTGCCAATACAGTCAGACTATTTCATCTGGGCTTCTTAGATCTAAGCTACTAACATGACTAAGTTTTCTTCTTTTTTTCTATGTTTCCTACTAGCCGGGACTTAC
ATGACTCCAGAGT[271]GTTTCAACATGGAGATTATTGGAGGGAGAGAAGTGTCCCCTCACTCCAGGCCGTTTATGGCGTCCCTGCAGTATGGCGGCGACCACATCTGCG
GGGGAGTGCTGATCCATCCTCAGTGGGTGCTCACAGCAGCCCACTGCCACTTGCG[6655]GTTTGCCAAAAGCCAGTCTTCCAAAGTGGTTTTAGGAGCACACTCTCTCT
CAAAGAATGAGGCCTCCAAGCAAACATTTGAGATTAAAAAATTCATACGATTCCCAGGATTTGCATTAGCCCCTAAATCAAACGATATTATGCTGGTTAAG[771]CTTCA
CACGGCCGCAATACTCAACAGACATGTCCAACTGCTCCACCCAAGGGCTAAAAATGATATTAAAGCTGGAACAAAATGCCAGGTTGTTGGCTGGGGAGCCACTGACCCAGA
AGGCTTAAGCCTTTCTGATACCCTGCGAGAAGTCACTGTCACTGTTATAAGTCGAAAAACGTGCAACAGCCGAGATTATTACAACCACAGCCCTGTTATAACTAGAACCAT
GCTATGTGCAGGAGACGCCAGAGGCCAGAAGGATTCCTGTCAG[1845]GGTGACTCAGGGGGCCCCTTGGTCTGCAAAGGTGCCTTTCATGCCTTAGTTTCTGGAGGTCC
CAAATGTGGTGATGCCAAGAAACCTGGAATCTACATCCTACTAACCCGGAAATTCCAAGCTTGGATCAAAAGCAACTTGGCCCCATCTCATGCAGACTAAGACTACAGATG
ATTTTCTTGGCTCTATCAGCTGCTTGTTTTCATTTTTGTTTCATTAATGTGTTCTACAGGCTAACTTATCTGCACAGGTACTTGAATGTAGTTAAAGTGGAGAACAGTCAA
GGTCCACTCCTGACCTGTTAGGACTGATTTTGTTGAGGAATCAAGTTCTTTTTCACATGTACCACTGATGTATTTCTTCTATGCTGCTTTTATTCTGAATAAAATTTAGAA
TAGACGAGTGTCTATTCATCTTATATGGAAATAAGATACAAAGAATGATATGTCCTGCAACTCAATCTACAACCCCACAAAGTGCAAAAAGATGATTAAAAAAGACAGTTG
TGGCATCACCTTCAA

Granzyme K					Start	Stop	Length	Splice sites		Coding region		UTR		
	Ori			Chromosome								Length	Start	Stop
Exon 1	Rev	1	124	20	25737822	25737759	64			1	64	60	25737882	25737823
Intron1								GTAAG	GGCAG					
Exon 2	Rev	125	272	20	25737487	25737340	148			65	212			
Intron 2								GTGAG	TTTAG					
Exon 3	Rev	273	423	20	25730684	25730534	151			213	363			
Intron 3								GTATG	GTCAG					

Exon 4	Rev	424	693	20	25729762	25729493	270			364	633			
Intron 4								GTAAG	TCCAG					
Exon 5	Rev	694	1214	20	25727647	25727486	162			634	795	359	25727485	25727127

Gene K is reverse strand and locates in Chromosome 20: 25,737,882 to 25,727,127. One transcript in this gene which consists of 5 exons has been identified and the length of cDNA and amino acid is 1214bp and 264residues, respectively.

(f). Granzyme M: Full length nucleotide sequence:

```
                                                                    -7 -6 -5 -4 -3 -2 -1 +1
                                                                    GlyAsnThrPheGluThrHisIleIleGlyGly
AGAACCGTGGAGGCCCCCCAGATCCAAGATGCTGCTCCTGCTGGTGGTCCTGGAAGCTCTGTGGGCAG[3394]GAGGCAACACCTTCGAGACCCACATCATCGGGGGTCG
AGACGCTGTCCCCCACTCACGCCCATACATGGTCTCGCTGCAGAAGTCGTCTGGCTCACACCAATGTGGTGGGGTGCTCCTGCACCAAAATTGGGTGTTGACAGCTGCCCA
CTGCCTGACCCAGCC[744]GACGCAGCAGCTGAGGCTTGTGCTGGGGCTTCATGTGCTGGGAGAGATCAGCCCTATCTACCGCATCAGGAAGGTGGTCCGGCACCCCGAA
TACAAGCCAGTCCCTCATCTGGAGAATGACCTCGCACTGCTAAAG[160]CTGGACGGGAAGGTGAAGCCCAGCAGGACCATCCAGCCCCTGGCGTTGCCCCGAGGGCGCC
AGATGGTGGCCACAGGCACCCGGTGCAGCCTGGCCGGCTGGGGCCTGACCCACCAGCCTGGGAACCTGGCCAGGGTGCTGCAGGAGCTGGACGTGCATGTGTTGGACACCA
GGATGTGCAACAACAGTCGGTTCTGGCACGGCAACATCAGCTCCCACATGATCTGCCTGGCAGCTGACTCCAAGAACCAGGCCCCCTGCAAG[402]GGGGACTCAGGAGG
GCCGGTGGTGTGCAAAAGAGGCCAAGTGGCTGGAATCCTGTCCTTCAGCTCTGAGAATTGCACCGACATCTTCAAACCCCCCGTGGCTGTCGCTGTGGCCCCCTACATGCC
CTGGATCAAGAAGGTCCTCCGCCACAATGGCTCACCTCCCTCACCTTGACCCTCCAGAGGTGGCCCTGATTCTGTGTTCCAAGGGGCATCCTGCAGCAGGGCAGCCAGGGG
CCAGGATGTATCAGGGGACCAATACACCATAATAATAAAACTGCTTGAGCCTCCTGCAGGCCTTGGGCCTCGGGTTTGCCACCTACA
```

Granzyme M					Start	Stop	Length	Splice sites		Coding region		UTR		
	Ori			Chromosome								Length	Start	Stop
Exon 1	For	1	68	7	42307080	42307119	68			1	40	28	42307052	42307079

Intron1								GTGAG	CCCAG						
Exon 2	For	69	231	7	42310514	42310676	163			41	203				
Intron 2								GTGAG	CCCAG						
Exon 3	For	232	367	7	42311421	42311556	136			204	339				
Intron 3								GTGCT	TATAG						
Exon 4	For	368	631	7	42311717	42311980	264			340	603				
Intron 4								GTGCG	CACAG						
Exon 5	For	632	954	7	42312383	42312556	323			604	777	149	42312557	42312705	

Gene M is forward strand and locates in Chromosome 7: 42,307,052 to 42,312,705. One transcript in this gene which consists of 5 exons has been identified and the length of cDNA and amino acid is 954bp and 258residues, respectively.

Red- 5'or 3' untranslated region; Yellow- The length of the intron; Dark- A nucleotide codon encodes the amino acid of catalytic triad

8.2 Appendix B- Comparison of bovine, human, murine and pig nucleotide sequences

(a). Granzyme A

```
            1          11         21         31         41         51
            .          .          .          .          .          .
BovineGZMA  ATGAGGAACT CCTCTACCTT TCTGGCAGCC ACTCTCTCAA TTGTCGTTTT TCTCCT---A
HumanGZMA   .......... ...A..GA.. .......T.. T........G .........C ......GCT.
MurineGZMA  .........G .....GGTCC C.G..GGC.A T....TG.T. C.C..C.... ...T..GCTT

            61         71         81         91         101        111
            .          .          .          .          .          .
BovineGZMA  ATTCCTGAAG ATCTCTGTGA AAAAATTATT GGAGGAAATC AAGTGACTCC TCATTCAAGA
HumanGZMA   .......... ..G....... .......... .........G ....A..... ..........
MurineGZMA  .......... GAGG...... ..G...C... ......G.CA CG..TGT... ...C......

            121        131        141        151        161        171
            .          .          .          .          .          .
BovineGZMA  CCCTACATGG TGCTACTT-- ----GATGGG GGAAACATCT GTGCCGGAGC TTTGATTGCC
HumanGZMA   .......... .C......AG TCTT..CA.A AA..C..... ....T..G.. .........A
MurineGZMA  ..G..T.... CT......AA ACTTAG.TCA AAT.C..... ....T..C.. ........AA

            181        191        201        211        221        231
            .          .          .          .          .          .
BovineGZMA  AAAGACTGGG TATTGACTGC AGCTCACTGT TCCCTGAACC AGAAATCCCA GATCATTCTT
HumanGZMA   .......... .G........ .......... AA.T.....A .A.GG..... .G........
MurineGZMA  ..GA...... .G........ T..C...... AA.G..GGAA ...G...TA. .T........

            241        251        261        271        281        291
            .          .          .          .          .          .
BovineGZMA  GGGGCCCACT CAAGAAACAA GGAAGAGCCT GAAAAACAGA TTATGTTTGT TAAGAAAGAG
HumanGZMA   .....T.... ...T..C..G .........A AC........ .A...C.... ..........
MurineGZMA  .....T.... ...TC..T.. .---.....A ...C...... .AT..AC... ........CA

            301        311        321        331        341        351
            .          .          .          .          .          .
BovineGZMA  TTTCCCTATC CATGCTATGA CCCGGACACA CATGAAGGCG ATCTTAAACT TCTAAAGCTG
HumanGZMA   .......... .......... ...A.C.... .GC.....T. .C........ .T..C.....
MurineGZMA  .......... .......... TGAAT.T... .G...G..G. ....AC.... .G..CG...A

            361        371        381        391        401        411
            .          .          .          .          .          .
BovineGZMA  AACAAAAAAG CAACACTTAA TAAAAACGTG GCTATCCTTC AGCTCCCAAA AGAGGGCAAA
HumanGZMA   .TGG...... ...A.A.... C...T.T... A......... .T..A..T.. .A....GG.C
MurineGZMA  ..G....... .....G.... C.G...T... .......... .C..A..T.. .A....AG.T

            421        431        441        451        461        471
            .          .          .          .          .          .
BovineGZMA  GATGTGGAAC CAGGAACTGC ATGTCGAGTT GCAGGGTGGG GACAGTTTTA CAATAATTCC
HumanGZMA   ......A... .......CAT G..C.A.... .......... .CAG.AC.C. .....G.G.A
MurineGZMA  ......A... .......CAG ...C.....A .....A.... .GAGA...GG ......G..A

           481        491        501        511        521        531
            .          .          .          .          .          .
BovineGZMA  CCTGTGT CCAAGATTTT GAGAGAAGTC AATGTCACCA TCATAGACAG AAAAATCTGCAAT
HumanGZMA   T..TG.. ..G.T.C.C. .......... ...A..... .......... ....G........
MurineGZMA  G..CCC. .TG.A.C.C. .......... ..CA....TG .......... ............

            541        551        561        571        581        591
            .          .          .          .          .          .
BovineGZMA  GATCAAT CACACTATAA TTATAATCCT GTGATTGGAC TGAATATGAT TTGTGCTGGAAGC
HumanGZMA   ....G.A AT........ ..T...C... .........A ........G. .............
MurineGZMA  ...G..A A......... ..T.C..... ..A....... .A..C..... ......A..GGA.

            601        611        621        631        641        651
```

```
            .          .          .          .          .          .
BovineGZMA  CTCCAAG GTGGAAAAGA CTCCTGCCAT GGAGATTCTG GAAGCCCTCT GATATGTAAAGAC
HumanGZMA   ....G.. ......G... ...G...A.. .......... ........T. .T.G..CG.G.GT
MurineGZMA  ....GT. .......G.. .......A.. ..G....... .C........ .C.....G.T.GT

            661        671        681        691        701        711
            .          .          .          .          .          .
BovineGZMA  ACTTTCA GAGGCATCAC TGCCTTTGGC ATTCCGGGGA GATGTGGAGA CCCTCGAGGGCCC
HumanGZMA   GT....C ....GG.... .T........ C..GAAAAT. A...C..... ......T.....T
MurineGZMA  .T...GC .......... CT.T......- --.GGA.A.. AG........ T.GC...T....T

            721        731        741        751        761        771
            .          .          .          .          .          .
BovineGZMA  GGCGTCT ATACACTTCT CTCAAAGAAA CACCTCAACT GGATAGTTAA GACTATGAAGCAA
HumanGZMA   ..T.... ...TT..... .......... .......... .....A...T ......C...GG.
MurineGZMA  ..T.... ....TT.C.. ....G.T... ........T. .....AAG.. ..T.......GGT

           781        791
            .          .
BovineGZMA  GCAGTTT AA
HumanGZMA   ....... ..
MurineGZMA  T.T..G. ..
```

(b). Granzyme O

```
                1          11         21         31         41         51
                .          .          .          .          .          .
BovineGZMO ATGAATATTC CTTTTCCTTT CTCTTTTCCT CCTGCCATTT GTCTCCTTCT AATTCCTGGA
PigGzmO    ...G.A.... .......... ..T....... G.......G. ........A. ..........

                61         71         81         91         101        111
                .          .          .          .          .          .
BovineGZMO GTTTTTCCAG TATCCTGCGA GGGAATTATA GGAGGAAATG AAGTGGCCCC TCACACAAGA
PigGzmO    .......... .......T.. C......... .......... .....T.A.. C........G

                121        131        141        151        161        171
                .          .          .          .          .          .
BovineGZMO CGCTACATGG CTCTAATCAA AGGGCTGAAA CTCTGTGCAG GGGCTTTAAT CAAAGAAAAC
PigGzmO    .......... ..T....TG. .......... G......... .......G.. ..........

                181        191        201        211        221        231
                .          .          .          .          .          .
BovineGZMO TGGGTGTTGA CAGCCGCTCA TTGTGACCTG AAGGGCAATC CTCAAGTTAT TCTTGGGGCC
PigGzmO    .......... ....T..... C......... ..A.....C. .......... ......A...

                241        251        261        271        281        291
                .          .          .          .          .          .
BovineGZMO CACTCTACAT CCCATAAAGA GAAACTTGAC CAAGTATTTT CCATTAAAAA GGCAATTCCC
PigGzmO    .......... .......... ....TA.... ..GAC..... .......... .........T

                301        311        321        331        341        351
                .          .          .          .          .          .
BovineGZMO TACCCATGCT TTGATCCACA GACATTTGAA GGGGATCTTC AACTACTTCA GCTGGAAGGT
PigGzmO    ..T.....T. .......... .......... .......... .G..G..... ..........

                361        371        381        391        401        411
                .          .          .          .          .          .
BovineGZMO AAAGCAACTA TGACCAAAGC TGTAGGAATA CTTCAGCTAC CAAGAACAGA AGACGATGTC
PigGzmO    .......... .......... .......... ...A...... .C.AC....G .A.A......

                481        491        501        511        521        531
                .          .          .          .          .          .
BovineGZMO AAACCCCACA CCAAGTGTCA TGTGGCAGGA TGGGGAAGCA CCAAAAAAGA CGCATGTCAA
PigGzmO    G......... .......... .......... .......... ...G...... .T.G..CA..

                541        551        561        571        581        591
                .          .          .          .          .          .
BovineGZMO ATGTCTAATG CCTTGAGAGA AGCCAACGTT ACAGTGATAG ATAGGAAAAT ATGCAATGAT
PigGzmO    ..T..A..CA .......... ..T....A.. ..T....... ..C.A..... G.........
```

```
                601        611        621        631        641        651
                .          .          .          .          .          .
BovineGZMO GCCCAGCACT ATAATTTTAA TCCAGTTATT GATCTCAGTA TGATCTGTGC TGGTGGTAGA
PigGzmO    .......... .......... .......... ..C..TG... .......... A.T.......

                661        671        681        691        701        711
                .          .          .          .          .          .
BovineGZMO AAAGGTGAAG ATGATTCATG TGAAGGGGAT TCTGGAAGTC CTCTGATATG TGATAATGTT
PigGzmO    .......... .......... ......A... .......... .......... ...C..CA..

                721        731        741        751        761        771
                .          .          .          .          .          .
BovineGZMO TTCAGAGGTG TCACTTCCTT TGGCAAGTGT GGTAATCCCC AGAAGCCTGG CATCTACATC
PigGzmO    ........G. .......... ...G...... .....C.... .......... .G.......T

                781        791        801        811        821        831
                .          .          .          .          .          .
BovineGZMO CTCCTTACCA AAAAACACCT CAACTGGATA AAGAAAACCA TTGCAGGAGC CATATAA
PigGzmO    .....C.... .......... .......... .......... .......... ......G
```

