IMMUNOLOGICAL ASSAYS RELEVANT TO DEFINITION OF BOVINE *THEILERIA PARVA*-SPECIFIC CYTOTOXIC $CD8^+$ T CELL RESPONSES

A thesis submitted for the degree of Doctor of Philosophy

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August 2012

ABSTRACT

A major objective in *Theileria parva* subunit vaccine development is to induce a vaccine antigen specific response mediated by cytotoxic $CD8^+$ T cells (CTL). Therefore it is essential to be able to measure the frequency of the responding $CD8^+$ T cells after vaccination and correlate it with a clinical outcome on challenge. Recently concluded immunogenicity and efficacy studies of *T. parva* specific CTL antigens showed successful induction of CTL responses in some animals, which correlated with reduced disease severity after challenge. To provide correlates of immunity antigen-specific $CD8^+$ T cell mediated IFN-γ responses and CTL lytic responses were measured over the course of the experiments. Several challenges presented in these trials aimed at optimising vaccine efficacy. While the IFN-γ ELISPOT is a sensitive and reliable assay widely used in vaccine research, the use of chromium/indium release assay remains to be the only assay in use that measures *T. parva*-specific CTL activity. Hence the overall goal of the study was to develop novel reagents and novel assays to identify parasite-specific $CD8^+$ T lymphocytes with lytic potential.

To address this objective, bovine perforin, granzymes A and B, as specific effector proteins expressed in activated CTL were cloned and expressed using a baculovirus expression system. Sequence analysis of the cloned cDNAs showed the isolated cDNA belonged to the perforin and granzyme sub-families respectively. Perforin cDNA demonstrated 85% homology to human perforin with presence of conserved regions resembling calcium binding motif, membrane attack complex component as well complement protein. The sequences encoded by the cloned granzyme A and B cDNAs have the features of a trypsin like serine protease and demonstrates over 70% homology to the human cDNA over the active enzyme region as well catalytic residues characteristic of serine proteases. The expressed polypeptides of all three proteins were used to produce specific antibodies for use as reagents in immunoassays including ELISpot and intracellular staining for flow cytometric analysis. While the antibodies showed reactivity to the recombinant proteins, these reagents displayed different functionality in the recognition of the native protein.

Peptide-major histocompatibility complexes (MHC) class I tetrameric complexes (tetramers) are proving invaluable as fluorescent reagents for enumeration, characterisation and isolation of peptide-specific $CD8^+$ T cells and have afforded advantages to phenotype antigen-specific T cells with minimal *in vitro* manipulation. Fluorescent bovine tetramers were shown to specifically stain antigen-specific CTL by directly binding the T cell receptor (TCR). Analyses of CD8 T-cell responses in live-vaccine immunised cattle also showed that this method is robust and demonstrates changes in the kinetics and specificity of the $CD8^+$ T cell response in primary and secondary infections with *T. parva*. On average, results of functional assays and tetramer staining followed parallel trends, measured roughly the same populations and allowed for surface and intracellular staining for CD8 T cell marker and perforin, respectively, demonstrating a method that reliably quantifies the frequency, phenotype and function of specific $CD8^+$ T cells. The technical simplicity, rapidity and ability of the flow cytometric technique described in this thesis to measure low frequency antigen-specific responses suggests that tetramer staining, combined with functional assays could be broadly applicable to the valuation of vaccination efficacy to determine which protocols are most successful in inducing CTL responses.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

ABTS	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)
BSA	bovine serum albumin
con A	concanavalin A
DAB	3,3' diaminobenzidine tetrahydrochloride
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
HEPES	*4*-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hr	hour(s)
IL	interleukin
IPTG	isopropyl-β-D-1-thiogalactopyranoside
min	minute(s)
PBS	phosphate buffered saline
PE	phycoerythrin
PerCP	peridinin chlorophyll protein
PMSF	phenylmethylsulfonyl fluoride
rpm	revolutions per minute
RPMI-	Roswell Park Memorial Institute- (tissue culture medium)
TEMED	tetramethylethylenediamine
xg	relative centrifugal force
Ni-NTA	nickel-nitriloacetic acid
DTT	dithiothreitol

Chapter Overview

Chapter 1: Introduction and Literature Review

Chapter 1 presents a review of the current understanding of the biology of *Theileria parva,* immune responses to infection, and how this knowledge has been applied in initiatives to develop effective vaccines against *T. parva*. It also provides an account of the basic principles of antigen recognition by $CD4^+$ and $CD8^+$ T cells, their activation and effector mechanisms as well as immunological assays deployed in the evaluation of specificity and magnitude of the T-cell response and how these relate to what is known about *T. parva*-specific immune responses. The chapter concludes by outlining the rationale and objectives of the present study.

Chapter 2: Cloning, Sequence Analysis and Expression of Bovine Perforin, Granzymes A and B

Chapter 2 outlines the standard methodologies and materials used in cloning and reports on the outcome of the cloning of bovine perforin, granzymes A and B cDNA from *T. parva*-specific cytotoxic T lymphocytes as well as the expression of recombinant proteins encoded by the respective cDNA.

Chapter 3: Generation of Antibodies to Bovine Perforin, Granzymes A and B for Assay Development

Chapter 3 presents data on the production and characterisation of antibodies generated against the recombinant proteins and synthetic peptides as well as outlining the antibody production materials and assays. This chapter also reports the use of these reagents in attempts to optimise bovine perforin ELISpot and ELISA assays.

Chapter 4: Identification of *T. parva* antigen specific $CD8^+$ T cells using peptide-MHC class I tetrameric complexes

Chapter 4 discusses data on the optimisation of a co-staining assay using bovine class I MHC-peptide tetramers and antibodies to CD8 or perforin, and its preliminary application, alongside IFN-γ ELISpot and standard [^{51}Cr] chromium- and [^{111}In] indium-release cytotoxicity assays, in the evaluation of $CD8^+$ T cell responses in cattle following immunisation and challenge. This chapter also outlines materials and methods used in this research including animal inoculation and sampling, T cell culture and assays.

Chapter 5: General Discussion and Conclusion

Chapter 5 discusses the key findings and limitations of the present study in the context of its overall objective and what is known in other systems while highlighting how the study contributes to the general understanding of this field.

Acknowledgments

The work described in this thesis was carried out at the International Livestock Research Institute (ILRI), Nairobi, Kenya, with funding support from the United Kingdom Government Department for International Development (DFID-UK), through a technical grant provided to ILRI. For this I am grateful to the ILRI Training and Outreach Department for the opportunity to carry out this research work.

I would like to acknowledge and thank Dr. Evans Taracha, who conceptualised the project and as my former ILRI supervisor, for his invested time, advice, useful discussions, contributions and patience in introducing immunology to me as critical and imaginative curiosity. I wish to express my sincere appreciation to Dr. Vish Nene, my current supervisor at ILRI for his support, encouragement and constructive critique in writing up this thesis. In addition, the guidance, support and interest shown by my supervisor at Brunel University, Dr. Su-Ling Li, is greatly appreciated. Other crucial contributors to development of this thesis include Dr. Simon Graham and Dr. Duncan Mwangi. Their tremendous knowledge base, useful comments in editing the thesis and thoughtful considerations were great assets to my understanding of immunology.

I wish to acknowledge Dr. Didier Colau (Ludwig Institute for Cancer Research, Brussels) for the collaboration on bovine tetramer construction and Dr. Simon Graham for providing some T cell samples and data discussed in this study. My sincere appreciation goes to all immediate and former members of the ILRI Molecular Biology, Immunology and Animal Unit groups particularly Dr. Roger Pelle, Dr. Yoshikazu Honda, Elias Awino, James Gachanja, John Wasilwa, Thomas Njoroge and Gideon Ndambuki for scientific advice and technical support. I also wish to acknowledge TreeStar Incorporated (USA) for provision of a free license for use of the FlowJo software through its FlowJo for Africa program in support of research within the African continent and Dots & Graphics (Kenya, Limited) for the assistance in developing figures found in this thesis and print-work.

Last but not least is a special appreciation to Dr. Florence Muli-Musiime, Dr. Jotham Musiime and Dr. Victor Konde, my role models and mentors who enabled this work to be concluded successfully. I also wish to thank most sincerely my family for their continued sacrifice and encouragement. A sincere thanks to my mother Monica Ndunge, for her unwavering faith and prayers, I could never have hoped to finish. To the memory of my late father, Josphat George Musembi, I dedicate this thesis.

Chapter 1: Introduction and Literature Review

1.1 The history and biology of *Theileria parva* and East Coast fever

1.1.1 Historical background of East Coast fever

East Coast fever was first reported in southern Africa in 1902. The origin of this virulent disease, initially diagnosed as Rhodesian red water, was traced to importations of cattle from the eastern Africa coastline and it was given the name East Coast fever (ECF). A devastating rinderpest epidemic in southern Africa in 1896 had led to the importation of cattle from a number of sources including eastern Africa to rebuild the cattle population. It appears that the disease was known to the inhabitants of eastern Africa long before it was reported in southern Africa but nothing was known of its aetiology. Koch (1897) (cited by Neitz, 1957) while investigating red water in cattle in German East Africa (now Tanzania) found erythrocytes of affected animals that were parasitized by minute rod-shaped, oval and round organisms which he considered to be young forms of *Babesia*. The importation of cattle coupled with the widespread use of transport oxen and the existence of common grazing grounds and outspans in the region proved major factors in the spread of ECF. Following the early outbreak and subsequent spread of the disease after the introduction of affected cattle from German East Africa (Tanzania), Koch, Theiler and others investigated the aetiology and transmission of the disease. The causal agent, which is now known as *Theileria parva* (Bettencourt *et al*., 1907), was originally named *Piroplasma kochi* (Stephens and Christophers 1903) and *Piroplasma parvum* (Theiler, 1904). Schizonts, commonly known as Koch blue bodies, were described by Koch (1905, 1906), but it was Gonder (1910, 1911) who conclusively showed that the Koch bodies represented the schizogonous phase of the developmental cycle of the parasite. Pioneering studies by Theiler and Lounsbury further identified *Rhipicephalus appendiculatus* (the brown ear tick) (Neumann, 1901) as the principal vector (Lounsbury, 1904; Theiler, 1908), which was already widespread in the region. The history of bovine theileriosis has been extensively reviewed in Norval, Perry and Young (1992).

1.1.2 Classification and life cycle of *Theileria parva*

1.1.2.1 Taxonomy

Theileria are tick-borne, intracellular protozoan parasites which infect wild and domestic ruminants although one species, *Theileria equi* is found in horses (Norval, Perry and Young, 1992; Mehlhorn and Schein, 1998; Bishop *et al*., 2004).

The taxonomy of *Theileria* has been a subject of review for many years. *Theileria* are currently classified in the class Sporozoa together with human pathogens, including *Plasmodium* and *Toxoplasma*. Along with other Sporozoa, *Theileria* has been included within the phylum, Apicomplexa based on the possession of an apical complex containing secretory organelles involved in invasion, or establishment, in the cells of their mammalian hosts. Analysis of the 18S ribosomal RNA gene sequences shows that the genus *Theileria* is phylogenetically most closely related to *Babesia*, a genus of tick-borne protozoans infective to the red cells of mammals including domestic livestock, and more distantly to the genus *Plasmodium* which cause malaria in humans and other species of vertebrates (Allsopp *et al*., 1994). The classification scheme originally suggested by Levine *et al*., 1980, is as follows:

Sub-kingdom: Protozoa

Phylum: Apicomplexa

Class: Sporozoea

Sub-class: Piroplasmia

Order: Piroplasmorida

Family: Theileridae

Genus: *Theileria*

Species: *Theileria parva*

At least thirty nine *Theileria* species have been described (Levine, 1988). Economically important *Theileria* species that infect cattle and small ruminants are transmitted by ixodid ticks of genera *Rhipicephalus*, *Amblyomma*, *Hyalomma* and *Haemaphysalis*. Table 1 summarizes the major *Theileria* species of economic importance in ruminants.

1.1.2.2 Life cycle

Theileria parva life cycle stages in the tick vector and bovine host have been described previously (Mehlhorn and Schein, 1984; Norval, Perry and Young, 1992; Bishop *et al*., 2004) and are summarised schematically in Figure 1. *T. parva* has a

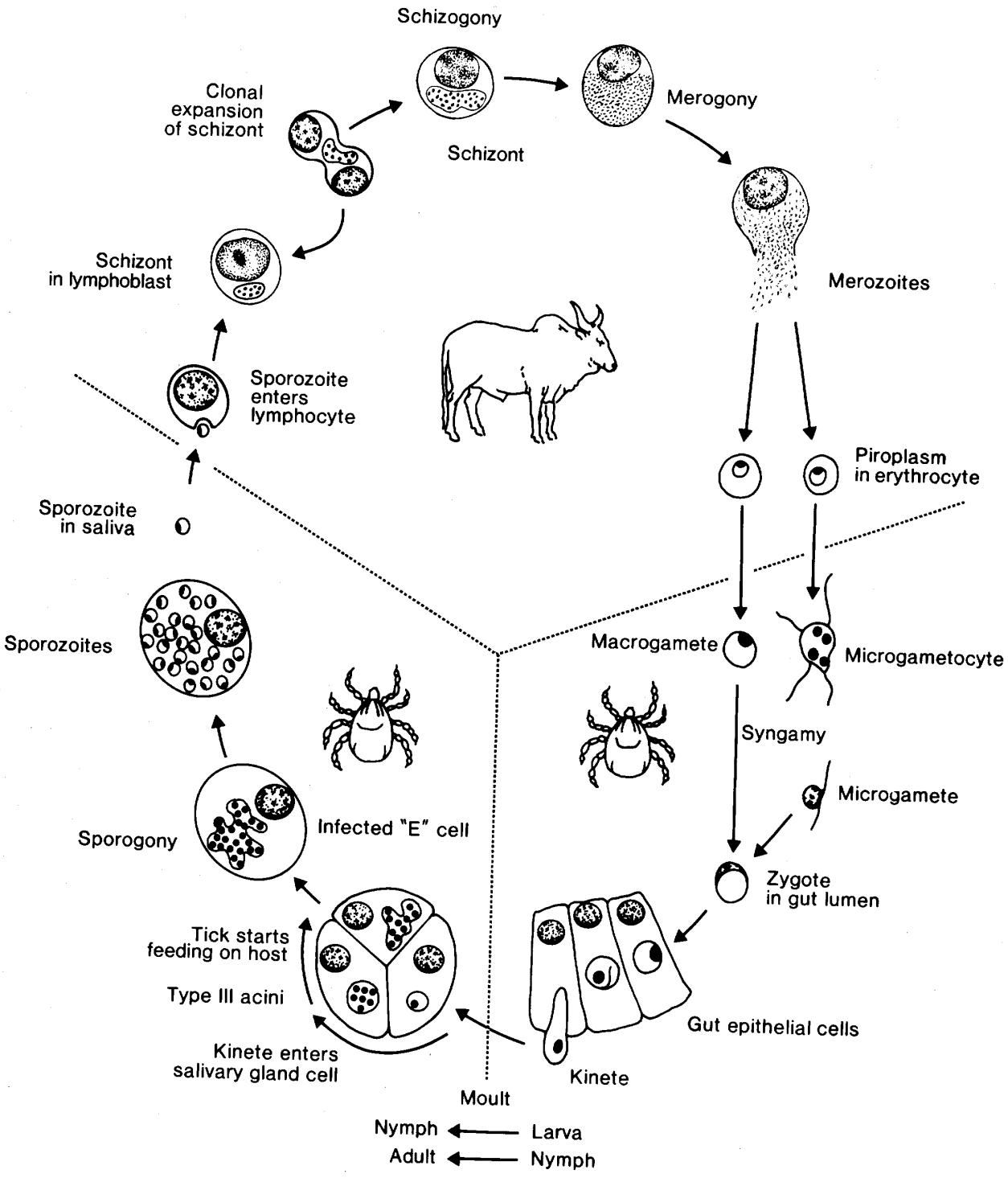
Table 1: *Theileria* species infective to domestic ruminants, their tick vectors, disease and distribution

Species	**Vector**	**Disease**	**Distribution**
T. parva	*R. appendiculatus* *R. zambesiensis* *R. duttoni*	East Coast fever, January disease, Corridor disease	Eastern, central and southern Africa
T. taurotragi	*R. appendiculatus* *R. zambesiensis* *R. pulchellus*	Benign theileriosis	Eastern, central and southern Africa
T. mutans	*Amblyomma spp.*	Benign theileriosis	Western, eastern, central and southern Africa, Caribbean Islands
T. velifera	*Amblyomma spp.*	Theileriosis	Western, eastern, central and southern Africa
T. annulata	*Hyalomma spp.*	Tropical theileriosis	Southern Europe, western, southern and eastern Asia
T. orientalis	*Haemaphysalis longicornis*	Oriental theileriosis	Japan, Korea
*T. lestoquardi**	*Hyalomma spp.*	Malignant ovine theileriosis	Asia and northern Africa
*T. ovis**	*Hyalomma spp.*	Benign ovine theileriosis	Asia

Adapted from Brown *et al*., (1990) and Bishop *et al*., (2004). * Indicates *Theileria* species infective to small ruminants (sheep and goats). Other species are infective to cattle.

Figure 1: Life cycle of *Theileria parva* in cattle and the ixodid tick, *Rhipicephalus appendiculatus*

Figure prepared by A.S. Young. Adapted from Norval *et al*., (1992).


Schizogony
Schizont
Merogony
Merozoites
Clonal expansion of schizont
Schizont in lymphoblast
Sporozoite enters lymphocyte
Sporozoite in saliva
Piroplasm in erythrocyte
Macrogamete
Microgametocyte
Syngamy
Microgamete
Zygote in gut lumen
Gut epithelial cells
Kinete
Kinete enters salivary gland cell
Type III acini
Tick starts feeding on host
Infected "E" cell
Sporogony
Sporozoites
Moult
Nymph ← Larva
Adult ← Nymph

typical apicomplexan life cycle that is predominantly haploid, with only a brief diploid phase in the tick. *T. parva* is transmitted by the three-host tick *Rhipicephalus appendiculatus*. Transmission occurs transtadially i.e. infection acquired by the larvae or nymph is subsequently transmitted, following moulting, by the nymph or adult stages of the tick, respectively (Lounsbury, 1904; Theiler, 1905, 1908). Larvae or nymphs get infected during feeding on infected cattle when they ingest piroplasm-infected erythrocytes. Lysis of infected erythrocytes within the tick gut releases piroplasms which differentiate into micro- (male) or macro- (female) gametes. These undergo syngamy to form diploid zygotes, which invade gut epithelial cells and differentiate to motile kinetes, a process that appears to be synchronised with the moulting of the tick (Young and Leitch, 1980). There is evidence that this process also entails a meiotic reduction division involving interchromosomal cross-events (Gauer *et al*., 1995; Morzaria *et al*., 1993; Katzer *et al*., 2006). The existence of sexual cycle in *T. parva* has been demonstrated in direct measurements of DNA content of single cells by fluorescence microscopy (Gauer *et al*., 1995) and analysis of laboratory crosses of Muguga and Marikebuni or Uganda isolates (Bishop *et al*., 2002). Evidence for genetic cross-over of *T. parva* in the tick has been obtained by tracking the segregation of several polymorphic sequences (Morzaria *et al*., 1992; 1993; 1995). Following tick moulting, kinetes are released into the haemocoele of the tick and migrate to the developing salivary gland, where they specifically invade E-cells of type III acini (Mehlhorn and Schein, 1984; Fawcett *et al*., 1982a). Parasites then remain quiescent or undergo limited development, until activated by the stimulus of tick feeding or ambient high temperature. As an infected adult tick or nymph feeds on its next host, the kinete undergoes further development within the infected cell to form a large synctial structure known as the sporoblast (Fawcett *et al*., 1982a; 1985). Each sporoblast has been estimated to give rise to 10^4 – 10^5 uninucleate haploid sporozoites, which are released from about the fourth day of feeding (Fawcett *et al*., 1982a).

Once sporozoites are inoculated, infection in the mammalian host is initiated when they gain entry into lymphocytes. *In vitro* observations indicate that sporozoites rapidly gain entry into host lymphocytes by receptor-mediated endocytosis which is completed within 10 min of cell contact (Fawcett *et al*., 1982b). The host cell membrane enveloping the parasite is then lysed and the parasite then comes to lie free in the cytosol. The intracellular sporozoite rapidly differentiates into a trophozoite

which continues to grow and by 48 – 72 hr it has undergone nuclear division to form a multi-nucleate schizont (Fawcett *et al*., 1984). At this time, the host cell is transformed to a state of uncontrolled proliferation. At each cell division, the parasite also divides so that both daughter cells are infected (Hulliger *et al*., 1964; Brown *et al*., 1973). Thus infection is established and becomes disseminated by clonal expansion of the small population of lymphocytes initially infected by the parasite. As the infection progresses, the mean number of nuclei per schizont increases and a proportion of the schizonts undergo merogony to form merozoites (Schein *et al*., 1978). The latter, upon release through cell lysis, infect erythrocytes and develop into piroplasms which are infective for the tick. Susceptible cattle die within three weeks of infection as a result of pathological changes associated with the invasion of tissues by the rapidly dividing infected lymphoblasts.

1.1.3 Epidemiological features of *Theileria parva*

Pathogenic *Theileria* are found worldwide but the economically important species are in the tropics and sub-tropics (Uilenberg, 1995). The emergence of the disease complex due to *T. parva* followed the introduction of *T. parva*-infected cattle into southern Africa where the disease had not previously been reported and where a large population of cattle was markedly susceptible. The extensive introduction of European breeds into ECF-endemic regions to improve livestock productivity was also a precipitating factor in the spread of the disease. ECF is enzootic in a large part of eastern and central Africa where it causes considerable economic losses in the cattle industry. The presence of disease closely follows the distribution of the tick vector, *R. appendiculatus*. However, in South Africa and Zimbabwe, the tick exists but the disease was eradicated early in the 20th century by rigorous quarantine measures, systematic dipping and slaughter of infected and contact cattle as well as fencing to prevent contact of cattle with buffalo (Lawrence, 1981). In some areas, the tick population is maintained throughout the year while in other areas there is seasonal variation in the number of ticks following temperature and rainfall patterns, further affecting the epidemiological state. *Theileria* species may also affect wild or feral ungulates (Burridge, 1975; Young *et al*., 1977; Uilenberg *et al*., 1982; Stagg *et al*., 1994) but are rarely associated with disease. Buffaloes are considered the definitive hosts of *T. parva*. The presence of the African buffalo (*Syncerus caffer*) acts as a constant reservoir of parasites from which infection can be maintained in the

resident tick population. In addition, the carrier state engendered in cattle following natural or experimental infections with *Theileria* provides an important reservoir of parasites that can be transmitted to susceptible animals (Young *et al*., 1978; 1986; Kariuki *et al*., 1995). The extent to which transmission of infection occurs from carrier cattle in the field remains to be determined. However, studies by Oura and colleagues (2004, 2007) have shown that some of the strains present in live vaccines can establish a persistent carrier state in immunised cattle that is accessible to the local tick population and is transmissible to unvaccinated cattle.

1.1.4 Genome characteristics of *Theileria parva*

In order to underpin studies of the biology of *T. parva*, its genome sequence was determined. The haploid *T. parva* nuclear genome is $8.3x10^6$ base pairs (bp) in length and consists of four chromosomes (Gardner *et al*., 2005). *T. parva* chromosomes contain one extremely A+T rich region (>97%), about 3000 bp in length, that is suggested to be the centromere. The regions between the telomeric repeats and the first protein-encoding gene are short, about 2900 bp on average, and do not contain other repeats; constituting much less structural complexity than that in *Plasmodium falciparum* where arrays of repeats extend up to 30000 bp. The *T. parva* nuclear genome contains about 4035 protein coding genes, which is fewer than that (5268) of *P. falciparum* but exhibits higher gene density, a greater proportion of genes with introns and shorter intergenic regions. Putative functions have been assigned to approximately 38% of the predicted proteins. Massively parallel signature sequencing (MPSS) analysis of the transcriptome suggests a low level of baseline transcription from most of the protein coding genes (Bishop *et al*., 2005). Like other apicomplexan organisms, *T. parva* contains two extrachromosomal DNAs, a mitochondrial DNA and a plastid DNA molecule (Kairo *et al*., 1994; Gardner *et al*., 2005). The complete sequencing and significant annotation of the *T. parva* genome has aided vaccine development efforts by contributing to the identification of schizont stage antigens that are targets of cell-mediated immunity (Graham *et al*., 2006). The genome sequence data has also facilitated efforts toward identification of parasite molecules involved in inducing reversible host leukocyte transformation and tumour-like behaviour of transformed parasitized cells (Shiels *et al*., 2006).

1.1.5 Parasite strain diversity

Reports from field observations and cross-immunity experiments have indicated that different strains of *T. parva* exist (Cunningham *et al*., 1974; Irvin *et al*., 1983). Indeed, early literature quotes three sub-types of *Theileria parva* namely, *T. parva parva*, *T. parva bovis* and *T. parva lawrencei* (Uilenberg, 1976, 1981; Lawrence, 1979; Uilenberg *et al*., 1982) as causative agents of classical East Coast fever, January disease and Corridor disease, respectively. Sub-type classification was based on the apparent epidemiological distinction and clinical differences of the diseases they produce; their morphological features and serological characteristics being indistinguishable. *T. p. bovis* and *T. p. parva* are primarily transmitted between cattle but the disease caused by *T. p. bovis* is less virulent. *T. p. lawrencei* is transmitted mainly from buffalo to cattle. Although *T. p. parva* and *T. p. lawrencei* both produce acute fatal diseases in cattle, the infections differ in the lower level of schizont parasitosis and much lower piroplasm parasitemia but higher virulence caused by *T. p. lawrencei* compared to *T. p .parva*. However, it is noteworthy that monoclonal antibodies and DNA probes which detect heterogeneity between isolates of the parasite do not reveal a clear distinction between the subtypes (Conrad *et al*., 1987a and b).

The diversity of parasite populations in the field is evident at both the antigenic and molecular levels. Studies using monoclonal antibodies (mAb) that react with polymorphic determinants on schizont antigens (Irvin *et al*., 1983; Minami *et al*., 1983; Conrad *et al*., 1987b) and parasite-specific DNA probes demonstrating restriction fragment length polymorphism in DNA from different parasite populations (Conrad *et al*., 1987a; Bishop, Sohanpal and Morzaria, 1993; Bishop *et al*., 1993; 1996) have corroborated reports of a lack of protection between distinct isolates of the parasite (Cunningham *et al*., 1974; Irvin *et al*., 1983). The heterogeneity of *T. parva* parasites is also reflected in their virulence with some isolates being highly pathogenic whereas others give rise to only mild infections (Koch *et al*., 1988). In addition, the duration of the carrier state varies following infection with different isolates (Skilton *et al*., 2002).

It is presumed that sexual recombination in *T. parva* contributes to the extensive polymorphism observed in field isolates (Morzaria *et al*., 1990; Katzer *et al*., 2006). Using a genome-wide panel of polymorphic markers, Katzer *et al*. (2006)

recently provided evidence for extensive genotypic diversity in a *T. parva* population derived from ticks fed on cattle infected with Marikebuni stock and Patel *et al*., (2011) have recently shown the presence of at least eight genotypes in a live *T. parva* sporozoite vaccine stabilate that is derived from three *T. parva* stocks. To what extent recombination occurs in *T. parva* in field populations is currently unknown. However the use of DNA satellite markers (Oura *et al*., 2003) in population studies of *T. parva* reveal a high level of diversity and frequency of infection of cattle with mixed genotypes (Oura *et al*., 2005; Odongo *et al*., 2006).

Little is known about the influence of host immunity on the genotypic diversity of *T. parva*. It has been reported that protective immunity against *T. parva* has considerable impact on the emergence of targeted parasites following challenge but fails to prevent their differentiation into transmissible forms (McKeever *et al*., 2006; Katzer *et al*., 2007). This suggests that in addition to the tick, the host and/or parasite factors may also play a role in the emergence of dominant genotypes.

1.1.6 The disease

1.1.6.1 Clinical features

Clinical aspects of ECF have been discussed in several papers including Shannon (1977), Jura and Losos (1980), Maxie *et al*., (1982) and Irvin and Mwamachi (1983). After a natural exposure to infestation by infected ticks, there is an incubation period of between eight and twenty five days, with an average of thirteen days, before clinical disease is observed (Theiler, 1904; Neitz, 1957; Irvin and Morrison, 1987). Clinical disease arises from invasion of lymphoid and non-lymphoid tissues by parasitized cells (Irvin and Morrison, 1987). The disease presents as a generalized lymphoproliferative disorder characterised by enlargement of lymph nodes and sustained high fever. In terminal cases, the animals develop severe pulmonary oedema seen clinically as acute dyspnea and frothing at the nostrils. The course and severity of the disease may vary depending on factors such as the virulence of the parasite strain, sporozoite infection rates in ticks and genetic background of infected animals. In general, indigenous cattle (*Bos indicus*) develop less severe disease and suffer lower levels of mortality than European breeds (*Bos taurus*) which are highly susceptible.

The pathology and pathogenesis of the disease was extensively reviewed in

Irvin and Morrison (1987). The major pathology of ECF is caused by invasion of tissues with parasitized cells, lysis of infected lymphoid cells by cytotoxic T lymphocytes and also extensive non-specific lymphocytolysis and tissue destruction (Morrison *et al*., 1981). Other lesions are evident in the kidney, and frequently, haemorrhage in the muscles and sub-cutaneous tissue is also observed. During the clinical phase of disease, parasites are evident as macro- and microschizonts in lymphoid cell-biopsies of the enlarged lymph nodes. Parallel blood smears may show piroplasms. Death can occur within three to four weeks of infection and high levels of mortality (>90%) have been seen in fully susceptible stock and calves less than six months old. Pulmonary oedema is evident at death.

1.1.6.2 Diagnosis

Several approaches to the diagnosis of ECF are routinely applied. Observation of the body condition, which progressively deteriorates with disease, coupled with knowledge of the disease history and reports of cattle movement or a change of management conditions may lead to a provisional diagnosis. In essence, diagnosis is dependent on identifying the parasite in the animal by use of Giemsa-stained thin blood smears for piroplasms and lymph node biopsy smears for schizonts. In an area where ECF is endemic, a blood smear is an essential early test.

A number of serodiagnostic techniques using schizont and piroplasm antigen and nucleic acid-based assays are available. The serodiagnostic tests consist of a schizont antigen indirect fluorescent antibody (IFA) test (Burridge and Kimber, 1972; Goddeeris *et al*., 1982), the piroplasm indirect haemagglutination (IHA) (Duffus and Wagner, 1984) and an enzyme-linked immunosorbent assay (ELISA) (Katende *et al*., 1998). The latter technique has been shown to provide a higher degree of sensitivity and specificity than IFA and IHA tests. Serological tests have been used for disease surveillance (FAO, 1975; Kiltz, 1985; Flach, 1988; Norval *et al*., 1985) although they are also of restricted value in disease surveys because of several limitations; antibodies are usually absent during the early phase of clinical disease and a strong antibody response does not persist. None of these serological tests can distinguish immunologically distinct strains of *T. parva*.

T. parva-specific repetitive DNA probes have been developed and are useful for the differentiation of *T. parva* from other *Theileria* species and for discrimination

of *T. parva* stocks by detection of restriction fragment length polymorphisms (Conrad *et al*., 1987a; Allsopp and Allsopp, 1988; Morzaria *et al*., 1990; Bishop *et al*., 1993; 1995; Collins, Allsopp and Allsopp, 2002). Newer molecular diagnostic tests particularly those based on reverse line blot hybridization (Gubbels *et al*., 1999) and PCR (Skilton *et al*., 2002) as well as genotyping using mini- and micro-satellite markers (Bishop *et al*., 1996; Oura *et al*., 2003; 2004; Odongo *et al*., 2006), are proving to be powerful tools for characterising parasite polymorphism, defining population genetics and generating epidemiological data.

1.1.6.3 Control of East Coast fever

While decimating herds of indigenous cattle, ECF is even a greater threat to improved exotic cattle breeds and is therefore limiting development of the livestock enterprise which often depends on cross-breed cattle. Control of ECF has depended primarily on breaking the transmission cycle between the cattle and the tick vector, *R. appendiculatus*. On the one hand, this has involved short-interval application of acaricides by regular dipping or spraying. However this method has major drawbacks including the development of resistance in ticks, food safety concerns and environmental contamination resulting from toxic residues (George *et al*., 2004). In addition, dipping facilities are frequently not operational because of lack of financial resources for maintenance, particularly in pastoral systems, which depend to a substantial degree on the informal economy. After nearly a century of acaricide utilization, it is widely believed that acaricides alone do not provide a sustainable solution to tick and tick-borne disease control (Norval, Perry and Young, 1993). On the other hand, treatment of clinically sick animals with antitheilerial drugs has been applied. Two theilericidal compounds namely naphthoquinones (Parvaquone [Clexon®; Coopers Animal Health] and its analogue Buparvaquone [Butalex®; Coopers Animal health]) and febrifugines (Halufuginone [Terti®; Hoechst/Roussel]) have been of considerable value in treating clinical theileriosis. However, not only are they expensive but their effective use require early diagnosis of the disease. Buparvaquone is widely and successfully used to treat early-stage infections of *T. parva* though its cost may be prohibitive for small farms and poor farmers (D'Haese, Penne and Elyn, 1999; Muraguri, Kiara and McHardy, 1999; Kivaria, 2006, 2007). Recent studies by Lizundia *et al*., (2009) are targeted at exploiting the *T. parva* apicoplast as a potential target for developing new antitheilerial drugs. Alternatives

will be urgently required in the event of buparvaquone resistance.

An alternative approach to the control of the disease by vaccination led to the development of the Infection and Treatment Method (ITM) of immunisation (Radley *et al*., 1975b; Morzaria *et al*., 1999). This involves the simultaneous administration of defined doses of cryopreserved sporozoites and long-acting oxytetracycline. This protocol is shown to result in solid immunity to challenge with the homologous parasite strain. To overcome the problem of breakthrough infections with heterologous strains, currently, the vaccine is administered as a 'cocktail' of three parasite isolates; Muguga, Kiambu-5 and Serengeti-transformed. In areas of greater disease transmission and larger herds, vaccination is the most cost effective strategy (Muraguri *et al*., 1998; D'Haese, Penne and Elyn, 1999).

However, though ITM was developed over thirty years ago, very little progress in the advancement of this technology has been made (Giulio *et al*., 2009). Several pilot trials of ITM have been performed and these have been reviewed (Uilenberg, 1999) and despite the effectiveness of the vaccine in inducing protection experimentally, until recently, adoption of ITM has been limited. The main practical constraints relate to the complex procedure of vaccine production and distribution which requires considerable infrastructure and specialized expertise. Each batch of sporozoites used in the vaccine needs to be produced by passage through ticks fed on infected cattle and the appropriate dose of sporozoites need to be determined by titration in cattle (reviewed in Giulio *et al*., 2009). The latter step is necessary because long-acting oxytetracycline fails to control infection initiated with higher doses of parasites. The use of an *in vivo* vaccine production system in cattle also introduces significant risk of contamination with extraneous bovine pathogens, necessitating rigorous quality control of each vaccine batch. The stocks of parasites are stored as cryopreserved stabilates in liquid nitrogen and since the organisms need to be administered shortly after thawing, a cold chain is required for distributing vaccine to regions distant from the site of production. Ideally, vaccine users should have access to therapeutic antitheilerial drugs to allow treatment of any animals in which the tetracycline does not effectively control the immunising infection. Related to the latter, an additional reason why ITM has not been adopted in some regions is the perceived risks associated with establishment of tick-transmissible carrier infections (Young *et al*., 1986, Bishop *et al*., 1992) with the 'foreign' vaccine parasite strains

and hence their introduction into local tick populations. Specifically, the main concern is that the introduced parasites may be more virulent or differ antigenically from the indigenous parasites.

In spite of all these shortcomings, and given the prevalence and economic importance of the disease as well as the lack of more sustainable control options, measures are being taken to address some of the limitations in order to allow quality-controlled bulk production, registration and field delivery of the vaccine. One such improvement was the provision of standardised vaccine stabilate (Morzaria *et al*., 1999). With an increased acceptance of ITM among livestock owners, the vaccine has since been deployed extensively in Tanzania (Melewas, 1999) and Uganda (Nsubuga-Mutaka, 1999) and to a lesser extent in Malawi and southern Zambia (Lynen *et al*., 2006; Homewood *et al*., 2006). More recently (2006, 2007), the ITM has been sanctioned for use in the Maasai ecosystem in Kenya (Personal communication, Dr Evans Taracha). The shift in policy on ITM deployment in Kenya was informed by the outcome of field trials in the Narok District of Rift Valley Province, which generated data on vaccine safety, efficacy and demand. Initiatives are underway to roll out the vaccine in other areas including the intensive dairy of central Kenya, extensive dairy of North Rift and other pastoral eco-zones of Northern Kenya. Recent developments pioneered by the Global Alliance for Livestock Veterinary Medicines (GALVMed) and ILRI has seen the production of a new batch of vaccine, the successful registration of the vaccine in Kenya, Malawi and Tanzania with Uganda expected to follow (DFID Research 2009 – 2010: providing research evidence that enables poverty eradication). GALVMed is also working to establish viable commercial production and delivery systems adequate for marketing and commercialization of the ECF vaccine.

1.2 Immune responses to *Theileria parva*

1.2.1 General features of the immune response

A great deal of investment in resources and time has led to the elucidation of the nature and specificity of the immune responses underpinning host immunity against *T. parva*. Cattle exposed to a single natural or experimental infection with *T. parva* sporozoites mount diverse immune responses to all the stages of the parasite. The role of immune responses against the piroplasm stage of the parasite has been

largely ignored because such responses, even if effective, could have little effect in preventing disease caused largely by the schizont stage of the parasite. Whereas antibody responses against sporozoites, schizonts and piroplasms have been detected in recovered cattle, albeit at low levels, such animals exhibited potent cell-mediated immune responses directed at schizont-infected cells (reviewed by Morrison *et al*., 1989; Morrison and McKeever, 2006). Further, these animals are immune to challenge with the same stock of the parasite, but only a proportion will withstand heterologous challenge (Neitz, 1957; Barnett, 1957; Wilde, 1967; Radley *et al*., 1975b; Radley, 1981; Irvin *et al*., 1983; Morrison *et al*., 1986).). Further in-depth studies have been conducted demonstrating an unambiguous role of cell-mediated immune responses directed against schizont-infected cells in *T. parva* immunity (Emery, 1981; Morrison *et al*., 1987; McKeever *et al*., 1994; Taracha *et al*., 1995a). It has been postulated that the parasite strain specificity of the $CD8^+$ T cell response is due to the heterogeneity of parasite antigens recognised by the host immune responses (Morrison and Goddeeris, 1990; McKeever and Morrison, 1990).

Under experimental conditions, all the methods that have been used to induce immunity have utilised infective material such as live sporozoites or schizont-infected cells (Brocklesby and Bailey, 1965; Pirie *et al*., 1970; Radley *et al*., 1975a and b; Schein and Voigt, 1979; Brown *et al*., 1981; Radley, 1981). Attempts to immunise cattle with irradiated non-infective sporozoites or heat-killed schizont-infected cells (Cunningham *et al*., 1973; Wagner *et al*., 1974; Emery, 1981) were unsuccessful. Taken together, these observations have supported the notion that the establishment of infection in cells of recipient cattle is necessary for successful development of immunity (reviewed by Morrison *et al*., 1986; 1989; Morrison and Goddeeris, 1990; McKeever and Morrison, 1990). Moreover, while the serum from immune cattle contains antibody against all stages of the parasite (Burridge and Kimber, 1972), antibody was not considered to play a role in the protection seen in recovered animals since the transfer of immune serum or concentrated gammaglobulins from immune to naïve cattle failed to protect against the disease (Muhammed *et al*., 1975). By contrast, immunity against *T. parva* was transferred adoptively between chimeric twin calves using thoracic duct leucocytes (Emery, 1981). Subsequent studies have however provided evidence that antibody responses against sporozoites may play a role in immunity, under certain circumstances (Musoke *et al*., 1992).

1.2.2 Immune responses to *Theileria parva* sporozoites

Following immunisation by ITM, low titres of anti-sporozoite antibodies are detected in the serum (Musoke *et al*., 1982). On the other hand, sera from cattle exposed to repeated high parasite challenge with large numbers of infected ticks under laboratory and field conditions contain high titres of sporozoite-specific antibodies (Musoke *et al*., 1982; 1984). Importantly, these high-titre anti-sporozoite antibodies have been demonstrated to neutralise sporozoite infectivity *in vitro* (Musoke *et al*., 1984). Antibodies against *T. parva* (Muguga) sporozoites neutralise the infectivity of homologous sporozoites as well as those of a variety of parasite stocks, some of which do not cross-protect following immunisation of cattle by ITM (Musoke *et al*., 1984). Hence, it was postulated that sporozoite antigens might be used in vaccination initiatives to induce immunity that protects against all parasite strains and prompted a search for the antigen(s) targeted by this antibody activity. Monoclonal antibodies (mAbs) were raised against purified sporozoites and a number of them exhibited neutralising activity *in vitro* (Musoke *et al*., 1984; Dobbelaere *et al*., 1984). One of the mAb was shown to recognise a surface coat antigen (p67) with a molecular weight of 67 kDa on the sporozoite (Dobbelaere *et al*., 1985), and furthermore the mAb could neutralise sporozoites of other *T. parva* strains (Musoke *et al*., 1984). Consequently, the gene encoding the p67 sporozoite antigen was cloned and expressed (Nene *et al*., 1992). Several recombinant forms of p67 expressed using bacterial or viral vectors have been generated and evaluated extensively in laboratory and field immunisation and challenge experiments (Musoke *et al*., 1992; Nene *et al*., 1995; 1996; Honda *et al*., 1998; Heussler *et al*., 1998; Bishop *et al*., 2003; Musoke *et al*., 2005; Kaba *et al*., 2005). Significantly, these experiments have demonstrated that 50% of immunised cattle were protected from severe disease following challenge. These findings provide a basis for the inclusion of p67 in a multi-component vaccine against *T. parva*.

1.2.3 Immune responses to *Theileria parva* schizont-infected cell

Schizont-infected cells constitute a critical developmental stage in the life cycle of *T. parva* in terms of pathogenicity and host immunity. Most of the pathology observed in ECF is attributed to schizont-parasitized cells. Yet, it is known that *Theileria* parasites have evolved the ability to remain in an intracellular location throughout their replication phase within leucocytes, and apparently there is no expression of parasite-encoded proteins on the surface of infected cells (reviewed by

Morrison *et al*., 1986; Newson *et al*., 1986). It is therefore evident that the parasite is not accessible to antibodies during this stage of infection, implicating a key role for cell-mediated immune responses in protective immunity against *T. parva*. This notion is supported by the intracytoplasmic location of the parasite favouring access of the parasite molecules to the class I MHC processing and presentation pathway for recognition by $CD8^+$ T cells.

However, schizont-specific antibodies can be detected using IFA and antigen ELISA in cattle following a single *T. parva* infection (Burridge and Kimber, 1972; Katende *et al*., 1998). Antibodies, most of which are IgG (Duffus and Wagner, 1974; Wagner *et al*., 1975), are first detected after 18-21 days and reach peak levels about 25-30 days after inoculation of sporozoites (Lohr and Ross, 1969). Antibody responses can be detected even in animals that develop only mild infections with *T. parva* (Burridge, 1971). Antibodies last for a variable period of time after recovery, depending on factors such as the establishment of a carrier state, chemotherapeutic intervention and presence or absence of re-challenge. Following recovery from ECF, some animals may have low antibody titres for four to six months but the antibodies may persist for more than one year following a single challenge. The presence in serum of anti-schizont antibody remains to be the most reliable measure of exposure to the parasite and the IFA and antigen-ELISA tests have been applied in national disease survey programmes (FAO, 1975; Kiltz, 1985; Flach, 1988; Norval *et al*., 1985).

Studies in immune cattle undergoing challenge have provided a better understanding of cell-mediated responses against the schizont-infected cell and their role in protective immunity. There is good evidence that protection is mediated by parasite-specific MHC class I restricted $CD8^+$ CTL (reviewed in McKeever and Morrison, 1990; 1994). CTL are detected transiently in the blood and efferent lymph of immune cattle under challenge at the time of parasite clearance (Eugui and Emery, 1981; Morrison *et al*., 1987b; McKeever *et al*., 1994). These are present at moderate frequency in immune animals and increase dramatically in number following challenge, peaking around the time that parasitosis is controlled, and they have been shown to be MHC class I restricted, have a $CD2^+CD4^-CD8^+$ phenotype (Emery Tenywa and Jack., 1981b; McKeever *et al*., 1994) and, in some instances parasite-strain specific (Goddeeris *et al*., 1986; Morrison *et al*., 1987b). In contrast, PBMCs

from naïve cattle undergoing lethal challenge exhibit only non-specific cytolytic activity (Emery *et al*., 1981; Houston *et al*., 2008).

Direct evidence indicating that CTL responses mediate immunity has been shown in two studies. The first of these studies involved application of a limiting dilution assay to analyse the parasite strain specificity of CTL responses in relation to cross-protection in individual animals immunised with the Muguga stock of *T. parva* and subsequently challenged with a cloned population of *T. parva* Marikebuni and vice versa (Taracha *et al*., 1995a). These studies showed that a proportion of animals immunised with either parasite exhibited cross-reactive memory CTL, whereas CTL detected in the remaining animals were specific for the homologous parasite. On challenge with the heterologous stock, those animals with cross-reactive CTL were solidly protected while those with strain-specific CTL showed moderate to severe reactions. The finding of a close association between parasite strain-specificity of the CTL response and protection against challenge provided strong evidence that CTL are important in mediating immunity. In the second study, the capacity of $CD8^+$ T cells to confer protection was examined by adoptively transferring lymphocytes enriched for $CD8^+$ T cells from immune to naïve monozygotic identical twin calves (McKeever *et al*., 1994). Lethally infected naive calves received $CD8^+$ T-cell enriched populations of cells or T cells depleted of the $CD8^+$ T cell subset derived from their immune monozygous twin partners to coincide with the emergence of patent schizont parasitosis. The calves that received cell populations enriched for $CD8^+$ T cells controlled the infection whereas the animals that received the population depleted of $CD8^+$ T cells underwent severe infection comparable to that in the challenge control. These findings indicated that parasite-specific $CD8^+$ T cells play an important role in the immunity against *T. parva*.

While the experiments described above provided evidence that $CD8^+$ T cells are important mediators of immunity, they do not exclude the possibility that other cells can also act as immune effectors and/or are required for induction of the $CD8^+$ T cell response. Infection with *T. parva* is known to result in strong $CD4^+$ T cell responses against the parasitized lymphoblasts (Baldwin *et al*., 1987; Brown *et al*., 1989). Further to this, *in vitro* experiments have indicated that $CD4^+$ T-cell derived factors are required for induction of CTL (Taracha, Awino and McKeever, 1997). In this study, purified populations of bovine immune and naïve $CD8^+$ T cells were co-

cultured with autologous *T. parva*-infected lymphoblasts in the presence or absence of immune $CD4^+$ T cells. It would be expected that culture of $CD8^+$ T cells from immune animals with *T. parva*-infected lymphoblasts would result in a population with detectable CTL activity. However, neither population developed CTL activity when cultured with *T. parva* infected lymphoblasts alone, whereas incorporation of immune and not naïve $CD4^+$ T cells in the cultures supported the generation of parasite-specific CTL from both immune and naïve $CD8^+$ precursors. This observation is consistent with those of previous studies that prolonged maintenance of parasite-specific CTL clones (Goddeeris, Morrison and Teale, 1986) required addition of exogenous T-cell growth factors or IL2 (Dobbelaere *et al*., 1988), and hence it is likely that these factors are provided *in vivo* by *T. parva*-specific $CD4^+$ T cells responding to *T. parva*-infected lymphoblasts. In addition to the release of soluble immunomodulatory factors, $CD4^+$ T cells have been shown to play a crucial role in the optimal stimulation of $CD8^+$ by activation of antigen presentation. This effect is mediated by ligation of CD40 on the surface of antigen presenting cells (APC) by CD40L (CD154) expressed on activated $CD4^+$ T cells (Mackey *et al*., 1998a and b; Shoenberger *et al*., 1998; Toes *et al*., 1998; Clarke, 2000; Diehl *et al*., 2000; Ma and Clark, 2009). In the context of *T. parva* infection, the important role of $CD4^+$ T cell responses in immunity may further be supported by recent findings from an immunogenicity study of potential CTL vaccine antigens in which antigen-specific $CD4^+$ T cell responses were undetected, and this appeared to coincide with the generally poor $CD8^+$ T cell responses (Graham *et al*., 2006).

Previous studies have established that bovine γδ T cells also respond to *T. parva* schizont-infected cells and that this population expands during the course of infection (Daubenberger *et al*., 1999). Findings from this study provided evidence that bovine γδ T cells participate in the early phase of an immune response against *T. parva*. These responses are MHC-unrestricted and cross-reactive between different parasite stocks. The precise role of these responses is unknown but it is possible that they may play a role in the innate arm of immunity to infection.

1.3 Molecular mechanism of antigen recognition by T cells

1.3.1 Mechanism of recognition of antigen by $CD4^+$ and $CD8^+$ T cells

It is now widely understood that $CD8^+$ and $CD4^+$ T cells recognise antigenic peptides bound to major histocompatibility complex (MHC) class I and II proteins, respectively, by virtue of their α/β heterodimeric T-cell receptor (TCR) (reviewed by Schwartz, 1985; Dembic *et al*., 1986; Saito *et al*., 1987). The current accepted model of antigen recognition by T cells proposes that an MHC molecule binds a fragment of antigen forming an MHC-peptide complex that is recognised by the TCR.

Direct peptide binding studies have shown that $CD4^+$ T cells of appropriate TCR consistently recognised bound peptide suggesting that the TCR recognises antigen in the form of processed peptides associated with MHC class II molecules. Further studies focusing on the mechanisms of peptide processing demonstrated that MHC class II bound peptides are generally derived from exogenous soluble proteins (Watts, 1997). Uptake of soluble antigens is achieved through non-specific mechanisms such as pinocytosis and phagocytosis or by specific receptor-mediated endocytosis by MHC class II-positive antigen-presenting cells (APC). Antigens are proteolytically degraded in endosomes into fragments (Shimonkevitz *et al*., 1983; Unanue, 1984; Unanue and Allen, 1987; reviewed in Watts, 1997), although unfolding of the intact protein without actual proteolysis has been shown to be sufficient to allow presentation of antigen in a number of cases (Streicher *et al*., 1984; Allen and Unanue, 1984; Berzofsky, 1985). The association of antigen and MHC class II is thought to take place in the endosome/lysosome system before the peptide/class II MHC complex is exported to the cell surface.

Several studies have also demonstrated that $CD8^+$ T cells recognise antigen in the form of processed peptides associated with MHC class I molecules (Townsend *et al*., 1985; Townsend *et al*., 1986a, b; Maryanski *et al*., 1986; Bastin *et al*., 1987; Braciale *et al*., 1987; Bodmer *et al*., 1988). Studies by Townsend and others working on influenza A virus provided the first clues to the nature of epitopes recognised by CTL. Mouse L cells transfected with overlapping deletion mutants of the influenza virus nucleoprotein gene, were used as targets to map CTL epitopes on the molecule. Further experiments using epitope-containing 13–16 mer synthetic peptides to

sensitise uninfected targets to lysis by antigen-specific CTL provided more evidence of $CD8^+$ TCR recognition of MHC class I -peptide complexes. Maryanski and other workers (1986) provided the evidence that this recognition of antigenic fragments by MHC class I -restricted CTL is a general feature not limited to antigens of intracellular pathogens. They showed that a MHC class I -restricted mouse CTL clone could lyse a mouse tumour cell line transfected with class I human leucocyte antigen (HLA) gene or pulsed with a 16-amino acid synthetic HLA peptide corresponding in sequence to an epitope recognised by a mouse CTL clone. There are several reports that the MHC class I -complexed peptides are generated from cytosolic proteins degraded in the proteasome and transported to the endoplasmic reticulum where a nascent MHC class I heavy and light (β2-microglobulin) chains assemble and bind the peptide prior to being exported to the surface of the APC (Townsend *et al*., 1986; Morrison *et al*., 1986; Germaine, 1986; Yewdell *et al*., 1988; reviewed in Pamer and Cresswell, 1998).