(c). Granzyme B

```
           1          11         21         31         41         51
           .          .          .          .          .          .
BovineGZMB ATGAAGCCTC TCCTGCTCCT GGTGGCCTTT CTCCTGACCC CCAGGGCAAA GGCAGGGGAG
HumanGZMB  ...C.A..AA .......T.. .C.......C ......CTG. ........G. T.........
MurineGZMB ......ATC. .......A.. .C..A....G TCT...G..T .....A.... ..........

           61         71         81         91         101        111
           .          .          .          .          .          .
BovineGZMB ATCATCGGGG GCCATGAAGC CAAGCCCCAC TCCCGCCCCT ACATGGCATA TCTTCAGTAC
HumanGZMB  .......... .A.....G.. .......... .......... .......T.. ....AT.AT.
MurineGZMB .......... .A.......T .......... ..T..A.... .......C.T A...TC.AT.

           121        131        141        151        161        171
           .          .          .          .          .          .
BovineGZMB TGGAATCAGG ATGTCCAGAG TAGGTGCGGT GGGTTCCTGG TTCGACAGGA CTTCGTGCTG
HumanGZMB  ...G.....A .GTCT.T..A G......... ..C......A .A...G.C.. ..........
MurineGZMB AA.G.....C .GCCTG..GC G.TA..T..G ..C.....TA .....G.... ...T......

           181        191        201        211        221        231
           .          .          .          .          .          .
BovineGZMB ACAGCCGCTC ACTGCAACGG AAGCTCAATC AAAGTCACCC TGGGGGCCCA CAACATCAAA
HumanGZMB  .....T.... ....TTGG.. ......C..A ..T......T .......... ...T......
MurineGZMB ..T..T.... ....TG.A.. ...TAT...A ..T.....TT .......... ..........

           241        251        261        271        281        291
           .          .          .          .          .          .
BovineGZMB CAGCAGGAGA GGACCCAGCA GGTCATCAGG GTGAGAAGAG CCATCAGCCA CCCTGACTAT
HumanGZMB  G.A......C C......... .T.T...CCT ....A....C .....CC... T..A.C....
MurineGZMB G.A.......  A......... A......CCT A..GT..A.T G...TCC... ...A......

           301        311        321        331        341        351
           .          .          .          .          .          .
BovineGZMB AATCCTAAGA ACTTCTCCAA CGACATCATG TTATTAAAGC TGGAGAGAAA GGCCAAGCAG
HumanGZMB  .......... .......... .......... C..C.GC... .......... ........G.
MurineGZMB .......... CA........ T......... C.GC...... ..A....T.. .......AG.

           361        371        381        391        401        411
           .          .          .          .          .          .
BovineGZMB ACATCAGCTG TGAAGCCCCT TAGTCTGCCC AGGGCCAAGG CCCGGGTGAA GCCAGGACAG
HumanGZMB  ..CAG..... ..C....... C..G..A..T ..CAA..... ...A...... ......G...
MurineGZMB ..TAG..... ...G...... C.AC...... ...CG...T. T.AAT..... .......G.T

           421        431        441        451        461        471
           .          .          .          .          .          .
BovineGZMB ACGTGCAGCG TGGCCGGCTG GGGG---AGG GACTCCACGG ACACCTACGC TGACACACTA
HumanGZMB  ..A.....T. .......... ....CAG.C. .C.C..CT.. GA.AAC..T. AC........
MurineGZMB GT....TAT. ....T..T.. ...AAGG.T. .C.C.A.T.. G..AA...T. AA....G...
```

```
           481        491        501        511        521        531
           .          .          .          .          .          .
BovineGZMB CAGGAGGTAA AGCTGATCGT GCAGGAGGAT CAAAAGTGTG AGGCCTACTT ACGCAACTTT
HumanGZMB  ..A.....G. ..A...CA.. ......A... .G......C. .AT.TG.... ....C.T.A.
MurineGZMB ..A.....TG ......CA.. A...A..... .GGG...... ..T....... TAAA..TCG.

           541        551        561        571        581        591
           .          .          .          .          .          .
BovineGZMB TATAACCGCG CCATCCAGCT GTGTGTGGGG GACCCAAAGA CAAAGAAAGC TTCCTTTCAG
HumanGZMB  ..CG..A.TA ....TG..T. ...C...... ......G... TT..A..GA. .......A..
MurineGZMB ..C...AAAA ...AT...A. A....C.... .......... .C..ACGT.. ........G.

           601        611        621        631        641        651
           .          .          .          .          .          .
BovineGZMB GGGGACTCGG GGGGCCCTCT CGTGTGTGAC AATGTGGCCC AGGGCATTGT CTCTTATGGA
HumanGZMB  ........T. .A........ T......A.. ..G....... .......... ...C......
MurineGZMB .....T..T. .A.....G.. T......A.A ..A.....TG CA.....A.. T..C......

           661        671        681        691        701        711
           .          .          .          .          .          .
BovineGZMB AAAAGAGATG GATCAACTCC ACGGGCCTTC ACCAAAGTCT CAAGTTTCCT GCCCTGGATA
HumanGZMB  CG..ACA... .CATGC.... ...A....G. .......... ....C..TG. A.A.......
MurineGZMB T.T.AG.... .T...C.... ...T..T... .......... .G......T. AT........

           721        731        741
           .          .          .
BovineGZMB AAGAAAACCA TGAAAAGCCT CTGA
HumanGZMB  .......... .....C..TA ..A.
MurineGZMB ........A. ........AG ..A.
```

(d). Granzyme H

```
           1          11         21         31         41         51
           .          .          .          .          .          .
BovineGZMH ATGCAGCTAC TCCTGCTCCT GATGGCCTTT CTTCTGCCTC CTGGG-CTGG GAGAGCCTTT
HumanGZMH  .......C.T ....C..... .T........ ......A.C. .....G.... ..C..-----

           61         71         81         91         101        111
           .          .          .          .          .          .
BovineGZMH TCTTTCAGAG GAGATCATTG GGGGCCATGA GGCCAAGCCC CACTCCCGCC CCTACATGGC
HumanGZMH  --------.. ........C. .......... .......... .......... ..........

           121        131        141        151        161        171
           .          .          .          .          .          .
BovineGZMH TTTCGTTCAG TTTCTGGGTG AGAAGAGTTG GAAGAGGTGT GGCGGTGTTC TCATACAAAA
HumanGZMH  C..T...... ......CAA. ........C. .......... .....CA.C. .AG.GAG...

           181        191        201        211        221        231
           .          .          .          .          .          .
BovineGZMH AGACTTTGTT CTGACAGCTG CTCACTGCAG AGGAAGCTCA ATCAATGTCA CCCTGGGGGC
HumanGZMH  G........G .......... ........CA G........C ..A....... ..T.......

           241        251        261        271        281        291
           .          .          .          .          .          .
BovineGZMH CCACAACATC AAACAGCAGG AGAGGACCCA ACAGGTCATC CAGGTGAAAA GAGCCATCCA
HumanGZMH  ......T... ..GG.A.... ..C....... G...T.T... .CT....... ..C......C

           301        311        321        331        341        351
           .          .          .          .          .          .
BovineGZMH CCACCCAGAC TATAATCCTA AGACCTTCTC CAACGACATC ATGTTACTGC AGCTGGAGAG
HumanGZMH  ...T....C. .......... ...A...... .......... ...C...... ..........

           361        371        381        391        401        411
           .          .          .          .          .          .
BovineGZMH AAAGGCCAAG CAGACATCAG CTGTGAAGCC CCTTAGTCTG CCCAAGGCCA AGGCCCAGGT
HumanGZMH  .......... TG...CA... .....CG... T..C..G..A ..T.GCAG.. ..........

           421        431        441        451        461        471
           .          .          .          .          .          .
BovineGZMH GAAGCCAGGA GAAGTGTGCA GTCTGGCCGG CTGGGGGAAG GTGGCCCTGG GCACTCCAGC
HumanGZMH  .........G C.GC...... ..G....T.. ......TT.T ..CT.AA..A .....TT...
```

```
           481        491        501        511        521        531
           .          .          .          .          .          .
BovineGZMH CACCACCCTG CAGGAGGTAG AGCTGACGGT TCAGGAGGAT CGGGTGTGTG AATCACTCAA
HumanGZMH  A.....A... .....A..GT T......A.. G...A....C T.CCA..... ..CGT...TT

           541        551        561        571        581        591
           .          .          .          .          .          .
BovineGZMH CCCCAGGAAC TACAGTCGGG CCACCCAGAT TTGTGTCGGG GACCCAAGGA AGGTGAAAAC
HumanGZMH  ..ATG.C..T .....CA.A. ....TG.... ......G... ..T....A.. ..ACAC.G..

           601        611        621        631        641        651
           .          .          .          .          .          .
BovineGZMH CGGCTTCAAG GGTGACTCCG GTGGACCCCT CGTGTGTAAA AAAGTGGTCC ATGGTATTTT
HumanGZMH  ...T...... ..G....... .G..G..... .........G G.C..A.C.. .A......C.

           661        671        681        691        701        711
           .          .          .          .          .          .
BovineGZMH CTCCTATGGA AAGACGAATG GGACACCTCC AGGAGTCTTC ACCCAGGTCT CACACTTCCT
HumanGZMH  .......... ..C.AA..A. .......... ........A. .T.A...... ..........

           721        731        741        751
                      .          .          .          .
BovineGZMH ACCCTGGATA AAGAGAACAA TGAAGCACCT CTAA
HumanGZMH  G......... .......... ......G... ....
```

(e). Granzyme K

```
           1          11         21         31         41         51
           .          .          .          .          .          .
BovineGZMK ATGACTAAGT TTTCTTCTTT TTTTCTATGT TTCCTACTAG CCGGGACTTA CATGACTCCA
HumanGZMK  .......... .......C.. ..C...G.T. ......A... TT...G.... T.......AT
MurineGZMK ...---.G.. .......A.G GGC...GGT. .C...GG.G. .T..CGT... T...T..T..

           61         71         81         91         101        111
           .          .          .          .          .          .
BovineGZMK GAGTGTTTCA ACATGGAGAT TATTGGAGGG AGAGAAGTGT CCCCTCACTC CAGGCCGTTT
HumanGZMK  .T........ .T.....A.. .......... .A........ .A.....T.. ......A...
MurineGZMK .........C .T.CT..A.. .......... ..G.....CC AG..G..T.. ......A...

           121        131        141        151        161        171
           .          .          .          .          .          .
BovineGZMK ATGGCGTCCC TGCAGTATGG CGGCGACCAC ATCTGCGGGG GAGTGCTGAT CCATCCTCAG
HumanGZMK  .....C...A .C........ ...AC.T... G.T..T..A. .T..T..... TG....A...
MurineGZMK .........A .C.....CC. .A..A.G..T ..T..T..A. ....C..... ...C..A...

           181        191        201        211        221        231
           .          .          .          .          .          .
BovineGZMK TGGGTGCTCA CAGCAGCCCA CTGCCACTTG CGGTTTGCCA AAAGCCAGTC TTCCAAAGTG
HumanGZMK  ........G. .......... ......A.AT ......A... ..G....... .C...CT...
MurineGZMK ........A. ....C..... ....T...CT T.....C... G.G....C.. .C...CC...

           241        251        261        271        281        291
           .          .          .          .          .          .
BovineGZMK GTTTTAGGAG CACACTCTCT CTCAAAGAAT GAGGCCTCCA AGCAAACATT TGAGATTAAA
HumanGZMK  ........C. .......... .......... .......... .A......C. G.....C...
MurineGZMK .......... ....T..... T..C...... ...C..ATG. ....G..... ...A......

           301        311        321        331        341        351
           .          .          .          .          .          .
BovineGZMK AAATTCATAC GATTCCCAGG ATTTGCATTA GCCCCTAAAT CAAACGATAT TATGCTGGTT
HumanGZMK  .....T.... C....T..A. .G..A...C. .AT...C... ....T..... C.........
MurineGZMK ..G.....C. C....T..C. .C..CAG.CC .GTT.CGC.. .GC.T..C.. C......A.A

           361        371        381        391        401        411
           .          .          .          .          .          .
BovineGZMK AAGCTTCACA CGGCCGCAAT ACTCAACAGA CATGTCCAAC TGCTCCACCC AAGGGCTAAA
HumanGZMK  ........A. .A.......A ......T.A. ......A.GA ........AT ...AT.C...
MurineGZMK .......G.. .T..T...GA ...A....AG A......... ....T....T GG.AT.C...

           421        431        441        451        461        471
```

```
           .           .           .           .           .           .
BovineGZMK AATGATATTA AAGCTGGAAC AAAATGCCAG GTTGTTGGCT GGGGAGCCAC TGACCCAGAA
HumanGZMK  .CCTC.C... G.T.....T. C......A.. ...AC..... .......... C..T.....T
MurineGZMK ..CT..C... G..A...G.. C......... ..GAC..... .....A.... CA.G.....T

           481         491         501         511         521         531
           .           .           .           .           .           .
BovineGZMK GGCTTAAGCC TTTCTGATAC CCTGCGAGAA GTCACTGTCA CTGTTATAAG TCGAAAAACG
HumanGZMK  TCA.....A. C......C.. .......... ........T. ....CC.... .......CTT
MurineGZMK CTG....C.G CC........ ....A..... ........T. .CA.C..... .A.....CGC

           541         551         561         571         581         591
           .           .           .           .           .           .
BovineGZMK TGCAACAGCC GAGATTATTA CAACCACAGC CCTGTTATAA CTAGAACCAT GCTATGTGCA
HumanGZMK  .......... A.AG...C.. ....GG.GA. ...T....C. .C.A.GA... .G.C......
MurineGZMK ..T....... A.AGC..C.. ........AA .......... .C.AGGA... .A........

           601         611         621         631         641         651
           .           .           .           .           .           .
BovineGZMK GGAGACGCCA GAGGCCAGAA GGATTCCTGT CAGGGTGACT CAGGGGGCCC CTTGGTCTGC
HumanGZMK  .....T.... A......... .......... A......... .......... ....A....T
MurineGZMK .....T.... ....T..A.. .........C A......... .T..T..... T...A.....

           661         671         681         691         701         711
           .           .           .           .           .           .
BovineGZMK AAAGGTGCCT TTCATGCCTT AGTTTCTGGA GGTCCCAAAT GTGGTGATGC CAAGAAACCT
HumanGZMK  .......T.. .C..C..TA. ...C...... ....ATG... ......T... ..CA..G...
MurineGZMK .....CAT.. .C......C. ...C...CAG ..CTAT.... ....CATC.. ...A..G...

           721         731         741         751         761         771
           .           .           .           .           .           .
BovineGZMK GGAATCTACA TCCTACTAAC CCGGAAATTC CAAGCTTGGA TCAAAAGCAA CTTGGCCCCA
HumanGZMK  .......... C...GT.... .AA.....A. ..GA...... .......... .C.T.T...G
MurineGZMK ........T. CG...T.... TAA.....A. ..GA.C.... .......... GC.T......

           781         791
           .           .
BovineGZMK TCTCATGCAG ACTAA
HumanGZMK  C.....A..A .T...
MurineGZMK ..A.G....C .T.G.
```

(f). Granzyme M

```
           1           11          21          31          41          51
           .           .           .           .           .           .
BovineGZMM ATG-----CT G--------- -CTCCTGCTG GTGGTCCTGG AAGCTCTGTG GGCAGGAGGC
HumanGZMM  ...GAGGC.. .CGTGTCTTC A..G...G.. C...C..... GG..C....- -...T....
MurineGZMM ...GAGGT.. .CTGGTCCCT G..G..A... C...C....A ..A.A..... .....C....

           61          71          81          91          101         111
           .           .           .           .           .           .
BovineGZMM AACACCTTCG AGACCCACAT CATCGGGGGT CGAGACGCTG TCCCCCACTC ACGCCCATAC
HumanGZMM  .G.T....T. G......G.. .........C ..G..G.TGA .......... G.....G...
MurineGZMM ....GA..T. .......G.. ...T...... .....G..A. ....G..... C.........

           121         131         141         151         161         171
           .           .           .           .           .           .
BovineGZMM ATGGTCTCGC TGCAGAAGTC GTCTGGCTCA CACCAATGTG GTGGGGTGCT CCTGCACCAA
HumanGZMM  ....C...A. ......GAA- --A......C ....TG..C. .G..T..C.. GG......C.
MurineGZMM ....C...T. .A.....AG- --.CAAG..C ..TGTG.... .G..A..C.. TG....T.GG

           181         191         201         211         221         231
           .           .           .           .           .           .
BovineGZMM AATTGGGTGT TGACAGCTGC CCACTGCCTG ACCCAGCCGA CGCAGCAGCT GAGGCTTGTG
HumanGZMM  ..G......C ....G..... .......... G......G.. T.GCC..... ......G...
MurineGZMM ..G.....A. .......... .......... T.TG.....C TA...A.C.. ..A...G...

           241         251         261         271         281         291
           .           .           .           .           .           .
BovineGZMM CTGGGGCT-- -------TCA TGTGCTGGGA GAGATCAGCC CTATCTACCG CATCAGGAAG
HumanGZMM  .........-- -------C.. CACC....AC AGCCC.G.T. TC.C..T..A .....A.GCA
```

```
MurineGZMM ..T..C..GC ACAACCTC.. ..AT..CCA. ..TCCTG... TC.C..T.TA ....C..G.A

           301        311        321        331        341        351
           .          .          .          .          .          .
BovineGZMM GTGGTCCGGC ACCCCGAATA CAAGCCAGTC CCTCATCTGG AGAATGACCT CGCACTGCTA
HumanGZMM  .CCA...A.. ....TCGC.. ......C... ...GCC.... ....C..... ...G.....T
MurineGZMM .CCA.TAAA. ....T.GC.. ...-------. .ACA.ATAT. ....C..... G........T

           361        371        381        391        401        411
           .          .          .          .          .          .
BovineGZMM AAGCTGGACG GGAAGGTGAA GCCCAGCAGG ACCATCCAGC CCCTGGCGTT GCCCCGAGGG
HumanGZMM  C......... ....A..... .......C.. .......G.. .GT....CC. ....A.TAA.
MurineGZMM .....A..TA .ACGA...C. ........A. .ATG..A.A. .A..A..TC. ...AA..AA.

           421        431        441        451        461        471
           .          .          .          .          .          .
BovineGZMM CGCCAG---A TGGTGGCCAC AGGCACCCGG TGCAGCCTGG CCGGCTGGGG CCTGACCCAC
HumanGZMM  .........-G .......AG. ...G..T... ......A... .......... G.........
MurineGZMM .C..GATCC. A.CC...AGA ...T...T.. ......ACA. .T........ AA........

           481        491        501        511        521        531
           .          .          .          .          .          .
BovineGZMM CAGCCTGGGA ACCTGGCCAG GGTGCTGCAG GAGCTGGACG TGCATGTGTT GGACACCAGG
HumanGZMM  ...GGC...C G....T..C. ........G. .........C .C..A...C. .......C.C
MurineGZMM ...GG....C C..G...... ..CC...... ...T....TC ...G....C. ...T...CAA

           541        551        561        571        581        591
           .          .          .          .          .          .
BovineGZMM ATGTGCAACA ACAGTCGGTT CTGGCACGGC AACATCAGCT CCCACATGAT CTGCCTGGCA
HumanGZMM  .....T.... ....C..C.. ....A..... .G.C..TC.C ..AG....G. .........G
MurineGZMM .....T.... ....C..C.. ....A....T GT.C...TAG A.AG....C. A...T.AAAG

           601        611        621        631        641        651
           .          .          .          .          .          .
BovineGZMM GCTGACTCCA AGAACCAGGC CCCCTGCAAG GGGGACTCAG GAGGGCCGGT GGTGTGCAAA
HumanGZMM  ..C....... ..G....... T......... ..T.....G. .C.....CC. ......TGGC
MurineGZMM ....GGAG.. ...G...A.. .......... ..T.....T. .......CC. ......TGGC

           661        671        681        691        701        711
           .          .          .          .          .          .
BovineGZMM AGAGGCCAAG TG---GCTGG AATCCTGTCC TTCAGCTCTG AGAATTGCAC CGACATCTTC
HumanGZMM  .A.....GG. ..TTG..CA. .G........ ........CA G.GTC..... T.........
MurineGZMM .A......G. ..---.A... G........T ........CA .A.CC..... A.........

           721        731        741        751        761        771
           .          .          .          .          .          .
BovineGZMM AAACCCCCCG TGGCTGTCGC TGTGGCCCCC TACATGCCCT GGATCAAGAA GGTCCTCCGC
HumanGZMM  ..G..T.... ....CAC... ......G..T ...G..T... ......G... ....AC.G..
MurineGZMM ..G..A..T. ....CACT.. ...A...... ....GCT... ......G... ....A.TG.T

           781        791        801
           .          .          .
BovineGZMM CAC-AATGGC TCACCTCCCT CACCTTGA
HumanGZMM  .G--.TC... C--------- -----...
MurineGZMM .G.TGG.CA. C.CAA..TT. GGT.-...
```

8.3 Appendix C – Solutions and media

All reagents were obtained from Sigma (Poole, Doreset, UK) unless otherwise stated. Double-distilled water (DDW) was sourced from a Millipore-RO 60 Plus unit (Millipore, Billerica, MA, USA)

C.1 Alsever's Solution 10x stock

• D-glucose	205.0 g	(113.8mM)
• Citric acid	5.5 g	(2.9mM)
• Sodium chloride	42.0	(71.9mM)
• Tris-sodium citrate Di-hydrate	80.0	(27.2mM)

Made up to a volume of 1 L with DDW

Made up to 1x solution by filter sterilizing 0.1 L stock solution using 0.45 µm Minisart single-use filter (Sartorius, Goettingen, Germany) and adding to 0.9 L of sterile DDW

C.2 Standard Culture Medium (SCM)

- RPMI 1640 medium + 20mM HEPES + L-Glutamine (Gibco, Paisley, UK)
- 10% Foetal bovine serum (Gibco, Paisley, UK)
- 100U/ml Penicillin, 100µg/ml streptomycin, 292µg/ml L-glutamine
- 5 x 10^{-5}M 2-Mercaptoethanol (2-ME)

C.3 FACS Medium

- RPMI 1640 medium + 20mM HEPES + L-Glutamine (Gibco, Paisley, UK)
- 2% Foetal bovine serum (Gibco, Paisley, UK)
- 0.2% Sodium Azide

C.4 Cytotoxic Medium

- RPMI 1640 medium + 20mM HEPES + L-Glutamine (Gibco, Paisley, UK)
- 5% Foetal bovine serum (Gibco, Paisley, UK)

C.5 SM-0005 PCR Buffer

- 45mM Tris-HCL (pH 8.8 at 25°C)
- 11mM Ammonium sulphate
- 4.5mM Magnesium chloride
- 0.113mg/ml Bovine serum albumin
- 4.4uM EDTA
- 1.0mM each of dATP, dCTP, dGTP and dTTP

Purchased from ABgene (Epsom, Surrey, UK)

C.6 Tris-acetate/EDTA electrophoresis buffer (TAE) 50x stock

• Trizma base	242g	(2M)
• Glacial acetic acid	57.1ml	(2M)
• EDTA	100ml of 0.5M solution	(50mM)

pH adjusted to 7.7-8.0 with glacial acetic acid if required, make up to a volume of 1L with distilled water

C.7 Loading Buffer for Agarose Gel Electrophoresis

- 15% w/v Ficoll (Type 400) in DDW with bromophenol blue (0.25% v/v) and xylene cyanol (0.25%) dyes

C.8 SOC medium

• Tryptone	20g
• Yeast Extract	5g
• Sodium chloride	0.5g
• Potassium chloride	0.19g
• Magnesium chloride	0.95g
• Glucose	3.6g

pH adjusted to 7.0 with 5M sodium hydroxide, made up to a volume of 1 L with DDW and autoclaved

C.9 Luria-Bertani (LB) Agar plates with ampicillin

- Tryptone 10g
- Yeast extract 5g
- Sodium chloride 10g
- Agar 15g

pH adjusted to 7.0 with 5M sodium hydroxide, make up to a volume of 1L with DDW and autoclaved

Upon use, LB agar was melted and ampicillin added to give a final concentration of 100ug/ml. The required amount of medium was poured into sterile Petri dished and allowed to set and dry. If LB/ampicillin/IPTG/X-Gal plates were required for blue/white colony selection, 100ul of 24mg/ml IPTG (Isopropyl β-D-1-thiogalactopyranoside) and 50ul of 50mg/ml XGal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) in 50ul of SOC medium (Appendix C.8) was applied to the surface of the medium and allowed to dry before application of any culture.

C.10 LB Medium

- Tryptone 10g
- Yeast extract 5g
- Sodium chloride 10g

pH adjusted to 7.0 with 5M sodium hydroxide, make up to a volume of 1L with DDW and autoclaved

C.11 PBS/EDTA 10x stock

- Sodium chloride 40.0g
- Potassium chloride 1.0g
- Sodium phosphate, dibasic 5.75g
- Potassium phosphate, monobasic 1.0g
- EDTA 1.12g

pH adjusted to 7.2 -7.4, made up to a volume of 500 ml with DDW.

Made up to 1x solution by filter sterilizing 100ml stock solution using 0.45 µm Minisart single-use filter (Sartorius, Goettingen, Germany), adding to 900ml of DDW and autoclaved

C. 12 Phosphate Buffered Saline (PBS)

- Sodium chloride 170.0mM
- Potassium chloride 3.4mM
- Sodium phosphate, dibasic 9.2mM
- Potassium phosphate, monobasic 1.8mM

pH adjusted to 6.8.

PBS tablets used according to manufacturer's protocol (Unipath LTD, Basingstoke, Hampshire, UK)

Chapter 9 References

Adrain, C., Duriez, P.J., Brumatti, G., Delivani, P. and Martin, S.J. (2006) The cytotoxic lymphocyte protease, granzyme B, targets the cytoskeleton and perturbs microtubule polymerization dynamics. J Biol Chem 281, 8118-25.

Alimonti, J.B., Shi, L., Baijal, P.K. and Greenberg, A.H. (2001) Granzyme B induces BID-mediated cytochrome c release and mitochondrial permeability transition. J Biol Chem 276, 6974-82.

Andrade, F., Bull, H.G., Thornberry, N.A., Ketner, G.W., Casciola-Rosen, L.A. and Rosen, A. (2001) Adenovirus L4-100K assembly protein is a granzyme B substrate that potently inhibits granzyme B-mediated cell death. Immunity 14, 751-61.

Andrade, F., Fellows, E., Jenne, D.E., Rosen, A. and Young, C.S. (2007) Granzyme H destroys the function of critical adenoviral proteins required for viral DNA replication and granzyme B inhibition. Embo J 26, 2148-57.

Andrade, F., Roy, S., Nicholson, D., Thornberry, N., Rosen, A. and Casciola-Rosen, L. (1998) Granzyme B directly and efficiently cleaves several downstream caspase substrates: implications for CTL-induced apoptosis. Immunity 8, 451-60.

Anthony, D.A., Andrews, D.M., Watt, S.V., Trapani, J.A. and Smyth, M.J. Functional dissection of the granzyme family: cell death and inflammation. Immunol Rev 235, 73-92.

Antonov, V.K., Vorotyntseva, T.I. and Zamolodchikova, T.S. (1992) [Duodenase--a new serine proteinase with unusual specificity]. Dokl Akad Nauk 324, 1318-22.

Atkinson, E.A. and Bleackley, R.C. (1995) Mechanisms of lysis by cytotoxic T cells. Crit Rev Immunol 15, 359-84.