Although MHC class I molecules primarily present endogenous antigens, exogenous antigen can be processed by antigen presenting cells and presented by MHC class I molecules to CD8+ T cells (Harding 1996; Cresswell *et al*., 2005; Rock and Shen 2005). This phenomenon was first described *in vivo* in a mouse model and termed cross-priming (Bevan, 1976). MHC class I presentation of exogenous antigens is the mechanism enabling professional antigen-presenting cells (APCs), in particular dendritic cells, to induce $CD8^+$ T-cell responses against viruses that do not have access to the classical pathway for MHC class I presentation in APCs (Arrode *et al*., 2000; Herr *et al*., 2000; Larsson *et al*., 2001, Tabi *et al*., 2001). In this process the APC acquires antigen through endocytic mechanisms, especially phagocytosis or macropinocytosis. MHC class I presentation of exogenous antigens by APCs can occur via at least 2 distinct mechanisms (Cresswell *et al*., 2005; Rock and Shen 2005). Antigens can be conveyed from the endocytic compartment into the cytosol in APCs (Pfeifer *et al*., 1993; Harding *et al*., 1994; Norbury *et al*., 1995; Kovacsovics-Bankowski *et al*., 1995; Reis e Sousa *et al*., 1995; Li *et al*., 2001). In the cytosol, antigens are degraded into oligopeptides and transported via transporter associated with antigen processing (TAP) into the endoplasmic reticulum (ER) for loading onto newly synthesized MHC class I molecules (Norbury *et al*., 1995; Kovacsovics *et al*., 1995; Sousa *et al*., 1995; Sigal and Rock, 2000; Li *et al*., 2001). Alternatively, antigens can be processed in endosomal compartments where peptides are generated,

acquired by recycling MHC class I molecules, and transported to the cell surface for presentation (Pfeifer *et al*., 1993; Harding *et al*., 1994).

Both MHC class I and II molecules display the capacity to bind stably to many different peptides to accommodate the full TCR repertoire of an individual. This is consistent with the findings that several peptides could bind to the same molecule class II and this binding could be correlated with recognition of the peptides by helper T cells in *in vitro* proliferation assays (Babbit *et al*., 1985; Buus *et al*., 1986, 1987; Guillet *et al*., 1986, 1987; Sette *et al*., 1987). In addition to showing the promiscuity of MHC class II molecules in peptide-binding, the findings also showed that most peptides competed for binding to the same class II molecule and those that had high avidity bound best. Similarly, Chen and Parham (1989) demonstrated direct binding of synthetic peptides on purified human leucocyte antigens (HLA) class I molecules. The promiscuity of the class I molecules in binding a wide range of peptides has been demonstrated in competition studies with synthetic peptides; simultaneous addition of competing peptides to an assay system led to inhibition of target cell lysis (Maryanski *et al*., 1988; Pala *et al*., 1988; Bodmer *et al*., 1989). The elucidation of the three-dimensional structure of the human MHC class I molecule, HLA-A2, obtained by X-ray crystallography revealed a cleft situated between two α-helices derived from the α1 and α2 domains of the molecule as described in detail by Bjorkman *et al*., (1987a,b). The cleft, incorporating many of the polymorphic residues of class I has been shown to be the binding site for peptides. Indeed, numerous examples of MHC class I -peptide complexes have since been successfully crystallised and their three-dimensional structures determined (Chen and Parham, 1989; Garboczi *et al*., 1992; Jones, 1997).

The concept that MHC restriction molecules function as receptors of broad specificity for a multitude of antigenic fragments was further demonstrated with the elucidation of the structure of a second human MHC class I molecule, HLA-Aw68 that revealed considerable similarity to HLA-A2 except for 11 amino acids substitution at polymorphic residues in the antigen-binding cleft. These substitutions were shown to alter the detailed shape and electrostatic charge of that binding site resulting in localised structural differences that provide the basis for allelic specificity in foreign antigen binding and the accompanying responsiveness or lack of it (Garrett *et al*., 1989). Subsequent studies in human and murine models have defined

polymorphic residues that are critical for the presentation of foreign peptide to CTL (McMichael *et al*., 1988; Nikolic-Zugic and Carbone 1990; Kier-Nielsen *et al*., 2002) and shown that changes at certain residues within the peptide binding cleft can affect antigen presentation by either interfering with peptide binding or presumably by changing peptide conformation of bound peptide. The structural basis for peptide binding to MHC class I molecules has been extensively investigated in humans and mice (Wilson *et al*., 1993; Madden, 1995), less so for other species (Speir *et al*., 2001; Koch *et al*., 2007). Less is known of the detailed cellular and molecular interactions of MHC class I molecules in cattle, an economic important species. In cattle peptide binding properties have been investigated for only a small number of MHC class I alleles (Hedge *et al*., 1995; Gaddum, Willis and Ellis, *et al*., 1996). Several $CD8^+$ T cell epitopes in *T. parva* have been identified recently (Graham *et al*., 2006, 2007, 2008). One of these was shown to be presented by the cattle MHC class I allele N*01301. Subsequent studies by Macdonald *et al*. (2010) perhaps present the first description of an MHC structure in cattle and the peptide binding properties of the cattle MHC class I allele N*01301 to an immunodominant 11mer *Theileria parva*-derived peptide. The overall structure of N*01301 is seen to be similar to that of other mammalian MHC class I molecules however the peptide is presented in a distinctive raised conformation generated by a hydrophobic ridge within the MHC peptide binding groove.

Another factor which may influence antigen recognition is the available T-cell antigen receptor repertoire (Deng *et al*., 1997), and constraints imposed by self tolerance (Yewdell and Bennick, 1999). The intrathymic selection events leading to the generation of T-cell repertoire involves expression of rearranged T-cell receptor genes and selection of T cells based on the avidity of their receptors for self-MHC molecules. Current evidence suggests that T cells with medium or high affinity receptors for self-MHC molecules are deleted as part of the process of induction of self tolerance. In addition, there is positive selection of T cells with low affinity receptors for self MHC and these give rise to a repertoire of T cells capable of reacting with foreign peptides plus self MHC with relatively high avidity. This is manifested by the capacity of the MHC to influence the responsiveness to certain antigens. Indeed, it has been shown that the specificity of CTL responses of mice and humans to virus-infected cells is influenced by their MHC phenotype (Zinkernagel and Doherty, 1979; Vitiello and Sherman, 1983; Townsend and McMichael, 1985).

Immunological dominance is a central feature observed particularly in $CD8^+$ T cell responses to pathogens most notably HIV-1, influenza and a number of herpes viruses (Zinkernagel and Doherty, 1979; Goulder *et al*., 1997; Butz and Bevan 1998b; Yewdell and Bennick, 1999; Crotzer *et al*., 2000). Few of the enormous number of peptides encoded by the pathogen elicit responses, and even among these few, responses are often skewed heavily to one or several peptides termed immunodominant determinants at the expense of the less preferred sub-dominant epitopes (Sercarz *et al*., 1993). A similar phenomenon has been postulated to exist in *T. parva* infections based on a number of observations. First, a direct role for class I MHC molecules in the generation of these responses has been confirmed by the use of a monoclonal antibody specific for a monomorphic determinant on bovine MHC class I to block the cytolytic activity against infected target cells (Morrison *et al*., 1987b). Second, by examining cytolytic activity of immune peripheral blood mononuclear cells against infected target cells derived from animals of various MHC phenotypes it was observed that responses appeared to be biased toward one or other of the haplotypes in each animal, and that within the group of animals tested, certain bovine leucocyte antigen (BoLA) specificities dominated as restricting elements (Morrison *et al*., 1987; Taracha *et al*., 1995a; Morrison, 1996). These features of the immune response were attributed to differences in the frequencies of CTL precursors with particular specificities for certain MHC-peptide combinations, and possibly also as a reflection of the relative affinities of different epitopes for the MHC binding site. Third, while the CTL response is largely parasite strain–specific, some animals can make a cross-reactive response to challenge with heterologous parasites (Goddeeris *et al*., 1990; Taracha *et al*., 1995a). Further, it was observed that the CTL response in majority of the animals was restricted by a product of only one of the parental class I haplotype indicating that the haplotype was capable of binding and presenting both strain-specific and conserved epitopes (Taracha *et al*., 1995b). In sum, MHC affinity, MHC-epitope avidity, T-cell repertoire and immunodominance are key factors that influence antigen recognition by T cells.

1.3.2 Bovine class I major histocompatibility complex genetics

MHC class I genes are among the most characterised polymorphic gene families. In humans, three distinct genomic loci encode class I human leucocyte antigens (HLA); HLA-A, HLA-B and HLA-C (Linert and Parham, 1996) allowing

individuals to express six different HLA class I molecules. By contrast, current evidence from mapping studies as well as haplotype and phylogenetic analyses in cattle suggests the number of distinct genomic loci are at least six and the number of different class I bovine leucocyte antigens that are expressed in individual animals are variable (Ellis *et al*., 1999; Ellis and Ballingall, 1999; Ellis, 2004; Birch *et al*., 2006; Babiuk *et al*., 2007). BoLA diversity has primarily been determined using serological reagents to analyse haplotypes, and over 50 distinct class I serological specificities have been defined (Davies *et al*., 1994). In contrast to the human MHC, bovine haplotypes differ from one another in both the number and composition of expressed classical class I genes, bearing one to three gene products (Birch *et al*., 2006). In addition, there are rare cases in which a haplotype expressing two genes may be associated with two serological specificities (Bensaid *et al*., 1991). Currently, there are nearly 95 classical class I gene full-length sequences. Most have not been assigned to a definitive locus and hence they are currently named in a single series prefixed N* (http://www.ebi.ac.uk/ipd/mhc/bola/; Ellis *et al*., 2005; Robinson *et al*., 2005).

Although there are limited functional data on cattle class I loci, peptide binding and CTL recognition has been determined for alleles encoded at five of the putative six loci, indicating that they can each function as classical class I genes (Bamford *et al*., 1995; Hegde *et al*., 1995; Gaddum *et al*., 2003; Graham *et al*., 2008). A very small number of cattle class I MHC haplotypes has been investigated for function. Table 2 highlights some of the alleles that are fully characterised. One of the functional consequences of cattle class I haplotype diversity is the expression levels of the functional products of all or most of the six loci. A number of cattle class I alleles have been identified that have significantly lower than usual levels of surface expression (Smith and Ellis, 1999). It has also been suggested that for class I haplotypes expressing two or three loci, one may have functional dominance over others based on criteria such as recognition by alloreactive mAb and CTL. There are data indicating that CTL responses to *T. parva* are often restricted by the products of one class I allele. Studies by Morrison *et al*. (1987), Goddeeris *et al*. (1990), Taracha *et al*. (1995b) showed that CTL responses to *T. parva* focused predominantly on one of four polymorphic class I products that were expressed in heterozygous animals. This is further supported by studies by Graham *et al*., (2008) and MacHugh *et al*., (2009) which showed that animals heterozygous for A18 consistently respond to a *T. parva* antigen presented by MHC class I allele N*01301 with over 75% of the

responding $CD8^+$ T cells being specific for the *T. parva* antigen in A18 homozygous animals (MacHugh *et al*., 2009). Thus despite its antigenic complexity, $CD8^+$ T cell responses induced by infection with the parasite show profound immunodominance of restricting haplotypes or class I alleles. Mechanisms underlying this phenomenon are unclear but with recent developments in technology more data relating to levels and functional role of MHC class I allelic polymorphism can be generated. Recently, it has been shown that MHC class I tetramer technology can be adapted for use in domestic animals by the successful construction of equine and bovine tetramers (Travis McGuire *et al*.; Shirley Ellis *et al*.; Didier Colau *et al*., unpublished data).

1.3.3 Events leading to activation of T cells

$CD4^+$ and $CD8^+$ T cells are known to play distinct roles in the function of the adaptive immune system. $CD4^+$ T cells are central in the initiation of both antigen-specific B-cell and $CD8^+$ T-cell responses by providing cognate and non-cognate helper signals (Singer and Hodes, 1983; Sprent *et al*., 1986; Ahmed, Butler and Bhatti, 1988). On the other hand, $CD8^+$ T cells are known to mediate immune surveillance against autologous cells undergoing malignant transformation or infected with intracellular pathogens and also to effect rejection of histo-incompatible tissues (Goddeeris *et al*., 1986; Rosenberg and Singer, 1988; Boon *et al*., 1994). To provide these functions, T cells need to differentiate from a resting precursor stage to activated effector cells. Robust activation of naïve T cells requires three signals (Bachmann *et al*., 1999a; Andreasen *et al*., 2000; reviewed by Schwartz, 2003). The first signal is delivered through binding of the TCR to peptide/MHC complexes on APCs in which the CD4 and CD8 surface proteins bind to non-polymorphic domains of MHC class II and I molecules, respectively. The second signal is provided by interaction between co-stimulatory molecules (CD80/86) on the professional APCs and their ligand CD28 on the responding T cells. The avidity of the TCR-peptide/MHC interaction coupled with the CD80/CD28 and CD86/CD28 engagement is stabilised by cell adhesion molecules and their ligands, namely, CD2/LFA-3 and LFA-1/ICAM-1, in a supramolecular adhesion cluster (Paul and Seder, 1994; Bromley *et al*., 2001). The essential role of co-stimulatory molecules has been demonstrated in blocking experiments using anti-CD80/CD86 antibodies or chimeric fusion proteins that recognise both ligands to induce T cell unresponsiveness (Bachmann *et al*., 1999b). Antigen recognition in the absence of co-stimulation inactivates naïve T cells thereby inducing a state of anergy expressed as the inability to produce IL-2. Anergic T cells fail to proliferate and

Table 2: Serologically-defined bovine class I haplotypes and expressed class I alleles.

Allele name	Local names	Associated serological specificity	Breed	Reference
N*00101	5.1	A10	Boran	Bensaid *et al*., Immunogenetics (1991)
N*00102	JSP.2	A10	Boran	Ellis *et al*., Immunogenetics (2005)
N*00103	JSP.3	A10	N'Dama	Ellis *et al*., Immunogenetics (2005)
N*00201	JSP.1	A10	Holstein	Pichowski *et al*., Immunogenetics (1996)
N*00301	KN104	KN104	Boran	Bensaid *et al*., Immunogenetics (1991)
N*01201	A10.2; T2a	A10	Holstein	Ellis *et al*., Immunogenetics (2005)
N*01301	HD6	A18 (A6)	Holstein	Ellis *et al*., Immunogenetics (1996)
N*01302	HD6.1	A18	Holstein	Ellis *et al*., Immunogenetics (2005)
N*01701	D18.2, AH11-A	A11,	Holstein,	Ellis *et al*., Immunogenetics (1999)
N*01801	D18.3	A11	Holstein	Ellis *et al*., Immunogenetics (1999)
N*01802	AH11-B	A11	Holstein	Unpublished
N*02001	MAN3	A12 (A30)	Angus	Ellis *et al*., Immunogenetics (1999)
N*02101	HD1	A31 (A30)	Holstein	Ellis *et al*., Immunogenetics (1996)
N*02201	HD7	A31 (A30)	Holstein	Ellis *et al*., Immunogenetics (1996)
N*02301	D18.4	A14	Holstein	Ellis *et al*., Immunogenetics (1999)
N*02401	D18.1	A14, A15	Holstein	Ellis *et al*., Immunogenetics (1999)
N*02501	D18.5	A14, A15	Holstein	Ellis *et al*., Immunogenetics (1999)

Haplotypes/alleles indicated in bold were used in the current study

differentiate into effector cells when they re-encounter antigen even in the presence of co-stimulation (Schwartz, 2003). Inflammatory cytokines, including IL-1, IL-6, IL-12, and IFN-γ provide a third signal that acts directly on T cells to optimally activate clonal expansion and differentiation (Curtsinger *et al*., 1999; Curtsinger and Mescher, 2010). Studies have also demonstrated a requirement for $CD4^+$ T-cell help during the generation of primary $CD8^+$ T cell responses, in particular CD40L-CD40 interaction. Activated T cells express CD40L that binds to CD40 on APCs to facilitate clonal expansion and differentiation (Mackey *et al*., 1998a and b; Shoenberger *et al*., 1998; Clarke, 2000; Diehl *et al*., 2000; Ma and Clark, 2009). In instances where priming of $CD8^+$ T cells requires the presence of $CD4^+$ T cells, a three-cell interaction model of activation has been proposed (Wagner *et al*., 1980) which entails that a $CD4^+$ T cell and a $CD8^+$ T cell simultaneously recognise their specific antigens on the same APC. This ensures that the activated $CD4^+$ T cell apart from providing the cognate (cell-cell contact signals) input, delivers the non-cognate (cytokine) signals in the immediate micro-environment of $CD8^+$ T cells and APCs. In addition to cytokines such as IL-2 and IL-7, among others, targeted at the $CD8^+$ T cells, activated $CD4^+$ T cells trigger APCs to express higher levels of co-stimulatory molecules (Andreasen *et al*., 2000; Blazevic, Truby and Shearer, 2001; Croft, 2003; Liang and Sha, 2002; Weninger, Manjunath and von Adrian, 2002; Seder and Ahmed, 2003).

Once signals are delivered, the T cells are stimulated to undergo clonal proliferation and differentiation into activated cells armed with effector molecules and immunological memory. $CD4^+$ T cells divide rapidly and differentiate into one of several T-helper (T_H) subtypes including T_H1, T_H2, T_H3, T_H17 or T_{FH} which secrete different cytokines to facilitate a different type of immune response. The mechanism by which $CD4^+$ T cells are directed into a particular subtype is poorly understood, though signalling patterns from the APC are thought to play an important role (Curtsinger 1999; Gutcher and Betcher, 2007). Effector $CD8^+$ T cells exhibit direct *ex vivo* effector function such as cytotoxicity and cytokine production upon TCR engagement (Harty, Tvinnereim and White, 2000; Harty and Badovinac, 2002; Woodland and Dutton, 2003). Memory T cells exhibit only minimal *ex vivo* activity if compared with effector cells but are able to produce cytokines with equivalent kinetics to effector T cells upon reactivation. Memory T cells typically express the cell surface protein CD45RO and comprises of two subtypes: central memory T cells (T_{CM}) and effector memory T cells (T_{EM}). Central memory T cells express CCR7 and

CD62L and preferentially reside in secondary lymphoid organs while effector memory T cells are devoid of these cell surface markers and preferentially localise in peripheral tissue (Sallusto *et al*., 1999; Weninger *et al*., 2001). Both subsets can however be found in blood and spleen. These cells display rapid mobilisation of the effector molecules upon antigen contact (Lalvani *et al*., 1997; Bachmann *et al*., 1999a; Veiga-Fernandes *et al*., 2001). Analyses suggest that memory T cells require reduced antigen levels and are less dependent on co-stimulation probably due to increased expression of adhesion molecules that enhance the TCR avidity, and higher precursor frequency of antigen-specific cells (Oehen *et al*., 1992; Busch *et al*., 1998; Ieezi *et al*., 1998; Bachmann *et al*., 1999a; Berard and Tough 2002).

With regard to stimulation of T cells by *T. parva*-infected cells, although a role for dendritic cells has been proposed in the priming of naïve T cells, parasitized T cells have been shown to express co-stimulatory molecules and are therefore capable of providing both a priming and recall APC function (Morrison and McKeever, 2006). As stated earlier, a requirement for antigen-specific $CD4^+$ T cells in the priming and recall of *T. parva*-specific $CD8^+$ T cell responses has been described *in vitro* (Taracha, Awino and McKeever, 1997).

1.3.4 Effector mechanisms operative in activated $CD8^+$ T cells

As aforementioned, $CD8^+$ T cells acquire effector function upon appropriate activation with APC-delivered specific antigen and accessory signals. Following several rounds (>8-10 cell divisions) of clonal expansion, $CD8^+$ T cells become immune effectors by virtue of gaining the capability to induce cytolysis of autologous infected cells and/or elaborate cytokines and chemokines that mediate several activities (Harty, Tvinnereim and White, 2000; Harty and Badovinac, 2002; Woodland and Dutton, 2003). $CD8^+$ T cells are heterogenous in terms of their mechanisms of effector function (Harty and Badovinac, 2002; Woodlands and Dutton, 2003) and other studies (Aandahl *et al*., 2003) have shown that CD7 expression defines $CD8^+$ T cell subpopulations capable of mediating cytolysis or cytokine secretion. The highly coordinated expression of both cytolytic and cytokine effector activities is tightly regulated through TCR-dependent signals.

$CD8^+$ T cells secrete cytokines including IFN-γ, TNF-α and TNF-β as well as chemokines, which contribute to host defence in several ways. These effector

molecules recruit and/or activate the microbicidal activities of effector cells such as macrophages and neutrophils. Cytokines also directly interfere with pathogen attachment, pathogen gene expression or may limit intracellular replication. For instance, IFN-γ inhibits viral replication directly and also induces class I MHC expression and macrophage activation. TNF-α and TNF-β can synergize with IFN-γ in macrophage activation and in killing some target cells through their interaction with TNF receptors on target cells.

On the other hand, $CD8^+$ T cells are able to induce cytolysis of infected cells by two distinct contact-dependent molecular pathways: the granule exocytosis pathway or through the interaction of the T-cell Fas-ligand (CD178) with the Fas (CD95) molecule on the target cell (Henkart, 1985; Kagi *et al*., 1994; Kagi *et al*., 1996; Zimmermann and Green, 2001; Barry and Beackley, 2002; Russell and Ley, 2002). A schematic representation of Fas-Fas ligand interaction and the granule exocytosis pathway is shown in Figure 2. Both of these pathways, activated in response to signals from the TCR stimulate the caspase cascade in the target cell leading to apoptotic death. Three distinct classes of cytotoxic proteins including perforin, granzymes A and B and granulysin have been identified from natural killer cells and cytotoxic CTL (Stenger *et al*., 1998; Ernst *et al*., 2000; Krensky *et al*., 2000; Ochoa *et al*., 2001; Smyth *et al*., 2001; Lieberman, 2003). Perforin is released on contact and polymerises in the presence of calcium to form pores in the target cell membrane lysing and killing target cells by necrosis (Voskoboinik, Smyth and Trapani, 2006); it also permits delivery of pro-apoptoptic proteases granzymes A and B into the target cell (Lowin *et al*., 1995; Trapani and Smyth, 2002; Trambas *et al*., 2003, Bolitho *et al*., 2007). Two competing models for delivery of granzymes have been proposed: diffusion of the granzymes through a plasma membrane perforin channel versus coendocytosis of perforin and granzymes with subsequent disruption of endosome membrane by perforin to release the granzymes. However, the precise molecular mechanism remains to be understood. The molecular mechanism by which perforin initiates polymerisation has been reviewed in Baran *et al*., (2009) and Podack (2009).

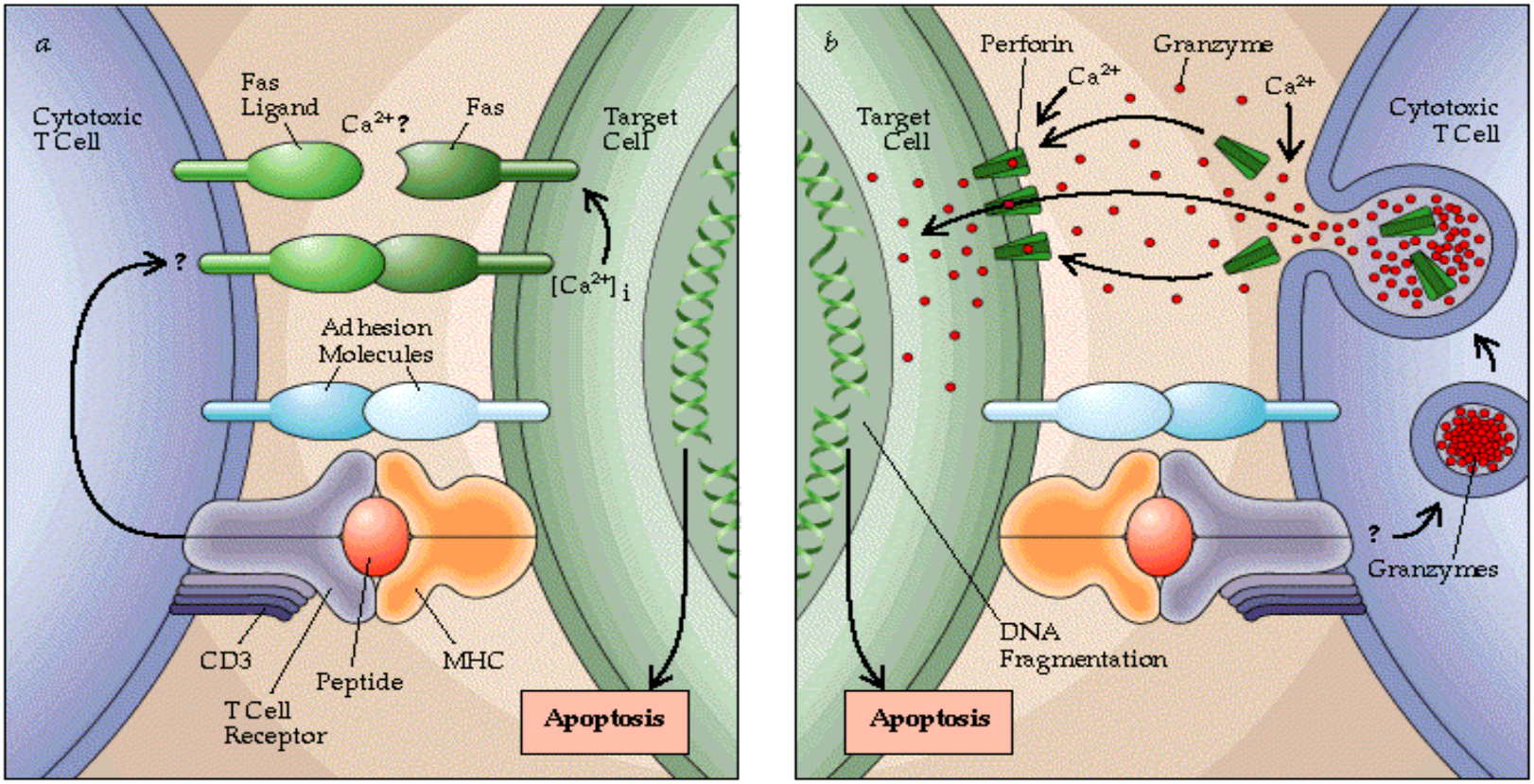
The cytotoxic proteins are contained in granules as demonstrated in experiments where purified granules from cytotoxic T cells when added to target cells *in vitro* resulted in lysis of the cells by creating pores in the lipid bi-layer. These

Figure 2: $CD8^+$ T lymphocyte effector mechanisms

a) Apoptosis through non-secretory Fas-Fas ligand interaction

b) Granule exocytosis of an effector cytotoxic $CD8^+$ lymphocyte

Figure sourced from Davidson College: Homepage Template for Biology students (http://www.bio.davidson.edu/../perforin.html)


a
Cytotoxic T Cell
Fas Ligand
Ca2+?
Fas
Target Cell
?
[Ca2+]i
Adhesion Molecules
CD3
Peptide
MHC
T Cell Receptor
Apoptosis
b
Perforin
Granzyme
Ca2+
Ca2+
Target Cell
Cytotoxic T Cell
?
Granzymes
DNA Fragmentation
Apoptosis

proteins are stored in the lytic granules in an active form after post-translational modification upon synthesis. Both perforin and granzymes are cleaved by signal peptidase to remove the signal peptide. In the case of granzyme, a second enzyme (DPPI) cleaves off a short pro-sequence, (Caputo *et al*., 1993, Mcguire Lipsky and Thiele 1993; Smyth, Mcguire and Thia, 1995; Pham *et al*., 1998; 1999) and it is glycosylated in the golgi apparatus (Griffiths and Isaac, 1997). Perforin is synthesised as a 70-kDa precursor that is cleaved at the carboxyl terminus to yield the active 60-kDa form (Uellner *et al*., 1997). Although stored in an active form, conditions within the granules prevent them from functioning until after their release. Other than the cytotoxic granules being specialised lysosomes (de Saint Basile and Fischer, 2001), Fraser *et al*., (2000) showed that calreticulin, a chaperone protein of the endoplasmic reticulum that is known to exist in CTL granules, inhibits perforin-mediated target cell damage raising the possibility that calreticulin acts as a regulatory molecule that dampens the effect of perforin by stabilising membranes to prevent excessive damage.

The highly controlled delivery of effector signals is coordinated by the antigen-specific TCR in three ways: firstly, it induces the stable binding of the effector cells to the specific target cells to create a tightly held, narrow space in which effector molecules can be concentrated; secondly, it focuses the delivery of effector molecules at the site of contact by inducing a re-orientation of the secretory apparatus of the effector cell; thirdly, it triggers their synthesis and/or release (Montoya *et al*., 2002; Dustin, 2003; Trambas and Griffiths, 2003).

The relative roles of $CD8^+$ T-cell-mediated cytolytic and cytokine effector mechanisms in host resistance to viral, bacterial and protozoan intracellular infections have been described in mouse experiments and to a lesser extent in humans (Harty, Tvinnereim and White, 2000; Nickell and Sharma, 2000). Using perforin-deficient mice and antibodies directed at IFN-γ, it has been demonstrated that cytotoxic and cytokine responses contribute differentially to host immunity against different infections. While lytic $CD8^+$ T cell responses were shown to be the mechanism of resistance against the protozoan *Encephalitozoon cuniculi*, clearance of *Trypanosoma cruzi* infection was dependent on IFN-γ responses (Kumar and Tarleton, 1998; Khan *et al*., 1999). By contrast, immunity against acute and secondary infection with *Toxoplasma gondii* was based on IFN-γ responses whereas clearance of *T. gondii* chronic infections relied on $CD8^+$ T cell lytic activity (Denkers *et al*., 1997).

Similarly, host resistance to *Listeria monocytogenes* and *Chlamydia* infections was shown to be dependent on lytic and cytokine-mediated $CD8^+$ T-cell responses, respectively (Harty and Bevan, 1992; Perry *et al*., 1999a and b). Concerning viral infections, both lytic and cytokine-mediated $CD8^+$ T cell responses appear to contribute synergistically to host resistance.

In the case of *T. parva* infections, it is likely that $CD8^+$ T-cell-based immunity against *T. parva* might be an interplay of different mechanisms, some of which hitherto are unknown. $CD8^+$ CTL directed against the schizont infected cell constitute the dominant protective bovine immune response (Morrison *et al*., 1987; McKeever *et al*., 1994; Taracha *et al*., 1995a) and antigen-primed $CD4^+$ cells are required for effective activation of $CD8^+$ T cells by parasitized cells (Taracha *et al*., 1997). The use of the IFN-γ ELISpot also indicates $CD8^+$ T cell-mediated IFN-γ responses that parallel the antigen specificity of the lytic response (Graham *et al*., 2006). However the actual contribution of cytokine-mediated T cell responses in the development of immunity to *T. parva* remains largely unknown. Indeed, there has been inconclusive evidence on the activity of IFN-γ against the early or established schizont-infected cells. The addition of IFN-γ to cultures of PBMC immediately following infection with *T. parva* sporozoites has no inhibitory effects on the development of the parasite or survival of the infected cells. Indeed it was found that many parasitized cell lines express IFN-γ (DeMartini and Baldwin, 1991; McKeever *et al*., *1997*; Morrison and McKeever, 2006).

1.3.5 Assays for detection of antigen-specific $CD8^+$ T cells

Several methods have been developed to analyse $CD8^+$ T cell responses and to study their involvement in the immune response to infection under natural infections and experimental vaccination. The best-established methods are based on the ability of cytotoxic $CD8^+$ T cells to lyse appropriate target cells *in vitro*. These have been performed either under limiting dilution assay (LDA) conditions to provide a quantitative measurement of antigen-specific CTL precursors or under bulk culture conditions unsuited for quantifying CTL responses (Carmichael *et al*., 1993; Lau *et al*., 1994; Shaefer-Weaver *et al*., 2003). Another set of assays has included those that measure antigen-stimulated T cell cytokine production, particularly the IFN-γ ELISpot (Czerkinsky *et al*., 1983; Lalvani *et al*., 1997; Schneider *et al*., 1999) and intracytoplasmic cytokine staining (Suni, Picker and Maino, 1998; Badovinac and

Harty, 2000; Ghanekar *et al*., 2001) techniques. A more recent generation of ELISpot assays includes those that are based on human and mouse perforin and granzyme B effector molecules (Rininsland *et al*., 2000; Ewen *et al*., 2005; Zuber *et al*., 2005). The other distinct set of assays employed in the study of $CD8^+$ T cell responses has involved the quantitation of individual TCR transcripts (Kalams *et al*., 1994; Moss *et al*., 1995) and determination of TCR Vβ chain usage (Walker *et al*., 1995).

A long-standing need for appropriate assays to enable accurate measurement of the specificity and magnitude of the $CD8^+$ T cell response in infections in general has been widely noted. While the LDA has provided useful information on the kinetics and duration of antigen-specific effector and memory cytotoxic $CD8^+$ T cells, serious concerns that this assay has considerably underestimated the clonal burst size have been reported (Altman *et al*., 1996; Ahmed and Gray, 1996; Busch *et al*., 1998; Murali-Krishna *et al*., 1998). It has been argued that the LDA, which relies on *in vitro* stimulation of antigen-specific $CD8^+$ T cells for several days prior to measuring cytotoxic activity, underestimates the cell numbers as not all potential $CD8^+$ T cell responders grow in culture to become effectors. This is particularly evident with regard to those cells obtained from animals at the peak of activation that may undergo activation-induced cell death in the course of culture (Murali-Krishna *et al*., 1998; Green, Droin and Pinkoski, 2003). In addition, it has been demonstrated that another major cause of underestimation of CTL numbers by the LDA is overgrowth of specific effectors by other cells, arising from use of high input responder cell numbers per well, and that low input cell numbers are less likely to be overgrown thus providing a closer estimate of the true CTL frequency (Goulder *et al*., 2000). A technique in which tetramers of fluorochrome bound MHC class I molecules assembled with a single antigenic peptide are used to stain $CD8^+$ T cells with specificity for the particular peptide- MHC class I complex (Altman *et al*., 1996) has proved a major advance in the study of antigen-specific T cells. Analysis of tetramer positive $CD8^+$ T cells by flow cytometry provides a method that reliably quantitates the number of specific $CD8^+$ T cells present in peripheral blood (Murali-Krishna *et al*., 1998; Tan *et al*., 1999; Goulder *et al*., 2000; Appay *et al*., 2000; Appay and Rowland-Jones, 2002). The combination of tetramer staining and other techniques such as CD8 surface marker staining, IFN-γ, perforin or granzyme B ELISpot assays that measure responses at the single cell level, has provided a powerful tool to generate precise information on phenotype, antigen specificity and function of $CD8^+$

T cell responses. Assays utilising tetramer staining in conjunction with surface molecules for naïve ($CD45RA^{+ve}CD62L^{+ve}CCR7^{+ve}$), central memory ($CD45RO^{+ve}CD62L^{+ve}CCR7^{+ve}$) and effector memory ($CD45RO^{+ve}CD62L^{-ve}CCR7^{-ve}$) T cell phenotypes have been useful in categorising antigen-specific T cells into naïve, memory and effector subpopulations, respectively (Callan *et al*., 1998, 2000; Champagne *et al*., 2001; Aandahl *et al*., 2003). Combination of tetramer staining with measurement of the production of cytokines such as IFN-γ, TNF-α, chemokines (RANTES) and cytotoxins (perforin) has provided a means of examining the functional phenotype of circulating $CD8^+$ T cells with specificity for a particular antigen at a single cell level (Wilson *et al*., 1998; Pittet *et al*., 2001a; Appay *et al*., 2000; Benito *et al*., 2004). In addition to this antigen induced cell surface expression of CD107a and b has been used as an indicator identifying T cells that had degranulated and released effector proteins from their lytic granules (Betts *et al*., 2003; Betts and Koup, 2004) and to distinguish effector and memory subsets of $CD8^+$ T cells (Wolint *et al*., 2004). Monoclonal antibodies to TCR Vβ regions used in combination with tetramers have further been used to study TCR Vβ usage (Wilson *et al*., 1998; Valmori *et al*., 2000).

In the case of evaluation of *T. parva*-specific $CD8^+$ T cell responses, lymphoproliferation, target cell lysis by limiting dilution and bulk cultures of T cells as well as surface marker staining of responding cells have been employed (Goddeeris and Morrison, 1988; Taracha *et al*., 1992). While useful information has been derived from assays of short-term LDA and bulk cultures as well as long-term T cell lines and clones, concerns relating to loss of certain specificities due to skewing of the response during prolonged and repeated stimulation have been noted. On the other hand, in addition to the shortcomings already associated with the LDA, it is also extremely tedious, technically demanding and variable. More recently, a high throughput IFN-γ ELISpot assay has been optimised and used in antigen identification and immunogenicity studies (Graham *et al*., 2006). While this assay is more sensitive and less variable than LDA, it was found not to be a predictive marker for protection in cattle immunised with defined antigens and challenged with *T. parva* sporozoites and did not correlate with cytolytic responses. The prospect of applying bovine tetramers and ELISpot and intracellular staining assays based on perforin and granzyme A/B or IFN-γ in the study of $CD8^+$ T cell responses following vaccination with candidate

antigens or infection with the parasite is compelling.

1.4 Initiatives in development of improved vaccines against *T. parva*

1.4.1 Identification of *T. parva* antigens

The practical limitations imposed by using live parasites for vaccination against *T. parva* have justified the research endeavours to develop sub-unit vaccines based on defined parasite antigens. As noted earlier, efforts have focused on understanding the immune responses that mediate immunity against *T. parva* and using immune probes to identify candidate antigens for vaccination (Musoke *et al*., 1984; 1992; Graham *et al*., 2006; reviewed by Morrison and McKeever, 2006) in line with initiatives in other parasite systems (Schofield *et al*., 1987; Zhang, Lonning and McGuire, 1998; Nacer *et al*., 2001; Gaddum *et al*., 2003). The discovery of p67 and success in induction of antibody responses capable of neutralising the infectivity of sporozoites has led to the field testing of a p67-based vaccine with potential to be included in a multi-component ECF vaccine. As previously mentioned, many studies have provided strong evidence that in animals immunised with live parasites, cellular immune responses directed towards parasitized leucocytes play an important role in immunity. Considerable effort has therefore been focused on the identification of *T. parva* schizont antigens that provoke protective immune responses, subsequently testing these candidate vaccine antigens for immunogenicity and efficacy. Two approaches to schizont antigen identification were adopted taking advantage of the recent complete *T. parva* (Muguga) genome sequence and annotation (Gardner *et al*., 2005) and optimisation of a high throughput screening system used to identify antigens recognised by human tumour cell-specific $CD8^+$ T cells (DePlaen *et al*., 1997). Both strategies were dependent on screening APCs transiently transfected with parasite cDNA using well characterised bovine CTL in a high throughput IFN-γ ELISpot assay (Graham *et al*., 2006). The two approaches were complementary to each other. The first one was based on selecting candidate genes with recognisable peptide signal motifs to allow access of the encoded protein to the host cell cytosol and class I MHC processing pathway. Since some protein antigens lacking a signal peptide are known to access the class I MHC pathway (Nacer *et al*., 2001), a randomly constructed schizont cDNA library was also used to maximise the chances of identifying CTL antigens.

Applying these methods, six *T. parva* antigens recognised by $CD8^+$ T cells from immune cattle of different MHC genotypes were identified (Graham *et al*., 2006; Graham *et al*., 2007). Analysis of the predicted amino acid sequences of the genes did not reveal any obvious functional relationship between the proteins (Gardner *et al*., 2005). Two (Tp1 and Tp2) of these proteins were annotated as hypothetical and four (Tp4; elongation translation initiation factor 1A, Tp5; ε-subunit of T complex protein 1, Tp7; heat shock protein 90, Tp8; cysteine proteinase) were annotated as in other systems. Interestingly, three (Tp4, Tp5 and Tp7) of the antigens did not contain recognisable signal peptides and would therefore not have been identified by the approach using the genome sequence. The identification of Tp9 and Tp10 as a $CD8^+$ T cell antigens has recently been described (MacHugh *et al*., 2011; Graham *et al*., unpublished data). Potential orthologs of these antigens exist in other major protozoan parasites including *T. annulata*, *Cryptosporidium parvum* and *P. falciparum* with clear implications for wider vaccine application. It is noteworthy that using similar strategies (Graham *et al*., 2006) recent work by MacHugh *et al*., (2011) has identified three *T. annulata* antigens recognised by $CD8^+$ T cells (Ta5, Ta9 and Ta11), two of which represent the orthologs of the *T. parva* antigens Tp5 and Tp9.

The relevance of responses to these antigens to the control of infections *in vivo* was examined by analysing $CD8^+$ T-cell responses following sporozoite challenge of immune cattle (Graham *et al*., 2006). The $CD8^+$ T-cell response elicited was shown to have memory $CD8^+$ T cells specific for the antigens. The kinetics of these responses to four (Tp1, Tp2, Tp5 and Tp8) of the antigens correlated well with those previously described for protective schizont-specific CTL in efferent lymph and peripheral blood after challenge of immune animals (Morrison *et al*., 1987; McKeever *et al*., 1994), whereas a delayed response was observed for one antigen (Tp4) and no boosting of Tp7-specific $CD8^+$ T cells was observed probably suggesting a delayed expression of Tp4 during the parasite development in the host and a low frequency of Tp7-specific responder cells. The observation that active $CD8^+$ CTL responses against four of these antigens are detected *in vivo* coinciding with the remission of the challenge infection indicates that they are likely to be involved in the protective response and appear to be appropriate candidates for use in immunisation.

While the adoptive cell transfer studies (McKeever *et al*., 1994) suggested that effector $CD8^+$ T cells were, on their own, adequate to resolve a primary *T. parva*

infection, it was not clear whether helper $CD4^+$ T cells were required in the induction of the $CD8^+$ CTL. This is an important question in terms of defining strategies for identification, formulation and delivery of vaccine candidate antigens. Indeed, Taracha and colleagues (1997) demonstrated in *in vitro* studies that resting $CD8^+$ T cells require $CD4^+$ T-cell helper input, in the presence of appropriate antigenic stimulation, to acquire effector activity. Further, it was shown that naïve and memory $CD8^+$ T cells displayed distinct requirements with the former needing cell-cell cognate interactions. The implication of this finding is that vaccine candidate antigen(s) targeting naïve $CD8^+$ T cells need to be co-presented with $CD4^+$ T cell-specific antigen(s) in order to effectively induce antigen-specific $CD8^+$ CTL in vaccines. Although the six *T. parva* antigens identified using defined $CD8^+$ CTL do contain MHC class I epitopes, these epitopes do not appear to be expressed by the MHC class II molecules on the surface of infected cells (Graham *et al*., 2006). However, it is possible that some of these CD4 epitopes on the Tp antigens are indeed presented by other APCs (dendritic cells) after they take up apoptotic TpM (cross-presentation). This might be more relevant for CD8 T cell induction. Efforts are underway to identify further antigens using parasite-specific $CD4^+$ T cells in line with previous reports (Baldwin, Goddeeris and Morrison, 1987; Brown *et al*., 1989).

1.4.2 Vaccine delivery platforms for evaluating immunogenicity and efficacy

A major obstacle in the development of vaccines against diseases such as tuberculosis, malaria, theileriosis, leishmaniasis and HIV-AIDS caused by intracellular pathogens that require cell-mediated immunity is the lack of an antigen-delivery strategy to elicit protective antigen-specific $CD8^+$ CTL. In this regard, although all currently licensed human and livestock vaccines are efficient at inducing antibody responses, only vaccines derived from live attenuated organisms efficiently induce cellular immunity. It should be noted, however, that widespread use of live attenuated vaccines might be precluded by practical constraints such as manufacturing and safety concerns. Different forms of vaccination strategies using bacterial vectors such as *Listeria monocytogenes*, viral vectors e.g., modified vaccinia virus Ankara (MVA) strain, canary pox, adenoviruses as well as plasmid DNA vectors have been used in vaccine immunogenicity and efficacy studies (Schneider *et al*., 1998, 1999; Degano *et al*., 1999; McShane *et al*., 2001; Gilbert *et al*., 1999, 2002). These prime-boost regimes have been shown to induce potent $CD4^+$ and $CD8^+$ T cell–mediated

IFN-γ and lytic responses in rodents (Schneider *et al*., 1998, 1999; Degano *et al*., 1999; McShane *et al*., 2001; Gilbert *et al*., 2002) and, to a lesser extent, in humans (McConkey *et al*., 2003). Testing of such prime/boost regimes in cattle has resulted in the induction of T-cell responses to defined antigens (Taracha *et al*., 2003; Vordermeier *et al*., 2004).

Genes encoding five (Tp1, Tp2, Tp4, Tp5 and Tp8) of the six CTL target antigens were engineered in plasmid DNA, canary pox and MVA viruses and tested in BoLA-defined cattle for immunogenicity and efficacy (Graham *et al*., 2006). Plasmid DNA/MVA or canary pox/MVA prime/boost vaccination protocols were used to immunise animals simultaneously with the five antigens in separate vaccine constructs and challenged, alongside naïve cattle, with a lethal dose of *T. parva* sporozoites. Significantly, cells obtained from 29% of the vaccinates displayed CTL activity against parasitized cells. By contrast, 79% of vaccinated cattle mounted antigen-specific $CD8^+$ T-cell IFN-γ responses, which were boosted in the majority of animals following sporozoite challenge. The significance of the lytic $CD8^+$ T cell responses was evident when correlated with the outcome to challenge. While the challenge controls suffered clinical ECF, only 9 of 19 IFN-γ responders survived, with seven animals having made lytic $CD8^+$ T cell responses. The association between the ability to mount a lytic response and survival was highly significant with the mean ECF reaction index, a statistical measurement of disease severity, significantly lower than that of CTL nonresponders. On the other hand, the high number (10/19) of IFN-γ responders that succumbed to challenge would suggest that these responses are not predictive of protection against severe ECF or fatality. While the data validated these antigens for an in-depth study on effective antigen delivery strategies to induce $CD8^+$ T-cell based immunity, the need for assays to detect immune responses that correlate with protection is equally of interest.

1.5 Rationale and objectives of the study

The current study aims at developing a platform of immunological assays for evaluating different parameters of *T. parva* antigen-specific $CD8^+$ T cell effectors of immunity that would serve as immunological endpoints of protection for application in vaccine development. In particular, the study seeks to develop novel assays for determining the fine specificity and magnitude of $CD8^+$ T cells with cytolytic activity utilizing class I MHC-peptide complexes as well as molecules of the granule

exocytosis pathway. The overall objective of this study focuses on establishing a bovine tetrameric staining methodology in conjunction with perforin and granzymes A/B intracellular staining and release assays as a more sensitive and rapid system for quantifying epitope-specific cytotoxic granule-mediated $CD8^+$ T cell responses. The specific objectives of the study include:

1. To clone and express genes encoding bovine perforin, granzymes A and B

2. To generate polyclonal and monoclonal antibodies to bovine perforin and granzymes A and B

3. To develop and optimise perforin and granzyme A and B-based ELISA and ELISpot assays

4. To develop and validate bovine tetramer staining methodology using *T. parva*-specific cytotoxic $CD8^+$ T cell lines and clones of defined specificities

5. To develop and validate flow cytometric assays combining double-staining with tetramer and antibodies to CD8, perforin and granzymes A/B using $CD8^+$ T cells of defined specificities

6. To detect and enumerate epitope-specific $CD8^+$ T lymphocytes expressing perforin, granzymes A and B in peripheral blood lymphocytes from live vaccine immunised animals before and after challenge.

Chapter 2: Cloning, Sequence Analysis and Expression of Bovine Perforin, Granzymes A and B

2.0 Introduction

CTL subsets of PBMC have the ability to recognise bind and lyse specific target cells. From morphologic analysis of CTL-target conjugates, it has been proposed that the lytic mechanism of CTL resides in their cytoplasmic granules. Analysis of the contents of granules from cytotoxic lymphocytes and natural killer cells has identified and isolated several proteins including perforin (also referred to as cytolysin) and granule specific serine proteases, granzymes A and B. In the case of *T. parva* infections, the actual role of perforin and granzymes is largely unknown although it has been demonstrated that cytotoxic $CD8^+$ T cell responses contribute to host immunity. The availability of perforin and granzyme A and B will facilitate studies on the role of perforin and granzymes in lymphocyte mediated cytotoxicity and the subsequent development of alternative assays for CTL. However purification of sufficient amounts of granule proteins has been a major obstacle for performing extensive analysis of the role of perforin in lysis of target cells as well as granzyme pathways for inducing apoptosis. Indeed the isolation of granules from bovine CTL has been unsuccessful (Francis Chuma *et al*., personal communication). Thus recombinant DNA approaches have been used to produce perforin and granzyme proteins in heterologous systems. Chapter 2 reports on the cloning, sequencing, structural analysis and expression of cDNAs that code for bovine perforin, granzyme A and B. The data shows that a full-length cDNA encoding bovine perforin and a cDNA fragment encoding bovine granzyme A has been cloned and sequenced, whereas a full-length cDNA with characteristics resembling those of the granzyme B family has been obtained. Further to this, using the baculovirus expression system, truncated forms of these bovine proteins have been prepared.

2.1 Materials and methods

2.1.1 Preparation of perforin, granzymes A and B single strand cDNA

Total RNA was isolated from $1x10^7$ bovine CTLs using the acid guanidinium thiocyanate-phenol-chloroform extraction (AGPC) method described by Chomczynski and Sacchi (1986). CTLs, generated and maintained as described in section 2.1.3.5.2 were harvested on day six post re-stimulation by centrifugation at 1500 rpm at 4°C for 10 min (Heraeus Megafuge, Model No. 2.0R) and washed twice in cold PBS pH 7.4. Cells were lysed by resuspending in 500 µl of denaturing solution (4M Guanidine thiocyanate, 25mM sodium citrate-pH 7.0, 0.5% Sarcosyl, 0.1M β-2 Mercaptoethanol), and RNA extracted by addition of 50 µl of 2 M sodium acetate, 500 µl of water-saturated phenol and 100 µl of chloroform-isoamyl alcohol mixture (49:1), in that order. After vortexing for 10 seconds, the mixture was incubated on ice for 15 min followed by centrifugation at 14000 rpm (Bench top Eppendorf centrifuge Model No. 5402) for 10 min at 4°C. The aqueous phase (containing the RNA) was transferred into a fresh eppendorf tube and mixed with 2.5 volumes of ice-cold 100% ethanol, and incubated on dry ice for 30 min to precipitate RNA. After centrifugation at 14000 rpm for 10 min at 4°C, the pellet was re-dissolved in 300 µl of denaturing solution. RNA was re-precipitated as before by addition of 750 µl of ice-cold 100% ethanol followed by incubation on dry ice. After further centrifugation, the pellet was washed with ice-cold 70% ethanol, dried overnight at room temperature and re-dissolved in 30 µl of RNase free water. The yield and integrity of the RNA was determined by agarose gel electrophoresis technique described by Pellé and Murphy (1993) as follows: 3 µl of the RNA sample was mixed with 7 µl of water (final volume 10 µl) to which 2 µl of sterile 6x loading buffer was added. The mixture was incubated at 75°C for 5 min to denature the RNA and immediately loaded onto a horizontal submarine gel. The gel was prepared by boiling 1.4 g agarose in 100 ml of 10 mM sodium phosphate buffer, pH 6.8, containing 1 µl of 10 mg/ml ethidium bromide. Electrophoresis was carried out at 3 to 7 V/cm in 10 mM sodium phosphate buffer containing 0.1 µg/ml ethidium bromide, while constant re-circulation of the buffer was maintained to prevent formation of a pH gradient. RNA was visualised on a 312 nm ultra-violet transilluminator (UVP Inc.). A digital image of the gel was captured with a computerised digital reader (UVP Inc).