Bade, B., Boettcher, H.E., Lohrmann, J., Hink-Schauer, C., Bratke, K., Jenne, D.E., Virchow, J.C., Jr. and Luttmann, W. (2005) Differential expression of the granzymes A, K and M and perforin in human peripheral blood lymphocytes. Int Immunol 17, 1419-28.

Badovinac, V.P. and Harty, J.T. (2000) Adaptive immunity and enhanced CD8+ T cell response to Listeria monocytogenes in the absence of perforin and IFN-gamma. J Immunol 164, 6444-52.

Baldwin, C.L., Black, S.J., Brown, W.C., Conrad, P.A., Goddeeris, B.M., Kinuthia, S.W., Lalor, P.A., MacHugh, N.D., Morrison, W.I., Morzaria, S.P. and et al.

(1988) Bovine T cells, B cells, and null cells are transformed by the protozoan parasite Theileria parva. Infect Immun 56, 462-7.

Baldwin, C.L., Goddeeris, B.M. and Morrison, W.I. (1987) Bovine helper T-cell clones specific for lymphocytes infected with Theileria parva (Muguga). Parasite Immunol 9, 499-513.

Ballingall, K.T., Mwangi, D.M., MacHugh, N.D., Taracha, E.L., Totte, P. and McKeever, D.J. (2000) A highly sensitive, non-radioactive assay for T cell activation in cattle: applications in screening for antigens recognised by CD4(+) and CD8(+) T cells. J Immunol Methods 239, 85-93.

Barry, M., Heibein, J.A., Pinkoski, M.J., Lee, S.F., Moyer, R.W., Green, D.R. and Bleackley, R.C. (2000) Granzyme B short-circuits the need for caspase 8 activity during granule-mediated cytotoxic T-lymphocyte killing by directly cleaving Bid. Mol Cell Biol 20, 3781-94.

Bell, J.K., Goetz, D.H., Mahrus, S., Harris, J.L., Fletterick, R.J. and Craik, C.S. (2003) The oligomeric structure of human granzyme A is a determinant of its extended substrate specificity. Nat Struct Biol 10, 527-34.

Beresford, P.J., Xia, Z., Greenberg, A.H. and Lieberman, J. (1999) Granzyme A loading induces rapid cytolysis and a novel form of DNA damage independently of caspase activation. Immunity 10, 585-94.

Beresford, P.J., Zhang, D., Oh, D.Y., Fan, Z., Greer, E.L., Russo, M.L., Jaju, M. and Lieberman, J. (2001) Granzyme A activates an endoplasmic reticulum-associated caspase-independent nuclease to induce single-stranded DNA nicks. J Biol Chem 276, 43285-93.

Berthou, C., Michel, L., Soulie, A., Jean-Louis, F., Flageul, B., Dubertret, L., Sigaux, F., Zhang, Y. and Sasportes, M. (1997) Acquisition of granzyme B and Fas ligand proteins by human keratinocytes contributes to epidermal cell defense. J Immunol 159, 5293-300.

Bird, C.H., Blink, E.J., Hirst, C.E., Buzza, M.S., Steele, P.M., Sun, J., Jans, D.A. and Bird, P.I. (2001) Nucleocytoplasmic distribution of the ovalbumin serpin PI-9 requires a nonconventional nuclear import pathway and the export factor Crm1. Mol Cell Biol 21, 5396-407.

Bird, C.H., Sutton, V.R., Sun, J., Hirst, C.E., Novak, A., Kumar, S., Trapani, J.A. and Bird, P.I. (1998) Selective regulation of apoptosis: the cytotoxic lymphocyte serpin proteinase inhibitor 9 protects against granzyme B-mediated apoptosis without perturbing the Fas cell death pathway. Mol Cell Biol 18, 6387-98.

Biron, C.A. (1994) Cytokines in the generation of immune responses to, and resolution of, virus infection. Curr Opin Immunol 6, 530-8.

Bladergroen, B.A., Meijer, C.J., ten Berge, R.L., Hack, C.E., Muris, J.J., Dukers, D.F., Chott, A., Kazama, Y., Oudejans, J.J., van Berkum, O. and Kummer, J.A. (2002) Expression of the granzyme B inhibitor, protease inhibitor 9, by tumor cells in patients with non-Hodgkin and Hodgkin lymphoma: a novel protective mechanism for tumor cells to circumvent the immune system? Blood 99, 232-7.

Bladergroen, B.A., Strik, M.C., Bovenschen, N., van Berkum, O., Scheffer, G.L., Meijer, C.J., Hack, C.E. and Kummer, J.A. (2001) The granzyme B inhibitor, protease inhibitor 9, is mainly expressed by dendritic cells and at immune-privileged sites. J Immunol 166, 3218-25.

Bleackley, R.C., Lobe, C.G., Duggan, B., Ehrman, N., Fregeau, C., Meier, M., Letellier, M., Havele, C., Shaw, J. and Paetkau, V. (1988) The isolation and characterization of a family of serine protease genes expressed in activated cytotoxic T lymphocytes. Immunol Rev 103, 5-19.

Boehm, U., Klamp, T., Groot, M. and Howard, J.C. (1997) Cellular responses to interferon-gamma. Annu Rev Immunol 15, 749-95.

Bossard, C., Belhadj, K., Reyes, F., Martin-Garcia, N., Berger, F., Kummer, J.A., Briere, J., Baglin, A.C., Cheze, S., Bosq, J., Ribrag, V., Gisselbrecht, C., Mounier, N. and Gaulard, P. (2007) Expression of the granzyme B inhibitor PI9 predicts outcome in nasal NK/T-cell lymphoma: results of a Western series of 48 patients treated with first-line polychemotherapy within the Groupe d'Etude des Lymphomes de l'Adulte (GELA) trials. Blood 109, 2183-9.

Bots, M., Offringa, R. and Medema, J.P. (2006) Does the serpin PI-9 protect tumor cells? Blood 107, 4974-5; author reply 4975.

Bovenschen, N., de Koning, P.J., Quadir, R., Broekhuizen, R., Damen, J.M., Froelich, C.J., Slijper, M. and Kummer, J.A. (2008) NK cell protease granzyme M targets alpha-tubulin and disorganizes the microtubule network. J Immunol 180, 8184-91.

Bovenschen, N. and Kummer, J.A. Orphan granzymes find a home. Immunol Rev 235, 117-27.

Bovenschen, N., Quadir, R., van den Berg, A.L., Brenkman, A.B., Vandenberghe, I., Devreese, B., Joore, J. and Kummer, J.A. (2009) Granzyme K displays highly restricted substrate specificity that only partially overlaps with granzyme A. J Biol Chem 284, 3504-12.

Bratke, K., Kuepper, M., Bade, B., Virchow, J.C., Jr. and Luttmann, W. (2005) Differential expression of human granzymes A, B, and K in natural killer cells and during CD8+ T cell differentiation in peripheral blood. Eur J Immunol 35, 2608-16.

Brown, G.R., McGuire, M.J. and Thiele, D.L. (1993) Dipeptidyl peptidase I is enriched in granules of in vitro- and in vivo-activated cytotoxic T lymphocytes. J Immunol 150, 4733-42.

Browne, K.A., Johnstone, R.W., Jans, D.A. and Trapani, J.A. (2000) 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 275, 39262-6.

Burkhardt, J.K., Hester, S., Lapham, C.K. and Argon, Y. (1990) The lytic granules of natural killer cells are dual-function organelles combining secretory and pre-lysosomal compartments. J Cell Biol 111, 2327-40.

Burridge, M.J., Morzaria, S.P., Cunningham, M.P. and Brown, C.G. (1972) Duration of immunity to East Coast fever (Theileria parva infection of cattle). Parasitology 64, 511-5.

Burset, M., Seledtsov, I.A. and Solovyev, V.V. (2000) Analysis of canonical and non-canonical splice sites in mammalian genomes. Nucleic Acids Res 28, 4364-75.

Buzza, M.S., Hirst, C.E., Bird, C.H., Hosking, P., McKendrick, J. and Bird, P.I. (2001) The granzyme B inhibitor, PI-9, is present in endothelial and mesothelial cells, suggesting that it protects bystander cells during immune responses. Cell Immunol 210, 21-9.

Buzza, M.S., Zamurs, L., Sun, J., Bird, C.H., Smith, A.I., Trapani, J.A., Froelich, C.J., Nice, E.C. and Bird, P.I. (2005) Extracellular matrix remodeling by human granzyme B via cleavage of vitronectin, fibronectin, and laminin. J Biol Chem 280, 23549-58.

Caputo, A., Garner, R.S., Winkler, U., Hudig, D. and Bleackley, R.C. (1993) Activation of recombinant murine cytotoxic cell proteinase-1 requires deletion of an amino-terminal dipeptide. J Biol Chem 268, 17672-5.

Caputo, A., James, M.N., Powers, J.C., Hudig, D. and Bleackley, R.C. (1994) Conversion of the substrate specificity of mouse proteinase granzyme B. Nat Struct Biol 1, 364-7.

Casciola-Rosen, L., Andrade, F., Ulanet, D., Wong, W.B. and Rosen, A. (1999) Cleavage by granzyme B is strongly predictive of autoantigen status: implications for initiation of autoimmunity. J Exp Med 190, 815-26.

Casciola-Rosen, L., Garcia-Calvo, M., Bull, H.G., Becker, J.W., Hines, T., Thornberry, N.A. and Rosen, A. (2007) Mouse and human granzyme B have distinct tetrapeptide specificities and abilities to recruit the bid pathway. J Biol Chem 282, 4545-52.

Chang, D.W., Xing, Z., Pan, Y., Algeciras-Schimnich, A., Barnhart, B.C., Yaish-Ohad, S., Peter, M.E. and Yang, X. (2002) c-FLIP(L) is a dual function

regulator for caspase-8 activation and CD95-mediated apoptosis. Embo J 21, 3704-14.

Chattopadhyay, P.K., Betts, M.R., Price, D.A., Gostick, E., Horton, H., Roederer, M. and De Rosa, S.C. (2009) The cytolytic enzymes granyzme A, granzyme B, and perforin: expression patterns, cell distribution, and their relationship to cell maturity and bright CD57 expression. J Leukoc Biol 85, 88-97.

Creemers, P. (1982) Lack of reactivity of sera from Theileria parva-infected and recovered cattle against cell membrane antigens of Theileria parva transformed cell lines. Vet Immunol Immunopathol 3, 427-38.

Cullen, S.P., Adrain, C., Luthi, A.U., Duriez, P.J. and Martin, S.J. (2007) Human and murine granzyme B exhibit divergent substrate preferences. J Cell Biol 176, 435-44.

Cullen, S.P., Afonina, I.S., Donadini, R., Luthi, A.U., Medema, J.P., Bird, P.I. and Martin, S.J. (2009) Nucleophosmin is cleaved and inactivated by the cytotoxic granule protease granzyme M during natural killer cell-mediated killing. J Biol Chem 284, 5137-47.

Curtsinger, J.M., Lins, D.C., Johnson, C.M. and Mescher, M.F. (2005) Signal 3 tolerant CD8 T cells degranulate in response to antigen but lack granzyme B to mediate cytolysis. J Immunol 175, 4392-9.

Darmon, A.J., Nicholson, D.W. and Bleackley, R.C. (1995) Activation of the apoptotic protease CPP32 by cytotoxic T-cell-derived granzyme B. Nature 377, 446-8.

Davis, J.E., Smyth, M.J. and Trapani, J.A. (2001) Granzyme A and B-deficient killer lymphocytes are defective in eliciting DNA fragmentation but retain potent in vivo anti-tumor capacity. Eur J Immunol 31, 39-47.

Davis, J.E., Sutton, V.R., Smyth, M.J. and Trapani, J.A. (2000) Dependence of granzyme B-mediated cell death on a pathway regulated by Bcl-2 or its viral homolog, BHRF1. Cell Death Differ 7, 973-83.

DeMartini, J.C. and Baldwin, C.L. (1991) Effects of gamma interferon, tumor necrosis factor alpha, and interleukin-2 on infection and proliferation of Theileria parva-infected bovine lymphoblasts and production of interferon by parasitized cells. Infect Immun 59, 4540-6.

DeMartini, J.C. and Moulton, J.E. (1973) Responses of the bovine lymphatic system to infection by Theileria parva. I. Histology and ultrastructure of lymph nodes in experimentally-infected calves. J Comp Pathol 83, 281-98.

Dive, C. and Hickman, J.A. (1991) Drug-target interactions: only the first step in the commitment to a programmed cell death? Br J Cancer 64, 192-6.

Dobbelaere, D.A., Spooner, P.R., Barry, W.C. and Irvin, A.D. (1984) Monoclonal antibody neutralizes the sporozoite stage of different Theileria parva stocks. Parasite Immunol 6, 361-70.

Duan, H., Chinnaiyan, A.M., Hudson, P.L., Wing, J.P., He, W.W. and Dixit, V.M. (1996) ICE-LAP3, a novel mammalian homologue of the Caenorhabditis elegans cell death protein Ced-3 is activated during Fas- and tumor necrosis factor-induced apoptosis. J Biol Chem 271, 1621-5.

Ebnet, K., Hausmann, M., Lehmann-Grube, F., Mullbacher, A., Kopf, M., Lamers, M. and Simon, M.M. (1995) Granzyme A-deficient mice retain potent cell-mediated cytotoxicity. Embo J 14, 4230-9.

Edwards, K.M., Kam, C.M., Powers, J.C. and Trapani, J.A. (1999) The human cytotoxic T cell granule serine protease granzyme H has chymotrypsin-like (chymase) activity and is taken up into cytoplasmic vesicles reminiscent of granzyme B-containing endosomes. J Biol Chem 274, 30468-73.

Ellis, S.A., Holmes, E.C., Staines, K.A., Smith, K.B., Stear, M.J., McKeever, D.J., MacHugh, N.D. and Morrison, W.I. (1999) Variation in the number of expressed MHC genes in different cattle class I haplotypes. Immunogenetics 50, 319-28.

Emery, D.L. (1981a) Adoptive transfer of immunity to infection with Theileria parva (East Coast fever) between cattle twins. Res Vet Sci 30, 364-7.

Emery, D.L. (1981b) Kinetics of infection with Theileria parva (East Coast fever) in the central lymph of cattle. Vet Parasitol 9, 1-16.

Emery, D.L., Eugui, E.M., Nelson, R.T. and Tenywa, T. (1981) Cell-mediated immune responses to Theileria parva (East Coast fever) during immunization and lethal infections in cattle. Immunology 43, 323-36.

Emery, D.L., MacHugh, N.D. and Morrison, W.I. (1988) Theileria parva (Muguga) infects bovine T-lymphocytes in vivo and induces coexpression of BoT4 and BoT8. Parasite Immunol 10, 379-91.