Single strand-cDNA (ss-cDNA) was synthesised from the total RNA using the reverse transcription kit (Superscript™Reverse Transcriptase, Invitrogen) with a slight modification of the manufacturer's protocol as follows: 25 µl of RNA was first mixed with 9.5 µl of water and heated at 75°C for 5 min to denature the RNA. After chilling on ice for 3 min and brief centrifugation at 10000 rpm (Bench-top Eppendorf centrifuge model No.5417) to collect the contents in the bottom of the tube, a master mix consisting of 5 µl oligo dT, 5 µl 10x buffer, 2.5 µl 10 mM dNTP mix (dATP, dTTP, dGTP, dCTP), 1 µl Ribonuclease inhibitor, 2 µl Reverse Transcriptase was then added to a final volume of 50 µl and the mixture incubated at 37°C for 1 hr followed by heating at 75°C for 10 min to inactivate the reaction. After synthesis, the ss-cDNA was stored at -20°C or diluted in 10mM Tris, pH 8.5, at a 1:10 dilution and stored at 4˚C to be used as template in PCR to generate perforin, granzyme A and B cDNA. The quality of ss-cDNA was determined by PCR using primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) a constitutively expressed gene, as described. Briefly, PCR reagents (Promega Madison, Wisconsin, USA) were mixed in a 96-well microtitre plate as follows: 1.5 µl 25 mM $Mgcl_2$, 2.5 µl 10x buffer for *Taq* polymerase, 0.5 µl 10 mM dNTP mix, 0.5 µl 100 ng/µl GAPDH forward primer, 0.5 µl 100 ng/µl GAPDH reverse primer, 5 U/ul (~0.125 µl) *Taq* DNA polymerase, 5 µl of ss-cDNA template and 14.375 µl sterile distilled water to a final volume of 25 µl. The PCR reactions were placed in a thermocycler (Minicycler™, MJ Research) for amplification using the following program: 94°C for 2 min for initial denaturation followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min and a final extension at 72°C for 5 min. The PCR products alongside DNA size markers were resolved and analysed in a 1.2% agarose gel and visualised under UV transillumination as previously described.

2.1.2 Cloning of perforin, granzymes A and B cDNA into pGEM®-T Easy

Gene-specific primers were designed to obtain the complete coding sequences of bovine perforin, granzymes A and B, based on homology to known human, mouse, rat and sheep sequences. Sequences encoding human, mouse, rat and sheep molecules where available were retrieved from the public database at NCBI (http://www.ncbi.nlm.nih.gov/entrez/query). Accession numbers of the retrieved sequences are shown in Table 3. The sequence data obtained was submitted for

Table 3: Gene accession numbers and sequences for primers used in cloning bovine perforin, granzymes A and B

Perforin, granzymes A and B gene identification				
Accession number	Size	Predicted function	Organism	Source
GI:190339	1668 bp	pore forming protein - perforin	*Homo sapiens*	GenBank
GI:34808711	1665 bp	pore forming protein - perforin	*Mus musculus*	GenBank
GI:59709458	1665 bp	pore forming protein - perforin	*Rattus norvegicus*	GenBank
GI:3559947	290 bp	pore forming protein - perforin	*Ovis aries*	GenBank
TC135848	1447 bp	EST showing significant similarity to perforin	*Bos taurus*	TIGRDB
GI:6996012	878 bp	serine esterase 3 – granzyme A	*Homo sapiens*	GenBank
GI:6754101	783 bp	granzyme A	*Mus musculus*	GenBank
GI:23618882	786 bp	granzyme A	*Rattus norvegicus*	GenBank
TC3999777	1002 bp	EST showing significant similarity to granzyme A	*Bos taurus*	TIGRDB
GI:20988817	744 bp	serine esterase 1 – granzyme B	*Homo sapiens*	GenBank
GI:7305122	744 bp	granzyme B	*Mus musculus*	GenBank
GI:31542925	747 bp	granzyme B	*Rattus norvegicus*	GenBank
TC134874	903 bp	EST showing significant similarity to granzyme B	*Bos taurus*	TIGRDB

ILRI oligo number	Primers for cloning cDNA Primer sequence 5' → 3' end	Target	Primer orientation
12782	CGC **GGA TCC** ATG GCA GCC CGT CTG CT	perforin	forward
12071	CAT CAC ATC ACA TTT ATT GGT CAG	perforin	reverse
9341	TCC TCA TTC AAG ACC	granzyme A	forward
9342	TCC TGG TTT CAC ATC	granzyme A	reverse
11848	CCG **GAA TTC** ATG GTC CTG CTC CTG CTC	granzyme B	forward
11849	CCG GCG GCC TCA CAC TGA TCC CTG GCG	granzyme B	reverse

Restriction enzyme recognition sequences are shown in bold letters. Several primer pairs were used (see Appendix I) but Table 3 shows only those pairs that generated the expected sequences.

BLASTN search of the TIGR *Bos taurus* gene index http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=cattle (formerly (http://tigrblast.tigr.org/tgi) to search for identical expressed sequence tags (ESTs) from available bovine gene sequences. Sequence alignments were done using ClustalW (http://www.ebi.ac.uk/Tools/clustalw/index.html) (shown in Appendix I) and PCR primers designed based on regions showing close to 100% nucleotide identity. Where full-length sequence data was available, primers were designed on the flanking 5' and 3' ends according to a homology block in the untranslated regions upstream of the ATG start codon and/or downstream of the TGA stop codon. Primers were obtained from MWG Germany or ILRI SEGOLI unit and the sequences are also shown in Table 3 and Appendix I whereas PCR reagents were obtained from Applied Biosystems, USA.

To amplify bovine perforin cDNA, the PCR reaction mixture was set up in a total volume of 50 µl as follows: 3.0 µl 25mM $MgCl_2$, 5.0 µl 10x Ampli*Taq* Gold *Taq* polymerase buffer, 1.0 µl 10mM dNTP mix, 1.0 µl 100 ng/µl forward primer, 1.0 µl 100 ng/µl reverse primer, 0.25 µl 0.25 U/µl Ampli*Taq*® DNA polymerase, 10 µl (or 5 µl) of template ss-cDNA and 28.75 µl (or 33.75 µl) sterile distilled water. PCR was carried out in Minicycler™, MJ Research thermocycler under the following conditions: 94°C for 1 min followed by 35 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 1.5 min and a final extension of 1 cycle of 72°C for 10 min. The resulting PCR products were analysed by gel electrophoresis using a 1.2% agarose gel stained with ethidium bromide and visualised under UV transillumination.

Bovine granzyme A and B cDNA were amplified essentially as described for bovine perforin with minor modifications. A 50-µl PCR reaction mix was set up as follows: 3.0 µl 25 mM $MgCl_2$, 5.0 µl 10x buffer for *Taq* polymerase, 1.0 µl 10 mM dNTP mix, 1.0 µl 100 ng/µl forward primer, 1.0 µl 100 ng/µl reverse primer, 0.25 U/µl (0.25 µl) *Taq* DNA polymerase, 5 µl of ss-cDNA and 33.75 µl sterile distilled water template. PCR reaction conditions for granzyme A were 94°C for 1 min followed by 30 cycles of 94°C for 1 min, 40°C for 1 min, 72°C for 1 min and a final extension of 1 cycle of 72°C for 5 min. The PCR cycling conditions for granzyme B amplification were as follows: 94°C for 1 min followed by 35 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 1 min and a final extension of 1 cycle of 72°C for 10

min. PCR products were resolved in a 1.2% gel, DNA fragments at expected sizes purified from the gel and cloned into pGEM®-T Easy vector for verification.

The PCR products at the expected molecular size were purified using the QIAquick Gel extraction kit (QIAGEN, Germany) as described in the QIAquick® Spin Handbook (2001) as follows: 50 µl of the PCR products were resolved in a 1.2% (w/v) agarose gel which was subsequently stained with ethidium bromide to visualize the DNA under UV transillumination. The DNA fragment band of interest was excised from the agarose gel using a scalpel blade under UV light, weighed and dissolved in 3 volumes of Buffer QG (contains Guanidine thiocyanate) to 1 volume of gel (100 mg of gel is approximately 100 µl) at 50°C for 10 min followed by addition of 1 gel volume of isopropanol. The solubilised sample was applied to the QIAquick spin column and centrifuged at 10000 rpm (Bench top Eppendorf Centrifuge) for 1 min. Bound DNA was washed once by adding 0.5 ml of buffer QG, followed by 0.75 ml of Buffer PE (contains ethanol). Residual wash fluid was removed by spinning the column for another 1 min, and DNA eluted with 30 µl of Buffer EB (10 mM Tris.Cl, pH 8.5) and quantified by determination of absorbance at 280 nm (Gene Quant II, Pharmacia, Biotech).

Perforin, granzymes A and B PCR products were ligated into the pGEM®-T Easy vector in a standard ligation reaction as follows: 5 µl 2x rapid ligation buffer, 1 µl pGEM®-T Easy vector (approximately 100 ng of vector DNA), 1 µl T4 DNA ligase and 3 µl (approximately 50 ng) of purified PCR product (total reaction volume 10 µl). The ligation sample was mixed gently, incubated overnight at 4°C and directly used in bacteria transformation. Analysis and output of sequence data was performed by applying web-based NCBI ORF Finder program (www.ncbi.nlm.nih.gov/gorf/gorf.html) using standard genetic codes. Protein analysis and alignment of homologous sequences was done using a web based program hosted at EMBL-EBI (http://www.ebi.ac.uk/InterProScan/index.html). and BoxShade software (http://www.ch.embnet.org/software/BOX.form.html).

2.1.3 Transformation of bacteria with plasmid DNA

2.1.3.1 Preparation of electrocompetent bacterial cells

Escherichia coli DH10α or JM109 bacteria cells from a frozen glycerol stock were recovered by streaking onto a 2xYT plate and grown overnight at 37°C. A start culture of the host strain was initiated by inoculating 50 ml of 2xYT medium with a single isolated bacterial colony which was then grown overnight at 37°C with shaking at 225 rpm in a rotatory shaker (Series 25D, New Brunswick Scientific Co. Inc. NJ, USA). A 10-ml aliquot of the overnight culture was then seeded into 500 ml of fresh 2xYT medium and grown at 37°C with continuous shaking until it reached an OD_{600nm} of 0.5-0.7. The culture was then chilled on ice for 30 min in the cold room, whose temperature was set at 10˚C, before being transferred into pre-chilled 50ml polypropylene tubes (BD Falcon™ USA). In all subsequent steps, the cells were maintained and handled on ice in the cold room. The cell pellets were recovered and washed 6 times with ice-cold 10% glycerol in sterile distilled water by centrifuging at 900xg (Model RC-3B, Sorval, Wilmington, DE, USA) for 15 min at 4˚C. Cell pellets were pooled gradually at each washing step and resuspended in 2 ml of ice-cold 10% glycerol in sterile distilled water after the final wash. Aliquots (100 µl) of the cell suspension were then dispensed into pre-chilled 0.5 ml eppendorf tubes and frozen in liquid nitrogen (snap freezing) for storage at -80˚C.

2.1.3.2 Electroporation of bacteria cells

A 100-µl aliquot of electrocompetent DH10α or JM109 bacteria cells was thawed on ice. The respective ligation reaction mix (5 µl) was added directly into the cell suspension, stirred gently and transferred into an ice-cold 0.2-cm gap electroporation cuvette (Bio-Rad laboratories, CA, USA). The cell suspension was then subjected to a high voltage discharge (15kV cm^{-1}) for approximately 5 seconds. One ml of chilled LB broth medium was immediately added to the cell suspension and mixed gently before being transferred into 10-ml polypropylene tube (Falcon®). The cell suspension was incubated for 1 hr at 37°C, with rotatory shaking at approximately 255 rpm (Series 25D, New Brunswick Scientific Co. Inc. NJ, USA). An aliquot of 100 µl of the transformation mix was plated onto pre-warmed (37°C) NZYCM agar plates containing ampicillin (50 µg/ml) and incubated overnight at 37°C.

Bacteria colonies containing the recombinant plasmid were identified by direct PCR screening. PCR reagents (Promega Madison, Wisconsin, USA) were mixed in a 96-well microtitre plate as follows: 1.5 µl 25 mM $MgCl_2$, 2.5 µl 10x *Taq* polymerase buffer, 0.5 µl 10 mM dNTP mix, 0.5 µl 100 ng/µl M13 forward primer, 0.5 µl 100 ng/µl M13 reverse primer, 0.125 µl 5 U/µl *Taq* DNA polymerase, and 19.375 µl sterile distilled water to a total reaction volume of 25 µl. Single bacterial colonies were added to the mix by picking each pre-labelled numbered colony with a sterile tip and transferring it into the respective well. Replicate colonies were streaked onto fresh NZYCM plates containing ampicillin (50 µg/ml) and incubated at 37°C for approximately 6 hr. The PCR reactions were placed in a thermocycler (Minicycler™, MJ Research, USA) for amplification using the following program: 94°C for 2 min for initial denaturation followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min and a final extension at 72°C for 5 min. The PCR products were resolved and analysed in a 1.2% agarose gel as described before. Bacterial colonies carrying the recombinant plasmid were grown overnight with vigorous shaking in 3 ml liquid broth medium containing ampicillin (50 µg/ml) for subsequent purification of plasmid DNA.

2.1.3.3 Plasmid DNA purification

Plasmid DNA was prepared by using the QIAprep® Spin Miniprep kit (QIAGEN, Germany) following manufacturer's instructions. Briefly, PCR positive replicate bacterial colonies were pelleted by spinning at 10000 rpm (Bench-top Eppendorf centrifuge, Model No. 5417) for 5 min at room temperature (~25˚C). Cell pellets were resuspended in 250 µl of Buffer P1 containing RNase A and subsequently lysed by addition of 250 µl of Buffer P2 which contains sodium hydroxide and sodium dodecyl sulphate (SDS). To avoid shearing of genomic DNA, the contents were mixed by inverting the tube 4-6 times. The lysis reaction was allowed to proceed at room temperature until the solution became clear and viscous or for a period not exceeding a maximum of 5 min. The lysis reaction was stopped by addition of 350 µl neutralisation buffer N3 (contains guanidine hydrochloride and acetic acid) which was mixed as before. Bacterial debris and precipitated proteins were removed by centrifuging at 14000 rpm (Bench-top Eppendorf centrifuge, Model No. 5417) at room temperature for 10 min. The supernatant was recovered and

transferred by decanting into a QIAprep spin column fitted into a 2-ml collection tube. The loaded column was then centrifuged at 14000 rpm (Bench-top Eppendorf centrifuge, Model No. 5417) for 60 seconds at room temperature and the flow through discarded. Column-bound plasmid DNA was washed twice by the addition of 500 µl of column wash buffer PB (contains guanidine hydrochloride and isopropanol) and once by the addition of 750 µl of buffer PE (contains ethanol) with centrifugation at 14000 rpm (Bench top Eppendorf centrifuge, model No. 5417) for 60 seconds at room temperature. After the final wash, the column was centrifuged for an additional 1 min to remove residual wash buffer. The column was transferred to a fresh eppendorf tube and bound DNA eluted by adding 50 µl of elution buffer (10 mM Tris.Cl, pH 8.5) to the centre of each QIAprep column followed by 1 min centrifugation. Plasmid DNA yield was determined by measuring absorbance at 260 nm and was stored at -20°C for subsequent use. The DNA insert was further verified by sequencing before use.

2.1.4 Protein expression in bacteria cells

2.1.4.1 Sub-cloning bovine perforin and granzyme B cDNA into pGEX-3T

Full-length cDNA inserts coding for bovine perforin and granzyme B were sub-cloned into pGEX-3T (Pharmacia Biotech, NJ, USA) in the appropriate reading frame and orientation. To facilitate sub-cloning of the target cDNA, appropriate restriction enzyme sites were introduced by PCR using primers flanking the 5' and 3' ends of the cDNA the resultant cDNA fragment prepared for cloning into pGEX-3T by digesting with the appropriate restriction enzyme.

As shown in Table 3, primer number 12782 was designed to introduce *Bam*HI (5' end) recognition sequence at the N-terminal of perforin cDNA. The resultant PCR product was subcloned into pGEM®-T Easy as previously mentioned in section 2.1.2. Consequently the recombinant pGEM®-T Easy/perforin cDNA plasmid was first digested with *Eco*RI restriction enzyme (New England Biolabs, MA, USA) following the manufacturer's guidelines. The perforin cDNA was recovered by resolving the reaction mix in a 0.8% agarose gel following which the 2kb-band was excised and purified from the gel essentially as described earlier. In parallel, 5 µg of the pGEX-3T plasmid vector was also digested with *Eco*RI. Digestion of the vector was determined by resolving 1 µl of the digest reaction in 1% agarose gel and staining with ethidium bromide to visualise plasmid DNA. Complete digestion results in a single band of

high molecular weight DNA at approximately 5 kb while incompletely digested plasmid DNA is manifested by multiple bands indicating the presence of linear and supercoiled plasmids. Linearised pGEX-3T plasmid vector was purified using QIAquick PCR purification kit (QIAGEN, Germany) in line with the manufacturer's protocol. Briefly, 5 volumes of Buffer PB were added and mixed with 1 volume of the digest reaction. The sample was applied to the QIAquick column and centrifuged at 14000 rpm (Bench top Eppendorf centrifuge Model No. 5417) for 1 min at room temperature. The column-bound plasmid DNA was then washed with 750 µl of Buffer PE under similar centrifugation conditions. Residual buffer was removed by an extra centrifugation step and bound DNA subsequently eluted with 50 µl of elution buffer (10 mM Tris-Cl, pH 8.5). To prevent re-ligation, the vector was further dephosphorylated using calf intestinal alkaline phosphatase (CIAP) (Promega USA) by adding 10 µl 10x CIAP reaction buffer, 2 µl CIAP and 38 µl sterile distilled water directly to the digested plasmid DNA. The dephosphorylation reaction mix was then incubated for 30 min at 37°C and the plasmid DNA recovered by purification using QIAquick PCR purification kit as described earlier. The *Eco*RI-digested perforin cDNA fragment and linearised plasmid was then digested with *Bam*HI restriction enzyme (New England Biolabs, MA, USA) as recommended and recovered as before. The perforin cDNA fragment was then ligated to the linearised pGEX-3T plasmid vector in a standard ligation reaction as follows: 1 µl 100 µg/ml vector DNA, 4 µl 10 µg/ml insert DNA, 1 µl 10x Ligase buffer, 1 µl T4 Ligase (Promega, USA) and 3 µl distilled water in total reaction volume of 10 µl in a 200-µl eppendorf tube. The contents were mixed and incubated overnight at 16°C. An aliquot of 5 µl of the ligation mix was used to transform electrocompetent JM109 bacteria cells as described earlier (Section 2.1.3.2). The resultant recombinant pGEX-3T/perforin cDNA vector was sequenced to verify the integrity of the open reading frame.

Granzyme B cDNA was sub-cloned in pGEX-3T vector at the *Bam*HI and *Sal*I sites. A set of primers, ILO 647597 and ILO 647598 (Appendix II), were designed based on the granzyme B cDNA sequence to generate *Bam*HI/ and *Sal*I restriction enzyme recognition sequences at the N- and C terminals of the cDNA, respectively. A typical 50-µl PCR reaction mix in a sterile 500 µl eppendorf tube included the following reagents: 3.0 µl 25mM $Mgcl_2$, 5.0 µl 10x *Taq* DNA polymerase buffer, 1.0 µl 10mM dNTP mix, 1.0 µl 100 ng/µl forward primer, 1.0 µl 100 ng/µl reverse

primer, 5 U/µl (~0.25 µl) Ampli*Taq*® Gold DNA polymerase, 0.5 µl template (10 ng/µl recombinant pGEM®-T Easy/granzyme B cDNA plasmid) and 38.25 µl sterile distilled water. The reaction mix was overlaid with a drop of mineral oil and incubated under the following conditions: 94°C for 2 min followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min and a final extension of 1 cycle of 72°C for 5 min (Minicycler™, MJ Research). A 5-µl aliquot of the PCR products was resolved in a 1.2% w/v TAE agarose gel along side DNA size markers and the DNA visualised by staining with ethidium bromide. PCR products were ligated into pGEM®-T Easy vector and purified as described previously. The resultant recombinant plasmid DNA was sequenced at ILRI (SEGOLI Unit) or Macrogen Inc (South Korea) using vector primers to ensure that no cloning artefacts or *Taq* polymerase induced mutations were generated during the amplification process. The recombinant plasmid was subsequently digested with BamHI and SalI, the granzyme B fragment bearing BamHI and SalI restriction sites recovered from gel as previously outlined. Linearised pGEX-3T vector DNA and *Bam*HI/*Sal*I digested granzyme B cDNA were ligated using T4 DNA ligase (Promega, USA) essentially as described for perforin.

2.1.4.3 Protein expression in *Escherichia coli*

Transformed JM109 cells were used for recombinant protein expression as described in QIAexpressionist ™ (A hand-book for high-level expression and purification of proteins, 2003). Briefly, start cultures were initiated by inoculating 2 ml of LB growth medium containing ampicillin (100 µg/ml) with a single colony-derived cells and grown overnight at 37°C with rotatory shaking at 225 rpm (25D, New Brunswick Scientific Co. Inc. NJ, USA). The overnight culture was expanded into a larger volume of fresh LB medium containing ampicillin and grown similarly. Typically, 500 µl of a 2-ml overnight culture was seeded into 50 ml of LB growth medium containing ampicillin. When the culture reached an OD_{600nm} of 0.5-0.7, protein expression was induced with the addition of IPTG to a final concentration of 2 mM or 10 mM and further incubation for 4-5 hr at 37°C with continuous shaking. A cell pellet recovered from 10 ml of the cell culture by centrifugation (Bench top Heraeus centrifuge) at 3000 rpm at room temperature for 20 min, was prepared for protein expression determination by resuspension in 1 ml 8M urea solution (Lysis Buffer B) containing a protease inhibitor (1 mM PMSF), lysed by sonication using a

micro-tip sonicator (Branson 3328, 50% duty cycle) for 1 min at 4°C and spun as before. The total lysate was recovered and analysed for protein content as described in Section 2.1.6.

2.1.5 Protein expression in insect cells using baculovirus

In order to produce perforin, granzymes A and B in insect cells, the Bac-to-Bac® expression system (Invitrogen ™ Life Technologies), assembled by first cloning the genes into a pFASTBac™HT B vector and then integrating into a baculovirus shuttle vector (bacmid), was applied as described in the Bac-to-Bac® Baculovirus Expression system manual (Invitrogen ™ Life Technologies).

2.1.5.1 Sub-cloning bovine perforin, granzymes A and B into pFASTBac™HT B

Primers were designed to introduce cleavage sites at the 5' and 3' ends for *Nco*I and *Sal*I, respectively of cDNA fragments of perforin and granzyme B as well as a partial fragment with a predicted coding sequence for granzyme A using primers indicated in Appendix II and cloned bovine cDNA (generated in section 2.1.2) as template. The amplified fragments were sub-cloned in pGEM®-T Easy vector and sequenced for verification. The cDNA fragments were then sub-cloned into pFASTBac™HT B at *Nco*I and *Sal*I sites following recommended protocols (New England Biolabs). Briefly, both recombinant pGEM®-T Easy and pFASTBac vectors were digested with *Nco*I and *Sal*I restriction enzymes (NEB). The linearised pFASTBac vector and the excised fragment were ligated together in a reaction mix containing T4 DNA ligase, and the resulting recombinant pFASTBac™HT B plasmid (rec-pFASTBac^{TM}HT.B) used to transform *E. coli* DH10α by electroporation. A GFP gene fragment was also fused in-frame to the C-terminal end of the target cDNA using *Sal*I and *Sph*I sites to function as an expression reporter gene. Hence, the recombinant pFASTBAC plasmid was isolated and digested with *Sal*I and *Sph*I restriction enzymes to allow cloning of the GFP cDNA. A schematic representation of the cloning process as well as the primers used is illustrated in Appendix III. The resulting GFP-containing recombinant pFASTBac (GFP-rec-pFASTBac™ HT B) was further used to transform *E. coli* DH10α. Plasmid DNA was isolated from ampicillin-resistant transformants and analysed by enzyme digestion using *Nco*I, *Sal*I and *Sph*I restriction enzymes or by PCR using M13 forward and reverse primers to confirm the presence of the insert. Sequencing was performed to verify that the cDNA was cloned

in-frame with the N-terminal ATG codon. The recombinant plasmid was then bulked and purified and stored at -20˚C until use.

2.1.5.2 Generation and isolation of recombinant bacmid

The rec-pFASTBac^{TM}HT.B or GFP-rec-pFASTBac™HTB plasmid DNA was used to transform chemically-competent DH10Bac™ *E. coli* cells (Invitrogen) as recommended in the manufacturer's guidelines. Briefly, 50 µl of competent cells were mixed with 1 µl of recombinant plasmid DNA (at a concentration of 1 µg/ml) and heat-shocked at 42°C for 45 seconds. The cells were resuspended in 900 µl of LB growth medium and plated out in three 10-fold serial dilutions (10^1, 10^2, 10^3) on LB agar plates containing 50 µg/ml kanamycin, 7 µg/ml gentamycin, 10 µg/ml tetracycline, 100 µg/ml bluo-gal, and 200 mM IPTG. Plates were incubated for 48 hr at 37°C. Colonies containing the recombinant bacmids were identified as white colonies in a background of blue colonies that harbour the unaltered bacmid. White colonies were re-streaked on fresh LB agar plates containing the appropriate antibiotics and substrates to confirm the white colour phenotype.

A single white colony was grown in 2 ml LB growth medium containing 50 µg/ml kanamycin, 7 µg/ml gentamycin and 10 µg/ml tetracycline at 37°C for 24 hr with gentle agitation (Series 25D, New Brunswick Scientific Co. Inc. NJ, USA) for aeration. Cells were harvested from 1.5 ml stationary-phase culture by centrifugation at 10,000 rpm for 5 min (Bench top Eppendorf centrifuge Model, 5417), recovered in 300 µl cell resuspension solution (15 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 µg/ml RNase A) then lysed by addition of 300 µl of cell lysis solution (0.2 N NaOH, 1% SDS) and incubation at room temperature for 5 min. Protein and genomic DNA was precipitated by addition of 300 µl of cold 3M KOH, pH 5.5 and the sample subsequently incubated on ice for 15 min. The white precipitate was removed by centrifugation at 14,000 rpm (Bench top Eppendorf centrifuge, Model 5417) for 10 min at 4°C resulting in a clear supernatant that was transferred into a microcentrifuge tube containing 10 µl of endotoxin-removal buffer (QIAGEN, Germany) and incubated on ice for 30 min. Endotoxin-free bacmid DNA was precipitated by addition of 800 µl of isopropanol with gentle agitation before the tube was placed on ice for 10 min. DNA was recovered by centrifugation at 14000 rpm (Bench-top Eppendorf centrifuge Model No. 5402) for 15 min at 4°C and the pellet washed by

further centrifugation as before in 500 µl endotoxin-free 70% ethanol then air-dried at room temperature for 15 min prior to dissolving it in 50 µl endotoxin-free TE buffer. An aliquot of 1 µl of the DNA was analysed by PCR to verify the presence of the target cDNA while 5 µl was used for transfection of insect cells to assess the quality of the DNA. Bacmid DNA was stored at 4°C or in 10-µl aliquots at -20°C to avoid repeated freeze-thawing.

2.1.5.3 Analysis of recombinant bacmid by PCR

PCR amplifications were performed in a total volume of 50 µl containing 1.0 µl of the purified bacmid DNA, 5.0 µl 10x *Taq* DNA polymerase PCR buffer, 6.0 µl 25 mM $MgCl_2$, 1.0 µl 10 mM dNTP mix, 1.0 µl 100 ng/ ml M13 forward, 1.0 µl 100 ng/µl M13 reverse primer and 0.5 µl 5 units/µl *Taq* polymerase and 34.5 µl sterile double distilled water. The cycling conditions were: initial denaturation at 94°C for 3 min, 35 cycles of denaturation at 94°C for 45 seconds, primer annealing at 55°C for 45 seconds and extension at 72°C for 5 min and a final extension cycle at 72°C for 7 min (GeneAmp, Applied Biosystems). A 5-µl aliquot of the PCR reaction was analysed by electrophoresis on a 1.2% agarose gel and DNA bands visualised by ethidium bromide staining and UV transillumination of the gel.

2.1.5.4 Culture and maintenance of insect cell lines

Spodoptera frugiperda Sf-21 insect cells were used as the host for the baculovirus transfer vectors while *Trichoplusia ni* High-Five™ BTI-TN-5B1-4 cells were used for protein expression. Sf-21 cells were maintained in supplemented insect cell growth medium (TC-100, Sigma Chemicals, UK) with 5% foetal bovine serum (Hyclone, UT, USA) while High-five cells were maintained in Express-Five™ serum-free growth medium (GIBCO Ltd., UK). Both growth media were supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 100 µg/ml gentamycin to make them complete.

For routine maintenance, cells were seeded and incubated at $2x10^4$- $5x10^5$ viable cells/cm^{-2} in 25-cm^2 (4-6 ml) or 75-cm^2 (15 ml) tissue culture flasks (Costar®). Cell culture flasks were kept in an humidified incubator at 27°C with loose caps to allow gaseous exchange. The cultures were kept in logarithmic growth phase by making sub-cultures when the monolayer reached 80% to 100% confluency; that is 2

to 4 days post-seeding. A portion of the cell suspension was removed and replaced with an equal volume of fresh growth medium. Cell cultures were discarded after approximately 30 passages. For cryopreservation, cells harvested at the peak of growth with a viability of >90%, were resuspended to a final cell density of $1x10^7$ – $2x10^7$ cells/ml in cold incomplete insect cell culture medium containing 7.5% DMSO, 10% FBS or 10% BSA and stored in 1-ml aliquots in cryotubes (Greiner, Germany) as master seed stocks. Vials were chilled at 4°C for 30 min before storage in liquid nitrogen.

To initiate fresh cultures, cells were recovered from the cryopreserved master seed stock by rapidly thawing the vials in a 37°C water-bath. The cell suspension was quickly transferred into a 10-ml polypropylene tube containing 8 ml of cold incomplete insect cell growth medium and washed two times before being transferred into pre-warmed complete growth medium at a minimal viable cell density of $3\text{-}5x10^5$ cells/ml. The culture was then maintained at a cell density of $3x10^5$ - 10^6 cells/ml for at least two sub-cultures after recovery then returned to the normal maintenance schedule.

Cell counts were done using a haemocytometer (Neubauer) and trypan blue dye exclusion. All operations except centrifugation and counting of cells were conducted in a horizontal laminar air flow hood.

2.1.5.5 Generation and amplification of recombinant baculovirus

Sf-21 cells were transfected with the recombinant bacmid using Cellfectin reagent (Invitrogen) in a 6-well tissue culture plate (Costar®) format according to the established protocol (*Guide to baculovirus expression vector systems (BEVS) and insect cell culture techniques*, http://www.invitrogen.com). Briefly, approximately $9x10^5$ cells in 2 ml of complete growth were seeded in each tissue culture plate well and allowed to attach for 1 hr at 27°C in a humidified incubator. An aliquot of 6 µl of Cellfectin reagent was diluted in 100 µl of unsupplemented TC-100 medium and mixed with 5 µl of purified recombinant bacmid diluted in 100 µl of the same medium. The DNA:Cellfectin mix was incubated for 1 hr at room temperature to allow the formation of DNA-lipid complexes. A further 800 µl of unsupplemented TC-100 medium was added to the tube containing the DNA-lipid complexes and this suspension mixed gently and then overlaid onto the Sf-21 cell monolayer. Prior to

adding the diluted DNA-lipid mix, the monolayer cells were washed once with 2 ml unsupplemented TC-100 medium. The cell culture containing the DNA-lipid complexes was then incubated at 27°C in a humidified incubator for 5 hr before the complexes were removed by aspiration and replaced with 2 ml of complete TC-100 medium in each well. The culture was incubated further at 27°C for 72 hr. Budding virus released into the growth medium was recovered into 10-ml polysytrene tubes (Sterilin, England) and centrifuged (Heraeus Megafuge, Model No. 2.0R) at 1500 rpm for 10 min at room temperature to remove cell debris and collect the supernatant in 5-ml polystyrene tubes (Sterilin, England). This small-scale low titre virus preparation termed P1 viral stock, was stored at 4˚C and protected from light until use.

To generate a high titre virus population, P1 was used to infect more Sf-21 cells resulting in a P2 viral stock. Briefly, aliquots of 6 ml of cells at a concentration of $2x10^6$ cells/ml were seeded in a 25-cm^2 tissue culture flask (Costar®) and allowed to form an adherent monolayer at room temperature. An aliquot of 500 µl of P1 virus stock was added to the cell monolayer and the culture maintained at 27˚C in a humidified incubator for 48 hr. The budded virus was recovered and stored as described before. All procedures were conducted under laboratory biosafety level 2 conditions.

2.1.5.6 Production and purification of recombinant perforin, granzymes A and B

Approximately 15-ml culture of *Trichoplusia ni* High Five™ cells at $2-3x10^6$ cells/ml was transferred into a 75-cm^2 tissue culture flask (Costar®) and the cells left to attach for at least 30 min at room temperature. The growth medium was removed by aspiration, and the cells rinsed once with fresh growth medium before adding a further 15 ml of fresh growth medium. The culture was then inoculated with 500 µl of the P2 viral stock and incubated at 27°C for 72 hr in a humidified incubator. After culture, cells containing the recombinant protein were collected in 10-ml polypropylene tubes (Sterilin, England) by centrifugation. The supernatant was discarded and the cell pellets washed once in chilled PBS.

The baculovirus expression vector contained N-terminal 6x-Histidine residues that allowed for the expression of the recombinant proteins as His-tag fusion proteins. This facilitated the purification of the recombinant fusion proteins using metal-chelating resin such as Ni-NTA (QIAGEN, Germany) essentially as described in

‘‘The QIAexpressionist™: A Handbook for High Level Expression and Purification of 6xHis-tagged Proteins’’ (2003). Briefly, chilled cell pellets were lysed in 5 ml lysis buffer (Lysis buffer A) containing 1mM PMSF by sonication for 1 min using a micro-tip sonicator (Branson 3328, 50% duty cycle) after which cell debri was removed by centrifugation (3000 rpm for 20 min at 10˚C; Bench top Heraeus centrifuge). His-tagged proteins were purified from the supernate using Ni-NTA resin. An aliquot of 200 µl of 50% Ni-NTA slurry was added to the supernate and mixed gently on a rolling mixer (Denley Roller Mixer) for 2 hr in the cold room. The Ni-NTA/sample admixture was then loaded onto a polypropylene column (Pierce Chemical Co., Rockford, US). The resin-bound His-tagged protein was washed three times with 5 ml of Wash buffer C. The bound protein was eluted sequentially with four aliquots of 500 µl Elution buffer E. Immediately after elution, 100 µl of 1M Tris-Cl, pH 8.0 was added to each of the eluates to neutralise the low pH of the elution buffer. An aliquot of the eluates was analysed for protein purity and integrity as described in section 2.2.6. The eluates were pooled and their concentration determined using the BCA protein assay kit (Pierce Chemical Co., Rockford, US) and stored at 4˚C until further use.

2.1.6 Protein expression analyses

Expression of recombinant proteins by the *E. coli* and baculovirus systems was assessed by Coomassie staining and Western blotting of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) resolved samples. Separation of proteins was done based on molecular size in the presence of SDS and 2-mercaptoethanol (Laemmli *et al*., 1970; Ausubel *et al*., 2001). Protein samples were resolved in a slab mini-gel (8.0 cm x. 10 cm x 0.5 mm) format. Separating and stacking gel solutions were prepared as shown in Appendix IV. The separating gel solution was slowly poured into a pre-assembled glass plate sandwich comprising two clean glass plates and 0.75 mm spacers (Hoefer Mighty Small SE 250/280) until the gels were 6 cm high allowing for 1.5 cm for the stacking gel. The separating gel was overlaid gently with distilled water and allowed to polymerise for 1 hr before the addition of the stacking gel solution. After pouring off the layer of water, the stacking solution was added and gel combs inserted carefully taking care not to trap air bubbles. The stacking gel was left to polymerise for 30 min. Once polymerised, the combs and spacers were removed and the sample wells rinsed with water followed by

the electrophoresis buffer. The assembled gels were then mounted in the electrophoresis tank containing electrophoresis buffer. To prepare the protein sample (crude *E. coli* cell lysate or Ni-NTA column eluates) for loading, 10 µl of the sample was diluted with 2 µl 6x sample buffer and boiled for 5 min in a microcentrifuge tube. The sample was then loaded into one well by carefully applying the sample as a thin layer at the bottom of the well. Electrophoresis was done at a constant current of 30 mA until the dye front reached the bottom of the gel lasting approximately 2 hr. SDS-PAGE resolved protein bands were visualised by staining with the protein precipitating 0.25% Coomassie Brilliant blue R-250 essentially as described previously (Wilson, 1983; Sambrook and Russell 2001). Briefly, the staining solution was prepared by dissolving the dye in a methanol:acetic acid mixture (500 ml methanol, 100 ml water, 100 ml acetic acid). After electrophoresis, the gel was immersed in 5 volumes of staining solution at room temperature for 1 hr while shaking on a rotating platform. The gel was then destained by soaking it in 30% methanol, 10% acetic solution (without the dye) for 2 hr while changing the destaining solution three or four times. After destaining, the gels were photographed and stored in water containing 20% glycerol. The composition of buffers and reagents for polyacrylamide separating and stacking gels is shown in Appendix IV. Prediction of the location of a protein band of interest on the gel in relation to a set of protein standards was guided by the calculated protein molecular weight of the cloned cDNA (http://www.sciencegateway.org/tools/proteinmw.htm).

For western blotting of SDS-PAGE resolved *E. coli* expressed proteins, crude bacteria cell lysates were separated in 12.5% polyacrylamide gels under reducing conditions and the proteins transferred onto a nitrocellulose membrane (PROTRAN, 0.2µm, Schleicherand Schuell) before addition of relevant antibody as described previously (Towbin, Staehelin and Gordon, 1979; Ausubel *et al*., 2001). The western blot sandwich was prepared as follows: the polyacrylamide gel containing the protein was laid on a sheet of transfer buffer pre-wetted filter paper. The uncovered side of the gel was then overlaid with a sheet of water pre-wetted filter paper. The nitrocellulose was then overlaid with another sheet of pre-wetted filter paper. This assembly was then sandwiched between two scotch brite pads, secured in a plastic support and the entire assembly placed in a tank containing transfer buffer such that the nitrocellulose paper faced the anodal (positively charged) side of the tank. Proteins were electrophoretically transferred overnight at 4°C using constant voltage

(15V) with continuous stirring of the transfer buffer to maintain a constant temperature gradient. Protein transfer was confirmed by incubation of the nitrocellulose filter in Ponceau S solution for 5 min at room temperature to stain the proteins, then visualised by destaining in water for 10 min at room temperature. To block non-specific binding sites, the nitrocellulose membrane was pre-incubated in 5% (w/v) non-fat dry milk in PBS for 30 min while shaking on a rocking platform, following which it was incubated with the anti-GST monoclonal antibody diluted 1:20000 in 0.1% (w/v) non-fat dry milk in PBS for 2 hr at room temperature or overnight at 4°C with shaking as before. The blot was then washed three times by immersing in PBS containing 0.1 % (w/v) Tween-20 for 10 minutes while shaking as before. After the final wash, the filter was incubated in goat anti-mouse immunoglobulins (Ig) coupled to alkaline phosphatase (Amersham) diluted 1:30000 in 0.1% (w/v) non-fat dry milk in PBS for 1 hr at room temperature then washed as before. Bound antibody was detected by addition of alkaline phophatase substrate buffer (Sigma) for 1-2 min or until a colour signal was observed. To stop the reaction, the filter was washed with a copious amount of running tap water and left to dry at room temperature.

2.2 Results

2.2.1 Cloning bovine perforin, granzymes A and B

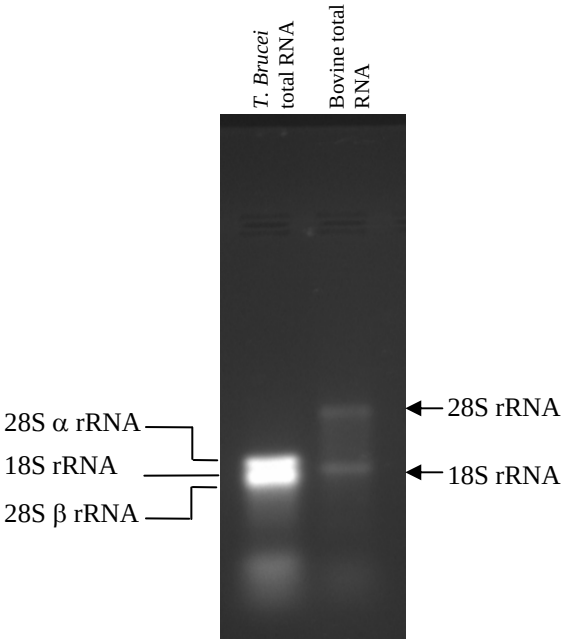
Cloning of bovine perforin, granzymes A and B was initiated by isolation of total RNA from characterised *T. parva* stimulated $CD8^+$ CTL, followed by synthesis of single strand cDNA (ss-cDNA) from the poly A^+ RNA and subsequent ligation of PCR amplified gene-specific DNA fragments into the pGEM-T vector. As shown in Figure 3, panel A, the yield and quality of the total RNA was determined by electrophoresis in a phosphate agarose gel in which it resolved into two distinct bands representing the 28S and 18S ribosomal RNA subunits which are characteristic eukaryotic total RNA. However the 28S rRNA did not appear 2-fold brighter than the 18S rRNA as expected of intact RNA indicating partial degradation of RNAs. Synthesis of ss-cDNA was assessed by the inclusion of bovine GAPDH as an internal indicator of full-length gene sequences (Figure 3, panel B). PCR amplification of the respective ss-cDNA and subsequent cloning into pGEM-T followed by sequencing and *in-silico* analysis of predicted protein revealed products of varying features.

BLASTN search of the TIGR *Bos taurus* Gene Index (now DFCI Gene Index) identified a 1447-bp *Bos taurus* EST, TC135848 (TC478084), with significant nucleotide similarity to GenBank sequences encoding human (gi:190339; 80%), mouse (gi:34808711; 73%), rat (gi:59709458; 74%) and sheep (gi:3559947; 96%) perforin. Perforin primers derived from the alignment of these sequences isolated a 2188 bp cDNA fragment (Figure 3, panel C) that contained a 1665-bp open reading frame with 99% identity to the orf in bovine EST TC135848 and encoding a protein of 554 amino acid residues (Figure 4a). Bioinformatic analysis of this polypeptide showed presence of conserved regions resembling a calcium-binding motif similar to the C2 domain of protein kinase C in the C-terminal region, membrane-attack complex components in the central one-third portion, and EGF-like domain and a leader (signal) peptide with a likely cleavage site between amino acid residues 20-21 (APA-PC) as shown in Figure 4b. It is evident that these features are consistent with those exhibited by members of the perforin family. BLASTP search of non-redundant protein sequences revealed 80%/91% amino acid/homology similarity of the cloned bovine sequence to pig perforin (Gene ID: 396575) and 78%/87% aminoacid/homology, 74%/85% aminoacid/homology similarity to horse (Gene ID:

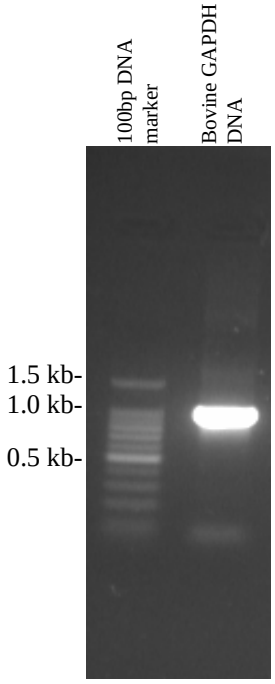
Figure 3: Generation of bovine cDNA and cloning bovine perforin granzymes A and B

Bovine total RNA was prepared from $1x10^7$ *T. parva* stimulated $CD8^+$ T cells by the AGPC extraction method. Panel A shows electrophoresis of bovine total RNA. RNA preparations (3 μl) were electrophoresed in phosphate agarose (1.4 % w/v) minigel and stained with ethidium bromide (Panel A). *T .brucei* total RNA was used as a control to verify the integrity of the bovine RNA preparation. The quality of first strand synthesis was assessed by PCR amplification of full length DNA sequence encoding bovine GAPDH (expected size 1.0kb). Five μl of the PCR reaction was resolved in a 1.2 % w/v agarose gel and the results shown in panel B. The single strand DNA was then used as the template in PCR using gene specific primers to amplify DNA sequences encoding bovine perforin (Panel C) granzymes A (Panel D) and B (Panel E). Panel C, D, E shows PCR products obtained using different gene-specific-primer pairs. Bands at the expected molecular weight (indicated by arrows) were purified from the gel and cloned into pGEM-T Easy for sequence verification as detailed in materials and methods.

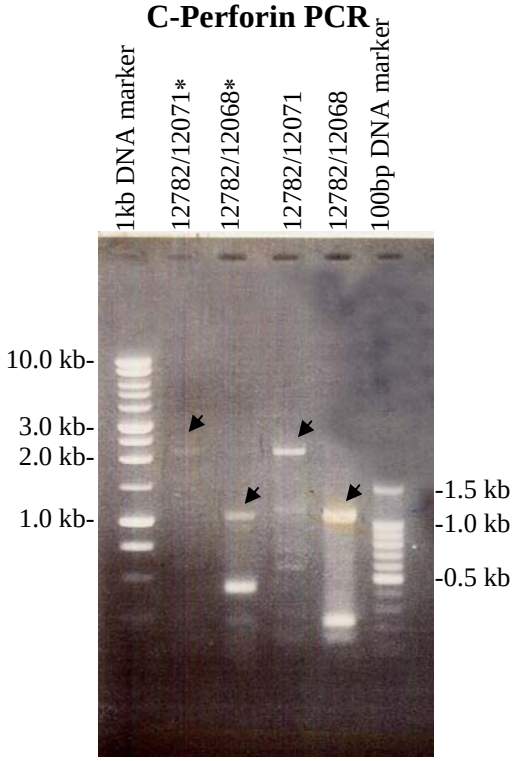
A-Total RNA



B-GAPDH PCR

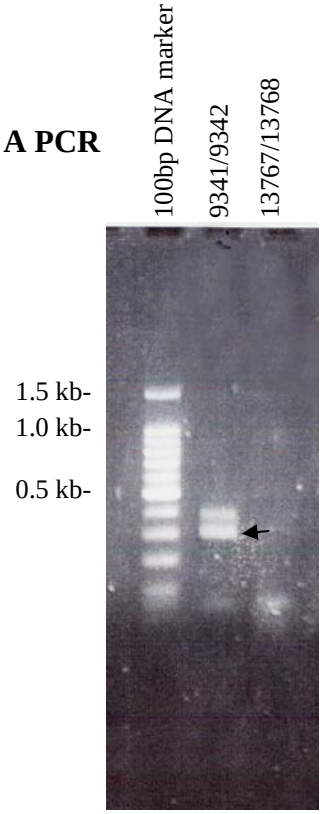


C-Perforin PCR



* 5µl of ss-DNA used in PCR mix

D-Granzyme A PCR



E-Granzyme B PCR

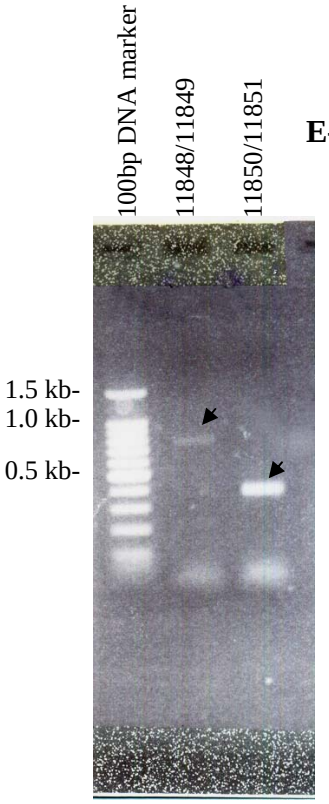


Figure 4a: Nucleotide sequence and predicted amino acid sequence of putative bovine perforin

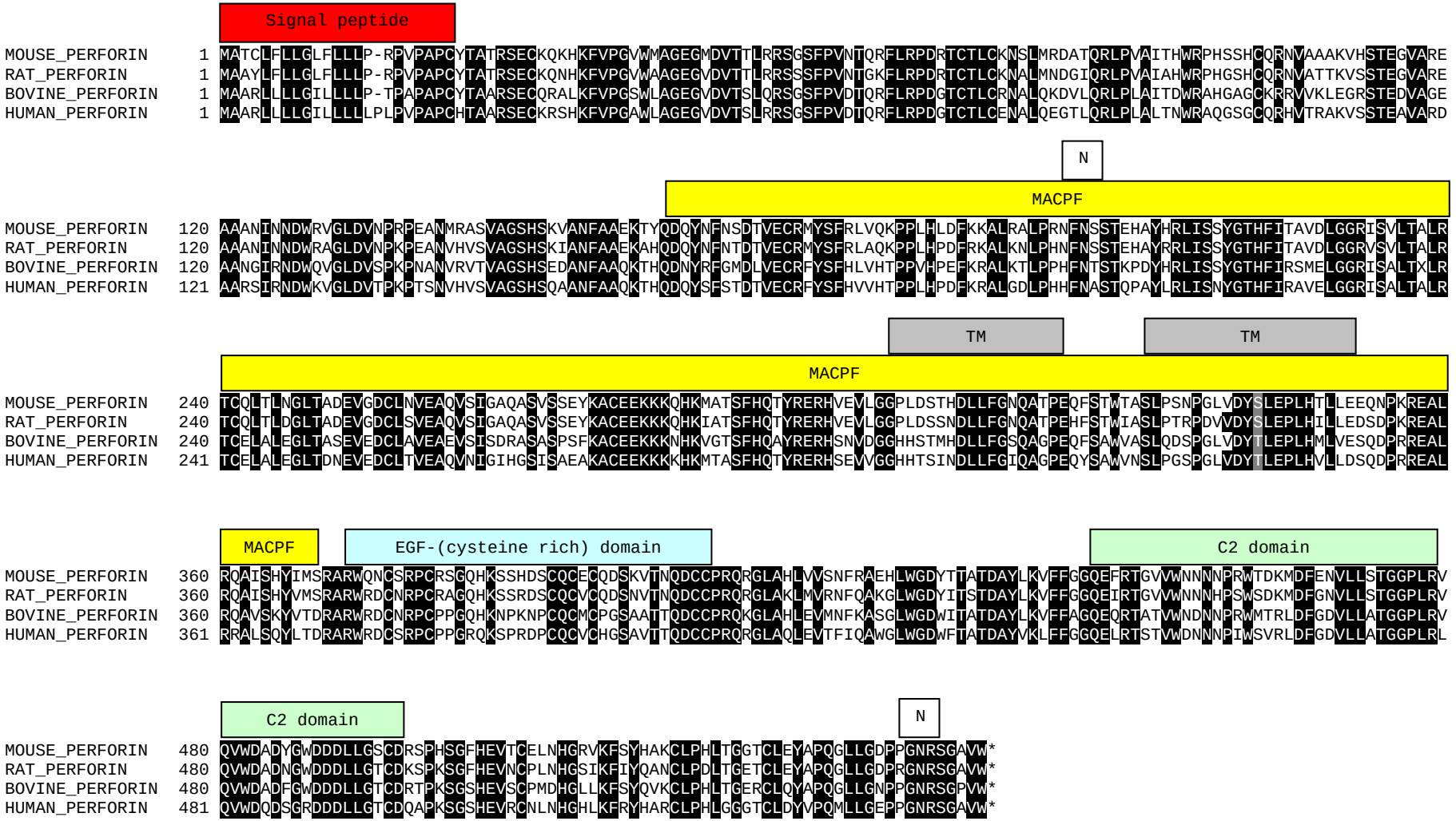
GenBank accession number FJ176744.

```
   1 atggcagcccgtctgctcctccttggcatcctcctgcttctgccc
     M  A  A  R  L  L  L  L  G  I  L  L  L  L  P
  46 acacctgcccctgccccctgctacacagccgcgcgctctgagtgc
     T  P  A  P  A  P  C  Y  T  A  A  R  S  E  C
  91 cagcgcgccctcaagtttgtgccaggctcctggctggcaggggag
     Q  R  A  L  K  F  V  P  G  S  W  L  A  G  E
 136 ggcgtggatgtgaccagcctccagcgctcaggctcgttcccagtg
     G  V  D  V  T  S  L  Q  R  S  G  S  F  P  V
 181 gacacacagcgtttcctgcggcccgacggcacttgcaccctctgc
     D  T  Q  R  F  L  R  P  D  G  T  C  T  L  C
 226 cgcaatgccctgcagaaggatgtcctccagcgcctgcccctggca
     R  N  A  L  Q  K  D  V  L  Q  R  L  P  L  A
 271 atcaccgactggcgtgcccacggagcgggctgcaagcgcagggtg
     I  T  D  W  R  A  H  G  A  G  C  K  R  R  V
 316 gtcaagctagagggccgctccaccgaggatgtggctggggaggcg
     V  K  L  E  G  R  S  T  E  D  V  A  G  E  A
 361 gccaacgggatccgcaacgactggcaggtggggctggacgtgtct
     A  N  G  I  R  N  D  W  Q  V  G  L  D  V  S
 406 cccaagccaaatgctaatgtccgtgtgacagtggcgggctcccac
     P  K  P  N  A  N  V  R  V  T  V  A  G  S  H
 451 tccgaggatgccaacttcgccgcccagaagactcaccaggacaac
     S  E  D  A  N  F  A  A  Q  K  T  H  Q  D  N
 496 taccgcttcggcatggacttagtggagtgtcgcttttacagtttt
     Y  R  F  G  M  D  L  V  E  C  R  F  Y  S  F
 541 cacctggtgcacactccccagtacaccctgagttcaagagggcc
     H  L  V  H  T  P  P  V  H  P  E  F  K  R  A
 586 ctcaagacactgccccccacttcaacacctccaccaagcccgac
     L  K  T  L  P  P  H  F  N  T  S  T  K  P  D
 631 taccacaggctcatctccagctacggaacccacttcatccggtcc
     Y  H  R  L  I  S  S  Y  G  T  H  F  I  R  S
 676 atggagctgggcggncgcatctcggccctcaccgnctgcgnacc
     M  E  L  G  G  R  I  S  A  L  T  X  L  R  T
 721 tgcgagctggccctggaggggctcacagccagcgaggtcgaggac
     C  E  L  A  L  E  G  L  T  A  S  E  V  E  D
 766 tgcctggctgtcgaggctgaggtcagcataagcgacagggccagt
     C  L  A  V  E  A  E  V  S  I  S  D  R  A  S
 811 gcctcgccatcgttcaaggcatgtgaggagaagaagaagaaccac
     A  S  P  S  F  K  A  C  E  E  K  K  K  N  H
 856 aaggtggggacctccttccaccaggcctaccgggagcgccattcc
     K  V  G  T  S  F  H  Q  A  Y  R  E  R  H  S

 901 aatgtcgatggtggccaccactcaaccatgcatgacctgctcttc
     N  V  D  G  G  H  H  S  T  M  H  D  L  L  F
 946 gggagccaggctgggcccgagcagttctcagcctgggtggcctca
     G  S  Q  A  G  P  E  Q  F  S  A  W  V  A  S
 991 ctgcaggacagccctggcctggtggactacacgctggagcctctg
     L  Q  D  S  P  G  L  V  D  Y  T  L  E  P  L
1036 cacatgcttgtggagagccaggacccgcggcgggaggccctcagg
     H  M  L  V  E  S  Q  D  P  R  R  E  A  L  R
1081 caggccgtgagcaagtacgtgactgacagggcacgctggagggac
     Q  A  V  S  K  Y  V  T  D  R  A  R  W  R  D
1126 tgcaaccgcccgtgccccccggggcaacacaagaacccgaagaac
     C  N  R  P  C  P  P  G  Q  H  K  N  P  K  N
1171 ccatgccagtgcatgtgtcctggttcagcagccaccacccaggac
     P  C  Q  C  M  C  P  G  S  A  A  T  T  Q  D
1216 tgctgtccccggcagaagggactggcccacctggaggtcatgaac
     C  C  P  R  Q  K  G  L  A  H  L  E  V  M  N
1261 ttcaaggcatcaggtctgtggggagactggatcactgccacggac
     F  K  A  S  G  L  W  G  D  W  I  T  A  T  D
1306 gcctatctgaaggtttttttcgccggccaggagcagaggaccgcc
     A  Y  L  K  V  F  F  A  G  Q  E  Q  R  T  A
1351 acagtatggaacgataacaaccccaggtggatgacgcggctggac
     T  V  W  N  D  N  N  P  R  W  M  T  R  L  D
1396 ttcggggatgtgctcctggccaccgggggccccctgagggtgcag
     F  G  D  V  L  L  A  T  G  G  P  L  R  V  Q
1441 gtctgggatgcagactttggctgggacgatgaccttcttggcact
     V  W  D  A  D  F  G  W  D  D  D  L  L  G  T
1486 tgtgaccgcaccccaaagtctggctcacatgaggtgtcatgcccc
     C  D  R  T  P  K  S  G  S  H  E  V  S  C  P
1531 atggaccacggtctcttgaaattctcctaccaggtcaaatgcttg
     M  D  H  G  L  L  K  F  S  Y  Q  V  K  C  L
1576 cctcacctgacgggggagaggtgcctgcagtatgccccccaaggg
     P  H  L  T  G  E  R  C  L  Q  Y  A  P  Q  G
1621 cttctggggaatcctccaggaaaccggagtgggccagtgtggtga
     L  L  G  N  P  P  G  N  R  S  G  P  V  W  *
```

Figure 4b: In-silico protein analysis of perforin cDNA

Sequence alignment of perforin proteins produced by ClustalW. GenBank Accession numbers of sequences are as follows NP_035203.2 (mouse perforin), NP_059026.2 (rat perforin), translated FJ176744 (putative bovine perforin) and AAA60167.1 (human perforin)



MACPF - membrane attack components/perforin
EGF - epidermal growth factor
N - putative N-linked (GlcNAc) glycosylation site
TM - transmembrane region
Conserved regions between proteins are shaded in black

100072744) and human (Gene ID: 5551) perforin, respectively.

On the other hand, BLASTN search of the TIGR bovine database identified TC399777, a 1002-bp Bos taurus EST with 68% nucleotide identity to human granzyme A (GI:6996012), 67% nucleotide identity to mouse granzyme A (GI:6754101) and 67% nucleotide identity to rat granzyme A (GI:23618882). Granzyme A primers generated a 322 bp cDNA clone (Figure 3, panel D) that contained a 303 bp open reading frame with 69% nucleotide identity to TC399777 and encoding a 100-amino acid polypeptide as shown in Figure 5a. BLASTP search indicated that this polypeptide was an internal coding sequence that mapped onto the N-terminal region generally between amino acid residues 40 to 145 of predicted granzyme A genes. Over this range the bovine cDNA showed 85%/93% amino acid/homology similarity to pig granzyme A (Gene ID: 100526762) as well as 78%/93% aminoacid/homology similarity and 75%/84% amino acid/homology similarity to horse (Gene ID: 100062497) and human (Gene ID: 3001) granzyme A, respectively. In silico analysis further showed that the cDNA displayed conserved domains that resemble trypsin-like serine protease of the granzyme family with the putative active site containing catalytic residues at H25 and D70 of the putative bovine granzyme A fragment as shown in Figure 5b. Attempts to clone a full-length bovine cDNA encoding granzyme A were however unsuccessful.

In regard to granzyme B, BLASTN search of the TIGR database identified TC134878, a 903 bp EST with 70% nucleotide similarity to human (GI:20988817), 70% nucleotide similarity to mouse (GI:7305122) and 68% nucleotide similarity to rat (GI:31542925) granzyme B. A 774-bp cDNA was cloned (Figure 3, panel E) and sequenced using the granzyme B primers shown in Table 3. Sequence analysis identified a coding sequence of 756 bp with a 95% nucleotide identity to TC134878 with a 251-amino acid long polypeptide reported in Figure 6a. BLASTP search of the protein database identified significant homology of this cDNA to to pig (GeneID:100233184; 61%/77% aminoacid/homology), horse (GeneID:10049805; 59%/76% aminoacid/homology) and human (GeneID:3002; 59%/74% aminoacid/homology) granzymes B. The bovine cDNA also showed significant homology to sheep mast cell proteinase-3 (Swiss-prot O46683; 90% nucleotide identity, 82/87% amino acid/homology) as well as bovine duodenase (GeneID:281731; 88/93% amino acid/homology) proteins. Structural analysis of the

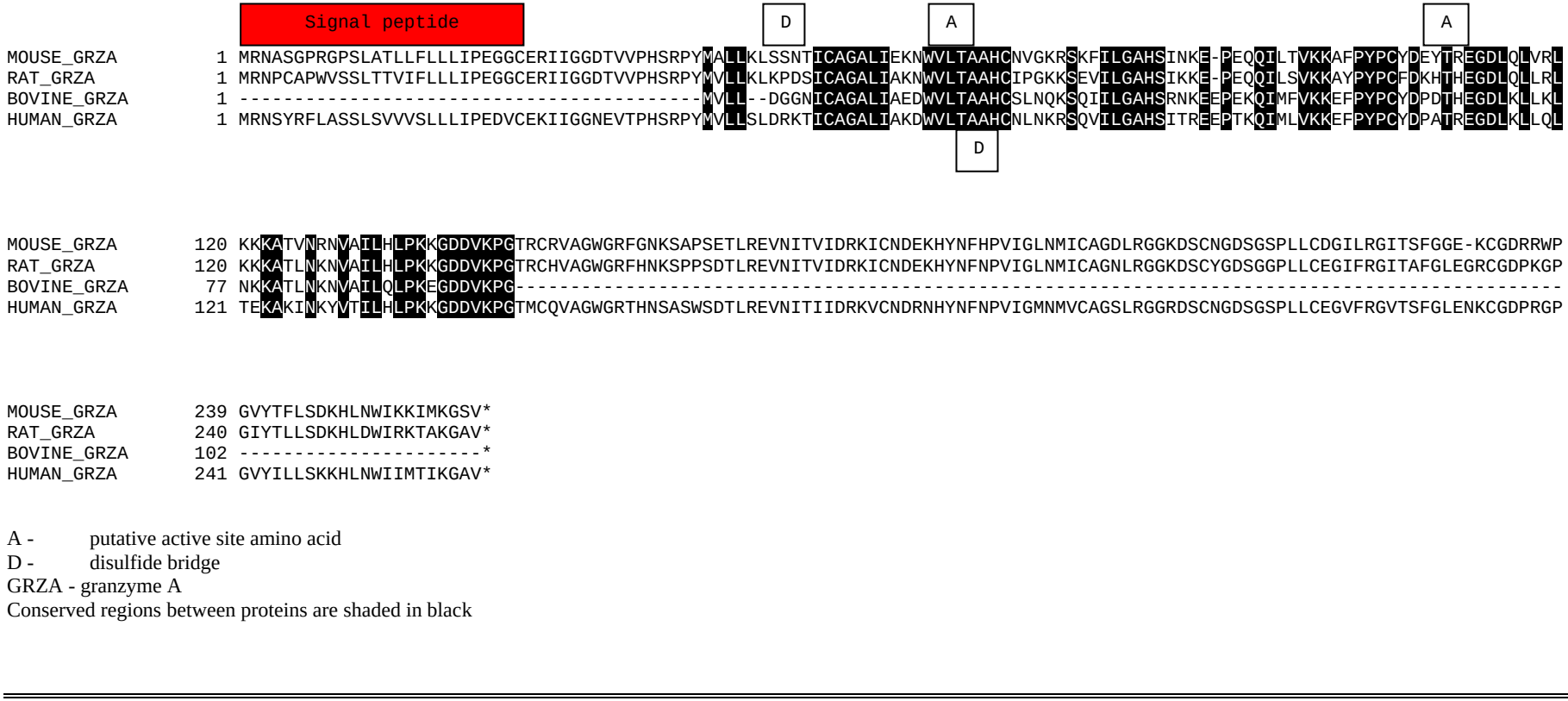
Figure 5a: Nucleotide sequence and predicted amino acid sequence of putative bovine granzyme A

GenBank accession number FJ176746.

```
  1 atggtgctacttgatgggggaaacatctgtgccggagctttgatt
    M  V  L  L  D  G  G  N  I  C  A  G  A  L  I
 46 gccgaagactgggtattgactgcagctcactgttccctgaaccag
    A  E  D  W  V  L  T  A  A  H  C  S  L  N  Q
 91 aaatcccagatcattcttggggcccactcaagaaacaaggaagag
    K  S  Q  I  I  L  G  A  H  S  R  N  K  E  E
136 cctgaaaaacagattatgtttgttaagaaagagtttccctatcca
    P  E  K  Q  I  M  F  V  K  K  E  F  P  Y  P
181 tgctatgacccggacacacatgaaggcgatcttaaacttctaaag
    C  Y  D  P  D  T  H  E  G  D  L  K  L  L  K
226 ctgaacaaaaaagcaacacttaataaaaacgtggctatccttcag
    L  N  K  K  A  T  L  N  K  N  V  A  I  L  Q
271 ctcccaaaagagggcgatgatgtgaaaccagga 303
    L  P  K  E  G  D  D  V  K  P  G
```

Figure 5b: In-silico protein analysis of granzyme A cDNA

Sequence alignment of granzyme A proteins produced by ClustalW. GenBank Accession numbers of sequences are as follows NP_034500.1 (mouse granzyme A), NP_803198.1 (rat granzyme A), translated FJ176746 (putative bovine granzyme A) and NP_006135.1 (human granzyme A)



A - putative active site amino acid
D - disulfide bridge
GRZA - granzyme A
Conserved regions between proteins are shaded in black

Figure 6a: Nucleotide sequence and predicted amino acid sequence of putative bovine granzyme B

GenBank accession number FJ176745.

```
  1 atggtcctgctcctgctcctggtggcccttctgtcccctaccagg
    M  V  L  L  L  L  L  V  A  L  L  S  P  T  R
 46 gaggcagggaaaatcatcgggggccacgaggccaagccacactcc
    E  A  G  K  I  I  G  G  H  E  A  K  P  H  S
 91 cgtccctacatggcgtttcttcaggtcaagacttcagggaaatct
    R  P  Y  M  A  F  L  Q  V  K  T  S  G  K  S
136 cacaactgtgggggtttcctcgtgcgtgaggacttcgtgctgaca
    H  N  C  G  G  F  L  V  R  E  D  F  V  L  T
181 gcagctcactgcctgggaagctcaatcagtgtcaccctgggggcc
    A  A  H  C  L  G  S  S  I  S  V  T  L  G  A
226 cacaacatcaaacaacgagagacgacccagcaggtcatcccagtg
    H  N  I  K  Q  R  E  T  T  Q  Q  V  I  P  V
271 agaagacccatcccccacccagactataatgatgagactttggcc
    R  R  P  I  P  H  P  D  Y  N  D  E  T  L  A
316 aacgacatcatgttactgaagctgactaggaaggctgacattacg
    N  D  I  M  L  L  K  L  T  R  K  A  D  I  T
361 gataaagtgagccccatcaatctgcccaggagcttggcgaaggtg
    D  K  V  S  P  I  N  L  P  R  S  L  A  K  V
406 aagacagggatgatgtgcagtgtggccggctgggggcgactgggg
    K  T  G  M  M  C  S  V  A  G  W  G  R  L  G
451 gtaaatatgccctctacagacaaactacaggaggtagatcttgaa
    V  N  M  P  S  T  D  K  L  Q  E  V  D  L  E
496 gtccaaaataagaagaaatgtaaggatcgcttccaagactacaat
    V  Q  N  K  K  K  C  K  D  R  F  Q  D  Y  N
541 gcctccatacagatatgtgctggagatccaagcaagaggaagagt
    A  S  I  Q  I  C  A  G  D  P  S  K  R  K  S
586 tctttcttgggtgactctgggggcccgcttgtgtgtaatggtgtg
    S  F  L  G  D  S  G  G  P  L  V  C  N  G  V
631 gcccagggcattgtgtcctatggaagagatgatgggacacctcca
    A  Q  G  I  V  S  Y  G  R  D  D  G  T  P  P
676 aatgtctacaccagaatctccagctttctgtcctggatccagaca
    N  V  Y  T  R  I  S  S  F  L  S  W  I  Q  T
721 acaatgagacggtacaaacgccagagatcagtgtga 756
    T  M  R  R  Y  K  R  Q  R  S  V  *
```

Figure 6b: In-silico protein analysis of granzyme B cDNA

Sequence alignment of granzyme B proteins produced by ClustalW. GenBank Accession numbers of sequences are as follows NP_776721.1 (bovine duodenase), NP_001009411.1 (sheep mast cell protease), translated FJ176745 (putative bovine granzyme B) and AAHH30195.1 (human granzyme B)

DP - activation dipeptide
A - putative active site amino acid
C - conserved region (granzymes)
GRZB - granzyme B
MCP - mast cell protease
Conserved regions between proteins are shaded in black

polypeptide predicted a trypsin- like serine protease showing typical features of zymogen activation, catalytic active and substrate-binding sites consisting of His, Asp and Serine in conserved domains resembling those of the granzyme subfamily. Further sequence analysis as shown in Figure 6b revealed a signal (leader) peptide (amino acid 1-17) with a likely cleavage site at positions 17 and 18 (REA-GK), followed by an amino-terminal activation dipeptide Glu-Lys (aa 19,20) and three highly conserved residues as central elements of the active site (H63, D107, S201). However in contrast to other granzymes, the dipeptide appears to be Gly-Lys and not Gly-Glu or Glu-Glu that is typically found on rodent and human granzymes. The residues at positions 1-4 after the activation dipeptide (typically Ile-Ile-Gly-Gly) and 9-16 (Pro-Ile-Thr-Ser-Arg-Pro-Tyr-Met-Ala) are similarly conserved.

Nucleotide and predicted protein sequences of bovine perforin, granzymes A and B were submitted (September 2008) to the GenBank sequence database and awarded FJ176744, FJ176746, FJ176745 GenBank codes. Amino acid full names and abbreviations are shown in Appendix V.

Recent analyses of the cloned coding sequences indicate that the perforin sequence shows 98% identity to the bovine sequence on NCBI accession number NM_001143735, (GeneID 369025) in the Bovine Genome Sequencing and Analysis Database (BGD) . This sequence is mapped onto chromosome 28.16 of the bovine genome and predicted to encode a transcript for perforin pore forming protein gene (BGD ID: BT19749). The granzyme A sequence obtained in this study showed identity to sequence 98% nucleotide identity to NM_001099095.1 encoding *Bos taurus* cytotoxic T lymphocyte serine esterase 3 or granzyme A (GeneID 539093) and mapped onto chromosome 20.18 of the bovine genome, a transcript for bovine cytotoxic T lymphocyte associated serine esterase-3 or granzyme A gene (BGD ID: BT14165). Unlike perforin and granzyme A, BLASTN search of BGD identified nucleotide similarity (E-value 0.0) to transcripts found on four genes- BT23865, 93%; BT18850, 93%; BT15925, 92%; BT13165, 92%, all mapped on chromosome 21.28 except for BT13165 whose genomic location is still unknown. Three of the transcripts, BT23865, BT15925 and BT13165 are predicted to encode bovine duodenase (NCBI reference 785952, 786126, 782827 respectively), whereas the transcript from BT18850 was identical to sequence in NM_174296.2, predicted to encode *Bos taurus* cytotoxic T lymphocyte associated serine esterase 1 or granzyme B

(GeneID 281731).

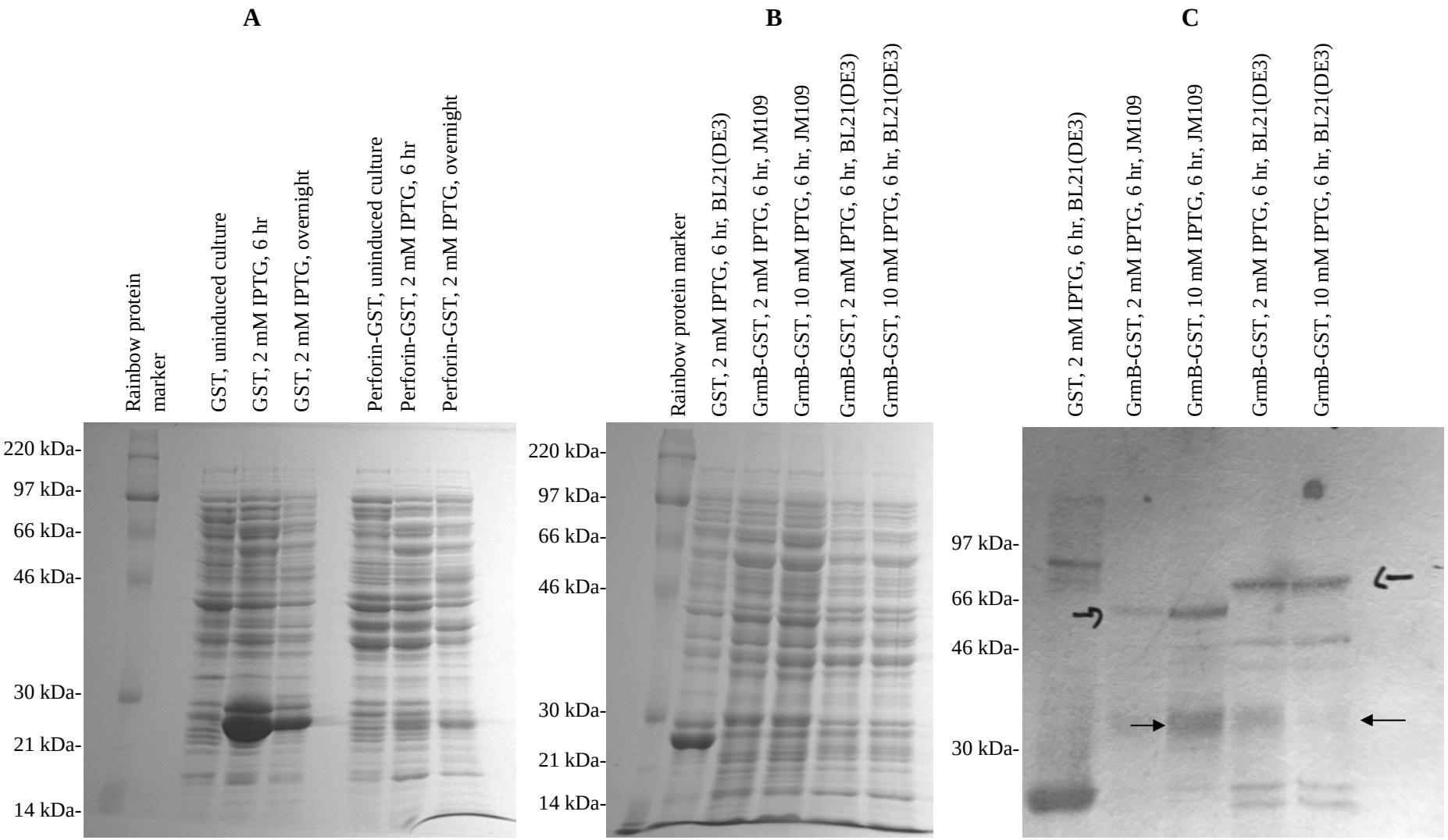
2.2.2 Expression of bovine perforin, granzymes A and B

Full-length cDNA inserts encoding bovine perforin and granzyme B were sub-cloned in the appropriate reading frame and orientation in the bacterial expression vector pGEX-3T. As stated in the Materials and Methods, the 2.1 kb perforin PCR fragment was sub-cloned into the *Bam*HI and *Eco*RI sites of the expression vector pGEX-3T to be expressed as glutathione-S-transferase (GST) fusion protein. *E. coli* JM109 was then transformed using the recombinant plasmid and induced to over-express protein. As shown in Figure 7, panel A, Coomassie staining of SDS-PAGE resolved whole cell lysates did not reveal any obvious expression of perforin-GST fusion protein, as a band at the expected molecular weight of approximately 81 kDa was either too faint or lacking. Similarly, western blot analysis of parallel nitrocellulose membranes using anti-GST antibodies failed to show any distinct perforin-GST fusion protein band (data not shown).

To express recombinant granzyme B, the bovine cDNA without its leader sequence (generates a 705 bp fragment) was directionally sub-cloned into pGEX-3T at the *Bam*HI and *Sal*I sites in-fusion with GST. The As with perforin, no obvious expression of granzyme B-GST fusion protein could be detected with Coomassie staining (Figure 7, panel B). However, immunoblot analysis of the bacterial cell lysate using anti-GST antibodies revealed low level expression of granzyme B-GST fusion protein at a predicted size of 52 kDa (Figure 7, panel C). The fusion protein appeared to undergo degradation as evidenced by the smears of GST reactive bands of various sizes on the western blot. Attempts to improve protein expression of perforin-GST and granzyme B-GST fusion proteins by optimisation of several parameters such as varying the concentration of IPTG (1-10 mM), the culture temperature (30°C vs. 37°C) and incubation time after induction (2 hr up to a maximum of 16 hr or overnight) or the cell type (expression in *E. coli* JM109 or BL21 (DE3) were not successful. While there was a decrease in expression levels of GST protein 16 hours following induction as compared to 6 hours of incubation time after, data obtained showed no significant differences in perforin-GST expression after 6 hours or 16 hours following addition of IPTG (Figure 7, panel A). Similarly there was no difference in granzyme B-GST protein expression level in JM109 or BL21 (DE3) or by using a different concentration of IPTG in the same cell types (data not shown).

Figure 7: Expression of GST fusion proteins

Whole cell lysates were electrophoresed and protein visualized by coomassie staining (A & B) or electro-transferred to nitrocellulose membrane and protein detected by anti-GST antibodies (C). Panel A shows perforin-GST fusion proteins expressed in JM109 cells and induced using IPTG while panel B shows expression of granzyme B-GST in JM109 or BL21 (DE3). Panel C shows immunoblot analysis of granzyme B-GST proteins showing various forms of the expressed protein as indicated by the arrows. Proteins from 7b (above) were electro-transferred onto nitrocellulose membrane and protein detected by anti-GST antibodies.


A
Rainbow protein marker
GST, uninduced culture
GST, 2 mM IPTG, 6 hr
GST, 2 mM IPTG, overnight
Perforin-GST, uninduced culture
Perforin-GST, 2 mM IPTG, 6 hr
Perforin-GST, 2 mM IPTG, overnight
220 kDa-
97 kDa-
66 kDa-
46 kDa-
30 kDa-
21 kDa-
14 kDa-
B
Rainbow protein marker
GST, 2 mM IPTG, 6 hr, BL21(DE3)
GrmB-GST, 2 mM IPTG, 6 hr, JM109
GrmB-GST, 10 mM IPTG, 6 hr, JM109
GrmB-GST, 2 mM IPTG, 6 hr, BL21(DE3)
GrmB-GST, 10 mM IPTG, 6 hr, BL21(DE3)
220 kDa-
97 kDa-
66 kDa-
46 kDa-
30 kDa-
21 kDa-
14 kDa-
C
GST, 2 mM IPTG, 6 hr, BL21(DE3)
GrmB-GST, 2 mM IPTG, 6 hr, JM109
GrmB-GST, 10 mM IPTG, 6 hr, JM109
GrmB-GST, 2 mM IPTG, 6 hr, BL21(DE3)
GrmB-GST, 10 mM IPTG, 6 hr, BL21(DE3)
97 kDa-
66 kDa-
46 kDa-
30 kDa-

Given the difficulties encountered with attempts to express recombinant proteins in bacteria, efforts were made to sub-clone the respective genes in the baculovirus expression vector, pFastBac™ HT-B and express the proteins in insect cells as overlapping truncated fragments. In the case of perforin, three cDNA fragments were generated by PCR consisting of the sequence beginning at 61 through to 661 (designated fragment I), 508 to 1107 (fragment II) and 1045 through 1662 (fragment III) of the open reading frame (shown in Appendix II). Using the cloned bovine perforin cDNA as template, cDNA fragments I and II, each sized 600 bp, were separately sub-cloned into the donor plasmid in-fusion with an N-terminal His-tag and with or without a C-terminal GFP and then incorporated into baculovirus via transposition onto a baculovirus shuttle vector as detailed in Materials and Methods. Cloning of cDNA fragment III was hindered by the presence of an *Nco*I recognition sequence at position 1529-1534 that generated cDNA fragments with similar ends when digested with *Nco*I restriction enzyme. Although cDNA fragment II also had another *Nco*I recognition sequence at position 674-679, it was possible to clone this fragment after partial digestion resulting in the larger sub-fragment (600bp) bearing *Nco*I and *Sal*I digested ends that was separated and purified from the smaller fragments (171bp and 429bp) generated from *Nco*I digestion. In contrast, partial digestion of cDNA fragment III generated three fragments of approximately 600bp, 500bp and 100bp resulting in poor resolution of the 600bp and 500bp fragments on agarose gel electrophoresis. Using GFP expression as a 'shotgun' approach to revealing target cDNA translation, perforin-GFP^{+ve} and perforin-GFP^{-ve} recombinant baculovirus-infected cultures were set up in parallel and the GFP^{+ve} culture viewed under a U.V. microscope. As shown in Figure 8, panel A, cultures infected with perforin-GFP^{+ve} recombinant baculovirus displayed green fluorescence indicating that the GFP gene had been successfully cloned in-frame with the perforin gene and that both cDNA had translated into protein. The diffuse pattern of GFP staining suggests that the proteins are retained in the cytoplasm. To detect individual proteins, the poly-histidine tagged proteins in perforin-GFP^{-ve} cultures were purified on Ni-NTA resin. Coomassie staining of SDS-PAGE resolved samples displayed a single polypeptide of approximately 30 kDa encompassing perforin fragment 1 compared to a predicted apparent molecular weight of 22 kDa. On the other hand perforin fragment 2 generated multiple (3) polypeptide products with an apparent molecular size range occurring between 21 kDa–30 kDa as compared to a predicted molecular weight of 22

Figure 8: Expression and purification of recombinant proteins from insect cells

Perforin, granzyme A and B were expressed as baculovirus derived proteins in insect cells as green fluorescence protein (GFP)-positive or $GFP^{negative}$ proteins. Column I shows typical $GFP^{positive}$ culture expressing GFP-fusion recombinant proteins when viewed under a UV microscope while column II shows Coomassie staining of SDS-PAGE resolved protein samples purified from $GFP^{negative}$ cultures. The bands of interest are shown by the arrows as follows

A - Lane 1: Rainbow Protein marker
Lane 2-6: Perforin fragment I;
Lane 7-11: Perforin fragment II

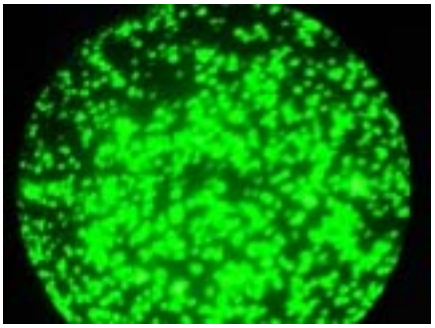
B - Lane 1: Rainbow Protein Marker
Lane 2-5: Granzyme A in lanes 2-5

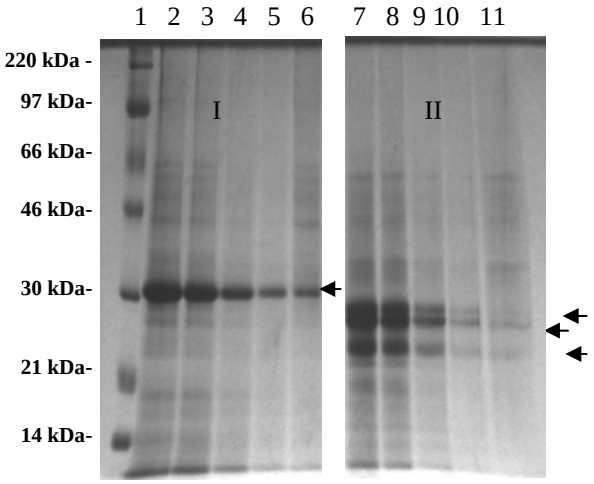
C - Lane 1: Rainbow Protein Marker
Lane 2 Granzyme B fragment I and II

I

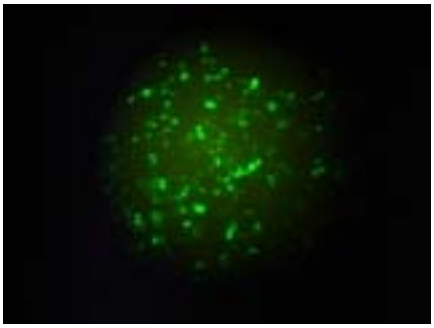
II

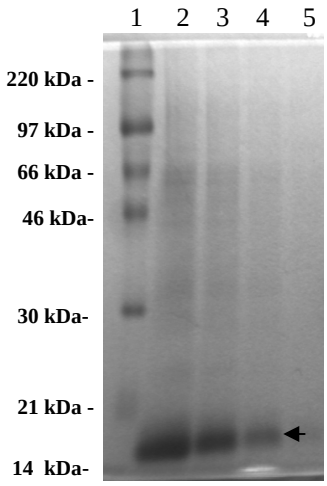
A - Perforin



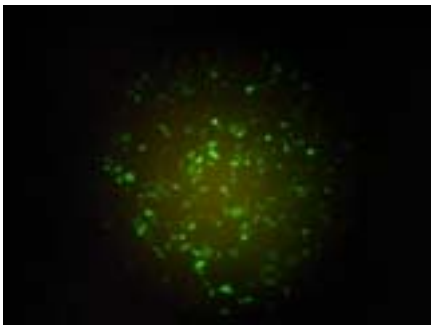


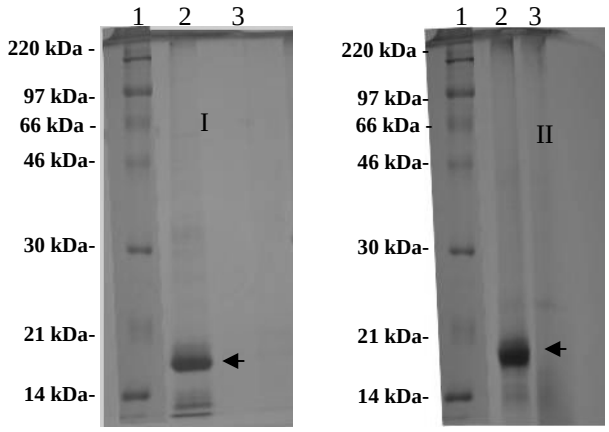
B - Granzyme A





C - Granzyme B





kDa. (Figure 8, panel A, column II).

Employing the same strategy the 303-bp granzyme A cDNA PCR fragment was sub-cloned into the *Nco*I and *Sal*I sites of pFastBac in-frame with the N-terminal His tag and the C-terminal GFP cDNA. Recombinant baculovirus particles with and without GFP were generated and used to infect insect cell cultures in parallel. As in the case of perforin, granzyme A-GFP^{+ve} but not granzyme A-GFP^{-ve} recombinant baculovirus-infected cultures displayed green fluorescence that was also diffuse in nature (Fig 8. panel B, column I). When Ni-NTA-purified SDS-PAGE separated granzyme A-GFP^{-ve} protein samples were subjected to Coomassie staining, they resolved as a single band of 14 kDa compatible with the molecular weight of 12 kDa, respectively (Figure 8, panel B, column II) With regards to granzyme B, a similar cloning and expression strategy was adopted. Two overlapping granzyme B cDNA fragments sized 450 bp and 321 bp, respectively, were separately sub-cloned into the pFastBac™ donor plasmid in-fusion with the N-terminal His tag and C-terminal GFP cDNA. It was observed that expression of the recombinant proteins was generally low and cultures 72 hours post infection were marked by an increase in cell death while the infectivity levels did not differ significantly with the use of a higher dose of virus particles. However as observed with the aforementioned polypeptides, the expressed granzyme B-GFP fusion protein showed a diffuse fluorescence pattern in the cytoplasm. Ni- NTA binding and elution purified proteins at an apparent molecular weight approximately 18 kDa which was in line with the predicted molecular weight of 17 kDa for granzyme B fragment I. In contrast the predicted molecular weight of granzyme B fragment II was shown to be 13 kDa, significantly smaller in size than the obtained fragments.

2.3 Discussion

Use of recombinant DNA techniques to select genes expressed preferentially in mouse or human CTL has cloned cDNA or RNA encoding perforin and trypsin-like serine proteases (Trapani *et al*., 1988; Liu *et al*., 1996). cDNA was generated from day-three *T. parva* antigen-stimulated bovine CTL when the mRNA should be upregulated. Nucleotide and amino acid sequence comparisons of the three bovine cDNAs and available gene sequence data strongly suggested that the cDNAs were the bovine homologues of human, mouse, rat perforin and granzymes A and B. Two of the cDNAs encoded full-length gene sequences with the third cDNA bearing an internal (partial) coding sequence.

The sequenced cDNA of 2188 base pairs was shown to contain an open reading frame of 554 amino acids (Figure 4) whose sequence displayed as expected considerable homology with certain functional domains-MACPF, EGF, and Ca^{2+} binding domains, found in the family of pore-forming proteins (Tschopp, Masson and Stanley, 1986; Lichtenheld *et al*., 1988; Ishikawa *et al*., 1999). These features suggest that the protein inserts into the lipid bi-layer of target cell membranes in the presence of Ca^{2+} after polymerization (Blumenthal *et al*., 1984; Young, Cohn and Podack, 1986). The amino acid sequence similarity within the conserved domains of the bovine cDNA and mouse, rat, and human sequences indicates that it is likely to be of the same gene family and hence the bovine homologue is likely to have the same functional activity as rat, mouse and human perforin.

With regard to bovine granzymes, the finding of the typical features of zymogen activation, conserved disulfide bridges, catalytic active and substrate binding sites consisting of His, Asp and Serine in conserved S1 peptidase domains conferring a substrate specificity in two of the cloned bovine cDNAs (322 bp and 774 bp) is consistent with the predicted activities of mouse and human granzymes A and B (Trapani, 2001). Despite the functional similarity between the putative bovine granzymes, the two serine proteases are about 36% identical at the amino acid level suggesting that they belong to different granzyme sub-families. This observation is consistent with what has been observed with murine granzymes (Trapani *et al*., 1988). Mouse, rat and human granzymes B are about 70% identical at the amino acid level, by contrast, amino acid sequence identity drops to approximately 30-40% when one compares granzymes from different sub-families such as granzyme A and B even

within the same species (Trapani, 2001).

The Bovine Genome Sequencing and Analysis consortium (2009) recently released the Bovine Genome Database project (http://BovineGenome.org) (Reese *et al*., 2010; Childers *et al*., 2011) which was not available at the time of the study. Analyses of the cloned bovine sequences showed that the putative bovine perforin, granzymes A and B cDNA maps on regions on chromosome 28, chromosome 20 and chromosome 21, respectively of the bovine genome. The structural and functional annotation of the corresponding genes predict a perforin-pore forming protein, cytotoxic T lymphocyte associated serine esterase and a CTL-associated serine esterase, respectively. However, the cDNA sequence predicted to encode bovine granzyme B also maps on regions encoding transcripts for bovine duodenase. This then questions the identity of the cloned cDNA. While it is likely to be a serine protease of the granzyme sub-family, there is a need for further functional analysis to determine the classification of the cloned cDNA. Work on cloning bovine perforin, granzymes A and B for this study was initiated in 2003/2004, a time period when bovine genome sequences were available as EST data from few sequencing work groups. As such the prevailing situation presented a major challenge with sequence data validation relying on sequences derived from other species.

Attempts to produce recombinant perforin, granzymes A and B in a prokaryotic system were not successful (Figure 7). While it is possible to argue that cloning of the perforin fragment with its leader sequence contributed to the lack of expression, deletion of the leader sequence while cloning granzyme B had minimal effect on successful expression of the protein in bacterial cells. Difficulties in expressing these proteins in prokaryotic systems have been reported. Apostolidis *et al*., (1995) reports the formation of an inactive aggregated protein product as a GST-granzyme B fusion product while Kummer *et al*., 1993 reports expression of an insoluble and inactive enzyme.

The baculovirus expression system, instead of prokaryotic systems, was hence chosen on the basis that recombinant proteins expressed in (eukaryotic) insect cells may resemble more closely the natural proteins produced by mammalian cells in terms of post-translational modifications (Luckow and Summers, 1988). Results obtained showed considerable production of recombinant perforin and granzymes A and B in High Five cells infected with recombinant baculovirus. Sufficient amounts of

recombinant perforin granzymes A and B, although not secreted into the culture medium, were readily purified in soluble form from infected High Five cell homogenate. This procedure offers some advantage over conventional purification of natural perforin from cultured CTL, as the latter requires expensive culture supplements (IL-2) and in our hands did not yield a signficant amount of perforin (Chuma, Francis *et al.,* personal communication). Although a detailed qualitative analysis is yet to be performed it is possible that differences in the biosynthesis of proteins between prokaryotic and eukaryotic cells may contribute to prevent protein expression in bacterial cells resulting in an inactive protein product if at all. For example Beresford *et al*., 1997 successfully expressed granzyme A as an inactive proenzyme exported to the bacterial periplasm by the replacement of the mammalian leader sequence with a bacterial leader sequence. Improved expression may also be achieved using mammalian expression vectors and cells such as pcDNA™ and COS cells which could also ensure generation of functional mammalian proteins.

Using the baculovirus expression system, it was possible to express truncated forms of perforin and granzyme B protein in addition to a granzyme A polypeptide (Figure 8, column II). These polypeptides were however only soluble in urea as attempts to re-solubilise the proteins were not successful resulting in insoluble precipitate. Whereas the predicted molecular weight of perforin fragments is 22 kDa the multiple perforin bands ranging between 21 kDa and 30 kDa seen in Figure 8 (panel A column I and II), suggest protein products at different levels of glycosylation during post-translational modification process. This is further supported by in-silico protein structural analysis of bovine perforin cDNA that identifies putative N-linked (GlcNAc) glycosylation sites (Figure 4b) a characteristic feature of perforin-like molecules (Lowry *et al*., 1989; Liu *et al*., 1996; Li *et al*., 2001).

An interesting observation concerning the production of recombinant perforin by insect cells is that intracellularly expressed recombinant perforin, although not segregated into membrane bound organelles, did not exert any detectable, harmful effect on the cells. Using GFP fluorescence as an indicator (Figure 8), it was observed that the insect cells remained viable and healthy until the death incurred by baculovirus four to five days after infection. Studies have shown significant lytic activities in truncated recombinant perforins suggesting that the lytic function in perforin lies in both the N-terminal and C terminal portion of the perforin molecule

(Liu *et al*., 1996; Li *et al*., 2001). It is suggested that mildly acidic pH and low Ca^{2+} concentration in the intracellular environment may prevent the endogenously produced recombinant perforin from damaging the host insect cell (Griffiths and Argon 1995). The lytic function of perforin is reported to be dependent on Ca^{2+} and a neutral pH while release of perforin from the granules upon granule exocytosis, the binding of perforin to the phospholipids bilayers and the homopolymerisation within the membranes of target cells are all calcium-dependent processes at a neutral pH (Blumenthal *et al*., 1984; Podack *et al*., 1985; Masson *et al*., 1990). As similar observations were made with expression of granzyme A and B, the low acidic pH of insect cell conditioned medium may contribute to the stability of the proteins allowing for the expression of granule proteases and lytic molecules.

In conclusion, bovine perforin- granzyme A/B- like molecules have been cloned sequenced and expressed. Although these molecules were expressed as fragments, it provides a basis for which the full-length, biologically active forms of the proteins could be cloned and expressed in heterologous systems to facilitate further structure and functional analysis of the perforin/granzyme pathways for inducing lysis/apoptosis of target cells in bovine CTL.

Chapter 3: Generation of Antibodies to Bovine Perforin, Granzymes A and B for Assay Development

3.0 Introduction

A body of information on *T. parva*-specific $CD8^+$ T cell responses has been generated by applying a number of immunological readouts. These include radioisotope release assays of CTL responses induced *in vivo* (Morrison, Godderis and Teale, 1987; Morrison *et al*., 1987; McKeever *et al*., 1994), short- and long-term limiting dilution assay (LDA) and bulk culture-generated effectors (Goddeeris, Morrison and Teale, 1986; Goddeeris and Morrison, 1988; Taracha *et al*., 1992; 1995a and b; McKeever *et al*., 1994) as well as *ex vivo* IFN-γ ELISpot analyses of T cells following immunisation and challenge (Graham *et al*., 2006). While assessment of T cell responses *ex vivo* by direct cytotoxicity is informative, this method may not detect responses of effector cells present at low frequencies in peripheral blood. On the other hand, whereas use of efferent lymph enhanced the detection of CTL in direct cytotoxicity assays (McKeever *et al*., 1994), the method of obtaining it through lymphatic duct cannulation is impractical for large-scale studies. Culturing of T cells to expand them to detectable frequencies may present an underestimate or highly selected specificities not reflecting the *in vivo* repertoire. *Ex vivo* IFN-γ ELISpot assays are highly sensitive and provide a more representative measure of the *in vivo* situation. However, Graham and co-workers (2006) observed that IFN-γ ELISPOT responses are not predictive of protection against *T. parva*, at least in the context of the five candidate antigens and antigen delivery systems tested, suggesting this response may not serve as immune correlates.

There is, therefore, a need for assay systems that are sensitive, rapid and relevant to protective immunity permitting the precise evaluation of *T. parva*-specific $CD8^+$ T cell responses in cattle following immunisation with candidate antigens and parasite challenge. Chapter 3 seeks to address two of the six objectives of the study focusing on the generation of antibodies to bovine perforin, granzymes A and B (proteins expressed in Chapter 2) and to evaluate the use of these reagents in development and validation of ELISpot, ELISA and intracellular staining assays. With the exception of perforin, application of mouse and human standardized assays to detect bovine granzymes A/B has not been successful (Simon Graham, personal communication). Preliminary studies had identified δG9 (BD Pharmingen), an anti-human perforin monoclonal antibody that cross reacts with bovine perforin shown by intracellular staining of CTL lines and this formed the basis for the generation of

additional anti-perforin antibodies for development of ELISpot and ELISA assays.

3.1 Materials and methods

3.1.1 Inoculation of laboratory animals with bovine recombinant proteins and peptides

Four- to six-week old male or female Balb/C mice and 6-week old male or female rats (Sprague-dawly) and adult New Zealand white male or female rabbits were used in the study. All animals were obtained from the ILRI Small Animal Unit, and used in accordance with the guidelines of the Institutional Animal Care and Use Committee. The animals were kept in a laboratory biosafety level I small animal holding facility with regular feeding and watering.

Bovine recombinant perforin, granzymes A and B as well as synthetic peptides (Pepscan Systems BV., Leystad, Netherlands) were formulated in TitreMax® Gold adjuvant (CytRx Corporation, Norcross, USA) according to the manufacturer's instructions. The 18-residue synthetic peptides were derived from the predicted amino acid sequences of perforin, granzymes A and B (Appendix VI). The antigenic regions within the molecules were selected using the Hopp and Woods (1981) algorithm which searches and identifies regions displaying properties that define likelihood of antigenicity (Appendix VII). The properties including hydrophilicity and accessibility to solvent are determined by scoring each amino acid with an antigenicity index. The higher the antigenicity index the more likely that antibody would recognise that group of amino acid residues. On the basis of this score, particular amino acid residues were selected to serve as templates for synthesis of peptides as shown in Table 4. The peptides were synthesised, covalently conjugated to keyhole limpet haemocyanin (KLH) carrier protein and shipped as lyophilised pellets. These were reconstituted to 10 mg/ml in sterile PBS and stored at -20°C.

Unless otherwise specified, relevant antigens in quantities indicated in Table 5 were emulsified in an equal volume of adjuvant. Water-in-oil emulsions of antigen in TiterMax® Gold adjuvant were prepared using the double hub needle method as follows: 0.5 volumes of aqueous antigen was added to 1 volume adjuvant oil phase, emulsified to a water-in-oil admixture, to which was added the remaining 0.5 volumes of aqueous antigen and further mixed to achieve a homogenous emulsion. Stability of

Table 4: List of KLH-conjugated peptides

Protein	Amino acid residue	Sequence
Perforin	262-279	EVSISDRASASPSFKACE
Perforin	446-463	EQRTATVWNDNNPRWMTR
Granzyme A	38-54	GAHSRNKEEPEKQIMFVK
Granzyme A	61-78	CYDPDTHEGDLKLLKLNK
Granzyme B	103-121	LANDIMLLKLTRKADITD
Granzyme B	149-166	LGVNMPSTDKLQEVDLEV

the water-in-oil emulsions was assessed by placing a drop of the emulsion onto the surface of water. The emulsion was ready for use when seen to hold together on the surface of water. Various routes of administration of the adjuvant-formulated antigens were used according to the manufacturer's instructions. For mice a total volume of 200 µl of the formulated antigen was administered subcutaneously in two sites (100 µl each) at the base of the tail and in the hind quadriceps. Rats were inoculated intramuscularly in the hind quadriceps with a total of 200 µl of formulated antigen. Rabbits received a total of 1.0 ml formulated antigen distributed in 6 injection sites as follows: four sites (both shoulders and both hind quadriceps) 200 µl each subcutaneously and two sites (both hind quadriceps) 100 µl each intramusculary. To evaluate the response to immunisation, blood samples were collected from the animals in weekly intervals beginning 7 days post-immunisation and serum IgG determined using indirect ELISA or immunoblot analysis. Blood for serum IgG detection studies was collected by tail vein bleeding for both rats and mice and by ear vein bleeding for rabbits. When serum IgG levels declined, usually three to four weeks post-inoculation, the animals were boosted in a similar manner. Immunisation regimes for the various animals is summarised in Table 5.

3.1.2 Production of polyclonal antibodies

Fourteen days after the last booster inoculation, non-heparinised blood was collected from the animals to prepare antisera. Rats were sacrificed by CO_2 asphyxiation and blood collected by a cardiac puncture while blood from the rabbits was collected by bleeding from the ear vein in accordance with the ILRI IACUC standard operating procedures. The blood was incubated at 37°C for 1 hr and 4°C overnight for coagulation and separation of serum to take place, respectively. The serum samples were transferred into fresh tubes and stored at -20°C for subsequent testing.

Immunoglobulins were purified from the serum samples using a DEAE-52 cellulose column. Briefly, DEAE-52 (Whatmann International, UK) was equilibrated by washing several times in 0.0175 M Phosphate buffer until pH 6.4 was achieved. The slurry was used to pack a 10-ml polypropylene column (Pierce Chemical Co., Rockford, USA) in order to prepare an 8-ml gel-bed of DEAE-52 per 1 ml of serum (in accordance with the Antibody Purification Handbook, Amersham Biosciences).