Emery, D.L., Morrison, W.I., Buscher, G. and Nelson, R.T. (1982) Generation of cell-mediated cytotoxicity to Theileria parva (East Coast fever) after inoculation of cattle with parasitized lymphoblasts. J Immunol 128, 195-200.

Estebanez-Perpina, E., Fuentes-Prior, P., Belorgey, D., Braun, M., Kiefersauer, R., Maskos, K., Huber, R., Rubin, H. and Bode, W. (2000) Crystal structure of the caspase activator human granzyme B, a proteinase highly specific for an Asp-P1 residue. Biol Chem 381, 1203-14.

Eugui, E.M. and Emery, D.L. (1981) Genetically restricted cell-mediated cytotoxicity in cattle immune to Theileria parva. Nature 290, 251-4.

Ewen, C., Kane, K.P., Shostak, I., Griebel, P.J., Bertram, E.M., Watts, T.H., Bleackley, R.C. and McElhaney, J.E. (2003) A novel cytotoxicity assay to evaluate antigen-specific CTL responses using a colorimetric substrate for Granzyme B. J Immunol Methods 276, 89-101.

Fan, Z., Beresford, P.J., Oh, D.Y., Zhang, D. and Lieberman, J. (2003a) Tumor suppressor NM23-H1 is a granzyme A-activated DNase during CTL-mediated apoptosis, and the nucleosome assembly protein SET is its inhibitor. Cell 112, 659-72.

Fan, Z., Beresford, P.J., Zhang, D., Xu, Z., Novina, C.D., Yoshida, A., Pommier, Y. and Lieberman, J. (2003b) Cleaving the oxidative repair protein Ape1 enhances cell death mediated by granzyme A. Nat Immunol 4, 145-53.

Fawcett, D., Musoke, A. and Voigt, W. (1984) Interaction of sporozoites of Theileria parva with bovine lymphocytes in vitro. I. Early events after invasion. Tissue Cell 16, 873-84.

Fawcett, D.W., Buscher, G. and Doxsey, S. (1982) Salivary gland of the tick vector of East Coast fever. III. The ultrastructure of sporogony in Theileria parva. Tissue Cell 14, 183-206.

Fellows, E., Gil-Parrado, S., Jenne, D.E. and Kurschus, F.C. (2007) Natural killer cell-derived human granzyme H induces an alternative, caspase-independent cell-death program. Blood 110, 544-52.

Froelich, C.J., Hanna, W.L., Poirier, G.G., Duriez, P.J., D'Amours, D., Salvesen, G.S., Alnemri, E.S., Earnshaw, W.C. and Shah, G.M. (1996a) 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 227, 658-65.

Froelich, C.J., Orth, K., Turbov, J., Seth, P., Gottlieb, R., Babior, B., Shah, G.M., Bleackley, R.C., Dixit, V.M. and Hanna, W. (1996b) 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 271, 29073-9.

Gallwitz, M. and Hellman, L. (2006) Rapid lineage-specific diversification of the mast cell chymase locus during mammalian evolution. Immunogenetics 58, 641-54.

Gallwitz, M., Reimer, J.M. and Hellman, L. (2006) Expansion of the mast cell chymase locus over the past 200 million years of mammalian evolution. Immunogenetics 58, 655-69.

Garcia-Calvo, M., Peterson, E.P., Leiting, B., Ruel, R., Nicholson, D.W. and Thornberry, N.A. (1998) Inhibition of human caspases by peptide-based and macromolecular inhibitors. J Biol Chem 273, 32608-13.

Garcia-Sanz, J.A., MacDonald, H.R., Jenne, D.E., Tschopp, J. and Nabholz, M. (1990) Cell specificity of granzyme gene expression. J Immunol 145, 3111-8.

Geysen, D., Bishop, R., Skilton, R., Dolan, T.T. and Morzaria, S. (1999) Molecular epidemiology of Theileria parva in the field. Trop Med Int Health 4, A21-7.

Godal, R., Keilholz, U., Uharek, L., Letsch, A., Asemissen, A.M., Busse, A., Na, I.K., Thiel, E. and Scheibenbogen, C. (2006) Lymphomas are sensitive to perforin-dependent cytotoxic pathways despite expression of PI-9 and overexpression of bcl-2. Blood 107, 3205-11.

Goddeeris, B.M., Morrison, W.I. and Teale, A.J. (1986a) Generation of bovine cytotoxic cell lines, specific for cells infected with the protozoan parasite Theileria parva and restricted by products of the major histocompatibility complex. Eur J Immunol 16, 1243-9.

Goddeeris, B.M., Morrison, W.I., Teale, A.J., Bensaid, A. and Baldwin, C.L. (1986b) Bovine cytotoxic T-cell clones specific for cells infected with the protozoan parasite Theileria parva: parasite strain specificity and class I major histocompatibility complex restriction. Proc Natl Acad Sci U S A 83, 5238-42.

Goddeeris, B.M., Morrison, W.I., Toye, P.G. and Bishop, R. (1990) Strain specificity of bovine Theileria parva-specific cytotoxic T cells is determined by the phenotype of the restricting class I MHC. Immunology 69, 38-44.

Goping, I.S., Barry, M., Liston, P., Sawchuk, T., Constantinescu, G., Michalak, K.M., Shostak, I., Roberts, D.L., Hunter, A.M., Korneluk, R. and Bleackley, R.C. (2003) Granzyme B-induced apoptosis requires both direct caspase activation and relief of caspase inhibition. Immunity 18, 355-65.

Gorak-Stolinska, P., Truman, J.P., Kemeny, D.M. and Noble, A. (2001) Activation-induced cell death of human T-cell subsets is mediated by Fas and granzyme B but is independent of TNF-alpha. J Leukoc Biol 70, 756-66.

Graham, S.P., Honda, Y., Pelle, R., Mwangi, D.M., Glew, E.J., de Villiers, E.P., Shah, T., Bishop, R., van der Bruggen, P., Nene, V. and Taracha, E.L. (2007) A novel strategy for the identification of antigens that are recognised by bovine MHC class I restricted cytotoxic T cells in a protozoan infection using reverse vaccinology. Immunome Res 3, 2.

Graham, S.P., Pelle, R., Honda, Y., Mwangi, D.M., Tonukari, N.J., Yamage, M., Glew, E.J., de Villiers, E.P., Shah, T., Bishop, R., Abuya, E., Awino, E., Gachanja, J., Luyai, A.E., Mbwika, F., Muthiani, A.M., Ndegwa, D.M., Njahira, M., Nyanjui, J.K., Onono, F.O., Osaso, J., Saya, R.M., Wildmann, C., Fraser, C.M., Maudlin, I., Gardner, M.J., Morzaria, S.P., Loosmore, S., Gilbert, S.C., Audonnet, J.C., van der Bruggen, P., Nene, V. and Taracha, E.L. (2006) Theileria parva candidate vaccine antigens recognized by immune bovine cytotoxic T lymphocytes. Proc Natl Acad Sci U S A 103, 3286-91.

Graham, S.P., Pelle, R., Yamage, M., Mwangi, D.M., Honda, Y., Mwakubambanya, R.S., de Villiers, E.P., Abuya, E., Awino, E., Gachanja, J., Mbwika, F., Muthiani, A.M., Muriuki, C., Nyanjui, J.K., Onono, F.O., Osaso, J., Riitho, V., Saya, R.M., Ellis, S.A., McKeever, D.J., MacHugh, N.D., Gilbert, S.C., Audonnet, J.C., Morrison, W.I., van der Bruggen, P. and Taracha, E.L. (2008) Characterization of the fine specificity of bovine CD8 T-cell responses to defined antigens from the protozoan parasite Theileria parva. Infect Immun 76, 685-94.

Griffith, T.S., Brunner, T., Fletcher, S.M., Green, D.R. and Ferguson, T.A. (1995) Fas ligand-induced apoptosis as a mechanism of immune privilege. Science 270, 1189-92.

Griffiths, G.M. (1995) The cell biology of CTL killing. Curr Opin Immunol 7, 343-8.

Griffiths, G.M. and Isaaz, S. (1993) Granzymes A and B are targeted to the lytic granules of lymphocytes by the mannose-6-phosphate receptor. J Cell Biol 120, 885-96.

Grossman, W.J., Revell, P.A., Lu, Z.H., Johnson, H., Bredemeyer, A.J. and Ley, T.J. (2003) The orphan granzymes of humans and mice. Curr Opin Immunol 15, 544-52.

Guergnon, J., Dessauge, F., Langsley, G. and Garcia, A. (2003) Apoptosis of Theileria-infected lymphocytes induced upon parasite death involves activation of caspases 9 and 3. Biochimie 85, 771-6.

Harari, A., Enders, F.B., Cellerai, C., Bart, P.A. and Pantaleo, G. (2009) Distinct profiles of cytotoxic granules in memory CD8 T cells correlate with function, differentiation stage, and antigen exposure. J Virol 83, 2862-71.

Harris, J.L., Peterson, E.P., Hudig, D., Thornberry, N.A. and Craik, C.S. (1998) Definition and redesign of the extended substrate specificity of granzyme B. J Biol Chem 273, 27364-73.

Harty, J.T. and Bevan, M.J. (1995) Specific immunity to Listeria monocytogenes in the absence of IFN gamma. Immunity 3, 109-17.

Hayes, M.P., Berrebi, G.A. and Henkart, P.A. (1989) Induction of target cell DNA release by the cytotoxic T lymphocyte granule protease granzyme A. J Exp Med 170, 933-46.

Heibein, J.A., Barry, M., Motyka, B. and Bleackley, R.C. (1999) Granzyme B-induced loss of mitochondrial inner membrane potential (Delta Psi m) and cytochrome c release are caspase independent. J Immunol 163, 4683-93.

Heibein, J.A., Goping, I.S., Barry, M., Pinkoski, M.J., Shore, G.C., Green, D.R. and Bleackley, R.C. (2000) Granzyme B-mediated cytochrome c release is regulated by the Bcl-2 family members bid and Bax. J Exp Med 192, 1391-402.

Henkart, P.A. and Sitkovsky, M.V. (1994) Cytotoxic lymphocytes. Two ways to kill target cells. Curr Biol 4, 923-5.

Henkart, P.A., Williams, M.S., Zacharchuk, C.M. and Sarin, A. (1997) Do CTL kill target cells by inducing apoptosis? Semin Immunol 9, 135-44.

Heusel, J.W., Wesselschmidt, R.L., Shresta, S., Russell, J.H. and Ley, T.J. (1994) Cytotoxic lymphocytes require granzyme B for the rapid induction of DNA fragmentation and apoptosis in allogeneic target cells. Cell 76, 977-87.

Heussler, V.T., Machado, J., Jr., Fernandez, P.C., Botteron, C., Chen, C.G., Pearse, M.J. and Dobbelaere, D.A. (1999) The intracellular parasite Theileria parva protects infected T cells from apoptosis. Proc Natl Acad Sci U S A 96, 7312-7.

Hill, M.M., Adrain, C., Duriez, P.J., Creagh, E.M. and Martin, S.J. (2004) Analysis of the composition, assembly kinetics and activity of native Apaf-1 apoptosomes. Embo J 23, 2134-45.

Hink-Schauer, C., Estebanez-Perpina, E., Kurschus, F.C., Bode, W. and Jenne, D.E. (2003) Crystal structure of the apoptosis-inducing human granzyme A dimer. Nat Struct Biol 10, 535-40.

Hink-Schauer, C., Estebanez-Perpina, E., Wilharm, E., Fuentes-Prior, P., Klinkert, W., Bode, W. and Jenne, D.E. (2002) The 2.2-A crystal structure of human pro-granzyme K reveals a rigid zymogen with unusual features. J Biol Chem 277, 50923-33.

Hirst, C.E., Buzza, M.S., Sutton, V.R., Trapani, J.A., Loveland, K.L. and Bird, P.I. (2001) 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 7, 1133-42.

Hou, Q., Zhao, T., Zhang, H., Lu, H., Zhang, Q., Sun, L. and Fan, Z. (2008) Granzyme H induces apoptosis of target tumor cells characterized by DNA fragmentation and Bid-dependent mitochondrial damage. Mol Immunol 45, 1044-55.

Houston, E.F., Taracha, E.L., Brackenbury, L., MacHugh, N.D., McKeever, D.J., Charleston, B. and Morrison, W.I. (2008) Infection of cattle with Theileria parva induces an early CD8 T cell response lacking appropriate effector function. Int J Parasitol 38, 1693-704.

Hudig, D., Allison, N.J., Pickett, T.M., Winkler, U., Kam, C.M. and Powers, J.C. (1991) The function of lymphocyte proteases. Inhibition and restoration of granule-mediated lysis with isocoumarin serine protease inhibitors. J Immunol 147, 1360-8.

Hulliger, L., Wilde, K.H., Brown, C.G. and Turner, L. (1964) Mode of Multiplication of Theileria in Cultures of Bovine Lymphocytic Cells. Nature 203, 728-30.

Irvin, A.D. and Mwamachi, D.M. (1983) Clinical and diagnostic features of East Coast fever (Theileria parva) infection of cattle. Vet Rec 113, 192-8.

Irvin, A.D., Ocama, J.G. and Spooner, P.R. (1982) Cycle of bovine lymphoblastoid cells parasitised by Theileria parva. Res Vet Sci 33, 298-304.

Isaaz, S., Baetz, K., Olsen, K., Podack, E. and Griffiths, G.M. (1995) Serial killing by cytotoxic T lymphocytes: T cell receptor triggers degranulation, re-filling of the lytic granules and secretion of lytic proteins via a non-granule pathway. Eur J Immunol 25, 1071-9.

Jenkins, M.R., Tsun, A., Stinchcombe, J.C. and Griffiths, G.M. (2009) The strength of T cell receptor signal controls the polarization of cytotoxic machinery to the immunological synapse. Immunity 31, 621-31.

Jenne, D.E. and Tschopp, J. (1988) Granzymes, a family of serine proteases released from granules of cytolytic T lymphocytes upon T cell receptor stimulation. Immunol Rev 103, 53-71.

Jiang, X., Orr, B.A., Kranz, D.M. and Shapiro, D.J. (2006) Estrogen induction of the granzyme B inhibitor, proteinase inhibitor 9, protects cells against apoptosis mediated by cytotoxic T lymphocytes and natural killer cells. Endocrinology 147, 1419-26.

Jiang, X. and Wang, X. (2000) Cytochrome c promotes caspase-9 activation by inducing nucleotide binding to Apaf-1. J Biol Chem 275, 31199-203.

Johnson, B.J., Costelloe, E.O., Fitzpatrick, D.R., Haanen, J.B., Schumacher, T.N., Brown, L.E. and Kelso, A. (2003a) Single-cell perforin and granzyme expression reveals the anatomical localization of effector CD8+ T cells in influenza virus-infected mice. Proc Natl Acad Sci U S A 100, 2657-62.

Johnson, H., Scorrano, L., Korsmeyer, S.J. and Ley, T.J. (2003b) Cell death induced by granzyme C. Blood 101, 3093-101.

Kagi, D., Ledermann, B., Burki, K., Hengartner, H. and Zinkernagel, R.M. (1994a) CD8+ T cell-mediated protection against an intracellular bacterium by perforin-dependent cytotoxicity. Eur J Immunol 24, 3068-72.

Kagi, D., Ledermann, B., Burki, K., Seiler, P., Odermatt, B., Olsen, K.J., Podack, E.R., Zinkernagel, R.M. and Hengartner, H. (1994b) Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. Nature 369, 31-7.

Kaiserman, D., Bird, C.H., Sun, J., Matthews, A., Ung, K., Whisstock, J.C., Thompson, P.E., Trapani, J.A. and Bird, P.I. (2006) The major human and

mouse granzymes are structurally and functionally divergent. J Cell Biol 175, 619-30.

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

Kariuki, D.P., Young, A.S., Morzaria, S.P., Lesan, A.C., Mining, S.K., Omwoyo, P., Wafula, J.L. and Molyneux, D.H. (1995) Theileria parva carrier state in naturally infected and artificially immunised cattle. Trop Anim Health Prod 27, 15-25.

Kataoka, T., Shinohara, N., Takayama, H., Takaku, K., Kondo, S., Yonehara, S. and Nagai, K. (1996) Concanamycin A, a powerful tool for characterization and estimation of contribution of perforin- and Fas-based lytic pathways in cell-mediated cytotoxicity. J Immunol 156, 3678-86.

Kataoka, T., Takaku, K., Magae, J., Shinohara, N., Takayama, H., Kondo, S. and Nagai, K. (1994) Acidification is essential for maintaining the structure and function of lytic granules of CTL. Effect of concanamycin A, an inhibitor of vacuolar type H(+)-ATPase, on CTL-mediated cytotoxicity. J Immunol 153, 3938-47.

Kaufmann, S.H. and Ladel, C.H. (1994) Role of T cell subsets in immunity against intracellular bacteria: experimental infections of knock-out mice with Listeria monocytogenes and Mycobacterium bovis BCG. Immunobiology 191, 509-19.

Kelly, J.M., O'Connor, M.D., Hulett, M.D., Thia, K.Y. and Smyth, M.J. (1996) Cloning and expression of the recombinant mouse natural killer cell granzyme Met-ase-1. Immunogenetics 44, 340-50.

Kelly, J.M., Waterhouse, N.J., Cretney, E., Browne, K.A., Ellis, S., Trapani, J.A. and Smyth, M.J. (2004) Granzyme M mediates a novel form of perforin-dependent cell death. J Biol Chem 279, 22236-42.

Kelso, A., Costelloe, E.O., Johnson, B.J., Groves, P., Buttigieg, K. and Fitzpatrick, D.R. (2002) The genes for perforin, granzymes A-C and IFN-gamma are differentially expressed in single CD8(+) T cells during primary activation. Int Immunol 14, 605-13.

Kienzle, N., Buttigieg, K., Groves, P., Kawula, T. and Kelso, A. (2002) A clonal culture system demonstrates that IL-4 induces a subpopulation of noncytolytic T cells with low CD8, perforin, and granzyme expression. J Immunol 168, 1672-81.

Kim, W.J., Kim, H., Suk, K. and Lee, W.H. (2007) Macrophages express granzyme B in the lesion areas of atherosclerosis and rheumatoid arthritis. Immunol Lett 111, 57-65.

Kojima, H., Shinohara, N., Hanaoka, S., Someya-Shirota, Y., Takagaki, Y., Ohno, H., Saito, T., Katayama, T., Yagita, H., Okumura, K. and et al. (1994) Two distinct pathways of specific killing revealed by perforin mutant cytotoxic T lymphocytes. Immunity 1, 357-64.

Korsmeyer, S.J., Gross, A., Harada, H., Zha, J., Wang, K., Yin, X.M., Wei, M. and Zinkel, S. (1999) Death and survival signals determine active/inactive conformations of pro-apoptotic BAX, BAD, and BID molecules. Cold Spring Harb Symp Quant Biol 64, 343-50.

Kuenzi, P., Schneider, P. and Dobbelaere, D.A. (2003) Theileria parva-transformed T cells show enhanced resistance to Fas/Fas ligand-induced apoptosis. J Immunol 171, 1224-31.

Kuwana, T., Mackey, M.R., Perkins, G., Ellisman, M.H., Latterich, M., Schneiter, R., Green, D.R. and Newmeyer, D.D. (2002) Bid, Bax, and lipids cooperate to form supramolecular openings in the outer mitochondrial membrane. Cell 111, 331-42.

Ladel, C.H., Flesch, I.E., Arnoldi, J. and Kaufmann, S.H. (1994) Studies with MHC-deficient knock-out mice reveal impact of both MHC I- and MHC II-dependent T cell responses on Listeria monocytogenes infection. J Immunol 153, 3116-22.

Lau, A.H. (1999) Apoptosis induced by cisplatin nephrotoxic injury. Kidney Int 56, 1295-8.

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

Lieberman, J. (2003) The ABCs of granule-mediated cytotoxicity: new weapons in the arsenal. Nat Rev Immunol 3, 361-70.

Lieberman, J. and Fan, Z. (2003) Nuclear war: the granzyme A-bomb. Curr Opin Immunol 15, 553-9.

Ling, M.M. and Robinson, B.H. (1997) Approaches to DNA mutagenesis: an overview. Anal Biochem 254, 157-78.

Lowin, B., Beermann, F., Schmidt, A. and Tschopp, J. (1994) A null mutation in the perforin gene impairs cytolytic T lymphocyte- and natural killer cell-mediated cytotoxicity. Proc Natl Acad Sci U S A 91, 11571-5.

Lowin, B., Mattman, C., Hahne, M. and Tschopp, J. (1996) Comparison of Fas(Apo-1/CD95)- and perforin-mediated cytotoxicity in primary T lymphocytes. Int Immunol 8, 57-63.

MacDonald, G., Shi, L., Vande Velde, C., Lieberman, J. and Greenberg, A.H. (1999) Mitochondria-dependent and -independent regulation of Granzyme B-induced apoptosis. J Exp Med 189, 131-44.

Macdonald, I.K., Harkiolaki, M., Hunt, L., Connelley, T., Carroll, A.V., MacHugh, N.D., Graham, S.P., Jones, E.Y., Morrison, W.I., Flower, D.R. and Ellis, S.A. (2010) MHC class I bound to an immunodominant Theileria parva epitope demonstrates unconventional presentation to T cell receptors. PLoS Pathog 6, e1001149.

MacHugh, N.D., Connelley, T., Graham, S.P., Pelle, R., Formisano, P., Taracha, E.L., Ellis, S.A., McKeever, D.J., Burrells, A. and Morrison, W.I. (2009) CD8+ T-cell responses to Theileria parva are preferentially directed to a single dominant antigen: Implications for parasite strain-specific immunity. Eur J Immunol 39, 2459-69.

Mahrus, S. and Craik, C.S. (2005) Selective chemical functional probes of granzymes A and B reveal granzyme B is a major effector of natural killer cell-mediated lysis of target cells. Chem Biol 12, 567-77.

Mahrus, S., Kisiel, W. and Craik, C.S. (2004) Granzyme M is a regulatory protease that inactivates proteinase inhibitor 9, an endogenous inhibitor of granzyme B. J Biol Chem 279, 54275-82.

Marcotty, T., Brandt, J., Billiouw, M., Chaka, G., Losson, B. and Berkvens, D. (2002) Immunisation against Theileria parva in eastern Zambia: influence of maternal antibodies and demonstration of the carrier status. Vet Parasitol 110, 45-56.

Maritim, A.C., Young, A.S., Lesan, A.C., Ndungu, S.G., Mutugi, J.J. and Stagg, D.A. (1989) Theilerial parasites isolated from carrier cattle after immunization with Theileria parva by the infection and treatment method. Parasitology 99 Pt 1, 139-47.

Martin, D.A., Siegel, R.M., Zheng, L. and Lenardo, M.J. (1998) Membrane oligomerization and cleavage activates the caspase-8 (FLICE/MACHalpha1) death signal. J Biol Chem 273, 4345-9.

Martin, S.J., Amarante-Mendes, G.P., Shi, L., Chuang, T.H., Casiano, C.A., O'Brien, G.A., Fitzgerald, P., Tan, E.M., Bokoch, G.M., Greenberg, A.H. and Green, D.R. (1996) 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 15, 2407-16.

Martinvalet, D., Zhu, P. and Lieberman, J. (2005) Granzyme A induces caspase-independent mitochondrial damage, a required first step for apoptosis. Immunity 22, 355-70.

Masson, D., Peters, P.J., Geuze, H.J., Borst, J. and Tschopp, J. (1990) Interaction of chondroitin sulfate with perforin and granzymes of cytolytic T-cells is dependent on pH. Biochemistry 29, 11229-35.

Masson, D. and Tschopp, J. (1987) A family of serine esterases in lytic granules of cytolytic T lymphocytes. Cell 49, 679-85.

McAleese, S.M., Pemberton, A.D., McGrath, M.E., Huntley, J.F. and Miller, H.R. (1998) Sheep mast-cell proteinases-1 and -3: cDNA cloning, primary structure and molecular modelling of the enzymes and further studies on substrate specificity. Biochem J 333 (Pt 3), 801-9.

McGuire, M.J., Lipsky, P.E. and Thiele, D.L. (1993) Generation of active myeloid and lymphoid granule serine proteases requires processing by the granule thiol protease dipeptidyl peptidase I. J Biol Chem 268, 2458-67.

McKeever, D.J., Nyanjui, J.K. and Ballingall, K.T. (1997) In vitro infection with Theileria parva is associated with IL10 expression in all bovine lymphocyte lineages. Parasite Immunol 19, 319-24.

McKeever, D.J., Taracha, E.L., Innes, E.L., MacHugh, N.D., Awino, E., Goddeeris, B.M. and Morrison, W.I. (1994) Adoptive transfer of immunity to Theileria parva in the CD8+ fraction of responding efferent lymph. Proc Natl Acad Sci U S A 91, 1959-63.

Medema, J.P., de Jong, J., Peltenburg, L.T., Verdegaal, E.M., Gorter, A., Bres, S.A., Franken, K.L., Hahne, M., Albar, J.P., Melief, C.J. and Offringa, R. (2001) Blockade of the granzyme B/perforin pathway through overexpression of the serine protease inhibitor PI-9/SPI-6 constitutes a mechanism for immune escape by tumors. Proc Natl Acad Sci U S A 98, 11515-20.

Medley, G.F., Perry, B.D. and Young, A.S. (1993) Preliminary analysis of the transmission dynamics of Theileria parva in eastern Africa. Parasitology 106 (Pt 3), 251-64.

Mehlhorn, H. and Shein, E. (1984) The piroplasms: life cycle and sexual stages. Adv Parasitol 23, 37-103.

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

Metkar, S.S., Wang, B., Aguilar-Santelises, M., Raja, S.M., Uhlin-Hansen, L., Podack, E., Trapani, J.A. and Froelich, C.J. (2002) Cytotoxic cell granule-mediated apoptosis: perforin delivers granzyme B-serglycin complexes into target cells without plasma membrane pore formation. Immunity 16, 417-28.

Mielke, M.E., Niedobitek, G., Stein, H. and Hahn, H. (1989) Acquired resistance to Listeria monocytogenes is mediated by Lyt-2+ T cells independently of the influx of monocytes into granulomatous lesions. J Exp Med 170, 589-94.

Montel, A.H., Bochan, M.R., Hobbs, J.A., Lynch, D.H. and Brahmi, Z. (1995) Fas involvement in cytotoxicity mediated by human NK cells. Cell Immunol 166, 236-46.

Morrison, W.I., Buscher, G., Murray, M., Emery, D.L., Masake, R.A., Cook, R.H. and Wells, P.W. (1981) Theileria parva: kinetics of infection in the lymphoid system of cattle. Exp Parasitol 52, 248-60.

Morrison, W.I., Goddeeris, B.M., Teale, A.J., Groocock, C.M., Kemp, S.J. and Stagg, D.A. (1987) Cytotoxic T-cells elicited in cattle challenged with Theileria parva (Muguga): evidence for restriction by class I MHC determinants and parasite strain specificity. Parasite Immunol 9, 563-78.

Morzaria, S.P., Irvin, A.D., Taracha, E., Spooner, P.R., Voigt, W.P., Fujinaga, T. and Katende, J. (1987) Immunization against East Coast fever: the use of selected stocks of Theileria parva for immunization of cattle exposed to field challenge. Vet Parasitol 23, 23-41.

Motyka, B., Korbutt, G., Pinkoski, M.J., Heibein, J.A., Caputo, A., Hobman, M., Barry, M., Shostak, I., Sawchuk, T., Holmes, C.F., Gauldie, J. and Bleackley, R.C. (2000) Mannose 6-phosphate/insulin-like growth factor II receptor is a death receptor for granzyme B during cytotoxic T cell-induced apoptosis. Cell 103, 491-500.

Muhammed, S.I., Lauerman, L.H., Jr. and Johnson, L.W. (1975) Effect of humoral antibodies on the course of Theileria parva infection (East Coast fever) of cattle. Am J Vet Res 36, 399-402.

Mukhebi, A.W., Perry, B.D. and Kruska, R. (1992) Estimated economics of theileriosis control in Africa. Preventive Veterinary Medicine 12, 73-85.

Mullbacher, A., Ebnet, K., Blanden, R.V., Hla, R.T., Stehle, T., Museteanu, C. and Simon, M.M. (1996) Granzyme A is critical for recovery of mice from infection with the natural cytopathic viral pathogen, ectromelia. Proc Natl Acad Sci U S A 93, 5783-7.

Mullbacher, A., Waring, P., Tha Hla, R., Tran, T., Chin, S., Stehle, T., Museteanu, C. and Simon, M.M. (1999) Granzymes are the essential downstream effector molecules for the control of primary virus infections by cytolytic leukocytes. Proc Natl Acad Sci U S A 96, 13950-5.

Muller-Eberhard, H.J. (1986) The membrane attack complex of complement. Annu Rev Immunol 4, 503-28.

Murphy, M.E., Moult, J., Bleackley, R.C., Gershenfeld, H., Weissman, I.L. and James, M.N. (1988) Comparative molecular model building of two serine proteinases from cytotoxic T lymphocytes. Proteins 4, 190-204.

Musoke, A.J., Nantulya, V.M., Buscher, G., Masake, R.A. and Otim, B. (1982) Bovine immune response to Theileria parva: neutralizing antibodies to sporozoites. Immunology 45, 663-8.

Musoke, A.J., Nantulya, V.M., Rurangirwa, F.R. and Buscher, G. (1984) Evidence for a common protective antigenic determinant on sporozoites of several Theileria parva strains. Immunology 52, 231-8.