Table 5: Details of immunisation of animals with adjuvant-formulated recombinant proteins and KLH-conjugated peptides

Antigen	Animal species (no. of animals/antigen)	Dosage levels		Week of booster immunisation		
Recombinant proteins		Primary immunisation	Secondary immunisation (no. of inoculations)			
Recombinant perforin	Mice (2)	50 µg	25 µg (2)	3	6	
	Rats (3)	100 µg	50 µg (2)	3	6	
Recombinant granzyme A	Mice (2)	50 µg	25 µg (2)	3	6	
	Rats (3)	100 µg	50 µg (2)	3	6	
Recombinant granzyme B	Mice (2)	50 µg	25 µg (2)	3	6	
	Rats (3)	100 µg	50 µg (2)	3	6	
KLH conjugated peptides						
Perforin $_{262-279}$	Mice (2)	500 µg	250 µg (2)	4	9	
	*Rabbits (2)	500 µg	250 µg (3)	4	9	14
Perforin$_{446-463}$	Mice (2)	500 µg	250 µg (2)	4	9	
	*Rabbits (2)	500 µg	250 µg (3)	4	9	14
Granzyme A$_{38-54}$	Mice (2)	500 µg	250 µg (2)	4	9	
	¥Rabbits (2)	500 µg	250 µg (3)	4	9	14
Granzyme A$_{61-78}$	Mice (2)	500 µg	250 µg (2)	4	9	
	¥Rabbits (2)	500 µg	250 µg (3)	4	9	14
Granzyme B$_{103-121}$	Mice (2)	500 µg	250 µg (2)	4	9	
	§Rabbits (2)	500 µg	250 µg (3)	4	9	14
Granzyme B $_{149-166}$	Mice (2)	500 µg	250 µg (2)	4	9	
	§Rabbits (2)	500 µg	250 µg (3)	4	9	14

*, §, ¥ A total of 6 rabbits were used. Each rabbit within the group was inoculated with both peptides. Antigens were reconstituted at 10mg/ml. Total volume of formulated antigen administered was 200µl for mice and rats and 1.0 ml for rabbits.

Serum was dialysed (Spectra/Por 4 MWCO 12-14kDa) overnight in 0.0175M Phosphate buffer, pH 6.4 (1-2 changes of buffer, volume of dialysis buffer at least 500-fold greater than than volume of sample) and centrifuged (Heraeus Megafuge Model No. 2.0R) at 450xg for 10 min to remove precipitated proteins. One ml of clarified serum was applied onto an 8-ml bed volume of DEAE-52 and 1 ml fractions of the flow through collected after the column void volume had flowed through the column. The absorbance of each fraction was determined at 280 nm. By adding phosphate buffer to the column, fractions were collected until the sample approached baseline absorbance. Samples showing peak absorbance were pooled and antibody concentrated using 10-kDa MWCO Centricon® tubes (Millipore, MA, USA). The antibodies were stored in 100 µl aliquots at -20˚C for subsequent evaluation and use.

3.1.3 Production of monoclonal antibodies

3.1.3.1 Fusion of myeloma cells with immune splenocytes

A master seed stock of mouse myeloma cells (X63-Ag) was recovered from liquid nitrogen storage by rapid thawing at 37°C before being transferred into a 10-ml polypropylene tube containing 5 ml cold complete culture medium comprising RPMI-1640 with 20 mM HEPES buffer supplemented with 10% (v/v) of gamma-irradiated, heat-inactivated FCS 50 µg/ml gentamicin 2mM L-glutamine $5x10^{-5}$ M 2-mercaptoethanol 240 IU/ml penicillin and 100 µg/ml streptomycin. Cells were recovered by centrifugation at 180 x g for 10 min at 4°C, the pellet resuspended in 5 ml culture medium and transferred into a 25-cm^2 tissue culture flask which was then incubated at 37°C in a humidified 5% CO_2 incubator. Cultures were maintained in logarithmic phase by sub-culturing every 2-3 days; two-thirds of the cell suspension were removed and replaced with an equal volume of fresh culture medium.

Three days prior to fusion and at least five weeks after the last immunisation, mice were subjected to an intraperitoneal inoculation with 25 µg of antigen in PBS. Fusion of splenocytes with the mouse X63-Ag myeloma cells was performed according to the described procedures (Pearson *et al*., 1990). Briefly, mice were euthanized by CO_2 asphyxiation and the spleen surgically removed into a stainless steel strainer in a sterile petri dish containing 10 ml complete culture medium where it was sliced into small pieces using sterile scissors and forceps. In circular movements, the spleen was pressed against the screen of the stainless steel strainer with the plastic

syringe plunger of a 3-cc syringe until only the fibrous tissue remained on top of the strainer screen. The suspension of spleen cells was then transferred into a sterile 10-ml polystyrene conical tube and allowed to stand for 3 min at room temperature for the large particles to settle. The top 95% of the cell suspension was transferred into a 50-ml polypropylene tube (Falcon®), centrifuged (Heraeus Megafuge, Model No. 2.0R) at 1500 rpm for 15 minutes at room temperature and the cell pellet resuspended in 10 ml of complete culture medium.

A cell suspension containing $2x10^7$ viable (determined by trypan blue exclusion) myeloma cells in logarithmic phase of growth was harvested and mixed with $2x10^8$ splenocytes in a 50-ml polypropylene tube (Falcon®). The cell suspension was then centrifuged (Heraeus Megafuge, Model No. 2.0R) at 1500 rpm for 15 minutes at room temperature, the cell pellet washed once with 10 ml serum-free RPMI-1640, recovered by centrifugation as before and drained thoroughly of culture medium. Cell fusion was induced by slowly adding 500 µl of 41.6% polyethylene glycol/15% dimethylsulfoxide (DMSO) in serum-free RPMI-1640 medium while gently swirling the tube for 1 min followed by addition of 500 µl 25% PEG in serum-free RPMI-1640 medium swirling gently as before for 2 min, and finally 4 ml of fusion medium (same as complete culture medium except that it contains 20% FCS) with similar agitation. An additional 20 ml of fusion medium was then added, mixed gently and the cell suspension dispensed in 1-ml per well aliquots into 24-well tissue culture plates which were incubated overnight at 37°C in a humidified 5% CO_2 incubator. After incubation 1-ml aliquots of HAT selective medium (fusion medium supplemented with 100 µM hypoxanthine, 100 µM aminopterin and 10 µM thymidine) were added into each well and the plate incubated as before. HAT medium was renewed every second day for 7 days while screening the well for growth of hybrids. This was done by carefully removing 1 ml of the culture medium and replacing it with an equal volume of HAT medium. After 7 days or once growth was observed, HT medium (fusion medium supplemented with 100 µM hypoxanthine, 10 µM thymidine) was used. When cell growth covered 10% to 50% of the well surface area, the hybridoma cell supernatants were screened for antibody by intracellular staining CTLs and those found positive were cloned by limiting dilution.

3.1.3.2 Cloning of hybridoma cell lines

The day prior to cloning, freshly harvested mouse thymocytes resuspended in complete culture medium comprising RPMI-1640 with HEPES and supplemented with 15% FBS and the same antibiotics as used above, were plated out in 100-µl aliquots into a 96-well flat bottom tissue culture plate and incubated overnight at 37°C in a humidified 5% CO_2 incubator. Cells from each well whose supernatant yielded antigen-specific antibody activity were harvested, resuspended at 500 viable cells/ml in culture medium, dispensed in 100 µl aliquots into the 96-well tissue culture plate containing thymocytes and incubated as before. Wells were screened for growth after 7 days and those containing single colonies were marked for screening the supernatant for specific antibody once cell growth was 10% to 50% confluent. Those found positive were expanded first into a separate well of a 24-well tissue culture plate that had been pre-incubated with 500 µl of thymocytes in complete culture medium then gradually scaled up to maintenance in a 25-cm^2 tissue culture flask while reducing the 15% FBS in the medium to 10%. Once established as stable cell lines, hybridomas were maintained and propagated in complete culture medium in a similar manner as described for the myeloma cell line. Cells were prepared for liquid nitrogen storage while the culture supernatant was harvested for determination of antibody activity, isotype and purification.

3.1.3.3 Monoclonal antibody isotype determination

Monoclonal antibody in supernatants of hybridoma cultures were assessed for class and sub-class type using the ImmunoPure Monoclonal Antibody Isotyping Kit (Pierce Chemical Co., Rockford, US). Nunc polysorp 96-well microtitre plates (Nunc, Denmark) were coated overnight at 4˚C with 50 µl of goat anti-mouse Ig (G, A and M) at a dilution of 1:50 in 0.1 M sodium bicarbonate buffer, pH 9.5. A total of ten microtiter wells were used to screen and determine the class/sub-class of one mAb; eight served as test and the remaining two being positive and negative controls respectively. The plates were washed once by adding and flicking off 200 µl of PBS containing 0.05% Tween-20 and blocking with 125 µl of PBS / 0.5% BSA at 37°C for 1 hr. After discarding the blocking solution, 50 µl of test culture supernatants were added to each of nine separate wells (well #1 - #9) of the plate and 50 µl of the positive control (mouse IgG_1) added to well #10. The plates were kept at 37°C for 1

hr. After the culture supernatants were discarded, plates were washed 4 times with PBS / 0.05% Tween-20 by adding and flicking off buffer. Aliquots of 50 µl of normal rabbit serum and sub-class- specific anti-mouse Ig were added to well #1 (negative control) and wells #2 - #9 (test) respectively, while 50 µl of anti-mouse IgG_1 was added to well #10 (positive control). The sub-class-specific anti-mouse Ig used were rabbit anti-mouse IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, IgA, IgM, Kappa light and Lambda light chain. The plates were incubated at 37°C for 1 hr and washed as before. Excess wash medium was removed by gently tapping plates on paper towels. An aliquot of 50 µl of goat anti-rabbit HRP conjugated-IgG was added and the plated incubated for 1 hr at 37°C. The plates were further washed as before and developed by adding 100 µl per well of ABTS substrate solution and the plates scored by visual inspection.

3.1.3.4 Monoclonal antibody purification

Monoclonal antibody were subsequently purified from culture supernatants by affinity chromatography essentially as described in the Antibody Purification Handbook, Amersham Biosciences (2003). Briefly, 50-ml aliquots of hybridoma culture supernatants were centrifuged at 180xg for 15 min to remove cell debri. Immunoglobulins in the clarified supernatants were concentrated by ammonium sulphate precipitation. Prior to this, 1 part of 1M Tris-HCl, pH 8.0 was added to 10 parts of the clarified hybridoma supernatant to maintain pH at 8.0. Saturated ammonium sulphate solution (100 g ammonium sulphate in 100 ml distilled water) was added dropwise up to 50% saturation while stirring gently. The solution was incubated at room temperature for 1 hr with continuous stirring and the precipitated Ig recovered by centrifuging at 180xg for 20 min at room temperature. The pellet was re-solubilised in 1 ml PBS, a 1.5-ml aliquot of resolubilised Ig applied on to an 8-ml bed volume of Sephadex G-25 column (Amersham Biosciences) and eluted with 1-ml fractions of 200 mM sodium phosphate buffer, pH 7.0 for IgG while IgM were eluted with 20 mM sodium phosphate and 0.8 M ammonium sulphate solution, pH 7.5.

A 1-ml HiTrap protein G HP column (Amersham Biosciences) was used for the purification of IgG. One-ml aliquots of de-salted antibody preparation were applied to the column previously equilibrated with 5 volumes of binding buffer (200 mM sodium phosphate buffer, pH 7.0). The column was washed with 10-column volumes of binding buffer or until no protein was detected in the eluate (determined

by UV absorbance at 280 nm). Column-bound antibodies were eluted with 5 column volumes of elution buffer (0.01 M glycine-HCl, pH 2.7) directly into a microcentrifuge tube containing 100 µl of neutralization buffer (1 M Tris-HCl, pH 9.0). Protein in eluted fractions was determined by UV absorbance at 280 nm. Positive fractions were pooled and stored at -20˚C in 100-µl aliquots.

Purification of IgM was done essentially as described for IgG but used 1-ml HiTrap IgM purification columns (Amersham, Biosciences). Columns were washed with 20 mM sodium phosphate, pH 7.5 containing 30% isopropanol and bound antibodies eluted with 20 mM sodium phosphate, pH 7.5 with no addition of neutralization buffer.

3.1.4. Evaluation of antibody activity

3.1.4.1 Testing using Indirect Enzyme Linked Immunosorbent (ELISA) Assay

Hybridoma and serum samples were evaluated for antigen-specific antibody activity using the ELISA technique essentially as described by Sambrook and Russell (2001). Nunc® polysorp 96-well microtitre plates (Nunc, Denmark) were coated overnight at 4°C with 50 µl or 100 µl of synthetic peptide, KLH, recombinant perforin, granzymes A or B (10 µg/ml) or CTL lysate (prepared by rapid freeze-thawing of bovine CTL re-suspended in serum-free tissue culture medium) in coating buffer (bicarbonate buffer, pH 9.6). The plates were washed by washing with 200 µl of PBS containing 0.05% Tween-20 (Washing buffer) prior to blocking with 200 µl PBS containing 1% casein and 0.05% Tween-20 (Blocking buffer) for 1 hr at room temperature. The blocking buffer was discarded, and 50 µl of hybridoma supernatant, mouse, rat or rabbit serum added at a 1:100 dilution in PBS containing 0.1% casein and 0.05% Tween-20 (antibody dilution buffer) prior to incubation for 2 hr at room temperature. After washing four times in washing buffer, 50–100 µl of the anti-mouse IgG or IgM-HRP conjugate (Amersham, UK), anti-rat IgG, or anti-rabbit IgG-HRP antibody conjugate (Serotec Antibodies Inc., Germany) diluted 1:10000 in antibody dilution buffer was added and incubated for 1 hr at room temperature. The plates were washed six times and developed by adding 100 µl of tetramethylbenzidine (TMB) peroxidase substrate solution (Sigma, UK) to the reaction mix. The intensity of the blue product was measured by a spectrophotometer at 405 nm. Alternatively, the enzyme-substrate reaction was stopped by the addition of an equal volume of 1N

HCL resulting in a yellow product whose intensity was measured at 450 nm.

3.1.4.2 Immunoblot analysis of antibodies against perforin, granzymes A and B

This assay was performed essentially as described for western blotting of SDS-PAGE resolved His-tagged proteins in section 2.2.6 but with slight modifications. After separation of the purified recombinant proteins using a 12.5% polyacrylamide gel and transfer onto nitrocellulose filters, blocking of non-specific binding sites was performed for 1 hr in 5% non-fat milk in PBS and the membranes incubated in 1:100 dilution of polyclonal sera followed by anti-mouse IgG HRP conjugate (Amersham) diluted 1:5000 in antibody dilution buffer or anti-rat IgG HRP conjugate (SEROTEC Antibodies) diluted 1:10000 in antibody dilution buffer. The blots were washed and bound antibody detected using diaminobenzidine (DAB) solution containing 0.1% hydrogen peroxide.

3.1.4.3 Intracellular staining of cytotoxic T cell lines

Hybridoma and serum samples were evaluated for antibody activity using the procedure described in section 4.1.4.8.2 but with slight modifications. Briefly, $4x10^5$ cells/well were washed with 200 µl of cold PBS and then fixed by resuspending in 200 µl 1% paraformaldehyde in PBS for 10 min at room temperature. After washing once with PBS, the cells were permeabilised by incubating in 200 µl of a solution containing 0.1% (w/v) saponin, 0.1% sodium azide, 10 mM HEPES (PBS-Sap), supplemented with 20% heat-inactivated normal goat serum (for Fc receptor blocking) for 30 min at room temperature. The cell pellet was recovered by centrifuging at 450xg for 5 min resuspended in residual fluid and incubated in 100 µl hybridoma supernatant or 100 µl of rabbit or rat serum diluted 1:50 in PBS-Sap for 30 min at 4°C. Cells were were recovered and washed twice by resuspension in 100 µl PBS-Sap by centrifugation as before. After the final wash, the supernatant was discarded, the pellet resuspended in residual fluid and incubated in 50 µl PE-conjugated anti-mouse (whole molecule), rat (anti IgG) or rabbit (IgG) immunoglobulins diluted 1:500 in PBS-Sap for for a further 30 min at 4°C. The cells were then washed twice with PBS-Sap as before and once with PBS before resuspension in 200 µl of FACS fixative (1% paraformaldehyde in PBS). For analysis, one colour flow cytometry acquisition was performed on a FACScan ™ flow cytometer using Cell Quest software. Viable cells were gated on FSC versus SSC and

10,000 – 20,000 events collected within this gate. FACS data was analysed using Cell Quest Software or Flow Jo (TreeStar, San Carlos) Software and displayed as dot plots of FSC and log fluorescence intensity on FL2.

3.2 Results

3.2.1 Antibodies reactive to perforin and granzymes A and B

Polyclonal antibody (pAb) was generated by immunisation of rats and mice with recombinant protein or KLH-conjugated peptides as indicated in Materials and Methods. A list of reagents that were generated from these immunisations is shown in Table 6. With the exception of anti-granzyme B, pAb generated by immunisation of rats and mice with recombinant proteins recognised the corresponding protein fragments expressed by baculovirus in western blot. Data shown in Figure 9a also indicates that rat pAb showed reactivity to the four bands of perforin fragments at the expected molecular weight ranging from 21 kDa to 30 kDa whereas mouse anti-perforin recognised only 2 forms of the perforin bands. However the 3rd booster immunisation particularly in the rats resulted in boosting of responses to other immunogneic insect-cell derived antigens in the protein preparation as shown by the smears on the western blot.

As aforementioned, various peptides predicted by computer analysis to be the most hydrophilic and potentially antigenic regions of perforin, granzyme A and B, were also injected into mice and rabbits for anti-peptide antibody production. Sera was collected during the immunisation protocol and assayed for peptide-specific antibody in ELISA. Figure 9b shows the level of antibody generated to individual peptides with the rabbit group immunised with a particular peptide combination. All peptides induced a reasonable peptide specific antibody response. However sera from Rabbit 10 and 12 produced notably a lower level of reactivity to $GrzA_{38-54}$/$GrzA_{61-78}$, and $GrzB_{103-121}$/Grz $B_{149-166}$, respectively. On the other hand all peptides induced a significantly lower level of specific antibody response in mice as shown in Figure 9c. This immunisation appeared to be unresponsive to subsequent booster inoculations with antigen contrary to observations in rabbits. Notably, anti-$GrzA_{38-54}$/$GrzA_{61-78}$ response was slightly elevated compared to the response induced by the other peptides.

As shown in Figure 9d and 9e, sera from rats and rabbits contained a significant level of antibody that reacted with the corresponding recombinant protein in ELISA. Based on the cut-off value for the lower detection limit it is apparent that

Table 6: Characterisation of antibodies against bovine perforin, granzymes A and B

Name	Type	Species	Immunogen	Purification	Reactivity[a]
Antibovine perforin	polyclonal	rat	recombinant perforin	serum	WB, ELISA
Antibovine granzyme A	polyclonal	rat	recombinant granzyme A	serum	WB, ELISA
Antibovine granzyme B	polyclonal	rat	recombinant granzyme B	serum	ELISA, FACS
Antibovine perforin	polyclonal	mouse	recombinant perforin	serum	WB, ELISA
Antibovine granzyme A	polyclonal	mouse	recombinant granzyme B	serum	WB, ELISA
Antibovine granzyme B	polyclonal	mouse	recombinant granzyme B	serum	ELISA
Antibovine perforin	polyclonal	rabbit	perforin peptides	serum	ELISA, FACS
Antibovine granzyme A	polyclonal	rabbit	granzyme aApeptides	serum	ELISA, FACS
Antibovine granzyme B	polyclonal	rabbit	granzyme B peptides	serum	ELISA, FACS
[b]14.F2	IgM	mouse	perforin peptide	hybridoma tissue culture supernatant	FACS
[b] 36.D3	IgG_3	mouse	perforin peptide	hybridoma tissue culture supernatant	FACS
[b] 17.H1*	IgG_1	mouse	perforin peptide	hybridoma tissue culture supernatant	ELISA
[b] 18.H3	IgG_1	mouse	perforin peptide	hybridoma tissue culture supernatant	ELISA
44.17	IgM	mouse	perforin peptide	hybridoma tissue culture supernatant	FACS
GA46	IgM	mouse	granzyme B peptide	hybridoma tissue culture supernatant	FACS
[c] GBA5	IgM	mouse	granzyme B peptide	hybridoma tissue culture supernatant	FACS
[c] GB29	IgM	mouse	granzyme B peptide	hybridoma tissue culture supernatant	FACS
[c] GB36	IgG_1	mouse	granzyme B peptide	hybridoma tissue culture supernatant	ELISA, FACS
[c] GB37	IgM	mouse	granzyme B peptide	hybridoma tissue culture supernatant	FACS

WB – western blot analysis
ELISA – enzyme linked immunosorbent assay
FACS – fluorescent-activated cell sorting (flow cytometry)
[a]recombinant proteins used as antigen in ELISA and immunoblotting
[b], [c] – generated from the same peptide immunogen

Figure 9a: Western blot analysis of mouse and rat polyclonal sera

Mouse polyclonal anti-sera (B) and rat polyclonal antisera (C) were used to detect recombinant perforin (21-30 kDa), granzyme A (approximately14 kDa) and granzyme B (approximately 17 kDa) antigen transferred onto nitrocellulose membranes as indicated by the arrows. The three strips in panel B and C correspond to the test bleed samples collected 7 days following primary immunisation (I), 1st booster immunisation (B1; Day 21), and 2nd booster immunisation (B2; Day 42) with recombinant antigen. Panel A shows the coomassie stained gel while panels B and C show the corresponding nitrocellulose filters. Blood was collected on day 8 after the secondary booster for purification of polyclonal antibody. Reactivity to protein at expected sizes is shown by the arrows.

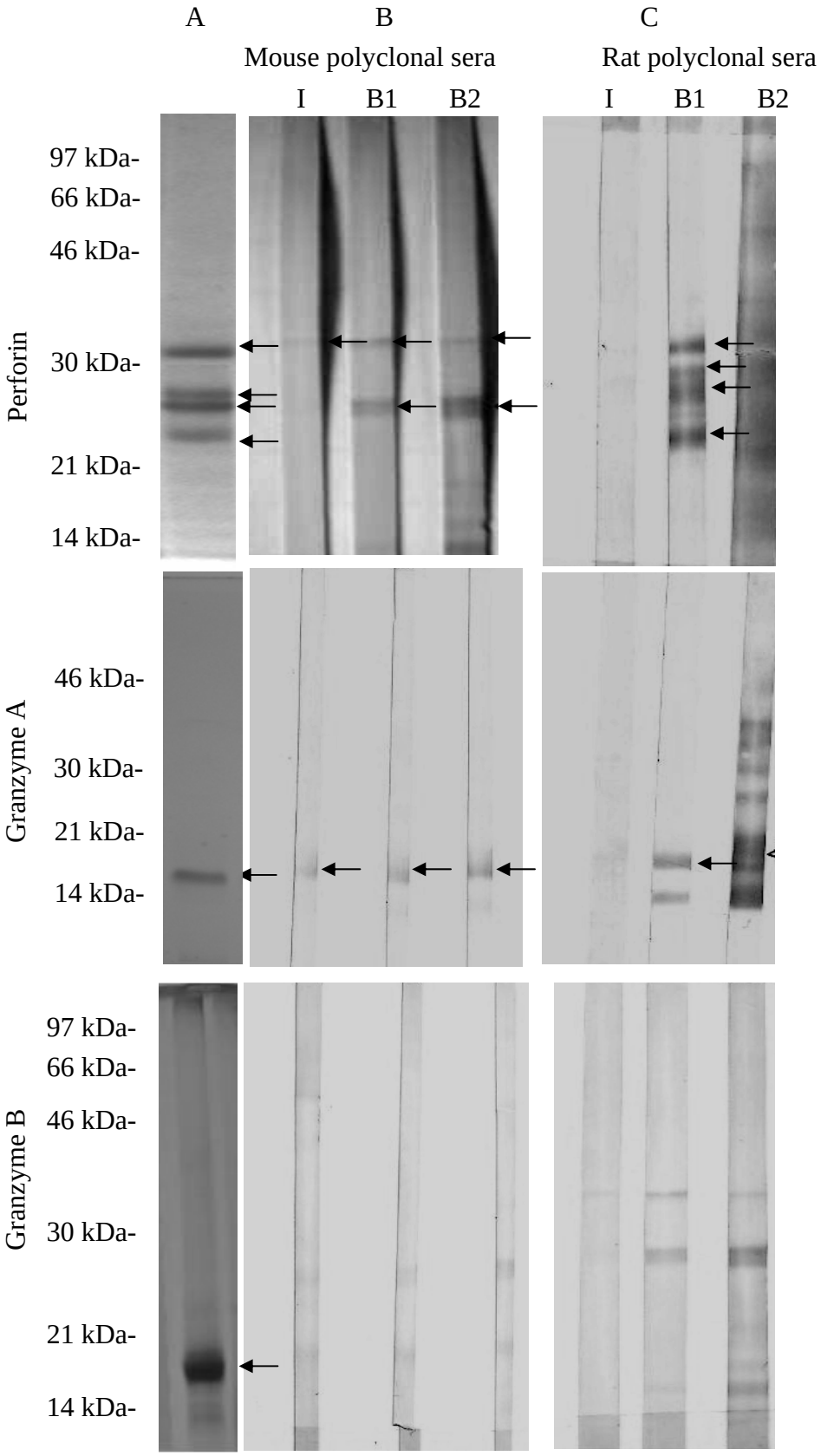

A
B
C
Mouse polyclonal sera
Rat polyclonal sera
I
B1
B2
I
B1
B2
Perforin
97 kDa-
66 kDa-
46 kDa-
30 kDa-
21 kDa-
14 kDa-
Granzyme A
46 kDa-
30 kDa-
21 kDa-
14 kDa-
Granzyme B
97 kDa-
66 kDa-
46 kDa-
30 kDa-
21 kDa-
14 kDa-

Figure 9b: Detecting anti-peptide antibodies in rabbit sera by ELISA

Un-conjugated peptide was used as the coating antigen and sera used at 1:25 dilution. Booster injections were administered on day 28 and 63 and day 98 (indicated by arrows). Blood was harvested 14 days (day 112) after the last booster immunisation for the purification of polyclonal antibody.

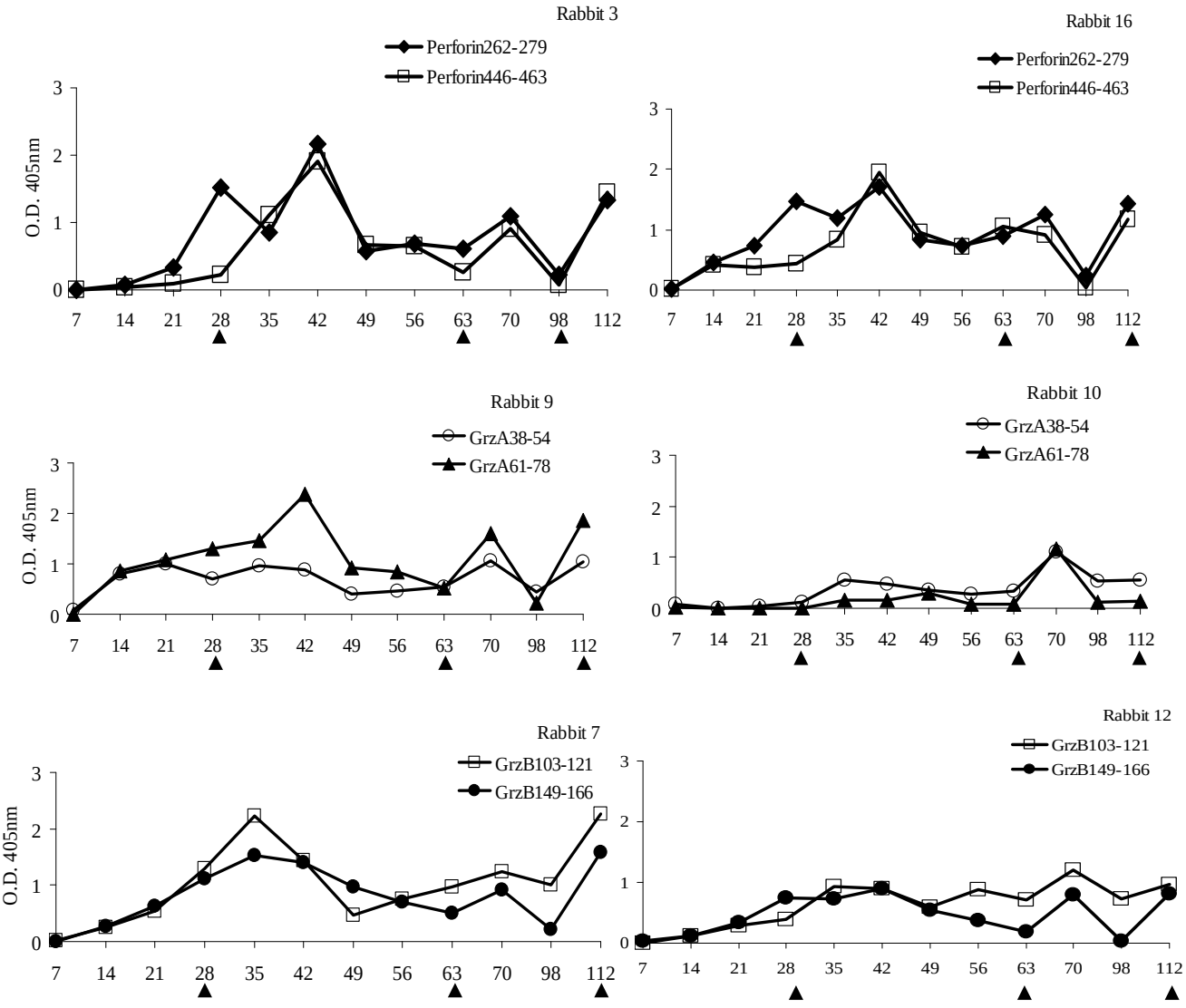

Rabbit 3
Perforin262-279
Perforin446-463
O.D. 405nm
Rabbit 16
Perforin262-279
Perforin446-463
Rabbit 9
GrzA38-54
GrzA61-78
O.D. 405nm
Rabbit 10
GrzA38-54
GrzA61-78
Rabbit 7
GrzB103-121
GrzB149-166
O.D. 405nm
Rabbit 12
GrzB103-121
GrzB149-166

Figure 9c: Detecting anti-peptide antibodies in mouse sera by ELISA

Unconjugated peptide was used as the coating antigen and sera used at 1:25 dilution. Booster injections were administered on day 28 and 63 as indicated by the arrows.

Analysis of anti-peptide antibody in mice

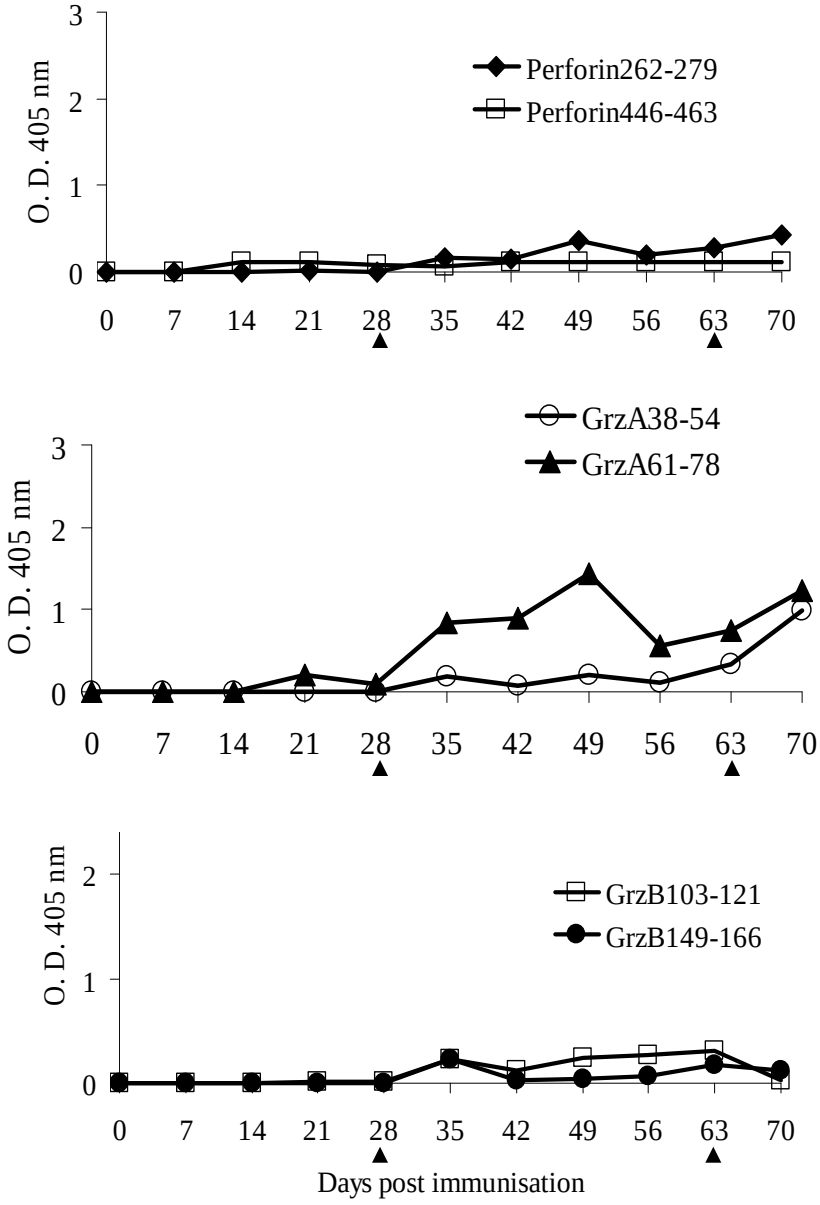


Figure 9d: Reactivity of rat polyclonal antibodies to recombinant protein

Perforin, granzyme A and granzyme B recombinant proteins were coated at a concentration of 1ug/well and the antibody used titrated in doubling dilution beginning at 1:100. The dashed line represents the cut-off for the lower detection limit as determined by antibodies binding to irrelevant antigen (mean OD value+2.6SD) as indicated below. The values shown are corrected for background staining using normal rabbit serum.

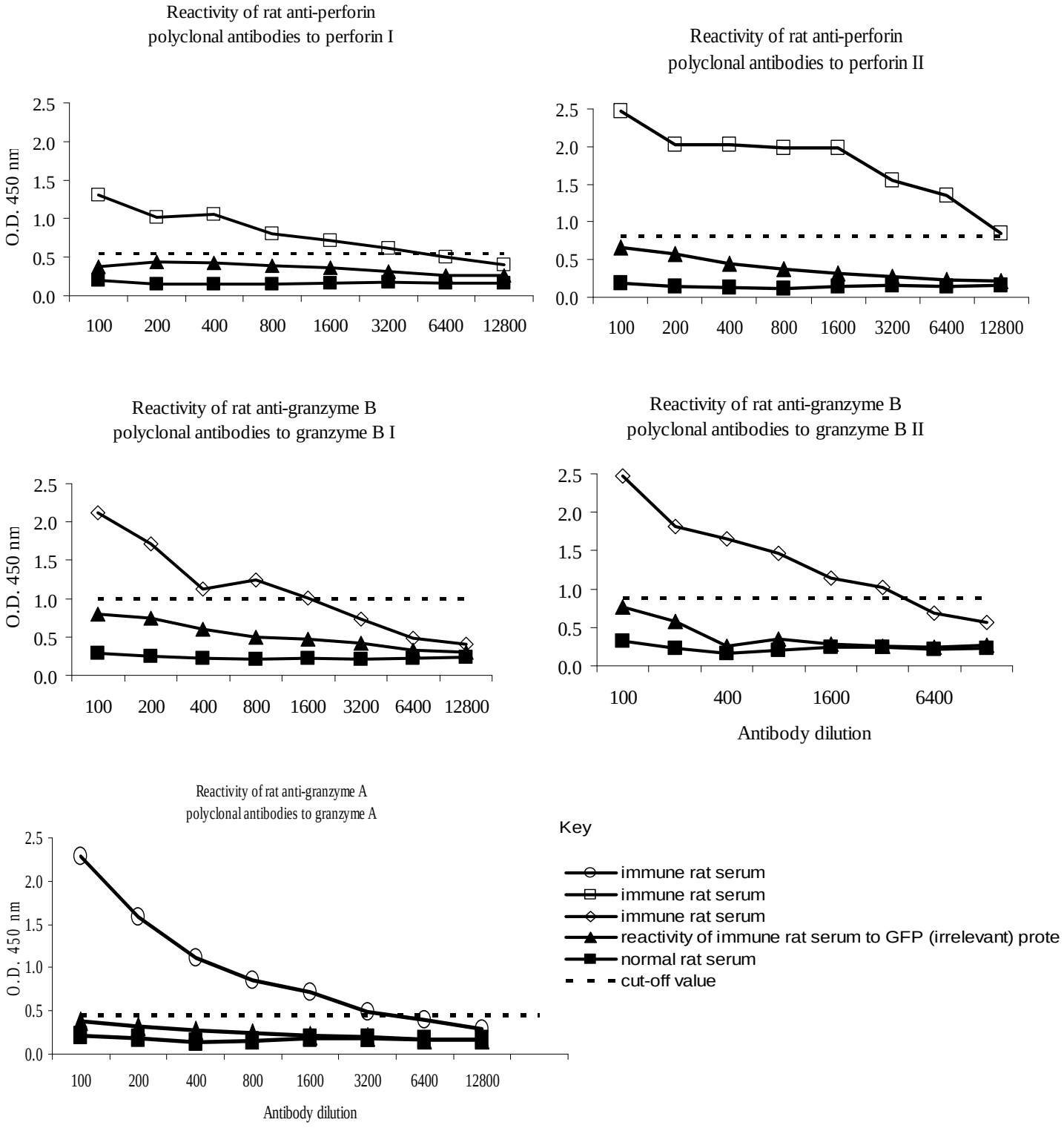
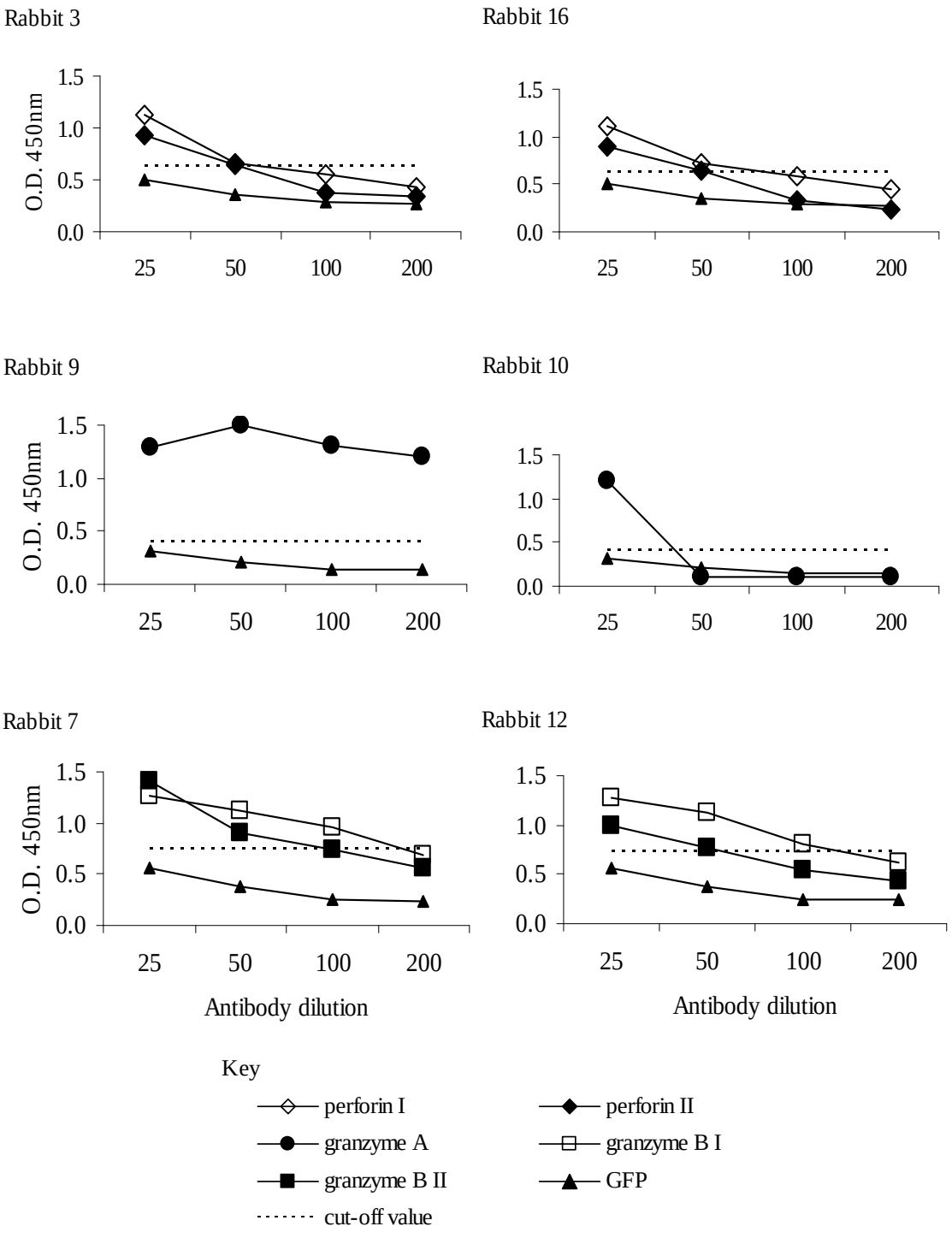

Reactivity of rat anti-perforin polyclonal antibodies to perforin I
Reactivity of rat anti-perforin polyclonal antibodies to perforin II
Reactivity of rat anti-granzyme B polyclonal antibodies to granzyme B I
Reactivity of rat anti-granzyme B polyclonal antibodies to granzyme B II
Reactivity of rat anti-granzyme A polyclonal antibodies to granzyme A
O.D. 450 nm
2.5
2.0
1.5
1.0
0.5
0.0
100
200
400
800
1600
3200
6400
12800
Antibody dilution
Key
immune rat serum
immune rat serum
immune rat serum
reactivity of immune rat serum to GFP (irrelevant) prote
normal rat serum
cut-off value

Figure 9e: Reactivity of rabbit polyclonal antibodies to recombinant protein

Perforin, granzyme A, granzyme B or GFP recombinant protein was coated at a concentration of 1 ug/well and rabbit antibodies titrated in doubling dilution beginning at 1:25. The dashed line represents the cut-off for the lower detection limit as determined by antibody binding to irrelevant antigen (GFP) (mean OD value+2.6SD). The values shown are corrected for background staining using normal rabbit serum.


Rabbit 3
Rabbit 16
Rabbit 9
Rabbit 10
Rabbit 7
Rabbit 12
O.D. 450nm
1.5
1.0
0.5
0.0
25
50
100
200
Antibody dilution
Key
perforin I
perforin II
granzyme A
granzyme B I
granzyme B II
GFP
cut-off value

the level of perforin II reacting antibody in the rat serum samples (1:11000) was significantly higher than the level of anti-perforin I specific antibodies (1:6000). Similarly the level of rat anti granzyme B I antibodies (1:6000) appeared to be slightly higher than granzyme B-II specific antibody (1:4000). Levels of granzyme A specific antibody (1:5000) were comparable to levels of perforin I at 1:6000 and granzyme B-I specific antibody at 1:4000. In contrast rabbit sera showed significantly lower reactivity levels with lower detection limits ranging from 1:50 to 1:200 (except for anti-granzyme A antibodies) compared to the rat pAb.

FACS analysis was done on CTLs to characterise the ability of the pAbs to bind native protein. Data shown in Figure 9f indicates that peptide specific rabbit antibodies showed marginal staining levels ranging from 4% - 20% positive staining CTLs. On the other hand rat anti-perforin pAbs did not bind to intracellular perforin and granzyme A whereas staining with rat anti-granzyme B resulted in a slight shift in fluorescence intensity of the cells with only 9% of CTLs staining positive above background levels.

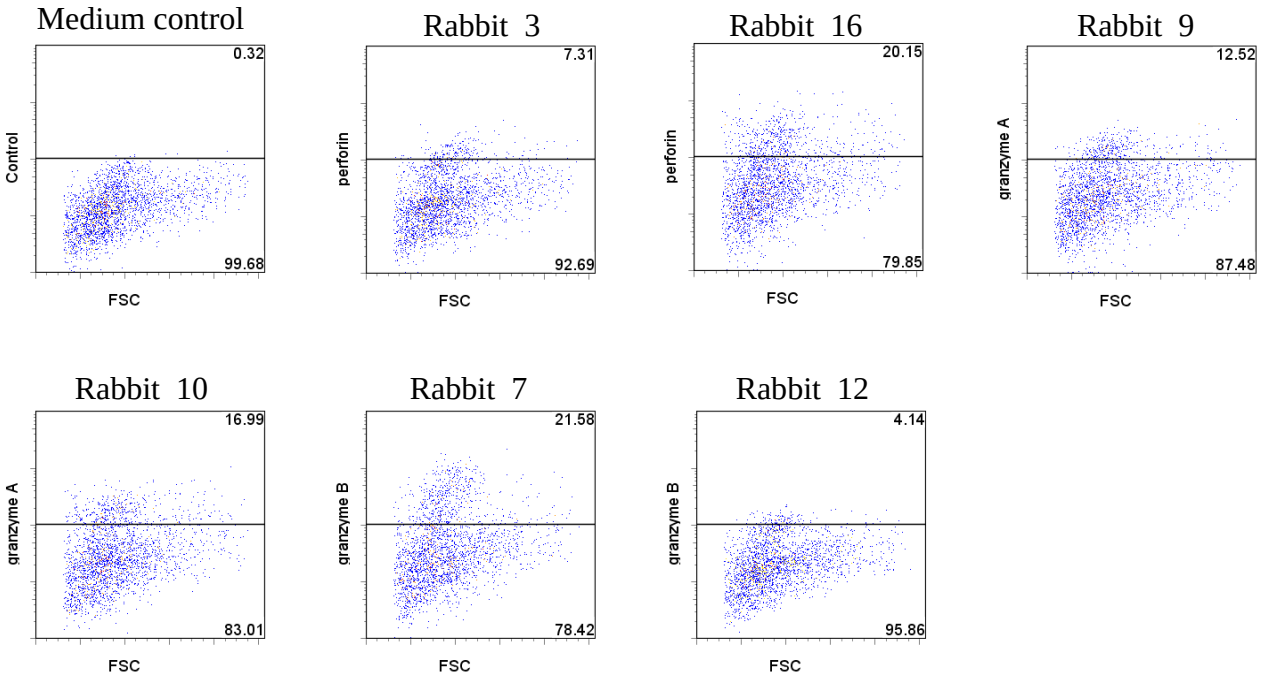
Attempts to generate hybridomas using splenocytes obtained from mice immunised with recombinant protein were unsuccessful. However screening of hybridoma clones and characterisation of anti-peptide monoclonal antibody (mAb) was conducted by ELISA and intracellular staining of CTL as described in Materials and Methods. Hybridomas that gave positive signals in the primary screening were cloned and further screened for reactivity to the recombinant protein by ELISA as well as to the native protein by ICS. This data is tabulated in Table 7. Generation of mAbs specific to peptides $GrzA_{61\text{-}78}$ and $GrzB_{149\text{-}166}$ were not successful. Out of 182 hybridoma clones generated, 10 were selected for further evaluation based on their peptide specificity, characteristic, staining of CTL (moderate (approximately 60%) or >90%), performance in ELISA and the original well that they were cloned from. Isotyping results (Figure 10a) showed 6/10 of the mAbs to be of IgM class while 3 were IgG_1 and 1 was of IgG_3 sub-type.

Monoclonal antibodies were tested for reactivity to the corresponding recombinant proteins by ELISA and to native protein by staining CTL. Commercially

Figure 9f: Reactivity of rabbit and rat polyclonal antibodies to native proteins

CTLs were stained for the expression of perforin and granzymes A/B using polyclonal antibodies raised in rabbits following immunisation with KLH-conjugated peptides (A) and antibodies raised in rats following immunisation with recombinant proteins (B).

A


Medium control
Control
0.32
99.68
FSC
Rabbit 3
perforin
7.31
92.69
FSC
Rabbit 16
perforin
20.15
79.85
FSC
Rabbit 9
granzyme A
12.52
87.48
FSC
Rabbit 10
granzyme A
16.99
83.01
FSC
Rabbit 7
granzyme B
21.58
78.42
FSC
Rabbit 12
granzyme B
4.14
95.86
FSC

B

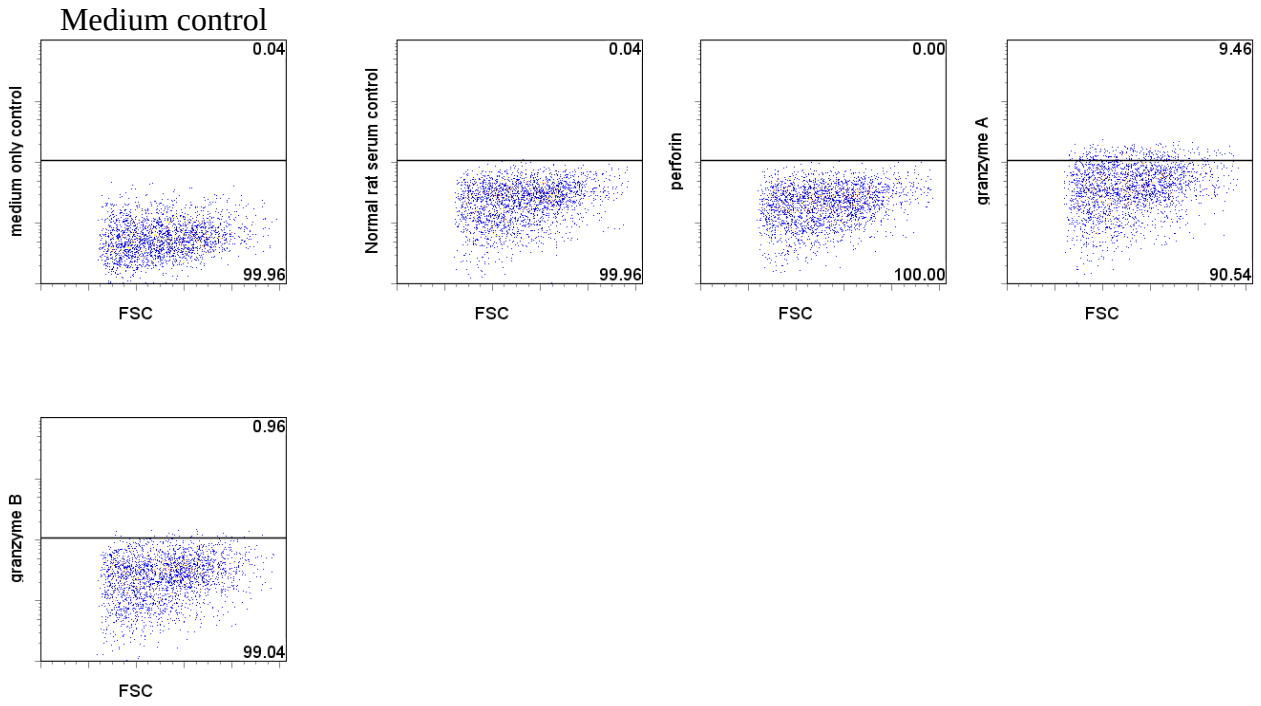

Medium control
medium only control
0.04
99.96
FSC
Normal rat serum control
0.04
99.96
FSC
perforin
0.00
100.00
FSC
granzyme A
9.46
90.54
FSC
granzyme B
0.96
99.04
FSC

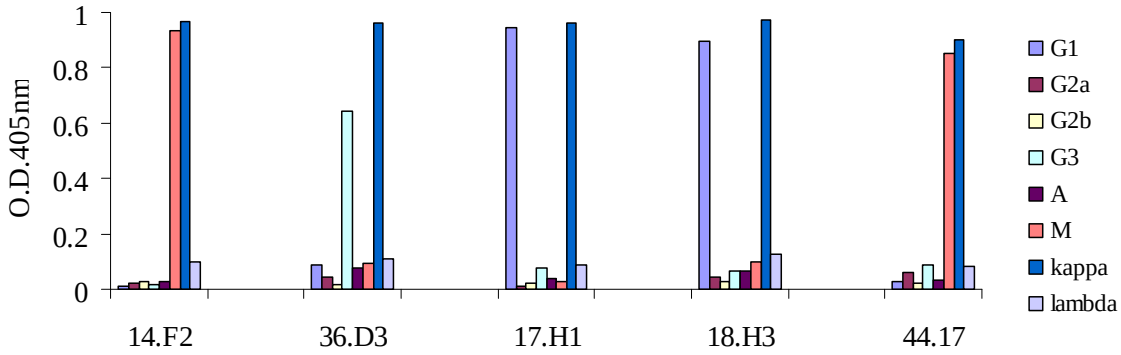
Table 7: Screening of hybridomas by intracellular staining of cytotoxic $CD8^+$ T cell lines and by indirect ELISA using recombinant protein

Antigen	Peptide	Number of positive wells (primary screening)	Number of positive wells (secondary screening)
		Screening by intracellular staining of CTL	
perforin	$Perforin_{262-279}$	15/48	24/189
perforin	$Perforin_{446-463}$	8/48	61/171
granzyme A	$GranzymeA_{38-54}$	10/48	30/247
granzyme B	$GranzymeB_{103-121}$	6/48	57/288
		Screening by indirect ELISA	
perforin	$Perforin_{262-279}$	2/48	9/32
perforin	$Perforin_{446-463}$	0/48	n/a
granzyme A	$GranzymeA_{38-54}$	0/48	n/a
granzyme B	$GranzymeB_{103-121}$	1/48	1/40

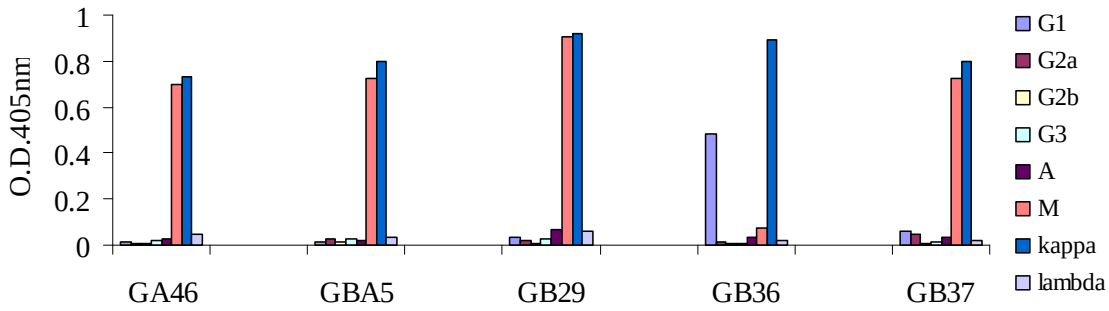
Figure 10a: Isotyping of monoclonal antibody

Screening and determination of the class/sub-class of mouse monoclonal antibody was done using an ELISA-based antigen-independent technique. Wells of the microtiter plates were coated with anti-mouse antibody to immobilise the antibody in supernatant samples applied to the antibody well. Captured mouse immunoglobulin class, sub-class and light chain was then identified by addition of rabbit anti-mouse heavy chain (anti-IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, IgA and IgM) or anti mouse light chain (anti-kappa and lambda) antibodies followed by HRP-conjugated goat anti-rabbit IgG. Results were visualised by the addition of TMB substrate and read quantitatively with a spectrophotometer at 405nm.