Musoke, A., Rowlands, J., Nene, V., Nyanjui, J., Katende, J., Spooner, P., Mwaura, S., Odongo, D., Nkonge, C., Mbogo, S., Bishop, R. and Morzaria, S. (2005) Subunit vaccine based on the p67 major surface protein of Theileria parva sporozoites reduces severity of infection derived from field tick challenge. Vaccine 23, 3084-95.

Nagata, S. and Golstein, P. (1995) The Fas death factor. Science 267, 1449-56.

Nakajima, H., Park, H.L. and Henkart, P.A. (1995) Synergistic roles of granzymes A and B in mediating target cell death by rat basophilic leukemia mast cell tumors also expressing cytolysin/perforin. J Exp Med 181, 1037-46.

Nickell, S.P. and Sharma, D. (2000) Trypanosoma cruzi: roles for perforin-dependent and perforin-independent immune mechanisms in acute resistance. Exp Parasitol 94, 207-16.

Odake, S., Kam, C.M., Narasimhan, L., Poe, M., Blake, J.T., Krahenbuhl, O., Tschopp, J. and Powers, J.C. (1991) Human and murine cytotoxic T lymphocyte serine proteases: subsite mapping with peptide thioester substrates and inhibition of enzyme activity and cytolysis by isocoumarins. Biochemistry 30, 2217-27.

Pao, L.I., Sumaria, N., Kelly, J.M., van Dommelen, S., Cretney, E., Wallace, M.E., Anthony, D.A., Uldrich, A.P., Godfrey, D.I., Papadimitriou, J.M., Mullbacher, A., Degli-Esposti, M.A. and Smyth, M.J. (2005) Functional analysis of granzyme M and its role in immunity to infection. J Immunol 175, 3235-43.

Pardo, J., Aguilo, J.I., Anel, A., Martin, P., Joeckel, L., Borner, C., Wallich, R., Mullbacher, A., Froelich, C.J. and Simon, M.M. (2009) The biology of cytotoxic cell granule exocytosis pathway: granzymes have evolved to induce cell death and inflammation. Microbes Infect 11, 452-9.

Pardo, J., Bosque, A., Brehm, R., Wallich, R., Naval, J., Mullbacher, A., Anel, A. and Simon, M.M. (2004) Apoptotic pathways are selectively activated by granzyme A and/or granzyme B in CTL-mediated target cell lysis. J Cell Biol 167, 457-68.

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

Pearson, T.W., Lundin, L.B., Dolan, T.T. and Stagg, D.A. (1979) Cell-mediated immunity to Theileria-transformed cell lines. Nature 281, 678-80.

Perona, J.J. and Craik, C.S. (1995) Structural basis of substrate specificity in the serine proteases. Protein Sci 4, 337-60.

Peters, P.J., Borst, J., Oorschot, V., Fukuda, M., Krahenbuhl, O., Tschopp, J., Slot, J.W. and Geuze, H.J. (1991) Cytotoxic T lymphocyte granules are secretory lysosomes, containing both perforin and granzymes. J Exp Med 173, 1099-109.

Pham, C.T. and Ley, T.J. (1999) Dipeptidyl peptidase I is required for the processing and activation of granzymes A and B in vivo. Proc Natl Acad Sci U S A 96, 8627-32.

Pham, C.T., MacIvor, D.M., Hug, B.A., Heusel, J.W. and Ley, T.J. (1996) Long-range disruption of gene expression by a selectable marker cassette. Proc Natl Acad Sci U S A 93, 13090-5.

Picard, V., Ersdal-Badju, E., Lu, A. and Bock, S.C. (1994) A rapid and efficient one-tube PCR-based mutagenesis technique using Pfu DNA polymerase. Nucleic Acids Res 22, 2587-91.

Pilat, D., Fink, T., Obermaier-Skrobanek, B., Zimmer, M., Wekerle, H., Lichter, P. and Jenne, D.E. (1994) The human Met-ase gene (GZMM): structure, sequence, and close physical linkage to the serine protease gene cluster on 19p13.3. Genomics 24, 445-50.

Pinkoski, M.J., Waterhouse, N.J., Heibein, J.A., Wolf, B.B., Kuwana, T., Goldstein, J.C., Newmeyer, D.D., Bleackley, R.C. and Green, D.R. (2001) Granzyme B-mediated apoptosis proceeds predominantly through a Bcl-2-inhibitable mitochondrial pathway. J Biol Chem 276, 12060-7.

Piuko, K., Bravo, I.G. and Muller, M. (2007) Identification and characterization of equine granzyme B. Vet Immunol Immunopathol 118, 239-51.

Podack, E.R. and Hengartner, H. (1989) Structure of perforin and its role in cytolysis. Year Immunol 6, 245-61.

Poe, M., Blake, J.T., Boulton, D.A., Gammon, M., Sigal, N.H., Wu, J.K. and Zweerink, H.J. (1991) Human cytotoxic lymphocyte granzyme B. Its purification from granules and the characterization of substrate and inhibitor specificity. J Biol Chem 266, 98-103.

Quan, L.T., Tewari, M., O'Rourke, K., Dixit, V., Snipas, S.J., Poirier, G.G., Ray, C., Pickup, D.J. and Salvesen, G.S. (1996) Proteolytic activation of the cell death protease Yama/CPP32 by granzyme B. Proc Natl Acad Sci U S A 93, 1972-6.

Radley, D.E., Brown, C.G.D., Burridge, M.J., Cunningham, M.P., Kirimi, I.M., Purnell, R.E. and Young, A.S. (1975a) East coast fever: 1. Chemoprophylactic immunization of cattle against Theileria parva (Muguga) and five theilerial strains. Veterinary Parasitology 1, 35-41.

Radley, D.E., Brown, C.G.D., Cunningham, M.P., Kimber, C.D., Musisi, F.L., Payne, R.C., Purnell, R.E., Stagg, S.M. and Young, A.S. (1975b) East coast fever: 3. Chemoprophylactic immunization of cattle using oxytetracycline and a combination of theilerial strains. Veterinary Parasitology 1, 51-60.

Radley, D.E., Young, A.S., Brown, C.G.D., Burridge, M.J., Cunningham, M.P., Musisi, F.L. and Purnell, R.E. (1975c) East coast fever: 2. Cross-immunity trials with a Kenya strain of Theileria lawrencei. Veterinary Parasitology 1, 43-50.

Redelman, D. and Hudig, D. (1980) The mechanism of cell-mediated cytotoxicity. I. Killing by murine cytotoxic T lymphocytes requires cell surface thiols and activated proteases. J Immunol 124, 870-8.

Riera, L., Gariglio, M., Valente, G., Mullbacher, A., Museteanu, C., Landolfo, S. and Simon, M.M. (2000) Murine cytomegalovirus replication in salivary glands is controlled by both perforin and granzymes during acute infection. Eur J Immunol 30, 1350-5.

Roberts, A.D., Ordway, D.J. and Orme, I.M. (1993) Listeria monocytogenes infection in beta 2 microglobulin-deficient mice. Infect Immun 61, 1113-6.

Rotonda, J., Garcia-Calvo, M., Bull, H.G., Geissler, W.M., McKeever, B.M., Willoughby, C.A., Thornberry, N.A. and Becker, J.W. (2001) The three-dimensional structure of human granzyme B compared to caspase-3, key mediators of cell death with cleavage specificity for aspartic acid in P1. Chem Biol 8, 357-68.

Rouvier, E., Luciani, M.F. and Golstein, P. (1993) Fas involvement in Ca(2+)-independent T cell-mediated cytotoxicity. J Exp Med 177, 195-200.

Ruhlmann, A., Kukla, D., Schwager, P., Bartels, K. and Huber, R. (1973) Structure of the complex formed by bovine trypsin and bovine pancreatic trypsin inhibitor. Crystal structure determination and stereochemistry of the contact region. J Mol Biol 77, 417-36.

Saito, S., Murakoshi, K., Kotake, S., Kamatani, N. and Tomatsu, T. (2008) Granzyme B induces apoptosis of chondrocytes with natural killer cell-like cytotoxicity in rheumatoid arthritis. J Rheumatol 35, 1932-43.

Salvesen, G.S. and Duckett, C.S. (2002) IAP proteins: blocking the road to death's door. Nat Rev Mol Cell Biol 3, 401-10.

Samsom, J.N., Langermans, J.A., Savelkoul, H.F. and van Furth, R. (1995) Tumour necrosis factor, but not interferon-gamma, is essential for acquired resistance to Listeria monocytogenes during a secondary infection in mice. Immunology 86, 256-62.

Sarin, A., Williams, M.S., Alexander-Miller, M.A., Berzofsky, J.A., Zacharchuk, C.M. and Henkart, P.A. (1997) Target cell lysis by CTL granule exocytosis is independent of ICE/Ced-3 family proteases. Immunity 6, 209-15.

Sarin, A., Wu, M.L. and Henkart, P.A. (1996) Different interleukin-1 beta converting enzyme (ICE) family protease requirements for the apoptotic death of T lymphocytes triggered by diverse stimuli. J Exp Med 184, 2445-50.

Sawada, M., Hayes, P. and Matsuyama, S. (2003) Cytoprotective membrane-permeable peptides designed from the Bax-binding domain of Ku70. Nat Cell Biol 5, 352-7.

Sayers, T.J., Lloyd, A.R., McVicar, D.W., O'Connor, M.D., Kelly, J.M., Carter, C.R., Wiltrout, T.A., Wiltrout, R.H. and Smyth, M.J. (1996) Cloning and expression of a second human natural killer cell granule tryptase, HNK-Tryp-2/granzyme 3. J Leukoc Biol 59, 763-8.

Schechter, I. and Berger, A. (1967) On the size of the active site in proteases. I. Papain. Biochem Biophys Res Commun 27, 157-62.

Schein, E., Warnecke, M. and Kirmse, P. (1977) Development of Theileria parva (Theiler, 1904) in the gut of Rhipicephalus appendiculatus (Neumann, 1901). Parasitology 75, 309-16.

Sedelies, K.A., Sayers, T.J., Edwards, K.M., Chen, W., Pellicci, D.G., Godfrey, D.I. and Trapani, J.A. (2004) Discordant regulation of granzyme H and granzyme B expression in human lymphocytes. J Biol Chem 279, 26581-7.

Sharif-Askari, E., Alam, A., Rheaume, E., Beresford, P.J., Scotto, C., Sharma, K., Lee, D., DeWolf, W.E., Nuttall, M.E., Lieberman, J. and Sekaly, R.P. (2001) Direct cleavage of the human DNA fragmentation factor-45 by granzyme B induces caspase-activated DNase release and DNA fragmentation. Embo J 20, 3101-13.

Shatry, A.M., Wilson, A.J., Varma, S. and Dolan, T.T. (1981) Sequential study of lymph node and splenic aspirates during Theileria parva infection in calves. Res Vet Sci 30, 181-4.

Shaw, M.K. (2003) Cell invasion by Theileria sporozoites. Trends Parasitol 19, 2-6.

Shi, L., Kam, C.M., Powers, J.C., Aebersold, R. and Greenberg, A.H. (1992a) Purification of three cytotoxic lymphocyte granule serine proteases that

induce apoptosis through distinct substrate and target cell interactions. J Exp Med 176, 1521-9.

Shi, L., Kraut, R.P., Aebersold, R. and Greenberg, A.H. (1992b) A natural killer cell granule protein that induces DNA fragmentation and apoptosis. J Exp Med 175, 553-66.

Shresta, S., MacIvor, D.M., Heusel, J.W., Russell, J.H. and Ley, T.J. (1995) Natural killer and lymphokine-activated killer cells require granzyme B for the rapid induction of apoptosis in susceptible target cells. Proc Natl Acad Sci U S A 92, 5679-83.

Simon, M.M., Hausmann, M., Tran, T., Ebnet, K., Tschopp, J., ThaHla, R. and Mullbacher, A. (1997) 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 186, 1781-6.

Simon, M.M., Kramer, M.D., Prester, M. and Gay, S. (1991) Mouse T-cell associated serine proteinase 1 degrades collagen type IV: a structural basis for the migration of lymphocytes through vascular basement membranes. Immunology 73, 117-9.

Simon, M.M., Prester, M., Nerz, G., Kramer, M.D. and Fruth, U. (1988) Release of biologically active fragments from human plasma-fibronectin by murine T cell-specific proteinase 1 (TSP-1). Biol Chem Hoppe Seyler 369 Suppl, 107-12.

Skilton, R.A., Bishop, R.P., Katende, J.M., Mwaura, S. and Morzaria, S.P. (2002) The persistence of Theileria parva infection in cattle immunized using two stocks which differ in their ability to induce a carrier state: analysis using a novel blood spot PCR assay. Parasitology 124, 265-76.

Slee, E.A., Adrain, C. and Martin, S.J. (1999) Serial killers: ordering caspase activation events in apoptosis. Cell Death Differ 6, 1067-74.

Smyth, M.J., Kelly, J.M., Sutton, V.R., Davis, J.E., Browne, K.A., Sayers, T.J. and Trapani, J.A. (2001) Unlocking the secrets of cytotoxic granule proteins. J Leukoc Biol 70, 18-29.

Smyth, M.J., McGuire, M.J. and Thia, K.Y. (1995a) Expression of recombinant human granzyme B. A processing and activation role for dipeptidyl peptidase I. J Immunol 154, 6299-305.

Smyth, M.J., O'Connor, M.D., Kelly, J.M., Ganesvaran, P., Thia, K.Y. and Trapani, J.A. (1995b) Expression of recombinant human Met-ase-1: a NK cell-specific granzyme. Biochem Biophys Res Commun 217, 675-83.

Smyth, M.J., O'Connor, M.D. and Trapani, J.A. (1996) Granzymes: a variety of serine protease specificities encoded by genetically distinct subfamilies. J Leukoc Biol 60, 555-62.

Stagg, D.A., Dolan, T.T., Leitch, B.L. and Young, A.S. (1981) The initial stages of infection of cattle cells with Theileria parva sporozoites in vitro. Parasitology 83, 191-7.

Stalder, T., Hahn, S. and Erb, P. (1994) Fas antigen is the major target molecule for CD4+ T cell-mediated cytotoxicity. J Immunol 152, 1127-33.

Strik, M.C., de Koning, P.J., Kleijmeer, M.J., Bladergroen, B.A., Wolbink, A.M., Griffith, J.M., Wouters, D., Fukuoka, Y., Schwartz, L.B., Hack, C.E., van Ham, S.M. and Kummer, J.A. (2007) Human mast cells produce and release the cytotoxic lymphocyte associated protease granzyme B upon activation. Mol Immunol 44, 3462-72.

Suda, T., Okazaki, T., Naito, Y., Yokota, T., Arai, N., Ozaki, S., Nakao, K. and Nagata, S. (1995) Expression of the Fas ligand in cells of T cell lineage. J Immunol 154, 3806-13.