Isotyping anti-perforin mAbs



Isotyping anti granzyme A and B mAbs



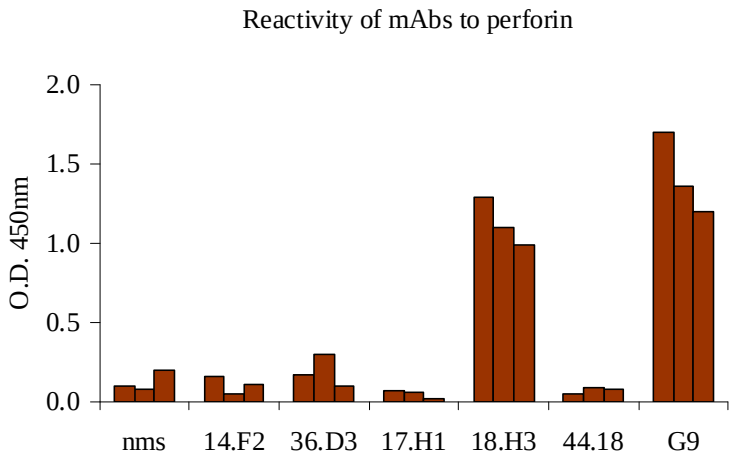
available mouse anti-human perforin (δG9; IgG_{2b}; BD Pharmingen), granzyme A (GA6; IgG_1; Serotec Antibodies, Germany) and granzyme B (2C5/F5; IgG_{2a}; BD Pharmingen) mAbs were used in parallel with purified mAbs in various assays. Results obtained (Figure 10b) showed that only mAb Perf18.H3, GrzB36.12 and anti-human perforin (δG9) showed reactivity to the specific recombinant proteins. In contrast, GA6 (anti-human granzyme A) and 2C5/F5 (anti-human granzyme B) did not cross-react with the bovine molecules. Further analysis of the reactivity of δG9 showed that the reactivity was specific to perforin fragment 2 (Figure 10c panel A) and the binding could be titrated when assayed with limiting antibody (Figure 10c panel B) or antigen (Figure 10c panel C). On the other hand, FACS analysis shown in Figure 10d, demonstrated variable levels of staining of CTL. This data is summarised in Table 8 and indicates the staining with Perf14.F2 (70%) and Perf44.17 (75%) was comparable to that of δG9 mAb (90%) whereas granzyme A specific mAb obtained 80% positive staining CTL and granzyme B specific antibody showed marginal staining levels ranging between 30%-50% positive staining of the same CTL. Although in limited quantity, sera that were obtained from the mice were also included in the assay. Data obtained indicated that immunisation with peptides generated anti-peptide antibodies that bind to regions on the corresponding native protein by positively staining CTL (Figure 10d). It is also interesting to note that the immunisation procedure generated mainly IgM class of antibodies. While these antibodies performed fairly well in FACS analysis, they were of limited function in ELISA assays.

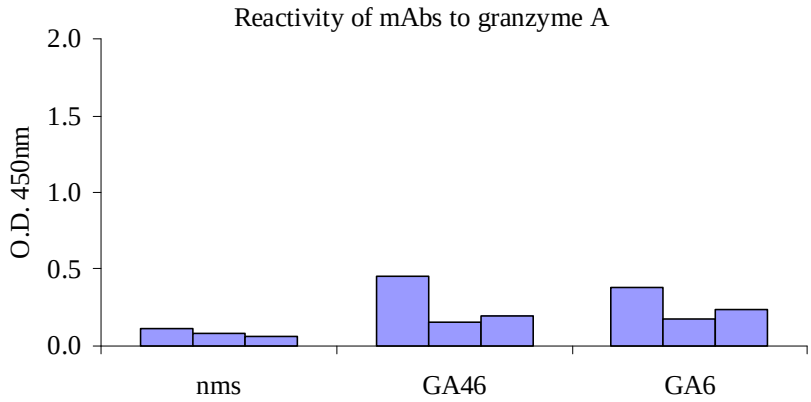
3.2.2 Attempts to optimize perforin and granzyme A/B ELISA and ELISpot assays

Polyclonal sera generated by immunisation of rats and mice were used in preliminary studies to assess the ability of perforin, granzymes A/B specific antibody to function as matched capture and detection pairs in a capture-ELISA with the corresponding recombinant protein or CTL lysate ($1x10^7$ CTL lysed by freeze thawing in 150 µl of PBS) as the source of antigen. Plates were adsorbed overnight with 50 µl of crude serum diluted 1:50 in bicarbonate buffer or 50 µl of affinity purified polyclonal and monoclonal antibody (100 µg/ml diluted in bicarbonate buffer). Commercial antibodies were diluted at 10 µg/ml. The plates were washed and blocked as described in section 3.1.4.1. Purified recombinant proteins or a cell lysate

Figure 10b: Reactivity of mAbs to recombinant protein

Perforin, granzyme A and granzyme B recombinant proteins were coated at a concentration of 1 ug/well and the antibody used titrated in doubling dilution beginning at 1:100. NMS – normal mouse serum.


Reactivity of mAbs to perforin
O.D. 450nm
2.0
1.5
1.0
0.5
0.0
nms
14.F2
36.D3
17.H1
18.H3
44.18
G9


Reactivity of mAbs to granzyme A
O.D. 450nm
2.0
1.5
1.0
0.5
0.0
nms
GA46
GA6

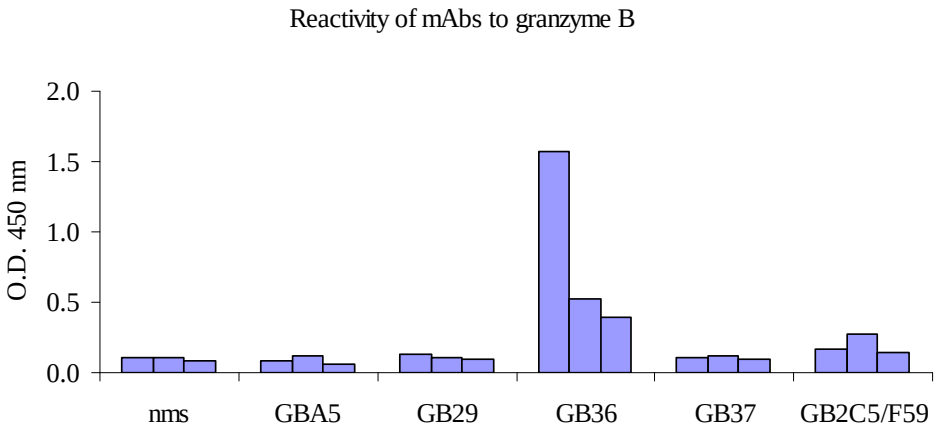
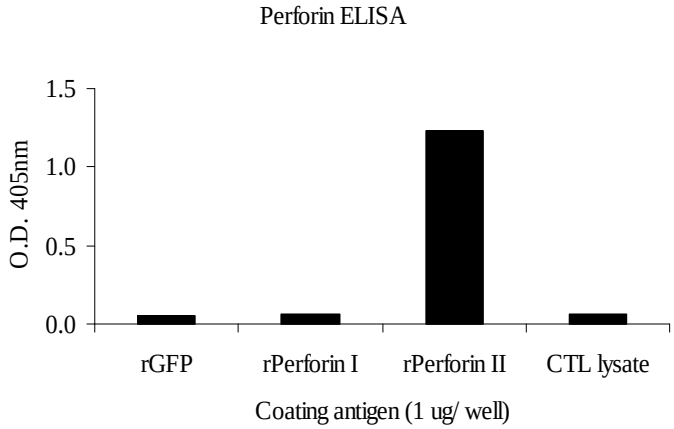

Reactivity of mAbs to granzyme B
O.D. 450 nm
2.0
1.5
1.0
0.5
0.0
nms
GBA5
GB29
GB36
GB37
GB2C5/F59

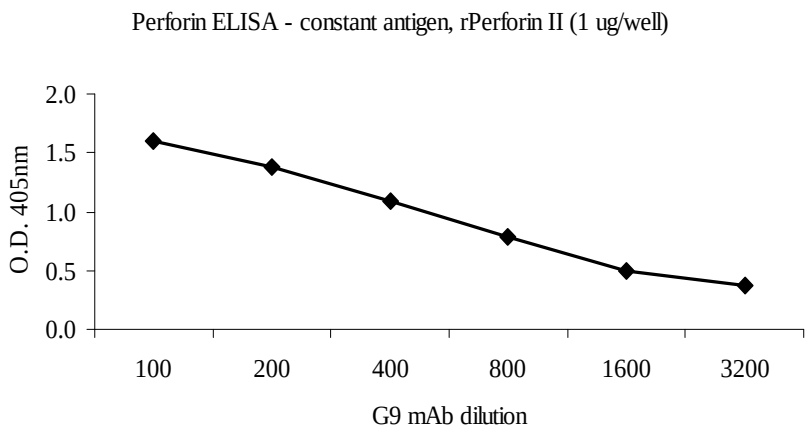
Figure 10c: Perforin ELISA

Bovine recombinant perforin purified from insect cells was coated at a 1 µg/well and mouse anti human perforin monoclonal antibody (δG9) at 1:400 dilution used to detect coated antigen in a direct ELISA (A) . To determine the specificity of the reaction antigen was coated at a constant dilution and the antibody used in doubling dilution beginning at 1:100 (B) or the coating antibody was titrated in doubling dilutions and the antibody used at a constant concentration of 1:400 (C). As shown in the figure, the specificity in binding of mAb δG9 to recombinant perforin protein was titratable. Concentration of the CTL lysate used was not determined. 100 µl aliquot of a total lysate ($1x10^7$ CTL lysed in 150 µl of PBS by freeze-thawing) was used to coat the plate. rPerforin – recombinant perforin fragment I and II

A


Perforin ELISA
O.D. 405nm
1.5
1.0
0.5
0.0
rGFP
rPerforin I
rPerforin II
CTL lysate
Coating antigen (1 ug/ well)

B


Perforin ELISA - constant antigen, rPerforin II (1 ug/well)
O.D. 405nm
2.0
1.5
1.0
0.5
0.0
100
200
400
800
1600
3200
G9 mAb dilution

C

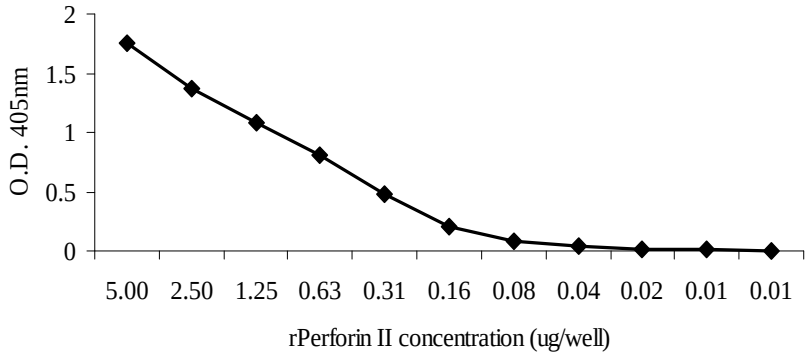

Perforin ELISA - G9 dilution 1:400
O.D. 405nm
2
1.5
1
0.5
0
5.00
2.50
1.25
0.63
0.31
0.16
0.08
0.04
0.02
0.01
0.01
rPerforin II concentration (ug/well)

Figure 10d: Reactivity of monoclonal antibodies to native proteins

CTLs were stained for the expression of perforin and granzymes A/B using the mAbs generated following immunisation of mice with KLH-conjugated peptides in parallel with commercially available anti-human perforin and granzyme A/B mAbs (δG9, GA6, GB2C5/F59) and anti-peptide polyclonal serum (pAbs) harvested from mice. pAb – polyclonal antibody.

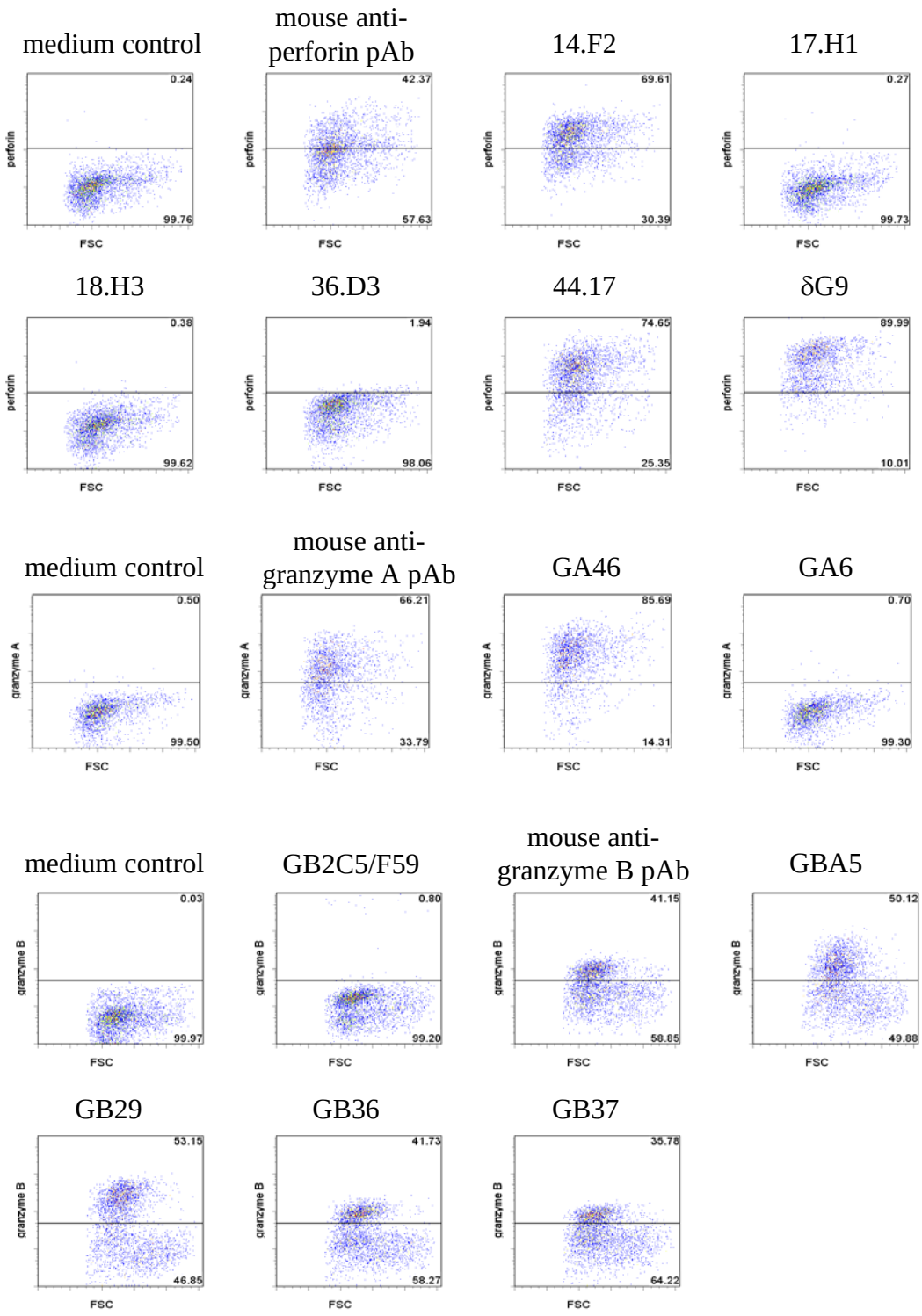

medium control
0.24
99.76
perforin
FSC
mouse anti-perforin pAb
42.37
57.63
14.F2
69.61
30.39
17.H1
0.27
99.73
18.H3
0.38
99.62
36.D3
1.94
98.06
44.17
74.65
25.35
δG9
89.99
10.01
medium control
0.50
99.50
granzyme A
mouse anti-granzyme A pAb
66.21
33.79
GA46
85.69
14.31
GA6
0.70
99.30
medium control
0.03
99.97
granzyme B
GB2C5/F59
0.80
99.20
mouse anti-granzyme B pAb
41.15
58.85
GBA5
50.12
49.88
GB29
53.15
46.85
GB36
41.73
58.27
GB37
35.78
64.22

Table 8: Characterisation of anti-peptide monoclonal antibody

Monoclonal antibody	Isotype	Assay	
		ELISA[a]	ICS (% positive staining)
14.F2	IgM	-	70
36.D3	IgG_3	-	2
17.H1	IgG_1	-	0
18.H3	IgG_1	+	0
44.17	IgM	-	75
δG9	**IgG_{2b}**	**+**	**90**
GA46	IgM	-	85
GA6	**IgG_1**	**-**	**0**
GBA5	IgM	-	50
GB29	IgM	-	53
GB36	IgG_1	+	42
GB37	IgM	-	36
GB2C5/F59	**IgG_{2a}**	**-**	**0**

Reactivity of the monoclonal antibodies to respective antigens was determined by intracellular staining of CTL for native protein and detection of recombinant protein by ELISA

Commercially available anti-human perforin, granzymes A and B are shown in bold.

[a]ELISA score is shown as positive (+) or negative (-).

Figure 11a: Evaluation of rat and mouse sera in sandwich ELISA

Mouse sera and rat sera were assessed for ability to capture and detect recombinant proteins in a sandwich ELISA format. Antigen was diluted serially in doubling dilution beginning from 1 μg/well while the detection antibody was used at 1:100 dilution. Lysate was prepared from $1x10^7$ CTL lysed in 150 μl of PBS. The antigen and polyclonal sera is shown in the individual graphs (I-VIII). The antibody combinations are as below:

Panel A:

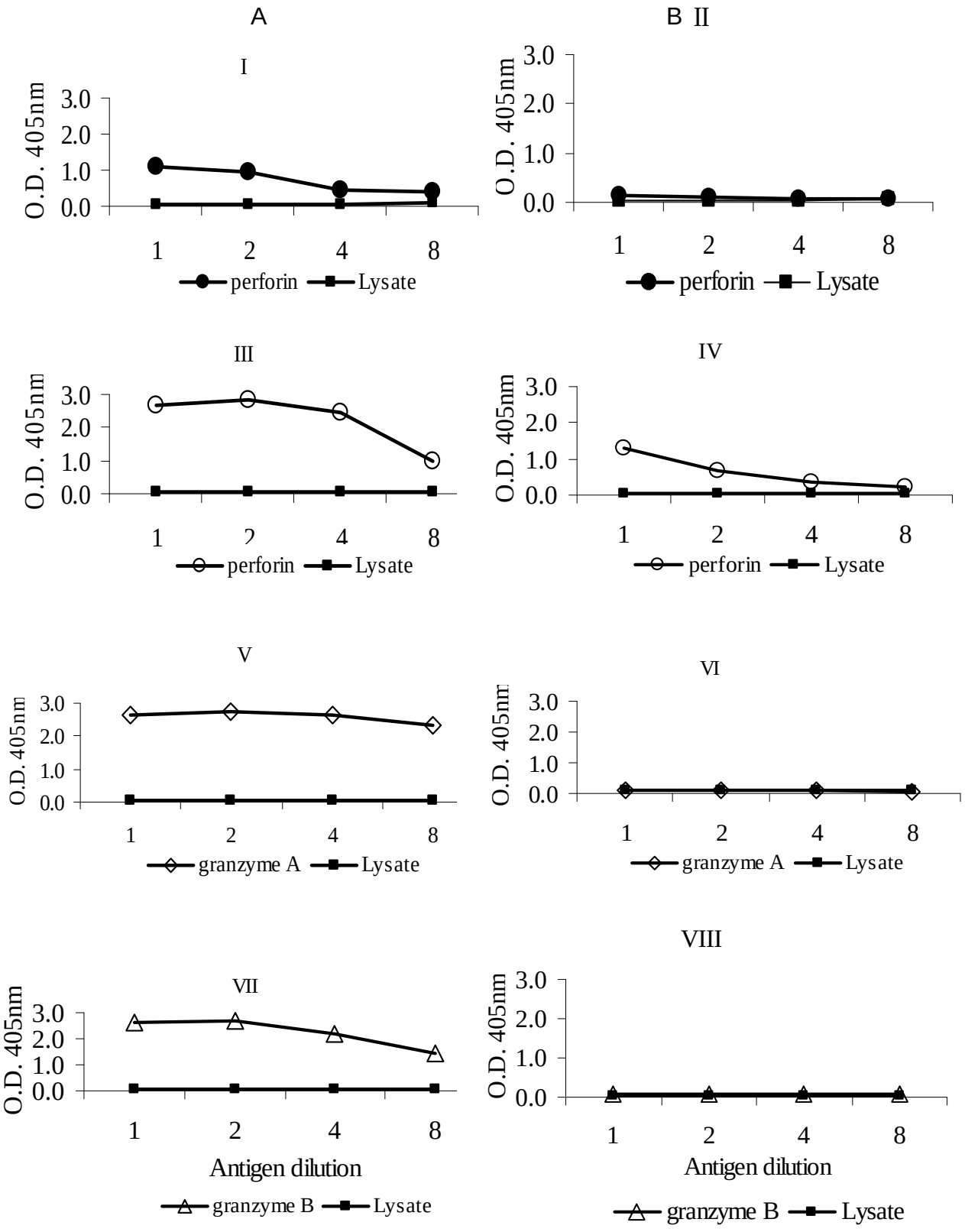
Coating antibody - Mouse sera (generated by immunisation with recombinant protein) except in graph I which is coated with δG9

Detecting antibody - Rat sera (generated by immunisation with recombinant protein)

Panel B:

Coating antibody - Rat sera (generated by immunisation with recombinant protein)

Detecting antibody - Mouse sera (generated by immunisation with recombinant protein) except in graph II which is detected with δG9

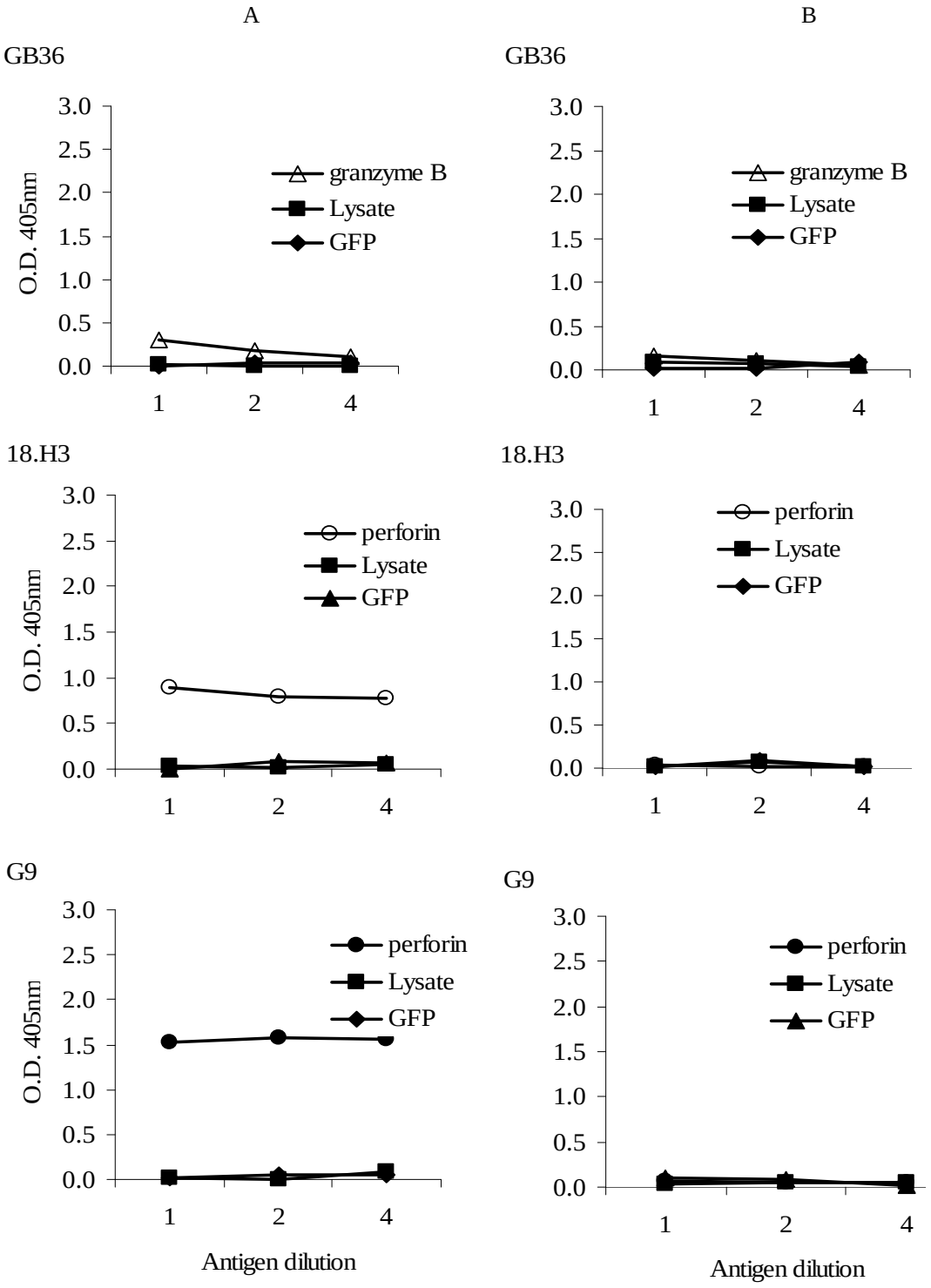

A
B II
I
III
IV
V
VI
VII
VIII
O.D. 405nm
3.0
2.0
1.0
0.0
1
2
4
8
perforin
Lysate
granzyme A
granzyme B
Antigen dilution

were diluted in antibody dilution buffer and incubated in the plate for 2 hrs hours at room temperature. After washing the second antibody was added (working concentrations as indicated previously but diluted in antibody dilution buffer) and incubated for 2 hrs at room temperature. The plates were washed and the appropriate HRP conjugated antibody as described in section 3.1.4.1 was then added and incubated for 1 hr. The plate was developed by addition of TMB substrate solution and the intensity of the resulting blue product measured at 405nm.

The use of mouse sera for capture and rat sera for detection resulted in assays displaying similar detection capacity as shown in Figure 11a and these antibody combinations could detect 500ng – 60ng of recombinant perforin, granzyme A or B but could not detect perforin nor granzymes in CTL lysates. As a control, δG9 was used in a parallel setup under the same assay conditions with similar results. Evaluation of reciprocal combination of pAb resulted in abrogation of the binding of mouse antibody to the recombinant granzyme A/B, inhibiting binding by nearly 50%. The inhibition of mouse antibody was also observed when rat sera were used as the capture antibody and δG9 as the detection mAb further validating observations obtained with rat and mouse anti-perforin antibody combination. Following the generation of anti-peptide mAb, combinations of mAb for capture or detection of recombinant proteins were analysed in a similar set up as that previously described (Figure 11b). Two mAbs, 18.H3 and GB36 that demonstrated applicability to an ELISA format were combined with corresponding rat or rabbit anti-peptide pAb. The use of mAb/rat pAb combination resulted in detection of 1 μg – 250 ng of recombinant perforin or granzyme B indicating a decrease in sensitivity of the assay compared to use of mouse polyclonal antibodies against the polypeptides. Not surprising was that mAb/rabbit (anti-peptide-pAb) combination did not capture recombinant protein. This would be expected as the repertoire of antibody is limited an 18-mer immunising peptide and it is highly possible that the antibodies are targeted to the same antigenic epitope. The intensity of the reaction observed with granzyme B antibodies was low as compared to intensities seen with the perforin assays, which were performed under identical conditions. Rabbit antibodies did not detect recombinant perforin captured by coating with δG9 while similar observations are made with anti-perforin antibody 18.H3. In contrast rat antibodies detected recombinant perforin using 18.H3 and δG9 as coating antibodies.

Figure11b: Evaluation of rat and rabbit sera and anti-peptide monoclonal antibody in sandwich ELISA

Plates were coated with the mAbs as indicated in the individual graphs and bound antigen detected using rat (panel A) and rabbit (panel B) polyclonal antibodies as indicated below. Recombinant antigen was serially diluted in doubling dilution beginning at 1 μg/well.

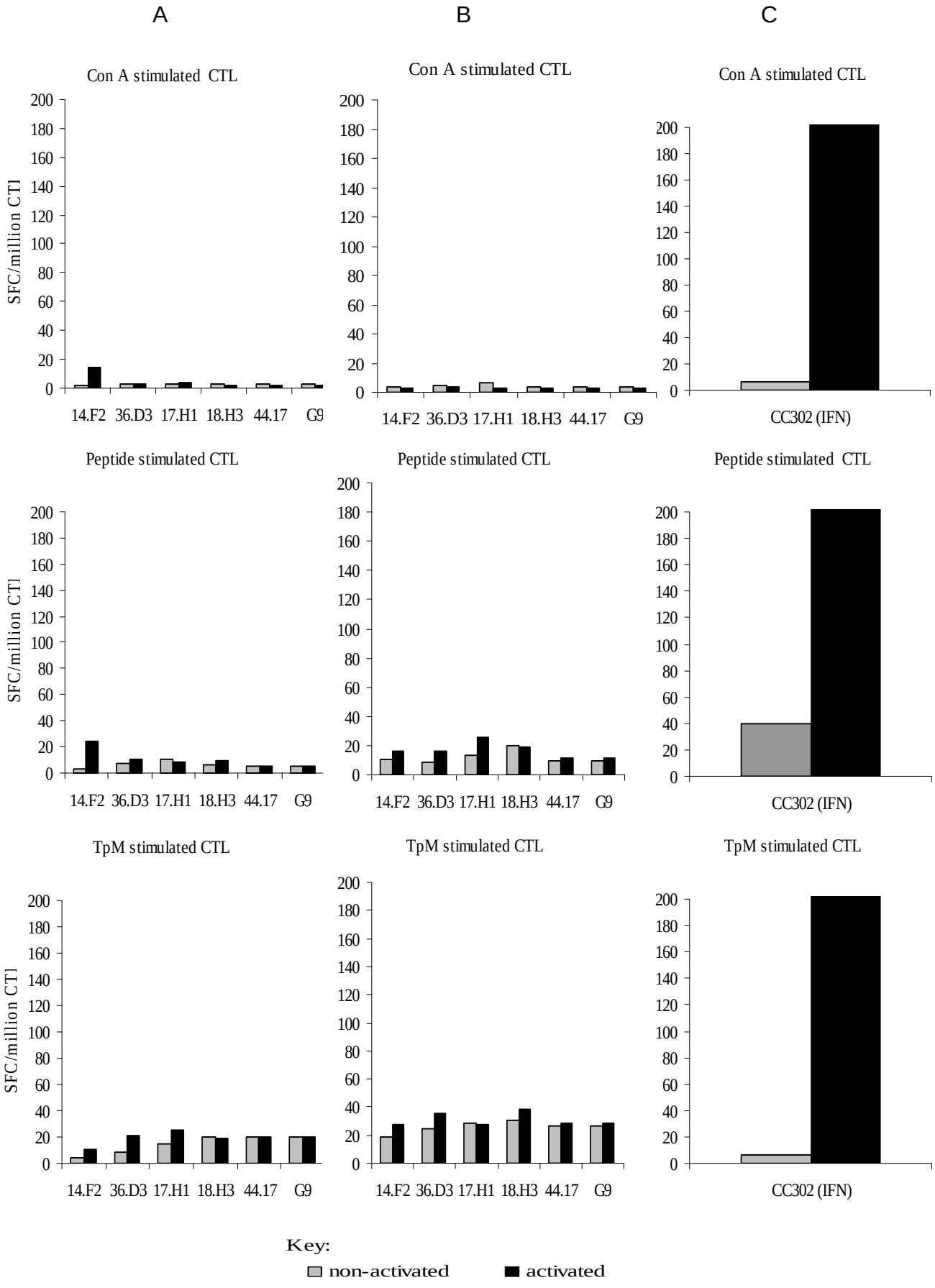

A
B
GB36
GB36
O.D. 405nm
granzyme B
Lysate
GFP
18.H3
18.H3
perforin
Lysate
GFP
G9
G9
Antigen dilution
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1
2
4

On the basis that some of the perforin specific mAb and rabbit anti- perforin peptide antibody generated stained CTL, indicative that the antibodies recognised native proteins, ELISpot assays were set up to determine the ability of these antibodies to capture and detect perforin released by CTL in response to antigen stimulation. CTLs were stimulated *in vitro* with concanavalin A (con A), TpM or peptide pulsed autologous skin fibroblasts. The ELISpot protocol used was similar to the IFN-γ ELISpot assay described in materials and methods (Chapter 4) but had several modifications outlined in perforin ELISpot assay described by Zuber *et al*., (2005). Briefly, MultiScreen IP 96-well PVDF membrane plates were pre-wetted with 50 µl of 70% ethanol in distilled water for 2 minutes at room temperature. The plates were then washed five times with 200 µl sterile water and coated with the specific antibodies (5 µg) overnight at 4°C. The plates were washed six times with sterile PBS and blocked for 1 hour at room temperature with 200 µl RPMI-1640 with 10% FCS. Fifty (50) µl of bovine CTL suspension containing $2.5x10^4$ cells, 50 µl of skin fibroblasts ($2.5x10^4$) and 10 µl of peptide diluted at 10 mg/ml were added in duplicate and the cells incubated in the plates overnight at 37°C and 5% CO_2. The cell suspension was discarded and the plates washed eight times with 200ml PBS. The second antibody of the appropriate antibody pair was added diluted in PBS containing 0.5% FCS was added and incubated at 1 hour at room temperature. After washing, the relevant secondary antibody conjugated to alkaline phosphatase was added and incubated for 1 hour at room temperature and washed as before. Filtered substrate BCIP/NBT plus was added and incubated for up to 30 minutes or until colour development was observed. The reaction was stopped by rinsing the plates in tap water, dried and analysed by using an automated AID Elispot Reader System (AID, Strassberg, Germany).

Results obtained showed use of mAbs and rabbit antibody did not detect perforin released in response to stimulation by peptide or TpM in spite of adequate stimulation of CTL as evidenced by IFN-γ release (Figure 12a). Data obtained was inconclusive as there were no significant differences between the background controls (unstimulated and con A stimulated CTL) and stimulated CTL samples. Results were also irreproducible with different experiments showing no spots to a few diffuse spots in repeat experiments using the same CTL cultures. To further evaluate strategies to

Figure 12a: Optimisation of perforin ELISpot - stimulation protocol

BV115 CTL were stimulated with Con A, specific peptide ($Tp1_{214-224}$) or autologous TpM (BV115 TpM) in a perforin ELISpot assay where the plates were coated with the 5 anti-bovine perforin mAbs and anti-human perforin mAb (δG9). Rat (Panel A) or Rabbit (Panel B) anti-perforin polyclonal serum was used as the detection antibody. A standard IFN-γ ELISpot (Panel C) was set up in parallel as a positive control. Spot forming units (SFU)/million CTL are shown along the Y-axis and the X-axis shows the coating antibody.


A
B
C
Con A stimulated CTL
Peptide stimulated CTL
TpM stimulated CTL
SFC/million CTL
0
20
40
60
80
100
120
140
160
180
200
14.F2
36.D3
17.H1
18.H3
44.17
G9
CC302 (IFN)
Key:
non-activated
activated

optimise the detection sensitivity the assay was done incorporating several variations to the protocol mentioned previously. These included altering the pre-activation conditions i.e. with or without ethanol pre-activation, activation with 35% ethanol vs. 75% ethanol and culturing the ELISpot for 20 hours, 24 hours and 48 hours to enhance the signal. Again the results obtained were inconclusive as indicated in Figures 12b and 12c that show data obtained from time point experiments and a typical image of a bovine perforin ELISpot plate after development.

Figure12b: Optimisation of perforin ELISpot - variation of incubation time

A standard perforin ELISpot assay of BV115 CTL stimulated with $Tp1_{214-224}$ peptide was set up using the anti-perforin mAbs and incubated for 20 hr, 30 hr and 48 hr before detection of bound perforin with Rat (A) or Rabbit (B) anti-perforin polyclonal antibodies. Panel C shows an IFN-γ ELISpot assay that was set up in parallel to the perforin ELISpot assay. Y-axis shows the SFU/million CTL while the X-axis shows the coating antibody used in the assay.

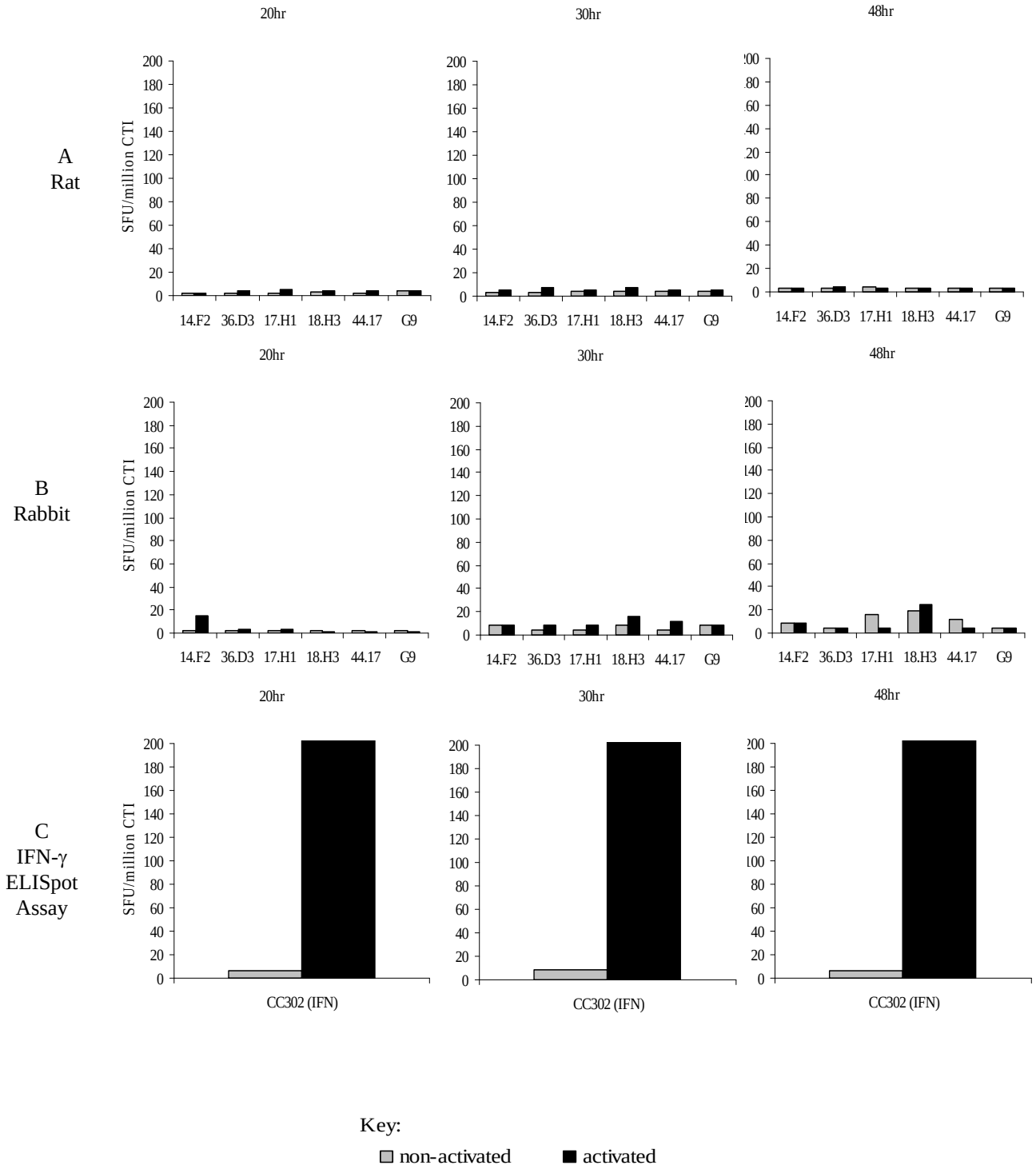
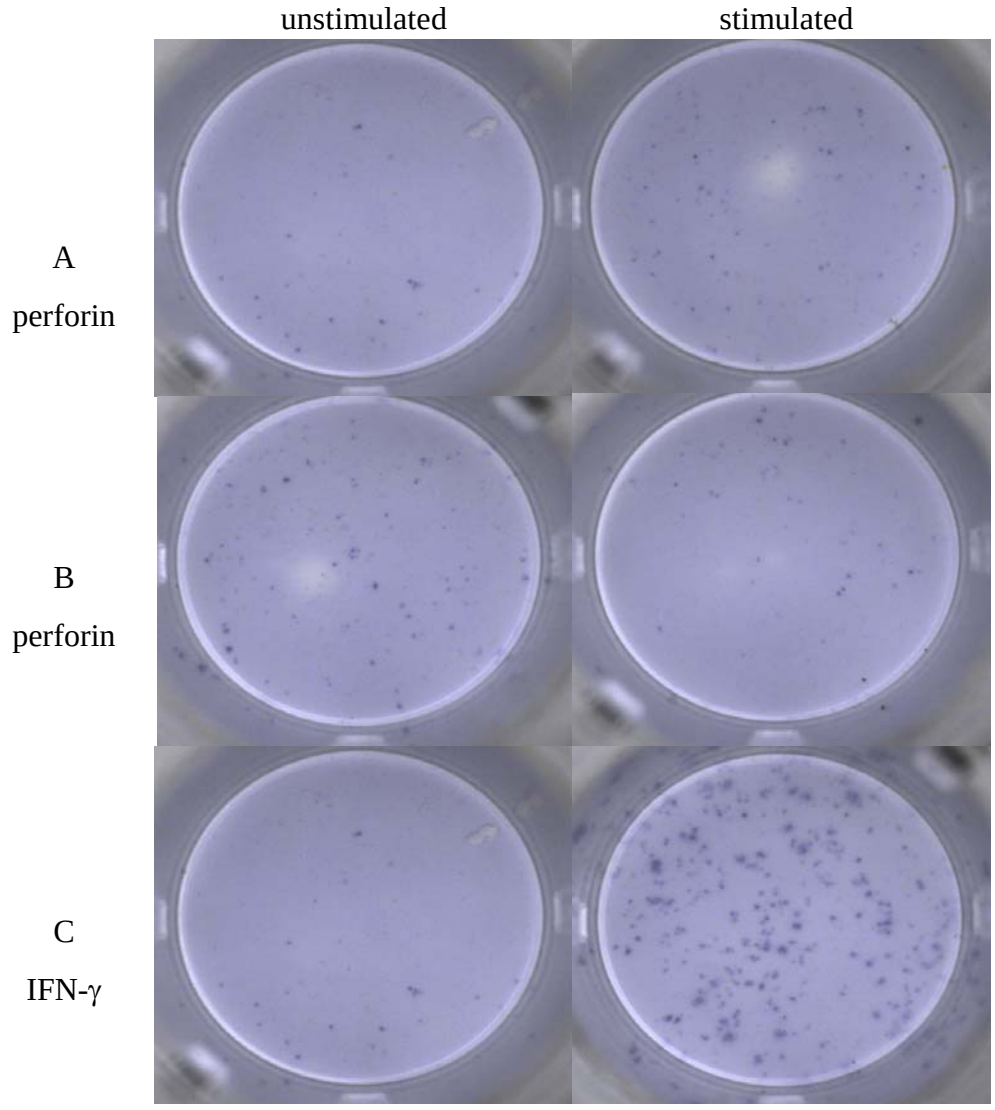

A
Rat
20hr
30hr
48hr
SFU/million CTI
14.F2
36.D3
17.H1
18.H3
44.17
G9
B
Rabbit
C
IFN-γ
ELISpot
Assay
CC302 (IFN)
Key:
non-activated
activated

Figure 12c: Development of perforin ELISpot - sample photograph

Typical images of bovine perforin ELISPOT (A & B) and bovine IFN-γ ELISpot (C) assay wells following development. Distinct spots are readily apparent in wells containing CTL that were stimulated and cultured overnight with peptide and developed for secretion of IFN-γ, whereas no obvious spots are detected in the wells containing non-stimulated cells. In contrast, spots obtained by staining for perforin secretion are few and diffuse with no clear difference between stimulated and un-stimulated wells.


unstimulated
stimulated
A
perforin
B
perforin
C
IFN-γ

3.3 Discussion

Perforin and granzymes A/B contained in cytolytic granules, is released by antigen-specific $CD8^+$ cytolytic T cells in host defence. Mice deficient in perforin display greatly impaired cytotoxicity *in vivo* (Kagi *et al*., 1994) as manifested by a reduced capacity to clear many viral and intracellular pathogens. The role of CTL in protective immunity against *T. parva* has also been extensively studied and is well documented. In-spite of its importance, the understanding of the molecular and cellular function of perforin and granzymes contained in CTL is limited. This in part is due to a lack of suitable tools for the study of CTL releasing perforin and granzymes. This study aimed to generate antibodies against disparate epitopes of bovine perforin, granzymes A and B, to allow the development of capture-based immunoassays for the same molecules.

Immunisation of rats and mice with peptides and recombinant protein induced antibody against multiple epitopes of the recombinant proteins (Figure 9, Figure11a). However peptide-immunisation induced a significantly lower level of specific antibody response as compared to immunisation with recombinant proteins which can be attributed to several factors. These include the inherent lack of immunogenicity of peptides, the choice of adjuvant and its efficacy in stimulating T-cells as well as the unresponsiveness of the animal due to tolerance to non-self antigen induced by the similarity of the bovine peptide sequence to mouse and rabbit sequences. Whereas an antibody response is also targeted to multiple epitopes on the recombinant protein, anti-peptide antibodies target epitopes on an 18-mer peptide which may also contribute to low levels of antibody. The limited ability of synthetic peptides to adhere to assay plates could also cause a poor read out of the assay used in determing the effectiveness of immunisation.

The above not-with-standing, results suggested that the antibody repertoire displayed overlapping epitope specificities as seen with the inhibition of mouse antibody binding when rat sera are used as the detection antibody for all the proteins (Figure 11). This is supported by the observation that mice appeared to generate antibodies against 2 of the 4 perforin polypeptides (Figure 9) and hence when the antibody combination is reversed in the ELISA assay (Figure 11) the rat pAb saturates the epitopes that were predominantly recognised by the mouse pAb. With respect to perforin, the cross reactivity of δG9 to bovine perforin not only validates the

expression of bovine perforin, it also emphasizes the importance of the region presented by the polypeptide in either the structure and/or function of the native protein. Given that the bovine perforin cDNA demonstrated on average 80% homology to human, mouse and rat perforin homologs (Figure 4b), the region defined by δG9 antibody is likely to be conserved across species. In contrast, it appears that the anti-human granzyme A and B mAbs used in this study are highly specific to the human proteins although human granzymes are about 60% identical at the amino acid level. Additionally, although granzyme structure is highly conserved across mammalian species, polyclonal antisera raised against purified granzyme B were found to be directed almost exclusively against the same region of the molecule (Apostolidis *et al* 1995).

The inability of the antibodies to bind to native perforin in CTL lysates is likely due to degradation of native perforin during cell disruption or perhaps the quantity released were below the detection limits of the assay given that the amount of protein in the lysate was not quantified. Differential sensitivity of ELISA systems have been reported (Tanguan and Killion, 1994; Zuber *et al*., 2005) moreover, the detection of bovine perforin in CTL supernatants using commercially available perforin ELISA kits have not been successful and this was attributed partly to the detection limits of the available reagents (Bateta, 2004).

Allowing for differences in antibody preparations (such as integrity of antibody after purification procedures) the differences observed between the reactivity of anti-perforin peptide and anti-granzyme peptide mAb/pAb combinations in ELISA (Figure 11b) is attributable to the resulting competitive binding and inhibition of the antibodies generated. These antibodies are likely to display overlapping specificities given that they are both generated by peptide immunisation. Further to this, the observation that rabbit anti-perforin peptide antibody did not detect recombinant perforin captured by δG9 and 18.H3 (Figure 11b) suggests that these two antibodies may display similar epitope specificities and by inference are targeted to antigenic molecules within if not the same region of the perforin molecule which would be defined by peptide $\text{Perforin}_{262\text{-}279}$ (Table 8). This may account for the loss of antibody binding due to steric hindrance or competitive binding.

Application of the antibodies that were generated in this study in FACS assays gave variable results as shown in Figures 9f and 10d. On one hand it is possible that

the rat pAb recognise linear epitopes whose structural presentation is altered or may be poorly accessible to antibody in the native molecule and hence their inability to bind to native perforin, granzymes A and B. On the other hand, antibodies such as δG9, 14.F2, GB36, GA46 and the rabbit anti-peptide antibodies recognise an epitope that is preserved and exposed in the recombinant proteins as well as the native form of the molecules. With respect to FACS data obtained with mAb (Figure 10d), the differences in the cell numbers staining positive for perforin, granzyme A and B, may be as a result of different expression levels of the two proteins in activated CTL as the assays were carried out using the same CTL population. Differential expression of perforin has been reported in several studies (Velotti *et al*., 1989) Garcia-sanz *et al*., 1990). With regard to the granzyme B antibodies, the peptides and consequently the recombinant protein used to immunise the animals has been shown in recent analysis (Chapter 2, results section) to have poor homology to granzyme B protein and it is likely that this contributed to the poor results obtained when staining CTL.