Suda, T., Takahashi, T., Golstein, P. and Nagata, S. (1993) Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. Cell 75, 1169-78.

Suidan, H.S., Bouvier, J., Schaerer, E., Stone, S.R., Monard, D. and Tschopp, J. (1994) Granzyme A released upon stimulation of cytotoxic T lymphocytes activates the thrombin receptor on neuronal cells and astrocytes. Proc Natl Acad Sci U S A 91, 8112-6.

Sun, J., Bird, C.H., Sutton, V., McDonald, L., Coughlin, P.B., De Jong, T.A., Trapani, J.A. and Bird, P.I. (1996) A cytosolic granzyme B inhibitor related to the viral apoptotic regulator cytokine response modifier A is present in cytotoxic lymphocytes. J Biol Chem 271, 27802-9.

Sun, J., Whisstock, J.C., Harriott, P., Walker, B., Novak, A., Thompson, P.E., Smith, A.I. and Bird, P.I. (2001) Importance of the P4' residue in human granzyme B inhibitors and substrates revealed by scanning mutagenesis of the proteinase inhibitor 9 reactive center loop. J Biol Chem 276, 15177-84.

Sutton, V.R., Davis, J.E., Cancilla, M., Johnstone, R.W., Ruefli, A.A., Sedelies, K., Browne, K.A. and Trapani, J.A. (2000) Initiation of apoptosis by granzyme B requires direct cleavage of bid, but not direct granzyme B-mediated caspase activation. J Exp Med 192, 1403-14.

Sutton, V.R., Wowk, M.E., Cancilla, M. and Trapani, J.A. (2003) Caspase activation by granzyme B is indirect, and caspase autoprocessing requires the release of proapoptotic mitochondrial factors. Immunity 18, 319-29.

Sweeney, E.A., Inokuchi, J. and Igarashi, Y. (1998) Inhibition of sphingolipid induced apoptosis by caspase inhibitors indicates that sphingosine acts in an earlier part of the apoptotic pathway than ceramide. FEBS Lett 425, 61-5.

Takahashi, T., Tanaka, M., Brannan, C.I., Jenkins, N.A., Copeland, N.G., Suda, T. and Nagata, S. (1994) Generalized lymphoproliferative disease in mice, caused by a point mutation in the Fas ligand. Cell 76, 969-76.

Takata, H. and Takiguchi, M. (2006) Three memory subsets of human CD8+ T cells differently expressing three cytolytic effector molecules. J Immunol 177, 4330-40.

Tamura, K., Dudley, J., Nei, M. and Kumar, S. (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 24, 1596-9.

Taracha, E.L., Awino, E. and McKeever, D.J. (1997) Distinct CD4+ T cell helper requirements in Theileria parva-immune and -naive bovine CTL precursors. J Immunol 159, 4539-45.

Taracha, E.L., Goddeeris, B.M., Morzaria, S.P. and Morrison, W.I. (1995a) Parasite strain specificity of precursor cytotoxic T cells in individual animals correlates with cross-protection in cattle challenged with Theileria parva. Infect Immun 63, 1258-62.

Taracha, E.L., Goddeeris, B.M., Teale, A.J., Kemp, S.J. and Morrison, W.I. (1995b) Parasite strain specificity of bovine cytotoxic T cell responses to Theileria parva is determined primarily by immunodominance. J Immunol 155, 4854-60.

ten Berge, R.L., Meijer, C.J., Dukers, D.F., Kummer, J.A., Bladergroen, B.A., Vos, W., Hack, C.E., Ossenkoppele, G.J. and Oudejans, J.J. (2002) Expression levels of apoptosis-related proteins predict clinical outcome in anaplastic large cell lymphoma. Blood 99, 4540-6.

Thomas, D.A., Du, C., Xu, M., Wang, X. and Ley, T.J. (2000) DFF45/ICAD can be directly processed by granzyme B during the induction of apoptosis. Immunity 12, 621-32.

Thomas, D.A., Scorrano, L., Putcha, G.V., Korsmeyer, S.J. and Ley, T.J. (2001) Granzyme B can cause mitochondrial depolarization and cell death in the absence of BID, BAX, and BAK. Proc Natl Acad Sci U S A 98, 14985-90.

Thornberry, N.A., Rano, T.A., Peterson, E.P., Rasper, D.M., Timkey, T., Garcia-Calvo, M., Houtzager, V.M., Nordstrom, P.A., Roy, S., Vaillancourt, J.P., Chapman, K.T. and Nicholson, D.W. (1997) A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis. J Biol Chem 272, 17907-11.

Toye, P.G., Goddeeris, B.M., Iams, K., Musoke, A.J. and Morrison, W.I. (1991) Characterization of a polymorphic immunodominant molecule in sporozoites and schizonts of Theileria parva. Parasite Immunol 13, 49-62.

Trapani, J.A. (2001) Granzymes: a family of lymphocyte granule serine proteases. Genome Biol 2, REVIEWS3014.

Trapani, J.A. and Bird, P.I. (2008) A renaissance in understanding the multiple and diverse functions of granzymes? Immunity 29, 665-7.

Trapani, J.A., Browne, K.A., Dawson, M. and Smyth, M.J. (1993) Immunopurification of functional Asp-ase (natural killer cell granzyme B) using a monoclonal antibody. Biochem Biophys Res Commun 195, 910-20.

Trapani, J.A., Jans, D.A., Jans, P.J., Smyth, M.J., Browne, K.A. and Sutton, V.R. (1998) Efficient nuclear targeting of granzyme B and the nuclear consequences of apoptosis induced by granzyme B and perforin are caspase-dependent, but cell death is caspase-independent. J Biol Chem 273, 27934-8.

Tschopp, C.M., Spiegl, N., Didichenko, S., Lutmann, W., Julius, P., Virchow, J.C., Hack, C.E. and Dahinden, C.A. (2006) Granzyme B, a novel mediator of allergic inflammation: its induction and release in blood basophils and human asthma. Blood 108, 2290-9.

Tyagi, R., Lai, R. and Duggleby, R.G. (2004) A new approach to 'megaprimer' polymerase chain reaction mutagenesis without an intermediate gel purification step. BMC Biotechnol 4, 2.

Uellner, R., Zvelebil, M.J., Hopkins, J., Jones, J., MacDougall, L.K., Morgan, B.P., Podack, E., Waterfield, M.D. and Griffiths, G.M. (1997) Perforin is activated by a proteolytic cleavage during biosynthesis which reveals a phospholipid-binding C2 domain. Embo J 16, 7287-96.

Uilenberg, G., Schreuder, B.E., Silayo, R.S. and Mpangala, C. (1976) Studies on Theileriidae (Sporozoa) in Tanzania. IV. A field trial on immunization against East Coast fever (Theileria parva infection of cattle). Tropenmed Parasitol 27, 329-36.

van Houdt, I.S., Oudejans, J.J., van den Eertwegh, A.J., Baars, A., Vos, W., Bladergroen, B.A., Rimoldi, D., Muris, J.J., Hooijberg, E., Gundy, C.M., Meijer, C.J. and Kummer, J.A. (2005) Expression of the apoptosis inhibitor protease inhibitor 9 predicts clinical outcome in vaccinated patients with stage III and IV melanoma. Clin Cancer Res 11, 6400-7.

Vassalli, P. (1992) The pathophysiology of tumor necrosis factors. Annu Rev Immunol 10, 411-52.

Vettel, U., Brunner, G., Bar-Shavit, R., Vlodavsky, I. and Kramer, M.D. (1993) Charge-dependent binding of granzyme A (MTSP-1) to basement membranes. Eur J Immunol 23, 279-82.

Vignaux, F., Vivier, E., Malissen, B., Depraetere, V., Nagata, S. and Golstein, P. (1995) TCR/CD3 coupling to Fas-based cytotoxicity. J Exp Med 181, 781-6.

von Heijne, G. (1986) A new method for predicting signal sequence cleavage sites. Nucleic Acids Res 14, 4683-90.

Wagner, G.G., Duffus, W.P. and Burridge, M.J. (1974) The specific immunoglobulin response in cattle immunized with isolated Theileria parva antigens. Parasitology 69, 43-53.

Walsh, C.M., Matloubian, M., Liu, C.C., Ueda, R., Kurahara, C.G., Christensen, J.L., Huang, M.T., Young, J.D., Ahmed, R. and Clark, W.R. (1994) Immune function in mice lacking the perforin gene. Proc Natl Acad Sci U S A 91, 10854-8.

Watanabe-Fukunaga, R., Brannan, C.I., Copeland, N.G., Jenkins, N.A. and Nagata, S. (1992a) Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. Nature 356, 314-7.

Watanabe-Fukunaga, R., Brannan, C.I., Itoh, N., Yonehara, S., Copeland, N.G., Jenkins, N.A. and Nagata, S. (1992b) The cDNA structure, expression, and chromosomal assignment of the mouse Fas antigen. J Immunol 148, 1274-9.

Waterhouse, N.J., Sutton, V.R., Sedelies, K.A., Ciccone, A., Jenkins, M., Turner, S.J., Bird, P.I. and Trapani, J.A. (2006) Cytotoxic T lymphocyte-induced killing in the absence of granzymes A and B is unique and distinct from both apoptosis and perforin-dependent lysis. J Cell Biol 173, 133-44.

Waterhouse, N.J. and Trapani, J.A. (2007) H is for helper: granzyme H helps granzyme B kill adenovirus-infected cells. Trends Immunol 28, 373-5.

Waugh, S.M., Harris, J.L., Fletterick, R. and Craik, C.S. (2000) The structure of the pro-apoptotic protease granzyme B reveals the molecular determinants of its specificity. Nat Struct Biol 7, 762-5.

White, D.W., Badovinac, V.P., Kollias, G. and Harty, J.T. (2000) Cutting edge: antilisterial activity of CD8+ T cells derived from TNF-deficient and TNF/perforin double-deficient mice. J Immunol 165, 5-9.

White, D.W. and Harty, J.T. (1998) Perforin-deficient CD8+ T cells provide immunity to Listeria monocytogenes by a mechanism that is independent of CD95 and IFN-gamma but requires TNF-alpha. J Immunol 160, 898-905.

Williams, N.S. and Engelhard, V.H. (1997) Perforin-dependent cytotoxic activity and lymphokine secretion by CD4+ T cells are regulated by CD8+ T cells. J Immunol 159, 2091-9.

Wolint, P., Betts, M.R., Koup, R.A. and Oxenius, A. (2004) Immediate cytotoxicity but not degranulation distinguishes effector and memory subsets of CD8+ T cells. J Exp Med 199, 925-36.

Wong, P. and Pamer, E.G. (2003) CD8 T cell responses to infectious pathogens. Annu Rev Immunol 21, 29-70.

Wu, L., Wang, L., Hua, G., Liu, K., Yang, X., Zhai, Y., Bartlam, M., Sun, F. and Fan, Z. (2009) Structural basis for proteolytic specificity of the human apoptosis-inducing granzyme M. J Immunol 183, 421-9.

Xia, Z., Kam, C.M., Huang, C., Powers, J.C., Mandle, R.J., Stevens, R.L. and Lieberman, J. (1998) Expression and purification of enzymatically active recombinant granzyme B in a baculovirus system. Biochem Biophys Res Commun 243, 384-9.

Yasukawa, M., Ohminami, H., Arai, J., Kasahara, Y., Ishida, Y. and Fujita, S. (2000) Granule exocytosis, and not the fas/fas ligand system, is the main pathway of cytotoxicity mediated by alloantigen-specific CD4(+) as well as CD8(+) cytotoxic T lymphocytes in humans. Blood 95, 2352-5.

Ye, W., Young, J.D. and Liu, C.C. (1996) Interleukin-15 induces the expression of mRNAs of cytolytic mediators and augments cytotoxic activities in primary murine lymphocytes. Cell Immunol 174, 54-62.

Yewdell, J.W. (2006) Confronting complexity: real-world immunodominance in antiviral CD8+ T cell responses. Immunity 25, 533-43.

Young, J.D., Nathan, C.F., Podack, E.R., Palladino, M.A. and Cohn, Z.A. (1986) Functional channel formation associated with cytotoxic T-cell granules. Proc Natl Acad Sci U S A 83, 150-4.

Zamolodchikova, T.S., Sokolova, E.A., Alexandrov, S.L., Mikhaleva, II, Prudchenko, I.A., Morozov, I.A., Kononenko, N.V., Mirgorodskaya, O.A., Da, U., Larionova, N.I., Pozdnev, V.F., Ghosh, D., Duax, W.L. and Vorotyntseva, T.I. (1997) Subcellular localization, substrate specificity and crystallization of duodenase, a potential activator of enteropeptidase. Eur J Biochem 249, 612-21.

Zamolodchikova, T.S., Sokolova, E.A. and Smirnova, E.V. (2003) Graspases--a special group of serine proteases of the chymotrypsin family that has lost a conserved active site disulfide bond. Biochemistry (Mosc) 68, 309-16.

Zamolodchikova, T.S., Vorotyntseva, T.I. and Antonov, V.K. (1995a) Duodenase, a new serine protease of unusual specificity from bovine duodenal mucosa. Purification and properties. Eur J Biochem 227, 866-72.

Zamolodchikova, T.S., Vorotyntseva, T.I., Nazimov, I.V. and Grishina, G.A. (1995b) Duodenase, a new serine protease of unusual specificity from bovine duodenal mucosa. Primary structure of the enzyme. Eur J Biochem 227, 873-9.

Zeng, R., Spolski, R., Finkelstein, S.E., Oh, S., Kovanen, P.E., Hinrichs, C.S., Pise-Masison, C.A., Radonovich, M.F., Brady, J.N., Restifo, N.P., Berzofsky, J.A. and Leonard, W.J. (2005) Synergy of IL-21 and IL-15 in regulating CD8+ T cell expansion and function. J Exp Med 201, 139-48.

Zhang, D., Beresford, P.J., Greenberg, A.H. and Lieberman, J. (2001a) Granzymes A and B directly cleave lamins and disrupt the nuclear lamina during granule-mediated cytolysis. Proc Natl Acad Sci U S A 98, 5746-51.

Zhang, D., Pasternack, M.S., Beresford, P.J., Wagner, L., Greenberg, A.H. and Lieberman, J. (2001b) Induction of rapid histone degradation by the cytotoxic T lymphocyte protease Granzyme A. J Biol Chem 276, 3683-90.

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

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

Zhou, M.Y. and Gomez-Sanchez, C.E. (2000) Universal TA cloning. Curr Issues Mol Biol 2, 1-7.

Zimmer, M., Medcalf, R.L., Fink, T.M., Mattmann, C., Lichter, P. and Jenne, D.E. (1992) Three human elastase-like genes coordinately expressed in the myelomonocyte lineage are organized as a single genetic locus on 19pter. Proc Natl Acad Sci U S A 89, 8215-9.