With regard to generation of monoclonal antibodies, it is of interest to determine why the resultant monoclonals were predominantly of IgM subclass. This may have been as a result of the adjuvant used or the immunisation procedure and therefore calls for testing of different adjuvant and immunisation regimes. The observation may also explain the disparity in the screening of hybridomas by ELISA and intracellular staining. While the latter identified hybridomas secreting all class types of immunoglobulins, the ELISA technique identified hybridomas secreting IgG subclass. Perhaps the same phenomenon occurred following immunisation of rabbits whereby the immune response did not mature well enough to generate IgG antibodies. This resulted in the poor staining of CTL where the secondary antibody used was anti-rabbit IgG-PE conjugate.

Challenges encountered in the development of perforin based ELISA/ELISpot assays and by extension granzyme based ELISA/ELISpot assay may be attributed in part to the limited functionality and repertoire of the antibodies that were generated. Antibodies that were generated were restricted to peptide epitopes while the rat polyclonal antibodies generated by immunisation of rats with protein were limited in their ability to bind to corresponding epitopes in the native protein. These challenges however are not unique to the bovine system. Similar set backs have been reported by other groups. For example Zuber *et al*., (2005) reports generation of perforin-specific

mAb that is of limited function in the ELISpot assay while Hameed *et al* (1992) developed an anti-perforin mAb that is restricted for use only in FACS analysis. Of the number of anti-human granzyme antibodies that have been generated, only a limited selection of 2-3 antibodies are used in ELISA assays while a single pair is shown to function in the granzyme ELISpot (Apostolidis *et al*., 1995). Of specific importance is the need for antibodies that recognise at least two distinct determinants of the molecule with an ability to recognise native protein as well as to function in different assay systems.

In conclusion, antibodies against bovine perforin, granzymes A and B have been generated. Although limited in function the antibodies generated in this study can be evaluated further and developed as anti-bovine perforin, granzyme A and B reagents. Given appropriate antibodies, the possibility of developing perforin based ELISA and ELISpot assays specific for the bovine system still exists as these have been successful in human and murine models. Related to this, δG9 antibody has been shown to cross-react to bovine perforin and can thus be further analysed and incorporated in assays for studies on bovine perforin.

Chapter 4: Identification of *Theileria parva* antigen specific $CD8^+$ T cells using peptide-Major histocompatibility complex class I tetrameric complexes

4.0 Introduction

Peptide-MHC class I (pMHC) tetrameric complexes are proving invaluable as fluorescent reagents for enumeration, characterisation and isolation of peptide-specific T-cells (Dunbar *et al*., 1998; Appay *et al*., 2000; Denkberg *et al*., 2001; Karanikas *et al*., 2003). Used in combination with functional assays such as cytokine secretion or intracellular staining of proteins (Murali Krishna *et al*., 1998; Callan *et al*., 1998; Wilson *et al*., 1998; Tan *et al*., 1999; Appay at al., 2000; Pittet *et al*., 2001a and b) this assay provides additional information on the functional heterogeneity of antigen-specific $CD8^+$ T-cells *ex vivo*. The use of the IFN-γ ELISpot as a means of examining the functional phenotype of circulating $CD8^+$ T-cells with specificity for a particular *T. parva* antigen has shown successful induction of IFN-γ antigen specific $CD8^+$ T cell responses following vaccination with defined *T. parva* antigens (Graham *et al*., 2006). However, it does not address their cytolytic function. The analysis of tetramer-positive $CD8^+$ T-cells by flow cytometry provides a method that reliably quantitates the number of specific $CD8^+$ T-cells present in peripheral blood and through co-staining for perforin, can provide additional information about their cytolytic potential.

Chapter 4 reports on validation of tetramers generated using antigenic peptides from *T. parva* by staining established T-cell lines and clones. It also describes a method that combines the use of tetramers and intracellular staining to examine the expression of perforin. The pMHC tetramers and intracellular staining for perforin has further been used to assess the T-cell response to specific antigens in animals following live vaccine immunisation and subsequent challenge with parasites. The functional relevance of tetramer staining is suggested by the correlation with other assays of CTL function, including ^{111}Indium-release assays and IFN-γ ELISpot. Tetramer staining is also used to examine antigen-specific T cells in cryopreserved samples obtained from animals vaccinated with defined *T. parva* antigens and challenged with live parasites (Graham *et al*., 2006, unpublished results).

4.1 Materials and methods

4.1.1 Cattle

A total of 10 cattle born and bred at ILRI and free from *T. parva*, were used in the study. The animals used in the study were selected on the basis of their bovine MHC phenotype by typing for bovine leucocyte antigens. Four of the 10 animals (BX063, BX064, BV115 and BZ144) had previously been immunised against *T. parva* by the infection and treatment method (ITM) conducted as part of another study (Graham *et al.,* personal communication). At the outset of the experiments all animals were clinically normal. A detailed animal list is shown in Table 9.

4.1.2 Bovine leucocyte antigen typing

The bovine leucocyte antigen (BoLA) class I phenotypes were determined using a combination of immunofluorescence staining with anti-BoLA monoclonal antibodies followed by flow cytometry as described in section 4.1.4.8 (Goddeeris and Morrison, 1988) and allele-specific PCR (Ellis *et al*., 1998). The expression of specific class I alleles was also confirmed functionally by testing the ability of cells from individual typed animals to present relevant peptide to CD8 T-cell lines of the appropriate BoLA restriction specificity. Lymphoblasts obtained by the stimulation of peripheral blood mononuclear cells with Con A (Sigma, Poole, United Kingdom) as described in section 4.1.4.4 were pulsed with serially titrated preparations of antigenic peptides for 1 hr at 37°C and then fixed in 0.1% glutaraldehyde (McKeever *et al*., 1992). Pulsed lymphoblasts ($2.5x10^4$ cells/well) were co-cultured with antigen-specific CD8 T cells ($2.5x10^5$ cells/well), and recognition was assessed by IFN-γ ELISpot assay (Graham *et al*., 2006) as described in section 4.1.4.7.

4.1.3 Immunisation and challenge

Five cattle (BB001, BB006, BB007, BB069, BB169) were immunised by ITM using cryopreserved viable *T. parva* (Muguga) (TpM) sporozoites and a slow-release formulation of antibiotic essentially as described previously (Radley *et al*., 1975a). The cryopreserved ILRI stabilate No. 4133 was pooled from plastic straws after rapid thawing in a water-bath at 37°C and a final dilution of 1:20 achieved using stabilate diluent (3.5% BSA, 7.5% glycerol 1ml/100ml Pen-Strep in D-MEM).

Table 9: Details of experimental cattle

Animal	MHC class I serotype[b]	Class I MHC restricting allele	Age[d] (months)	Breed
BB001	A10-KN104/A18	N*00101	17	Friesian-Boran cross
		N*01301		
BB006	A10/-	N*01201	17	Friesian-Boran cross
BB007	A18/-	N*01301	17	Friesian-Boran cross
BB069	A10-KN104[c]	N*00101	19	Boran
BB070	A10-KN104[c]	N*00101	17	Boran
BB0169	A10-KN104[c]	N*00101	15	Boran
BX063[a]	A10-KN104[c]	N*00101	74	Boran
BX063[a]	A10-KN104[c]	N*00101	74	Boran
BV115[a]	A10/A18	N*01301	unknown	Friesian
		N*01201	unknown	
BZ144[a]	A10-KN104/-	N*00101	37	Boran

[a] *T. parva* immune cattle – cattle previously immunised by ITM. BX063 and BX064 are identical twins
[b] a minus sign indicates that the MHC haplotype is unknown
[c] homozygous for MHC serotype
[d] age at time of experiment

A lethal dose of 1 ml of diluted stabilate was administered sub-cutaneously below the right ear. Long-acting oxytetracycline hydrochloride (Alamycine LA 300, Norbrook Pharmaceuticals) was inoculated deep in the gluteal muscles at 20 mg/kg body weight immediately after administration of the stabilate. Following inoculation with sporozoites, cattle were monitored clinically for temperature and parasitosis by microscopic examination of giemsa-stained smears of needle aspirates of local lymph nodes. Examination for parasitosis and measurement of rectal temperature were performed daily starting from 5 days after inoculation with sporozoites until recovery. A rectal temperature of 39.5ºC or higher, associated with schizont parasitosis, was considered a pyrexic response and treatment using an antitheilerial drug Butalex® (Coopers Animal health) administered at 2.5mg/kg body weight. Recovery of animals was defined as disappearance of schizonts from lymph nodes, a rectal temperature below 39.5ºC, and a clinically healthy appearance.

Homologous challenge infections were achieved by a lethal dose of 1 ml (1:20) of Stabilate No. 4133 administered as described. Examination for parasitosis and measurement of rectal temperature was also performed daily starting from 5 days to day 15 after challenge.

4.1.4 Generation, cloning and characterisation of bovine cytotoxic T cells specific for *Theileria parva*

4.1.4.1 Media

Tissue culture media used for growth and maintenance of *T. parva*-infected cell lines and *Theileria*-specific short-term T cell cultures or established polyclonal and clonal T cell lines were prepared as described by Goddeeris and Morrison (1988). Complete culture medium for growth of parasitized cells consisted of RPMI-1640 with 20 mM HEPES buffer (Flow laboratories, Ltd., Irvine Scotland) supplemented with 10% (v/v) of gamma-irradiated, heat-inactivated (56˚C, 35 min then stored at 4˚C) foetal calf serum (FCS) (Hyclone, Logan, UT), 50 μg/ml gentamicin (Flow Laboratories, Irvine Scotland), 2mM L-glutamine (GIBCO, Paisley Scotland), $5x10^{-5}$ M 2-mercaptoethanol (Merck, Darmstadt, Germany) 240 IU/ml penicillin and 100 μg/ml streptomycin. Culture medium used in *Theileria*-specific T cell cultures was as described above but without HEPES buffer. Culture medium used in cytotoxicity assays comprised of RPMI-1640 medium with HEPES buffer supplemented with 5%

(v/v) of heat-inactivated FCS.

4.1.4.2 Preparation of bovine peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated from blood collected in Alsever's solution by density gradient centrifugation on Ficoll-paque® (Pharmacia, Uppsala, Sweden) essentially as described by Goddeeris and Morrison (1988). Briefly, blood was aseptically collected from the jugular vein into a syringe containing an equal volume of Alsever's solution to form a mixture that was then layered onto Ficoll-paque solution and centrifuged (Model RC 3B, Sorval, DE) at 900xg for 30 min at room temperature with the brakes off. Cells at the interface between the plasma phase and the Ficoll-paque® were collected and transferred into a 50-ml polypropylene tube (Falcon®) and pelleted by centrifugation at 450xg for 10 min at room temperature. Residual red blood cells were lysed by incubating the cell pellet in Tris-ammonium chloride buffer pre-warmed at 37°C for 10 min. The cells were then washed three times with Alsever's solution by centrifuging at 180xg for 10 min at room temperature before being resuspended in the appropriate cell culture medium or buffer. Viable cell count was determined by trypan blue dye-exclusion using a haemocytometer. All operations except centrifugation and counting of cells were conducted in a horizontal laminar flow hood.

4.1.4.3 Establishment and maintenance of *Theileria parva*-infected cell lines

Parasitized cell lines were established by infection of PBMC *in vitro* with *T. parva* sporozoites. An aliquot of sporozoite suspension derived by homogenizing dissected tick salivary glands in HEPES buffered-culture medium was added to a cell pellet containing $2x10^7$ PBMC in a 10-ml polycarbonate tube (Nunc) to give a final volume of 200 µl. The sporozoite-cell mixture was incubated at 37˚C in a humidified 5% CO_2 incubator for 1.5 hr while mixing the contents every 30 min. The volume was then made up to 10 ml with the culture medium and centrifuged at 180xg for 10 min at room temperature. The pelleted cells were resuspended in 5 ml of the culture medium and transferred into a 25 cm^2-tissue culture flask (Costar®, Cambridge, MA) which was then incubated in a humidified 5% CO_2 incubator at 37°C. From about 2-3 days cultures were observed under an inverted microscope for appearance of lymphoblasts. Cytospin smears were also made and stained with Giemsa (Merck, Darmstadt, Germany) to confirm the presence of *Theileria* schizonts in the

lymphoblasts. The cultures were maintained in logarithmic growth phase by making sub-cultures when cell numbers reached $1x10^6$ cells/ml; that is, every 2 days, two-thirds of the cell suspension was removed and replaced with an equal volume of fresh culture medium.

Once cell lines were established, stabilates were prepared and frozen in liquid nitrogen. A cell suspension containing $5x10^6$ infected cells was spun at 180xg for 10 min at room temperature and the pellet resuspended gently in 1 ml of pre-chilled (4˚C) culture medium (without HEPES). An aliquot of 1 ml of pre-chilled FCS containing 20% dimethylsulfoxide (DMSO) (Merck, Germany) was added gently to the cell suspension. Aliquots of 1 ml were distributed in cryogenic vials (Nunc), suspended overnight in the vapour phase of liquid nitrogen and subsequently immersed in liquid nitrogen for storage.

In order to recover the stabilate from liquid nitrogen, the cryogenic vial was quickly thawed by immersing in a water-bath at 37°C and subsequently placed on ice. The cells were then gently transferred into a 10-ml centrifuge tube containing 5 ml pre-chilled complete culture medium. The cell suspension was centrifuged at 180xg for 10 min at 4°C. After discarding the supernatant, the cell pellet was resuspensed in 5 ml of complete culture medium and transferred into a 25-cm^2 tissue culture flask. The flask was incubated at 37°C in a humidified CO_2 incubator and the cell line was maintained in logarithmic growth phase as described before.

4.1.4.4 Establishment of uninfected lymphoblastoid cell lines

Uninfected lymphoblasts for use as control target cells in cytotoxicity assays were obtained from PBMC as described by Goddeeris and Morrison (1988). A suspension of PBMC containing $2x10^6$ cells/ml of complete culture medium without HEPES buffer was distributed in 1-ml aliquots into a 24-well culture plate. Aliquots of 1 ml complete culture medium containing 5 μg/ml Con A was added to each well and the cultures incubated at 37°C in a humidified incubator for 5 days. The cultured cells were harvested and resuspended at $5x10^5$ viable cells/ml of culture medium containing 10U/ml human recombinant IL2 (Sigma) or 1% bovine recombinant IL2 (ILRI in-house reagent) and 5 ml transferred into a 25-cm^2 tissue culture flask. The cell culture was maintained by making sub-cultures every 3 to 4 days; three-quarters of the cell suspension was removed and replaced with fresh complete culture medium

containing IL2. One day before use of uninfected lymphoblasts as target cells, complete culture medium containing IL2 and 0.1M alpha-methyl-D-mannoside was added to neutralise any residual Con A present.

4.1.4.5 Generation *Theileria parva*-specific $CD8^+$ T cells

4.1.4.5.1 Generation of polyclonal T-cell lines

The methodology applied was essentially as described by Goddeeris and Morrison (1988). Briefly, aliquots of $4x10^6$ PBMC and $2x10^5$ irradiated autologous *T. parva*-infected cells were added in a total volume of 2 ml complete culture medium (without HEPES) to the wells of a 24-well tissue culture plate (Costar®). The parasitized stimulator cells were irradiated by exposure to 50 Gy of gamma irradiation from a ^{137}Cesium source. Cultures were then incubated at 37°C in a humidified 5% CO_2 incubator. After incubation for 7 days, viable cells were recovered from the cultures by centrifugation (450xg, 20 min at room temperature) over Ficoll-paque® remove dead cells. Viable cells were then resuspended at $4x10^6$ cells/ml in complete culture medium and re-stimulated with irradiated autologous infected cells as described before. After 7 days incubation, viable cells were separated and restimulated a third time as before; using aliquots of $2x10^6$ restimulated cells/well with $4x10^5$ irradiated (50 Gy) autologous infected cells and $2x10^6$ irradiated (50 Gy) autologous PBMC as filler cells. Cytotoxicity assays (^{51}Chromium-release assays) were conducted on effector cells after each stimulation. The cells were cloned after the third stimulation, when maximal levels of killing had been achieved. Alongside the cloning, the remaining cells were maintained in culture by periodic re-stimulation to generate established polyclonal CTL lines.

4.1.4.5.2 Generation of cloned T-cell lines

The procedures followed for cloning were as previously described by Goddeeris and Morrison (1988). Prior to cloning, $CD4^+$ T cells were depleted from re-stimulated cultures by complement-mediated lysis with mAb IL-A12 (Baldwin *et al*., 1986) to enrich for the $CD8^+$ T cells known to mediate CTL activity. Briefly, $1x10^7$ viable cells in 1 ml of complete culture medium were incubated with 500 µl of sterile mAb (at 1:250 dilution of murine ascetic fluid in complete culture medium) for 40 min at 4°C. An aliquot of 500 µl of sterile rabbit complement solution was added to the mixture which was further incubated for 40 min at 37°C before adding 8 ml of

culture medium and centrifuging at 180xg for 10 min at room temperature. The cell pellet was washed twice by adding 10 ml culture medium and centrifuging as before then resuspended in 2 ml culture medium containing 1% bovine recombinant IL-2 (ILRI in-house reagent) and transferred into a well of a 24-well culture plate and incubated overnight at 37°C in a humidified 5% CO_2 incubator.

Following the overnight incubation, the cells were recovered by centrifugation, counted and adjusted to concentrations of 30, 10 and 3 cells/ml in culture medium. Aliquots of 100 µl of each dilution were dispensed into the wells of 96-well micro-titre round-bottom tissue culture plates (Costar®); 5 plates of each dilution at 0.3 cells/well and 1 cell/well and 2 plates at 3 cells/well were prepared. Aliquots of 100 µl of a cell suspension containing $5x10^4$ irradiated autologous infected cells/ml, $2x10^5$ irradiated autologous PBMC/ml and 1% IL-2 in complete culture medium was added to each well. The culture plates were incubated at 37°C in a humidified 5% CO_2 incubator. After about two weeks of incubation, wells were screened for cell growth and those wells with obvious cell pellets were selected and half of the cells in each well analysed for cytotoxicity on infected target cells in an ^{111}Indium oxine-release assay.

Wells that exhibited cytotoxic activity and originated from a cell concentration that gave rise to cell growth in less than 30% of the wells were selected for further study. These cloned cells were resuspended in culture medium at concentrations estimated to be between 500 and 5000 cells/ml; aliquots of 100 µl of cloned cells were distributed in the wells of 96-well round bottom tissue culture plates. Into each well, was added 100 µl of stimulator cell suspension containing $5x10^3$ – $1x10^4$ autologous irradiated infected cells and 1% IL-2 in culture medium. The plates were incubated as described before for 10 – 14 days. After this period of incubation, cloned cells were further sub-cultured as described above but at a responder-to-stimulator ratio of 1:10. Expanded cloned cells were analysed for cytotoxicity using ^{51}Cr-release assay. Aliquots of the cloned cells were also cryopreserved in liquid nitrogen.

4.1.4.6 Cytotoxicity assays

4.1.4.6.1 [^{51}Cr] Chromium-release assay

The ^{51}Cr-release assay was used to determine cytotoxicity of short-term cultures of PBMC, polyclonal T cell lines or CTL clones against *T. parva*-infected

lymphoblasts or Con-A induced lymphoblasts (peptide-pulsed or unpulsed) used as target cells essentially as described previously (Goddeeris, Morrison and Teale, 1986; Goddeeris and Morrison, 1988; Graham *et al*., 2006; 2008). Briefly, $2x10^6$ target cells were radio-labelled at 37°C for 1 hr with 100 µl containing 100 µCi of [^{51}Cr] sodium chromate (Amersham, UK). Target cells were then washed four times in 5ml of cytoxicity medium (HEPES-buffered RPMI-1640 supplemented with 5% FCS) and resuspended in 2 ml of the cytotoxicity medium to give a final concentration of $1x10^6$ cells/ml. For assays utilising short-term bulk PBMC cultures, viable effector cells were resuspended at $1x10^7$ cells/ml in cytotoxicity medium. Aliquots of 200 µl of two-fold dilutions of the effector cell suspension were plated in duplicate into the wells of a 96-well flat-bottom culture plate (Costar®). Radiolabelled target cells were added in each well in aliquots of 50 µl containing $5x10^4$ cells to give an effector-to-target cell ratio ranging from 40:1 to 1.25:1. Assays of cytotoxicity with polyclonal $CD8^+$ T cell lines and cloned T cells utilised 96-well V-bottom tissue culture plates (Costar®). Aliquots of 50 µl of radiolabelled target cell suspensions containing $2.5x10^4$ cells were added to each well. The effector cells were suspended at $1x10^6$ cells/ml and aliquots of 100 µl were added to duplicate wells giving effector target ratios ranging from 4:1 to 0.125:1. To determine the spontaneous release of radioisotope, aliquots of 50 µl of each target cell suspension were added in triplicate wells containing 200 µl (or 100 µl) of cytotoxicity medium only. Maximum release of the radiolabel was determined by adding in triplicate wells, 50 µl of the labelled target cell suspension to 200 µl (or 100 µl) of 1% (v/v) Tween-20 in water.

After 4 hr incubation at 37°C in a humidified 5% CO_2 incubator, cells in each well were resuspended and spun at 450xg for 5 min at room temperature to separate the cells from the released radio-label. Aliquots of 75 µl or 125 µl were harvested from each well and transferred into sample vials for assessing gamma emission using a gamma counter (Gamma Counter, Gamma 5500, Beckman, Geneva, Switzerland). Percent-specific cytotoxic release of ^{51}Cr (hereafter referred to as % cytotoxicity) was calculated from the formula: (ER – SR)/(MR - SR) x 100, where ER is the mean experimental release, SR is the mean spontaneous release and MR is the mean maximum release.

4.1.4.6.2 [^{111}In] Indium oxine-release assay

The ^{111}In-release assay was used to screen for cytotoxic activity in T cell clones during and after the cloning process as well as assessing cytotoxicity in *ex vivo* PBMC essentially, as described previously (Goddeeris, Morrison and Teale, 1986; Goddeeris and Morrison, 1988). To assay for cytotoxic activity in T cell clones, $1x10^6$ target cells were radio-labelled in 50 µl of cytotoxicity medium with 5 µCi of [^{111}In] indium oxine (Amersham, UK) by incubating for 15 min at 37°C. The required volume of radiolabel was calculated for each occasion based on the reference date as ^{111}In has a half-life of 2.8 days. The labelled cells were washed six times with cytotoxicity medium essentially as described for the ^{51}Cr-labelled cells, and resuspended in 10 ml of cytotoxicity medium to give a concentration of $1x10^5$ cells/ml. Target cells were autologous *Theileria*-infected lymphoblasts, peptide-pulsed Con A-stimulated lymphoblasts and unpulsed Con A lymphoblasts which served as the negative control (Goddeeris and Morrison, 1988).

Ex vivo PBMC were resuspended at $4x10^6$ cells/ml while established T-cell clones at $4x10^5$ cells/ml and aliquots of 100 µl of two-fold dilutions of the effector cells were plated in duplicate in 96-well V-bottom microtitre plates (Costar®), followed by 50 µl containing $5x10^3$ radio-labelled target cells resulting in effector-to-target cell ratio ranging from 80:1 to 10:1 and 8:1 to 1:1 for PBMC and T-cell clones, respectively. For assays utilising T-cell clones in a cloning process an aliquot of 100 µl was transferred directly from the selected wells into single wells of 96-well, V-bottom plates containing $5x10^3$ radiolabelled target cells. The cells were pelleted and incubated for 4 hr at 37°C in a humidified 5% CO_2 incubator. The remaining aspects of the assay, including determination of spontaneous release and maximum release and harvesting of the supernatants was carried out as described for the ^{51}Cr-release assay.

4.1.4.7 Interferon-γ Enzyme Linked Immunospot Assay

The IFN-γ ELISpot assay was applied to quantify the frequency of peptide-specific IFN-γ secreting $CD8^+$ T cells and assess the kinetics of the response in cattle undergoing immunisation and challenge with *T. parva*, essentially as described previously (Taracha *et al*., 2003; Graham *et al*., 2006). Briefly, sterile 96-well microtitre plates (MAIPS4510, Millipore, MA, USA) were pre-coated overnight at

4°C with 50 µl-aliquots of mouse anti-bovine IFN-γ mAb (CC302; IgG_1; Serotec Antibodies, UK) diluted at 1 µg/ml in coating carbonate buffer, pH 9.6. Plates were washed twice with unsupplemented RPMI-1640 medium and blocked with 50 µl/well of RPMI-1640 supplemented with 10% FCS for 2 hr at 37°C. Aliquots of 50 µl containing $2.5x10^5$ responder PBMC were added per well followed by peptides in 50 µl-aliquots at a final concentration of 1 µg/ml. A control well with responder cells in medium alone and another well with responder cells with Con A at a final concentration of 5 µg/ml were included as background and positive controls. All samples were plated out in duplicate. The plates were kept for 20 hr at 37°C in a humidified 5% CO_2 incubator.

After the cells were discarded, the plates were washed four times with distilled water containing 0.05% Tween-20 and then four times with PBS-0.05% Tween-20, each time with gently shaking of the plates for 20 seconds before the wash fluid was flicked off. Excess wash buffer was removed by gently tapping plates on paper towels. A second rabbit anti-bovine IFN-γ antibody (ILRI in-house reagent) diluted at 1:1500 in PBS-0.05% Tween-20/ 0.1% BSA was added in 50-µl aliquots, and the plates incubated for 1 hr at room temperature. A further four washes with PBS-0.05% Tween-20 were performed without shaking the plates. Fifty-microlitre aliquots of an anti-rabbit IgG (α-chain specific)-alkaline phosphatase conjugate (RG-96; Sigma Chemicals, UK) diluted at 1:2000 in PBS- 0.05% Tween-20/ 0.1% BSA were added to the plates and incubated for 1 hr at room temperature. Plates were further washed six times with PBS-0.05% Tween-20 and developed with 50 µl/well of SigmaFast™ BCIP/NBT substrate (Sigma Chemicals, UK) solution at room temperature for 10 min in the dark to allow for spot development. The reaction was stopped by rinsing the plates with copious amounts of water. The plates were air-dried and spots counted in each well using an automated ELISPOT plate reader (AID ELISpot Reader system, Germany). After the ELISpot reader count, spots were manually edited to ensure that only "true" positive spots were included. After correction for background signals as determined in the absence of any antigen, results were expressed as spot-forming cells (SFC)/million PBMC.

4.1.4.8 Immunofluorescent staining and flow cytometry

4.1.4.8.1 Immunofluorescence surface staining

Suspensions of PBMC were stained with mAb specific for BoLA class I antigens (see Appendix VIII) using the indirect immunofluorescence technique and analysed using a fluorescence-activated cell sorter (FACScan™, Beckton Dickinson, USA). To stain the cells, aliquots of 50 µl of FACS medium (RPMI-1640 medium with HEPES and supplemented with 5% heat-inactivated horse serum [Sigma]) containing approximately $4x10^5$ cells, were added to 50 µl of mAb diluted at 1:500 in FACS medium in a 96-well round-bottom tissue culture plate (Costar®) and incubated for 30 min at 4°C, essentially as described previously (Lalor *et al*., 1986; Goddeeris *et al*., 1988) cells were then washed three times, by resuspension in 100 µl of FACS medium and centrifugation (Heraeus Megafuge, Model No. 2.0R) at 1500 rpm for 2 min at room temperature. After the final wash, the supernatant was discarded, the pellet resuspended in residual fluid by vortexing, 50 µl of a 1:200 final concentration of FITC-conjugated or PE-conjugated goat anti-mouse Ig added and incubated for a further 30 min at 4°C. The cells were then washed three times as before. The cell samples were finally resuspended in 200 µl of FACS fixative (1% paraformaldehyde in PBS) and analysed on the FACS machine.

One colour flow cytometry acquisition was performed on a FACScan ™ flow cytometer using Cell Quest software. Viable cells were gated on FSC versus SSC and 10,000 – 20,000 events collected within this gate. FACS data was analysed using Cell Quest Software or Flow Jo (TreeStar, San Carlos) Software and displayed as dot plots of FSC and log fluorescence intensity on FL1 or FL2.

4.1.4.8.2 Intracytoplasmic staining for perforin

This procedure was based on methods described previously by Jung *et al.,* (1993). Briefly, $4x10^5$ cells/well were washed with 200 µl of cold PBS and then fixed by resuspending in 200 µl 1% paraformaldehyde in PBS for 10 min at room temperature. After washing once with PBS, the cells were permeabilised by incubating in 200 µl of a solution containing 0.1% (w/v) saponin, 0.1% sodium azide, 10 mM HEPES (PBS-Sap), supplemented with 20% heat-inactivated normal goat serum (for Fc receptor blocking) for 30 min at room temperature. The cell pellet was

recovered by centrifuging at 450xg for 2 min and the cell sample stained for expression of perforin by incubating in 25 µl of mouse anti-human perforin monoclonal antibody δG9 (BD Pharmingen), diluted 1:250 in PBS-Sap, for 30 min at 4°C. Cells were washed twice by resuspension in 100 µl PBS-Sap and centrifugation as before. After the final wash, the supernatant was discarded, the pellet was resuspended in residual fluid and incubated in 25 µl FITC-conjugated or PE-conjugated anti-mouse Ig diluted 1:200 or 1:500, respectively, for 30 min at 4°C. The cells were washed as before then washed once with PBS and finally resuspended in 200 µl FACS fixative (1% paraformaldehyde) for FACS analysis as described in Section 4.1.4.8.1.

4.1.4.8.3 BoLA class I-peptide tetramer staining

BoLA class I-peptide complexes were synthesised and tetramerised by collaborators (*Didier Colau et al*.) at the Ludwig Institute for Cancer Research, Brussels, Belgium and shipped as PE conjugated tetramers for use at ILRI. Four BoLA class I-peptide tetrameric complexes were constructed in accordance with established protocols (http://research.yerkes.emory.edu/tetramer_core/protocol.html). Details of synthetic peptides and BoLA class I restricting elements are described in Table 10. Briefly, the assembly of the bovine tetramers first involved the cloning and expression of the BoLA heavy and light chains. BoLA N*00101, N*01301 and N*01201 cDNA clones were used as a templates to amplify the sequence coding for the extracellular domains (amino acid 1–279 of the mature protein) of the heavy chains. The PCR products were cloned into a vector derived from pET3D (Stratagene) and containing a *Bir*A biotinylation site in-frame with the 3'-end of the BoLA sequences. A cDNA clone containing the bovine beta-2 (β-2m) microglobulin gene was used to amplify the sequence coding for the mature β-2m protein (amino acid 21-118). The PCR product was cloned into a derivative of plasmid pET9A (Novagen). Recombinant proteins were expressed in *E. coli* strain BL21 (DE3) (Stratagene). Recombinant BoLA/peptide molecules were folded *in vitro* as previously described (Altman *et al*., 1996). Soluble complexes were purified by gel filtration and biotinylated using the BirA enzyme (Avidity LCC, Denver, CO). PE-labelled tetramers were produced by mixing the biotinylated complexes with streptavidin-PE (BD Pharmingen) (Dr. Didier Colau, LICR, personal communication).

Table 10: Cytotoxic $CD8^+$ T cell epitopes and bovine tetramers

BoLA class I peptide complex	Peptide sequence	Peptide size
BoLA-N*01301-$Tp1_{214-224}$	VGYPKVKEEML	11-mer
BoLA-N*01201-$Tp2_{98-106}$	QSLVCVLMK	9-mer
BoLA-N*00101-$Tp4_{328-336}$	TGASIQTTL	9-mer
BoLA-N*00101-$Tp8_{379-387}$	CGAELNHFL	9-mer

Four peptides corresponding to antigenic epitopes on four *T. parva* antigens, Tp1, Tp2, Tp4 and Tp8, previously identified to be the targets of CD8 T cell responses of *T. parva* immune cattle (Graham *et al*., 2006; 2008) were used in this study. The epitopes, as defined by the minimal peptide length that induced maximal T cell activity and their restricting MHC class I alleles are shown above. $Tp4_{328-336}$ and $Tp8_{379-387}$ are restricted by the same class I allele (N*00101; A10-$KN104^+$ haplotype), $Tp1_{214-224}$ is restricted by (N*01301; $A18^+$ haplotype) while $Tp2_{98-106}$ is shown to be restricted by N*01201 associated with $A10^+$ MHC class I haplotype. Peptides were synthesised by Mimotopes (Clayton, Australia; $Tp1_{214-224}$) and PepScan Systems (Netherlands; $Tp4_{328-336}$, $Tp8_{379-387}$, $Tp2_{98-106}$) and shipped as lyophilised pellets which were reconstituted by resuspending in a solution of 50% (v/v) DNA synthesis grade acetonitrile-water (Applied Biosystems, UK), aliquoted and stored at -20°C and subsequently diluted in sterile RPMI-1640 for use in IFN-γ ELISpot, CTL lytic activity assays as described.

Tetramers obtained from LICR were used to stain CTL prepared from cattle bearing the relevant MHC (Graham *et al*.). Briefly, $4x10^5$ CTL/well were washed twice by resuspension in 100 µl-aliquots of PBS-0.5% BSA, 0.1% sodium azide (PBS-BSA) and centrifugation at 450xg at room temperature for 2 min to recover the sample. After the final wash, the cell pellet was resuspended in residual fluid and incubated in 25 µl of tetramer at the appropriate dilution at room temperature for 30 min in the dark. The cells were washed twice as before and resuspended in 200 µl of PBS-BSA for FACS analysis.

4.1.4.8.4 Immunofluorescence double staining technique

Tetramer/CD8 and perforin/CD8 double staining was conducted on cell samples essentially as described previously (Appay *et al*., 2000; Meidenbauer *et al*., 2001; Denkberg *et al*., 2001). Briefly $4x10^5$/well of PBMC or CTL was centrifuged at 450xg for 2 min and washed twice with cold PBS-BSA. To avoid blocking of tetramer binding by anti-CD8 antibody, the cells were first incubated with 25µl of 40nm of tetramer in PBS containing 0.5% BSA and 0.1% sodium azide (PBS-BSA) for 10 min at room temperature in the dark followed by addition of 25 µl anti-CD8 (IL-A51; IgG_1) mAb at 1:250 dilution in PBS-BSA and incubated for a further 20 min in the dark, at room temperature. Cells were then washed twice by the addition of 150 µl aliquots of PBS-BSA and centrifugation as before. After the final wash, the cell pellet was resuspended in residual fluid and 25 µl of FITC conjugated anti-mouse IgG_1 (Southern Biotech) diluted 1:200 in PBS-BSA or 1 µl of anti-mouse IgG_1 PerCP reagent (X56; IgG_1; BD Immunocytometry Systems) added and incubated for a further 30 min. The cells were then washed twice as before and resuspended in 200 µl of FACS.

Two colour flow cytometric acquisition was performed on a FACScan™ flow cytometer (Beckton Dickinson) using CellQuest software (BD Biosciences, Immunocytometry Systems) or FACSCanto II using FACS Diva software as described previously in section 4.1.4.8.

For perforin/CD8 staining, $4x10^5$cells/well were fixed and permeabilised as previously described in Section 4.1.4.8.2. After permeabilisation the cell pellet was recovered by centrifugation and incubated in 25 µl of anti-human perforin monoclonal antibody δG9 (IgG_{2b}) diluted 1:250 in PBS-Saponin and 25 µl of IL-A105 (IgG_{2a})

diluted 1:250 in PBS-Sap at 4°C for 30 min. Cells were washed twice with PBS-Sap as described previously and incubated in 50 μl of a 1:200 final concentration of FITC-conjugated anti-mouse IgG_{2a} and PE-conjugated anti-mouse IgG_{2b} for 30 min at room temperature. The cells were washed two times with PBS-Sap and once with PBS by centrifugation and resuspended in 200 μl FACS fixative for two-colour flow cytometric acquisition and analysis as described before. IL-A105 (IgG_{2b}) was used in place of IL-A51 (IgG_1) because when used with the anti-IgG_{2a} FITC reagent it showed better discrimination of double positive staining cells in FACS profile analysis as compared to profiles obtained using the anti-IgG_1 FITC reagent.

4.2 Results

4.2.1 Studies with defined polyclonal and cloned CTL lines

4.2.1.1 T cell receptor staining using BoLA class I-peptide complexes

BoLA class I-peptide complexes were synthesised and tetramers prepared at the Ludwig Institute for Cancer Research, Brussels, Belgium and shipped as PE conjugates for use at ILRI. Tetramer binding to antigen-specific TCRs was examined by staining CTL lines and clones showing the appropriate specificity as indicated in Table 11 and titrated to determine the optimum concentration for use in staining. These CTL lines and clones generated after TpM restimulation of PBMC from *T. parva* immune animals, had shown specificity for Tp1 (VGYPKVKEEML), Tp2 (QSLVCVLMK), or Tp8 (CGAELNHFL) peptides using the IFN-γ ELISpot assay and exhibited specific lytic activity against autologous immortalised skin fibroblasts (iSF) transfected with the appropriate *T. parva* cDNA or COS-7 cells transfected with the restricting BoLA and peptide (Graham *et al*., 2006). CTL specific for Tp4 had not been successfully generated at the time of the study.

Cell staining was carried out 14 days after the last restimulation. As shown in Figure 13, over 80% staining of BV115 clone 27, BV115 clone 20 and BX064 clone 5 was observed with the Tp1, Tp2 and Tp8 tetramers, respectively, in comparison with a less than 1% background non-specific staining observed with an irrelevant CTL clone which was used as the negative control. The relevant tetramers were also able to discriminate and identify antigen-specific T cells in CTL lines. Additionally, the binding was titrated and covered a similar concentration range. Optimal staining was achieved at a tetramer dilution of 1/500 – 1/1000 corresponding to a final concentration of 20-10nm of tetramer, respectively. This was the lowest concentration of tetramer that yielded the highest percentage of tetramer positive events. These experiments demonstrate the specificity and potential usefulness of tetrameric class I-peptide complexes in the identification of antigen-specific T cells.

Further analysis of the $Tp1_{214\text{-}224}$, $Tp2_{98\text{-}106}$ and $Tp8_{379\text{-}387}$ specific CTL clones stained with tetramers revealed the presence of two distinct T cell sub-sets in the flow cytometry dot plots corresponding to tetramer ‘high’ and tetramer ‘low’ staining T

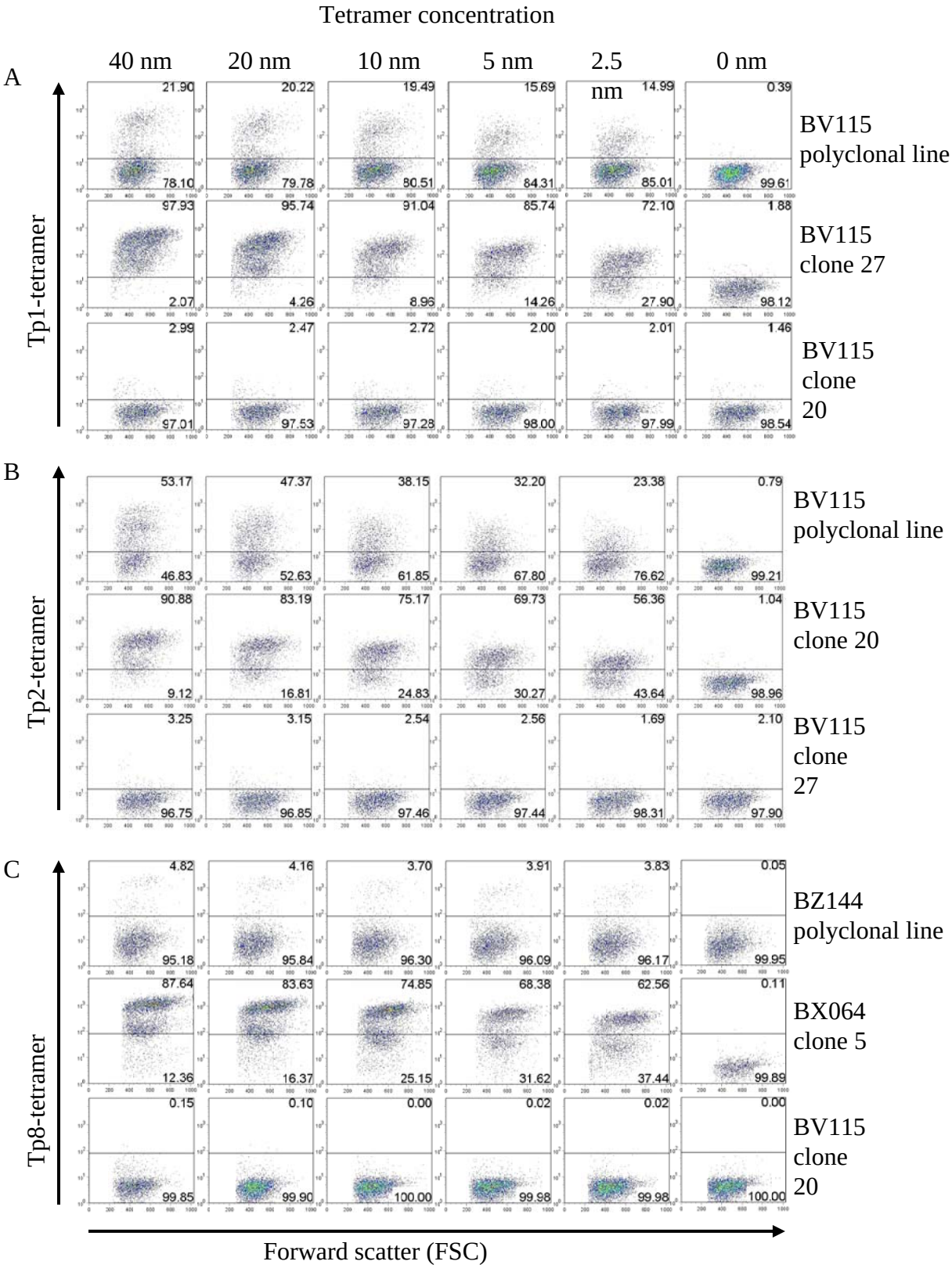
Table 11: Cytotoxic $CD8^+$ T cells used in determining specificity of tetramers

Animal source	$CD8^+$ CTL	Specificity			
		$Tp1_{214-224}$	$Tp2_{98-106}$	$Tp8_{379-387}$	Restricting class I BoLA
BV115	A10/A18 restricted polyclonal line		+		N*01201
		+			N*01301
BZ144	A10/KN104 restricted polyclonal line			+	N*00101
BV115	A10/A18 restricted clone 27	+			N*01301
BV115	A10/A18 restricted clone 20		+		N*01201
BX064	A10/KN104 specific clone 5			+	N*00101

Specificity of the CTL is denoted by (+).

Figure 13: Validation of bovine tetramers

Specific cytotoxic $CD8^+$ T cell lines and clones were stained with BoLA-N*01301-$Tp1_{214-224}$ tetramer (A) BoLA-N*1201-$Tp2_{49-59}$ tetramer (B) and BoLA-N*00101-$Tp8_{379-387}$ tetramer (C) and analysed by flow cytometry. The data is displayed as dot plots showing forward scatter (FSC) versus tetramer positive cells on FL2 phycoethrin (PE).


Tetramer concentration
40 nm
20 nm
10 nm
5 nm
2.5 nm
0 nm
A
Tp1-tetramer
21.90
78.10
20.22
79.78
19.49
80.51
15.69
84.31
14.99
85.01
0.39
99.61
97.93
2.07
95.74
4.26
91.04
8.96
85.74
14.26
72.10
27.90
1.88
98.12
2.99
97.01
2.47
97.53
2.72
97.28
2.00
98.00
2.01
97.99
1.46
98.54
BV115 polyclonal line
BV115 clone 27
BV115 clone 20
B
Tp2-tetramer
53.17
46.83
47.37
52.63
38.15
61.85
32.20
67.80
23.38
76.62
0.79
99.21
90.88
9.12
83.19
16.81
75.17
24.83
69.73
30.27
56.36
43.64
1.04
98.96
3.25
96.75
3.15
96.85
2.54
97.46
2.56
97.44
1.69
98.31
2.10
97.90
BV115 polyclonal line
BV115 clone 20
BV115 clone 27
C
Tp8-tetramer
4.82
95.18
4.16
95.84
3.70
96.30
3.91
96.09
3.83
96.17
0.05
99.95
87.64
12.36
83.63
16.37
74.85
25.15
68.38
31.62
62.56
37.44
0.11
99.89
0.15
99.85
0.10
99.90
0.00
100.00
0.02
99.98
0.02
99.98
0.00
100.00
BZ144 polyclonal line
BX064 clone 5
BV115 clone 20
Forward scatter (FSC)

cells. Dilution of tetramers gave progressively lower percentage values for the tetramer$^+$ T cell sets with nearly 1.5-fold difference between percent epitope specific T cell population recovered at saturating tetramer (40nm) staining conditions and limiting tetramer (2.5nm) dilution conditions. This observation suggests that the cell lines were not clonal but epitope-specific T cell sub-sets within the Tp1, Tp2, and Tp8 epitope-specific response with differing degress of expression of the particular TCR as defined by the interaction of TCR and pMHC complex.

4.2.1.2 Double staining with CD8 monoclonal antibody and peptide MHC class I tetramers

To phenotype the tetramer positive population, BV115 A10/A18 restricted- and BZ144 A10-KN104 restricted polyclonal T cell lines were stained with tetramer followed by anti-CD8 antibody (IL-A51). Results obtained showed that tetramer positive cells are CD8 positive and the proportions of tetramer$^+$CD8$^+$ T cells were comparable to tetramer$^+$ T cells. The dot blots also showed two distinct populations of the tetramer$^+$CD8$^+$ T cells corresponding to tetramer^{high}CD8$^+$ and tetramer^{low}CD8$^+$ staining T cell populations (Figure 14). Further to this, data obtained using Tp1 and Tp2 tetramers to stain BV115 polyclonal CTL line, also indicates that the CTL line comprises of CTL of other peptide specificities with Tp1 and Tp2 antigen-specific T cells accounting for nearly 50% or more of the CTL population. The same argument may be proposed for the composition the BZ144 CTL line, with Tp8-specific CTL accounting for 18% of the CTL population with the remainder being CTL of other peptide specificities.

4.2.1.3 Combination of tetramer staining and intracellular staining for perforin

Tetramer positive populations were stained for the expression of perforin using anti-human perforin antibody (δG9). A potential hindrance to intracellular staining of perforin in tetramer positive cells is the need for fixation and permeabilisation of the cells whose effect on the TCR and subsequently tetramer binding had not been established. Figure 15a shows staining of the BV115 polyclonal T cell line with Tp1- and Tp2- tetramers before or after fixation with 1% PFA and permeabilisation with 0.1% saponin in PBS solution. A near complete loss of tetramer binding was seen in cells pre-treated with PFA and saponin. However, cells stained with tetramers before fixation/permeabilisation maintained comparable levels of

Figure 14: Immunofluorescence double staining of cytotoxic $CD8^+$ T cell lines with tetramer and anti-CD8 antibody

BV115 polyclonal CTL and BZ144 polyclonal CTL were double stained with 20 nm of BoLA-N*01301 Tp1-tetramer, BoLA N*01301 Tp2-tetramer, 5 nm of BoLA-N*00101-Tp8 tetramer respectively and anti CD8 antibody (IL-A51; IgG_1). Dot plots are shown as log fluorescence intensity of CD8:FITC versus Tetramer:PE

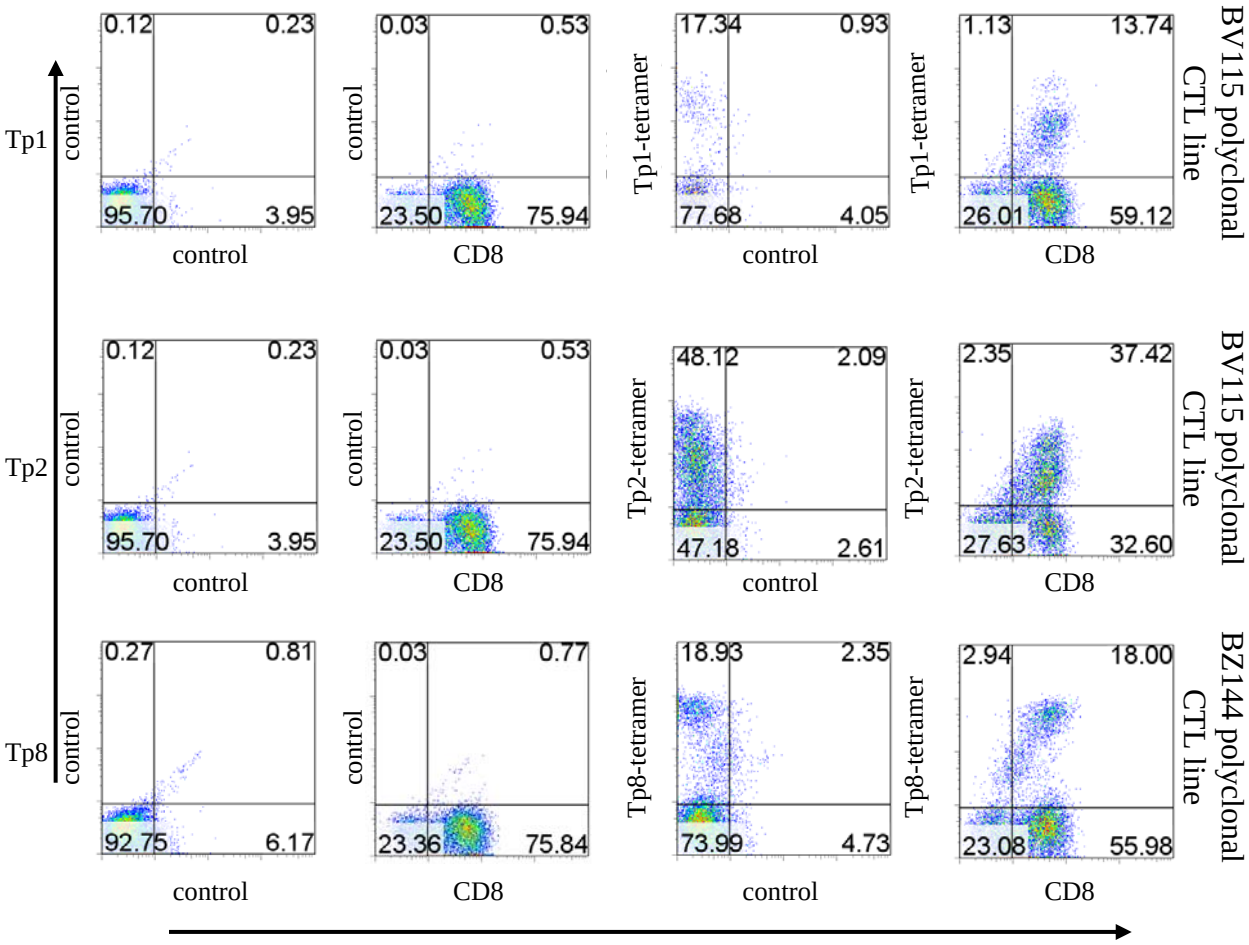
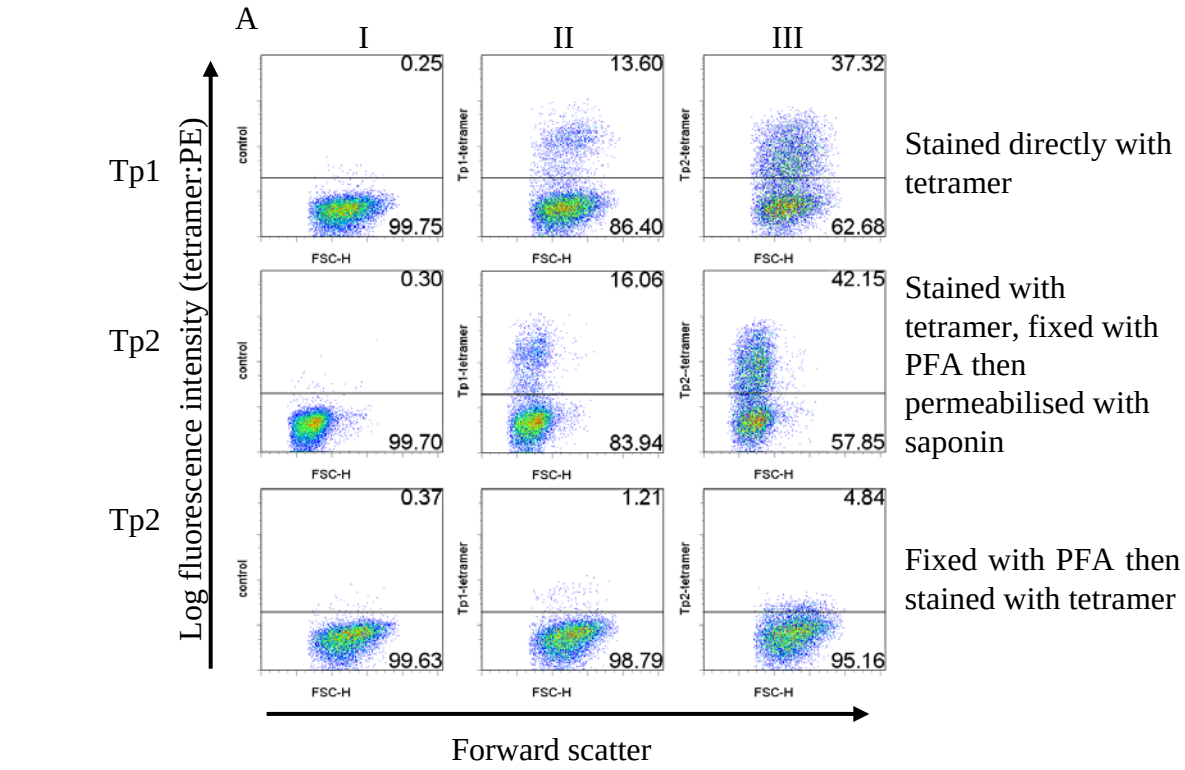
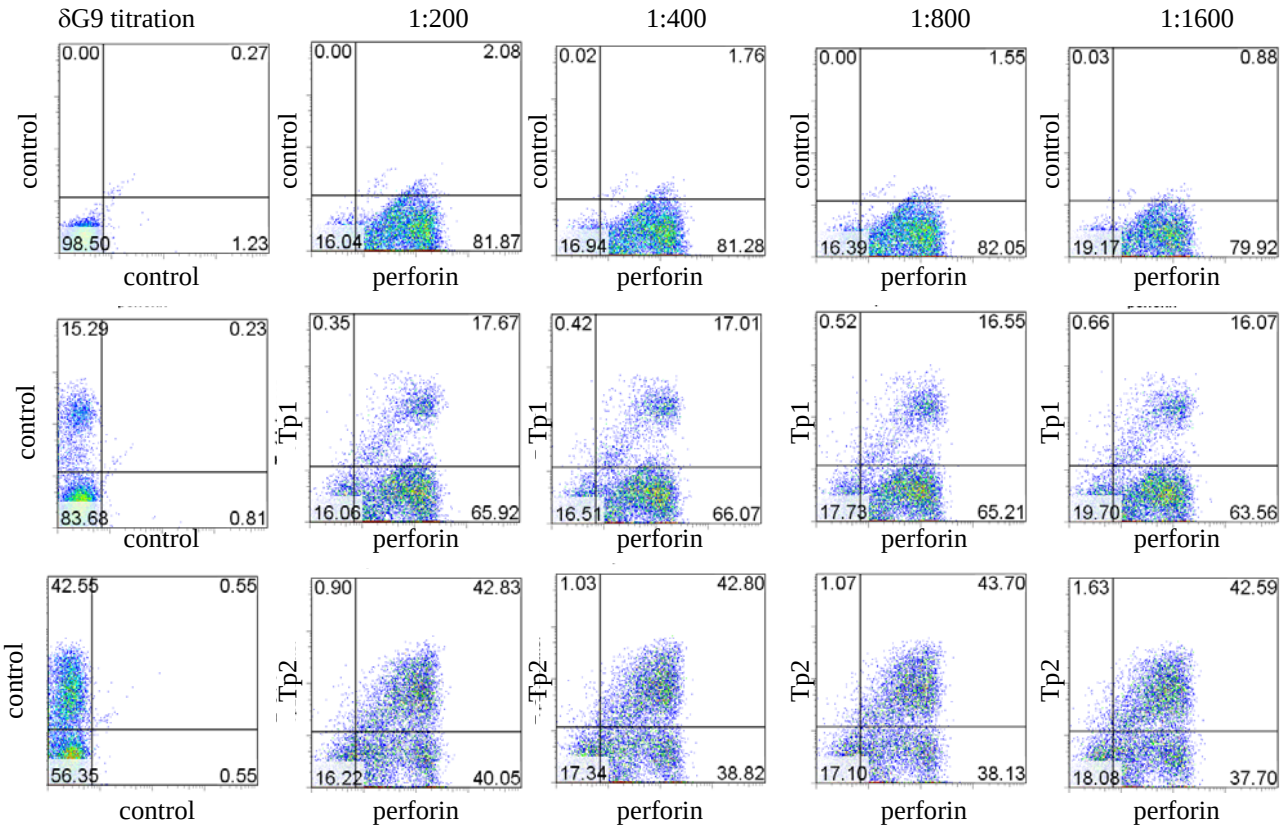

Tp1
Tp2
Tp8
control
control
0.12
0.23
95.70
3.95
0.03
0.53
23.50
75.94
17.34
0.93
77.68
4.05
1.13
13.74
26.01
59.12
48.12
2.09
47.18
2.61
2.35
37.42
27.63
32.60
0.27
0.81
92.75
6.17
0.03
0.77
23.36
75.84
18.93
2.35
73.99
4.73
2.94
18.00
23.08
55.98
Tp1-tetramer
Tp2-tetramer
Tp8-tetramer
CD8
BV115 polyclonal CTL line
BZ144 polyclonal CTL line

Figure 15: Double immunofluorescence staining for tetramer and intracellular perforin

To assess the effect of fixation and permeabilisation BV115 polyclonal CTL lines (columnsI II and III) were fixed and permeabilised as required for intracellular staining before or after staining with Tp1- or Tp2-tetramers (A). Panel B shows BV115 polyclonal CTL shown in column III stained for intracellular perforin using titrating amounts of mouse anti-perforin antibody (δG9, IgG_{2b}) and 20 nm Tp1- or Tp2-tetramer. PFA – paraformaldehyde


A
I
II
III
Tp1
Tp2
Tp2
Log fluorescence intensity (tetramer:PE)
Forward scatter
control
Tp1-tetramer
Tp2-tetramer
FSC-H
0.25
99.75
13.60
86.40
37.32
62.68
0.30
99.70
16.06
83.94
42.15
57.85
0.37
99.63
1.21
98.79
4.84
95.16
Stained directly with tetramer
Stained with tetramer, fixed with PFA then permeabilised with saponin
Fixed with PFA then stained with tetramer


B
δG9 titration
1:200
1:400
1:800
1:1600
control
perforin
Tp1
Tp2
0.00
0.27
98.50
1.23
0.00
2.08
16.04
81.87
0.02
1.76
16.94
81.28
0.00
1.55
16.39
82.05
0.03
0.88
19.17
79.92
15.29
0.23
83.68
0.81
0.35
17.67
16.06
65.92
0.42
17.01
16.51
66.07
0.52
16.55
17.73
65.21
0.66
16.07
19.70
63.56
42.55
0.55
56.35
0.55
0.90
42.83
16.22
40.05
1.03
42.80
17.34
38.82
1.07
43.70
17.10
38.13
1.63
42.59
18.08
37.70

staining although a slight increase in tetramer$^+$ populations was noted. An optimised protocol for combined tetramer and intracellular perforin staining was used to stain the polyclonal T cell lines for expression of perforin. Results obtained showed that tetramer$^+$ cells expressed perforin and the level of Tp1 and Tp2 tetramer$^+$perforin$^+$ T cells was comparable to tetramer$^+$ cells (Figure 15b). The proportion of Tp1 and Tp2-antigen specific CTL expressing perforin in the BV115 CTL line was additionally comparable to that obtained by costaining tetramer with CD8 antibody as shown in Figure 14.

4.2.2 Studies with *ex vivo* T cells

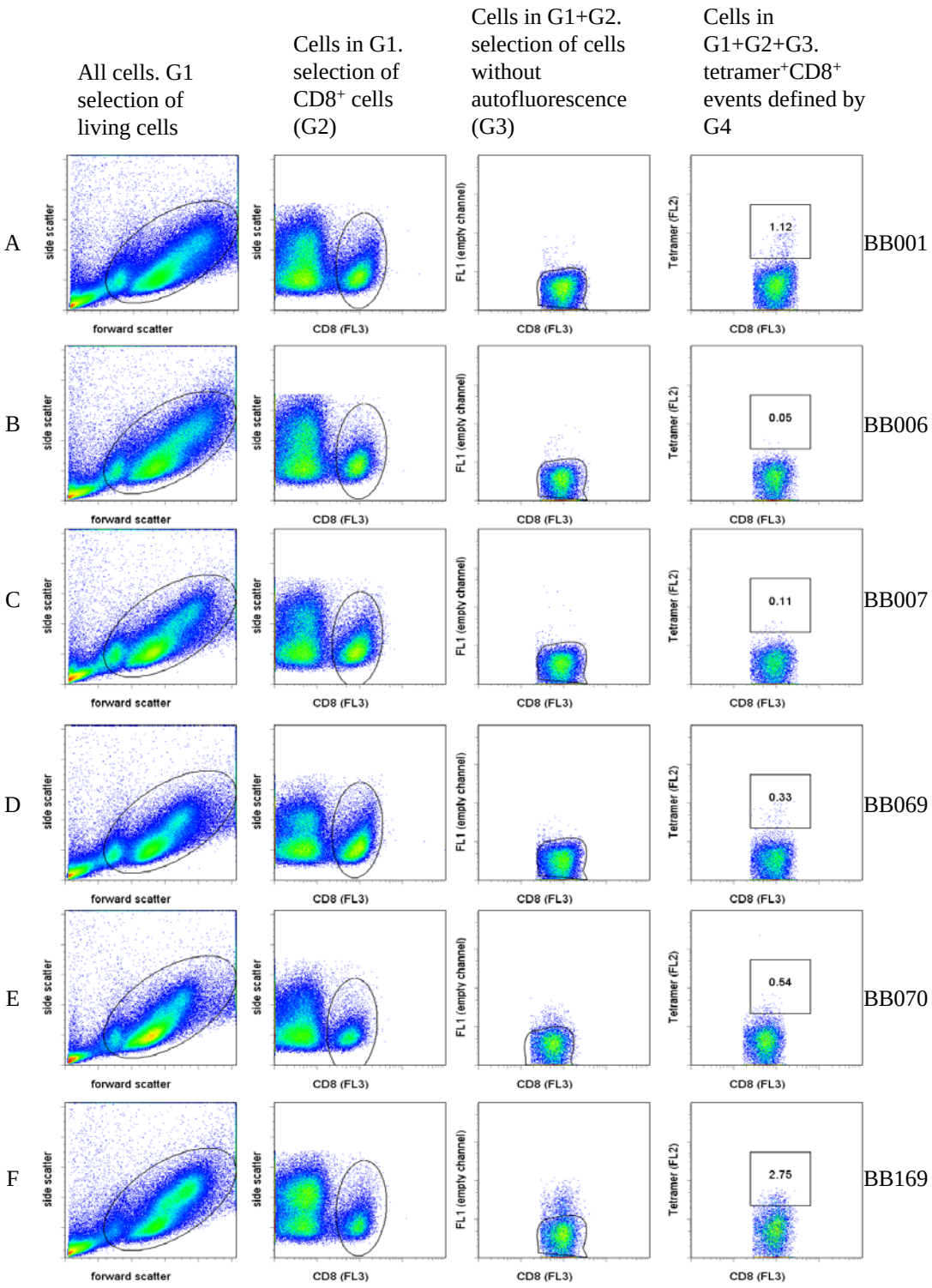
4.2.2.1 Elimination of non-specific tetramer binding events

To analyse Tp1, Tp2, Tp4 and Tp8 specific T cell responses in animals following immunisation and challenge, PBMC were collected on alternate days and stained with tetramer and anti-CD8 monoclonal antibody. One of the early observations in analysis of the results was high background staining obtained when staining PBMC as compared to staining T cell lines and clones. This required a gating strategy to minimise non-specific tetramer binding events. The logic of the selection of tetramer-labelled cells is shown in Figure 16. Live cells were first gated based on scatter light distribution in an SSC vs FSC dot plot. For each sample approximately 100,000 events were acquired within this gate (G1), with the appropriate compensation (spectral overlap) adjustment for fluorescein on FL1, phycoerythrin on FL2 and PerCP on FL3. Events within G1 were next gated with an additional region in an SSC vs FL-3 (CD8) dot plot (G2). To improve specificity, cells emitting high autofluorescence were gated out by displaying the cells in the FL-1 "empty channel" vs CD8 (FL-3) (G3) using a regional gate. A final region was placed around tetramer positive events that emerged as a discernible cluster of tetramer$^+$ T above the negative population in a tetramer (FL-2) vs CD8 (FL-3) dot plot (G4). Assay validation was further improved by staining samples with irrelevant tetramers. Tetramer positive cells were expressed as frequencies of parent CD8$^+$ population and lymphocyte (live gate) population as percentages as well reciprocal frequencies. Precursor frequencies were determined as a function of staining $4x10^5$ cells/well.

Using this strategy sources of non-specific binding were eliminated and compound gating also permitted the use of a liberal lymphocyte scatter panel that

Figure 16: Compound gating used to identify tetramer^{+}CD8^{+} T cells

Peripheral blood mononucleated cells (PBMC) from respective animals were stained with tetramer followed by anti-CD8 monoclonal antibody using two-colour flow cytometry. To obtain a clearly discernible cluster of tetramer^{+} cells (G4) gates within an extended live lymphocyte light scatter (G1), CD8^{+} gate (G2) and selection of cells without autofluorescence (G3) were used. Results are shown for six independent PBMC samples from BB001, BB006, BB007, BB069, BB070 and BB169 (A-F) stained with BoLA N*00101-Tp8-tetramer and ILA-51.


All cells. G1 selection of living cells
Cells in G1. selection of CD8+ cells (G2)
Cells in G1+G2. selection of cells without autofluorescence (G3)
Cells in G1+G2+G3. tetramer+CD8+ events defined by G4
A
B
C
D
E
F
BB001
BB006
BB007
BB069
BB070
BB169
side scatter
forward scatter
CD8 (FL3)
FL1 (empty channel)
Tetramer (FL2)
1.12
0.05
0.11
0.33
0.54
2.75

does not exclude large activated T cells. However, a 'smear' of tetramer$^+$CD8$^+$ cells was often detected, which included a significant number of cells with 'low' and 'high' and low tetramer staining intensity. Generally better compensation was seen using PE and PerCP fluorochromes visualised by a large separation between positive and negative populations as compared to FITC and PE double stains.

Using the above strategy a distinct population of Tp1 tetramer$^+$CD8$^+$ T and Tp8 tetramer$^+$CD8$^+$ T cells was repeatedly stained in PBMC samples from A18 and A10-KN104 animals, respectively. However, it was not possible to demonstrate a clearly discernable cluster of Tp2 and Tp4 tetramer$^+$CD8$^+$ T cells which were at least one log above the mean fluorescence intensity of the negative population in PBMC samples from the respective BoLA matched animals. For all tetramers, low levels of tetramer$^+$CD8$^+$ T cells were detected in PBMC of BoLA-mismatched donors. As these events were non-specific by definition, this data set was used to empirically determine the lower limit of detection of the assay as reported in section 4.2.2.3.

4.2.2.2 Log transformation of data and the use of geometric mean

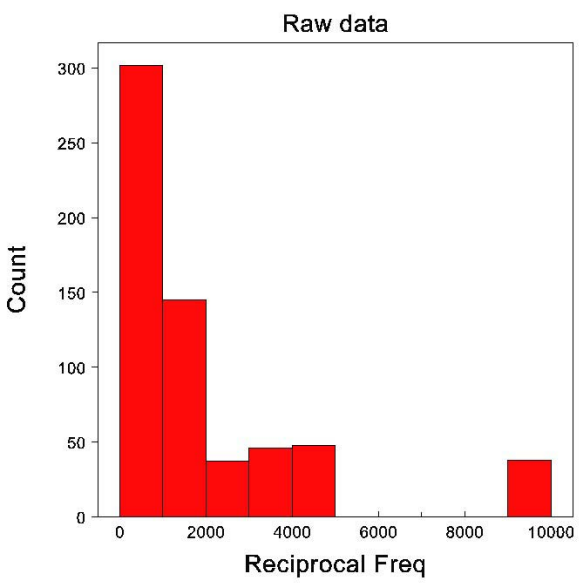
Examination of the raw data using normal probability plots showed that the raw data, expressed as reciprocal frequencies, was left skewed and did not conform to a normal distribution invalidating the use of parametric tests for data analysis and interpretation. For the four tetramers used in this study, taking the log of the reciprocal frequency was seen to bring data into conformation with a normal distribution with the mode and median values closer to the mean (Figure 17). Log-transformation of the reciprocal frequencies resulted in more symmetric data distribution with the mode and median values closer to the mean. Accordingly, descriptive statistics (means, standard deviation (SD), confidence intervals (CI) and statistical analyses (Student's t-test) were performed on log-transformed reciprocal frequencies.

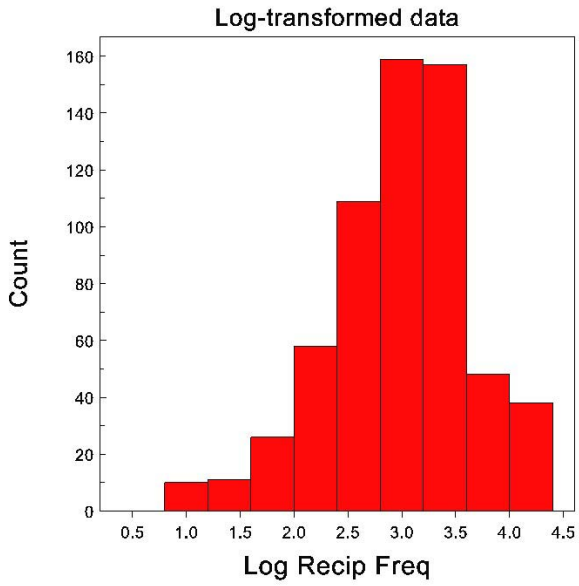
4.2.2.3 Definition of the lower limit of detection of tetramer positive cells

Despite application of the gating strategy described in section 4.2.2.1, low levels of tetramer$^+$CD8$^+$ T cells were detected in PBMC of BoLA-mismatched donors. As these events were considered non-specific, a cut-off for the lower detection limit of each of the tetramers was established at the upper 99th percentile of geometric mean frequency of tetramer$^+$CD8$^+$ T cells in BoLA mismatched samples

Figure 17: Tetramer frequency data are log normally distributed

Reciprocal frequency data (left) and log-transformed reciprocal frequency data (right) from the analysis using the four tetramers are displayed as frequency histograms. Without log-transformation, the data are poorly represented by the normal distribution. This invalidates the use of parametric tests and renders the mean and standard deviation (SD) poor descriptors of the data set.


Raw data
Count
300
250
200
150
100
50
0
0
2000
4000
6000
8000
10000
Reciprocal Freq


Log-transformed data
Count
160
140
120
100
80
60
40
20
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
Log Recip Freq

(geometric mean + 2.6SD). The limit of detection of BoLA-N*01301-Tp1 tetramer was determined to be 1/7440, and 1/3768, 1/4068 and 1/12287 for BoLA-N*01201-Tp2, BoLA-N*00101-Tp4 and BoLA-N*00101-Tp8 tetramers,respectively (Table 12a).

These cut offs were then applied to data obtained from testing BoLA matched animals to determine differences between positive and negative events (Table 12b). For Tp1 and Tp8 tetramers, the detectable frequencies of Tp1-tetramer$^+$CD8$^+$ and Tp8-tetramer$^+$CD8$^+$ events were significantly higher than the established cut-off ($p<0.001$) with a mean of 1/862 and 1/6931 for Tp1-tetramer$^+$CD8$^+$ and Tp8-tetramer$^+$CD8$^+$ events, respectively. However the difference between most Tp2-tetramer$^+$CD8$^+$ (mean=1/2861), Tp4-tetramer$^+$CD8$^+$ (mean=1/5712) and negative events was not statistically significant with less than 50% of Tp2-tetramer$^+$CD8$^+$ and Tp4-tetramer$^+$CD8$^+$ events above the cut-off values. These two tetramers were characterised by non-specific binding and ambiguous staining of CD8$^+$ populations making it difficult to clearly determine tetramer positive events suggesting that the sensitivity of staining with these tetramers may be just below threshold levels.

4.2.3 Evaluation of antigen-specific CD8$^+$ T cells in cattle after live vaccine immunisation and challenge with *T. parva* sporozoites

Five animals, BB001, BB006, BB007, BB069 and BB169 were immunised by ITM and challenged in this study whereas BB070, BV115 and BX063, (the latter two already ITM immunised in earlier studies) were challenged with sporozoites. All seven ITM immunised animals survived as expected whereas BB070, an animal not previously exposed to *T. parva* nor ITM vaccinated, succumbed to ECF 21 days following challenge with *T. parva*. Five of the ITM animals, BB001, BB006, BB007, BB069 and BB169 were protected against secondary infection with *T. parva* while BV115 and BX063 remained immune to infection after repeated challenge. As mentioned in materials and methods, BV115 and BX063 had previously been immunised by ITM and challenged for use in other studies. Experimental animal records are shown in Appendix IX. Of the five animals immunised during the study two animals, BB001 and BB006, were characterised by a a vaccine break-through infection during the course of immunisation which necessitated treatment with Butalex.

Table 12a: Summary statistics of background levels

Tetramer	Geometric mean P<0.001	Std error of means	Cut-off (geometric mean+2.6SD)
BoLA-N*01301-Tp1	3.872 (7438)	0.07	7440
BoLA-N*01201-Tp2	3.577 (3766)	0.06	3768
BoLA-N*00101-Tp4	3.610 (4063)	0.06	4064
BoLA-N*00101-Tp8	4.090 (12286)	0.04	12287

Reciprocal frequencies of the geometric mean are shown in parentheses

Table 12b: Means of tetramer^{+}CD8^{+} events

Tetramer	Reciprocal frequency	Geometric mean P<0.01	Std error of means	P-probability
BoLA-N*01301-Tp1	862	2.940	0.115	<0.001
BoLA-N*01201-Tp2	2861	3.4580	0.078	NS
BoLA-N*00101-Tp4	5712	3.757	0.05	NS
BoLA-N*00101-Tp8	6931	3.841	0.06	<0.001

P-probability is derived from general linear regression to determine differences between positive events and negative events.

Tetrameric complexes were used for a quantitative analysis of the intensity and kinetics of *T. parva* specific $CD8^+$ T cell response in relation to *T. parva* infection following ITM vaccination as well as after a subsequent challenge infection.

The results obtained showed that the epitopes on *T. parva* antigens Tp1, Tp2, Tp4 and Tp8 are recognised by animals expressing the relevant MHC class I restricting genotype. The data obtained and presented hereafter also shows that *T. parva* specific $CD8^+$ T cells are circulating following vaccination as well as challenge infection and the appearance of $tetramer^+CD8^+$ T cells coincided with the demonstration of schizont parasitosis in lymph node biopsies shown in Appendix IX. Additionally the antigen-specific $CD8^+$ T-cells were shown to express perforin and correlated with peptide-specific cytotoxic activity in bulk cultures, a described below.

4.2.3.1 Intensity and kinetics of *T. parva* antigen specific $CD8^+$ T cell activity in PBMC

Almost all animals generated significant CD8 T cell responses to antigens for which they expressed the restricting class I molecule. The average frequencies of Tp1-, Tp2-, Tp4- and Tp8- specific $CD8^+$ T cells are reflected and summarised in Table 13a and Table 13b as well as Figure 18a. FACs dot-plot profiles are shown in Appendix X. The responses to Tp2 and Tp4 were generally poor with the significant values being close to the mean threshold value. T cells reactive with the BoLA-N*01301 restricted (VGYPKVKEEML) peptide from Tp1 antigen were detected in PBMC ranging between 0.12% and 8.76% of $CD8^+$ T cells from the two $A18^+$ animals before challenge and in all 3 animals after challenge. T cells reactive with the BoLA N*00101 restricted (CGAELNHFL) peptide from Tp8 antigen was detected in all four A10-$KN104^+$ cattle before and after challenge and ranged from 0.04% to 6.76% of circulating $CD8^+$ T cells. The results obtained above indicate that in peripheral blood a frequency of T cells specific for a single *T. parva* epitope to be estimated at a range between 1:2876 to as high as 1:36 (for the Tp1 specific T cells) or 1:12518-1:68 (for Tp8 specific T cells) of the total lymphocyte population. The frequency of T cells specific for the BoLA-N*00101 restricted Tp4 peptide (TGASIQTTL) was significantly lower ranging from at 0.11% - 1.74% of circulating $CD8^+$ T cells (1:3964-1:280) and was detected in all four A10-$KN104^+$ animals after immunisation. However significant levels of Tp4 specific CD8 T cells were observed after challenge in only three of the four A10-$KN104^+$ cattle and even in these cattle,

Table 13a: Average frequency of Tp1- Tp2- Tp4 and Tp8 specific $CD8^+$ T cells in peripheral blood in individual animals after ITM vaccination and sporozoite challenge

Average statistics of antigen specific $CD8^+$ T cell responses in individual animals after post live-vaccine immunisation and sporozoite challenge. (confidence level 95%)								
	Post live vaccine immunisastion				Post sporozoite challenge			
	Tp1	Tp2	Tp4	Tp8	Tp1	Tp2	Tp4	Tp8
BB001	ND		1 :3280 s.e. 0.12 P=0.002 n=18	1:1644 s.e. 0.21 P=0.025 n=18	1:1810 s.e 0.15 P=0.003 n=13		1:9166 s.e. 0.18 P= 0.004 n=14	1:6296 s.e. 0.06 P=0.001 n=14
BB006		1 :2610 s.e. 0.10 P=0.002 n=14				1:3136 s.e. 0.12 P=0.003 n=14		
BB007	1 :249 s.e. 0.16 P=0.004 n=14				1:997 s.e. 0.17 P=0.004 n=12			
BB069			1:3845 s.e. 0.13 P=0.003 n=18	1:1487 s.e. 0.18 P=0.004 n=19			1:8887 s.e 0.15 P=0.003 n=14	1:10162 s.e 0.01 P=0.002 n=14
BB070			1:5965 s.e. 0.11 P=0.002 n=19	1:17700 s.e 0.01 P=0.002 n=19			1:10427 s.e. 0.139 P=0.003 n=14	1:16218 s.e 0.08 P=0.002 n=14
BB169			1 :3038 s.e. 0.12 P=0.0025 n=19	1:10798 s.e. 0.11 P=0.002 n=18			1:10149 s.e. 0.13 P=0.003 n=14	1;18064 s.e. 0.11 P=0..002 n=14
BV115*	ND	ND			1:1663 s.e. 0.21 P=0.001 n=5	1:7580 s.e. 0.12 P=0.003 n=5		
BX063*			ND	ND			1:2430 s.e. 0.3 P=0.001 n=14	1:2656 s.e 0.008 P=0.002 n=12

Average frequencies are given as reciprocal frequencies
n-samples collected over a 2-3 week period following immunization or sporozoite challenge
s.e.- standard error of means
p - probability
ND not determined.
*Animals were not immunised because they were already *T. parva* immune having been immunised by ITM in previous studies. However these two animals were subjected to sporozoite challenge

Table 13b: Antigen specificity of $CD8^+$ T cells in animals after live-vaccine immunisation and challenge

Class I BoLA restricting allele (associated serotype)	Animal	Antigen specific $CD8^+$ T cells in PBMC[b]							
		Post-immunisation				Post-challenge			
		Tp1	Tp2	Tp4	Tp8	Tp1	Tp2	Tp4	Tp8
N*01301 (A18)	BB001	ND				+			
	BB007	+				+			
	BV115[a]	ND				+			
N*01201 (T2a)	BB006		Φ				Φ		
(A10)	BV115[a]		ND				BDL		
N*00101 (A10-KN104)	BB001			Φ	+			BDL	+
	BB069			Φ	+			BDL	Φ
	BB070[c]			BDL	BDL			BDL	BDL
	BB169			Φ	Φ			BDL	BDL
	BX063[a]			ND	ND			+	+

Responses are scored as positive, weak or below detection limits by comparing the average post-immunisation and post challenge frequencies in each individual animal to the threshold determined for the tetramer shown in Table 12a.

[a] *T. parva*-immune animals

[b] $CD8^+$ T cells detected by tetramer staining indicated as positive (+) or negative (-)

[c] non-immunised control animal challenged with sporozoites

Φ responses were present but weak nearing threshold values

ND – not determined

BDL – below detection limit

the responses appeared to be poor with frequencies observed being close to cut-off values. Tp2 specific responses appeared to be short lived with BoLA-N*01201 restricted Tp2 (QSLVCVLMK) peptide reactive cells when detected at significant levels, were at a range between 0.14%-0.75% of circulating $CD8^+$ T cells (1:2648-1:750) in the two expressing N*01201 class I restricting genotype. No antigen specific $CD8^+$ T cells were detected in BB070, a non-immune animal that was used as a negative control in the study.

The onset, duration, magnitude and peaking of the $CD8^+$ T cell response following ITM immunisation differed from animal to animal as well as the antigen. Tp1 specific T-cells were first detected 12 days after immunisation and increased significantly peaking by day 19 to constitute 8.76% of circulating CD8 T cells in BB007, while Tp2 responses were in most instances undetectable in the two animals expressing BoLA N*1201 although in BB006 significant levels were detected from day 17, albeit at a low frequency of approximately 1:1000 lymphocytes. Tp4 and Tp8 specific CD8 T cell responses were detected between days 9-12. Significant responses to Tp4 were observed at frequencies between 1:2000-1:5000 lymphocytes on average while Tp8 responses were most significant in two animals, BB001 and BB069, and were observed to peak at 1:223 (1.73% of $CD8^+$ T cells) and 1:68 (6.76% of $CD8^+$ T cells), on days 20 and 17 respectively. Tp8 responses in BB169 remained on average at 1:5500 lymphocytes after immunisation. After challenge infection the magnitudes of the responses were variable with the three animals expressing BoLA N*01301, (BB001, BV115 and BB007) showing a clear recall of Tp1 responses. Tp1-specific responses increased from pre-challenge levels by day 7/9 although the magnitude of the responses was below that observed after immunisation. Tp2– and Tp4– specific responses remained below detection limits at most sampling time points while there was no significant increase in Tp8-specific T cells from pre-challenge levels although frequencies remained just above threshold values.

4.2.3.2 Immunodominance hierarchy of $CD8^+$ T cell responses after live vaccine immunisation and parasite challenge

In animals that responded to more than one antigen, comparing the relative numbers of T cells in response to Tp1, Tp2, Tp4 and Tp8 was used to determine the immunodominance hierarchy of the $CD8^+$ T cell responses directed against multiple pathogen-derived epitopes. Figure 18b shows the relative numbers of Tp1, Tp2, Tp4

Figure 18a: Antigen-specific $CD8^+$ T cell responses in animals after live vaccine immunisation and subsequent challenge with sporozoites

Peripheral blood mononucleated cells (PBMC) harvested from animals after live vaccine immunisation (panel A) and subsequent challenge with *T. parva* sporozoites (panel B) were stained with Tp1- Tp2- Tp4- or Tp8- tetramers followed by anti-CD8 antibody (ILA-51). Antigen-specific $CD8^+$ T cell frequencies are determined per $4x10^5$ cells that were stained in a well. FACS dot plots are shown in Appendix X.

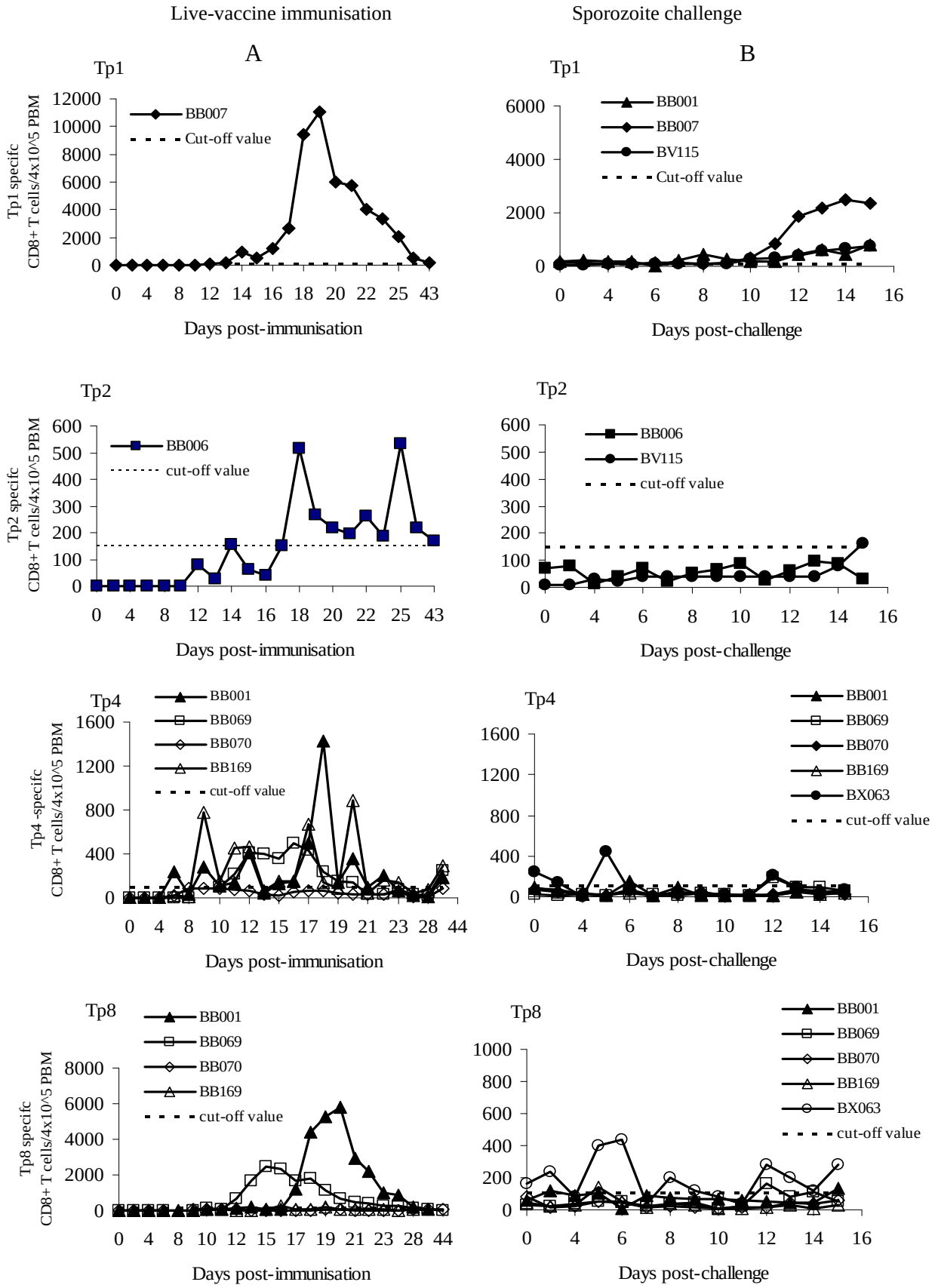
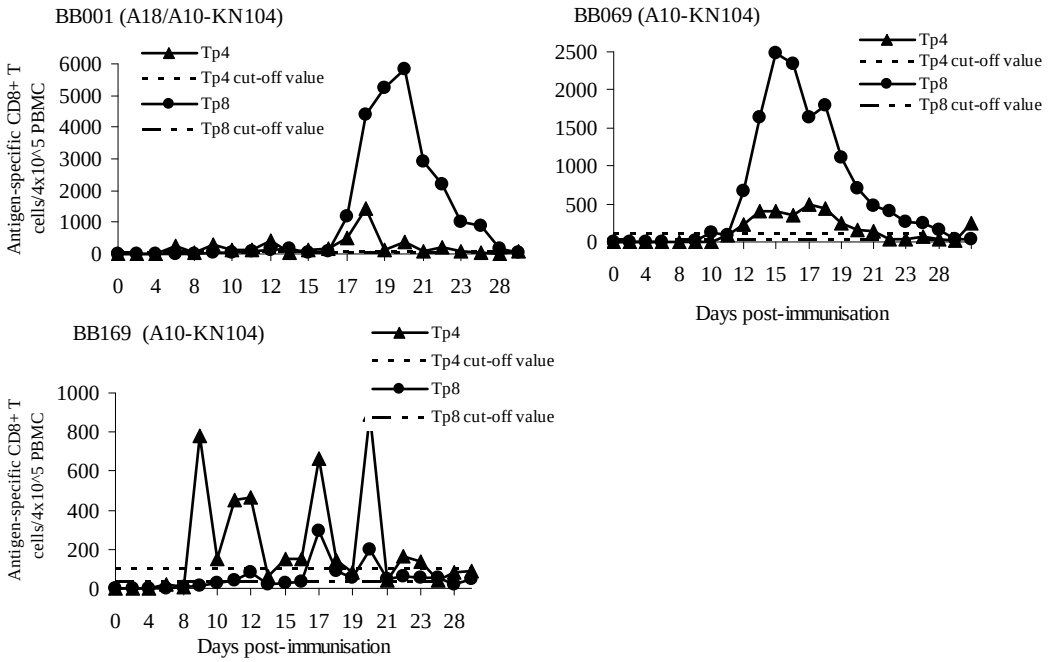

Live-vaccine immunisation
Sporozoite challenge
A
B
Tp1
Tp1 specifc CD8+ T cells/4x10^5 PBM
BB007
Cut-off value
Days post-immunisation
BB001
BB007
BV115
Cut-off value
Days post-challenge
Tp2
Tp2 specifc CD8+ T cells/4x10^5 PBM
BB006
cut-off value
Days post-immunisation
BB006
BV115
cut-off value
Days post-challenge
Tp4
Tp4 -specifc CD8+ T cells/4x10^5 PBM
BB001
BB069
BB070
BB169
cut-off value
Days post-immunisation
BX063
Days post-challenge
Tp8
Tp8 specifc CD8+ T cells/4x10^5 PBM
Days post-immunisation
Days post-challenge

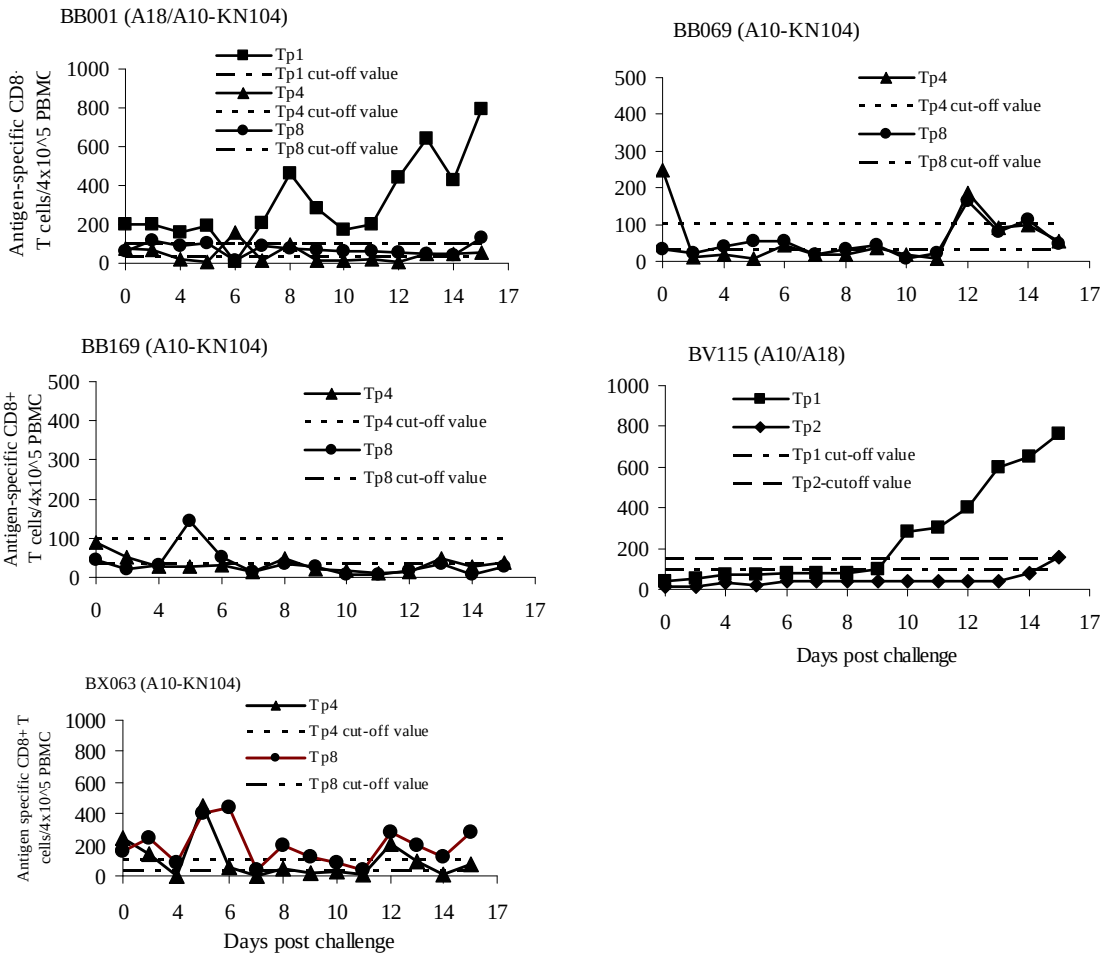
Figure 18b: Immunodominance hierarchy of antigen-specific $CD8^+$ T cell responses in animals expressing more than one defined class I BoLA allele

Peripheral blood mononucleated cells (PBMC) harvested from animals after live-vaccine immunisation (panel A) and subsequent challenge with *T. parva* sporozoites (panel B) were stained with Tp1- Tp2- Tp4- or Tp8- tetramers followed by anti-CD8 antibody (ILA-51). Antigen-specific $CD8^+$ T cell frequencies are determined per 4 x 10^5 PBMC that were stained in a well.

Panel A: Live-vaccine immunisation



Panel B: sporozoite challenge



and Tp8 epitope-specific $CD8^+$tetramer$^+$ cells T cells in individual animals after live vaccine immunisation and secondary challenge with parasite. BoLA N*00101-restricted Tp8 responses are shown to dominate over BoLA N*00101-restricted Tp4 responses during priming as shown in A10-$KN104^+$ animals except in BB169 where Tp4 epitope-specific $CD8^+$ T cell responses appear to dominate. Data comparing BoLA N*01301-restricted Tp1, BoLA N*01201-restricted Tp2, and BoLA N*00101-restricted Tp4 and Tp8 responses after immunisation was not available for analysis in this experiment.

Comparison of the $CD8^+$ T cell response after secondary challenge showed that memory T cells recognising epitopes derived from Tp4 and Tp8 did not expand as vigorously compared to the primary response and the levels remained just within or below the threshold detection limits. In contrast, BoLA N*01301-restricted Tp1 specific T cells expanded during the secondary challenge and were the dominant T cell population among the tested epitope specificities. This is well illustrated in BB001 whose MHC serotype $A18^+$/A10-$KN104^+$ is associated with the restricting alleles BoLA N*01301, BoLA N*00101. If precursor frequency is merely the critical factor in determination of immunodominance of the Tp1-specific recall response then it is possible to hypothesise that BoLA N*01301-restricted Tp1 response also dominates during the primary challenge. This dominance of the Tp1-specific response also appears to be maintained after multiple challenge events as illustrated in BV115 whose MHC serotype $A18^+$/$A10^+$ is associated with the restricting alleles BoLA N*01301 and BoLA N*01201.

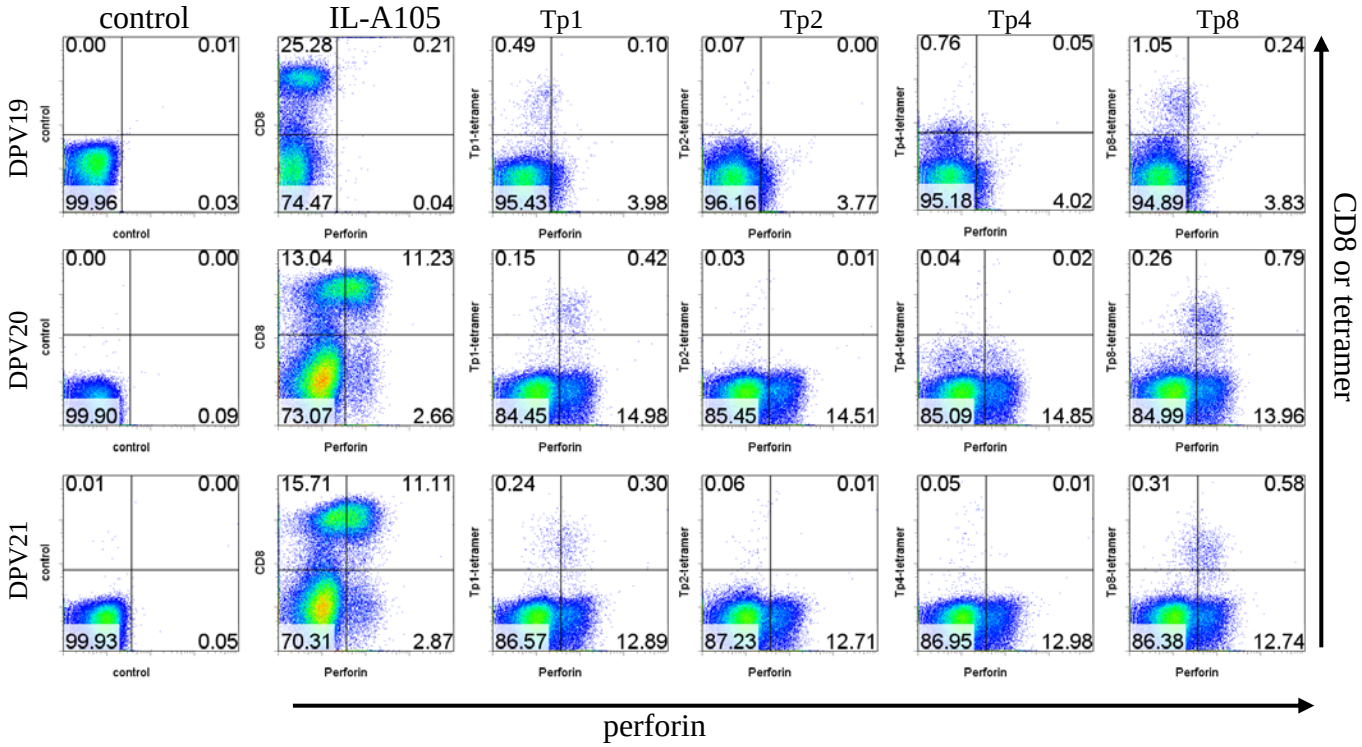
4.2.3.3 *T. parva* tetramer$^+$ CD8$^+$ T cells express perforin and are cytolytic

The optimized protocol for combined tetramer and intracellular staining was used to examine the phenotype of circulating $CD8^+$ T-cells specific for Tp1, Tp2, Tp4 and Tp8 in PBMC collected on days 19, 20 and 21 post vaccination were stained with tetramer and anti-perforin antibodies as well as anti-CD8 and anti-perforin antibodies. Results obtained and shown in Figure 19 demonstrated Tp1- and Tp8-tetramer$^+$ perforin expressing T cells. The frequencies of tetramer$^+$perforin$^+$ T-cells were comparable to the frequency of tetramer$^+$$CD8^+$ T-cells. Perforin staining levels in PBMC were however noted to be significantly reduced as compared to that stained in CTL lines and clones (Figure 15a, Section 4.2.1.3).

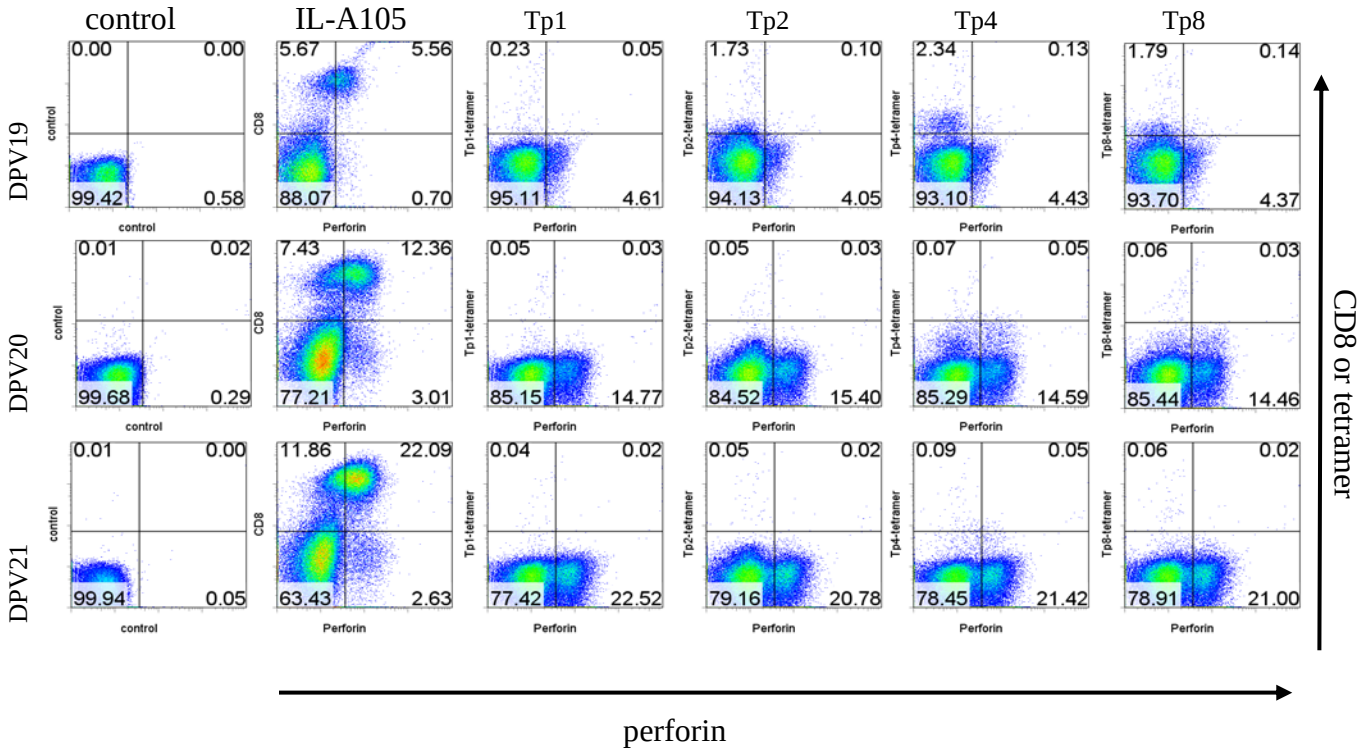
Figure 19: Staining of peripheral blood mononucleated cells with tetramer and anti-perforin antibodies

Peripheral blood mononucleated cells harvested on days 19, 20 and 21 post live vaccine immunisation were stained with IL-A105 (anti-CD8 monoclonal antibody; IgG_{2a}) , Tp1- Tp2- Tp4- or Tp8- tetramers and anti-perforin antibody (δG9; IgG_{2b}).Dot plots show log flourescence intensity of perforin-FITC:FL1 and tetramer or CD8-PE:FL2

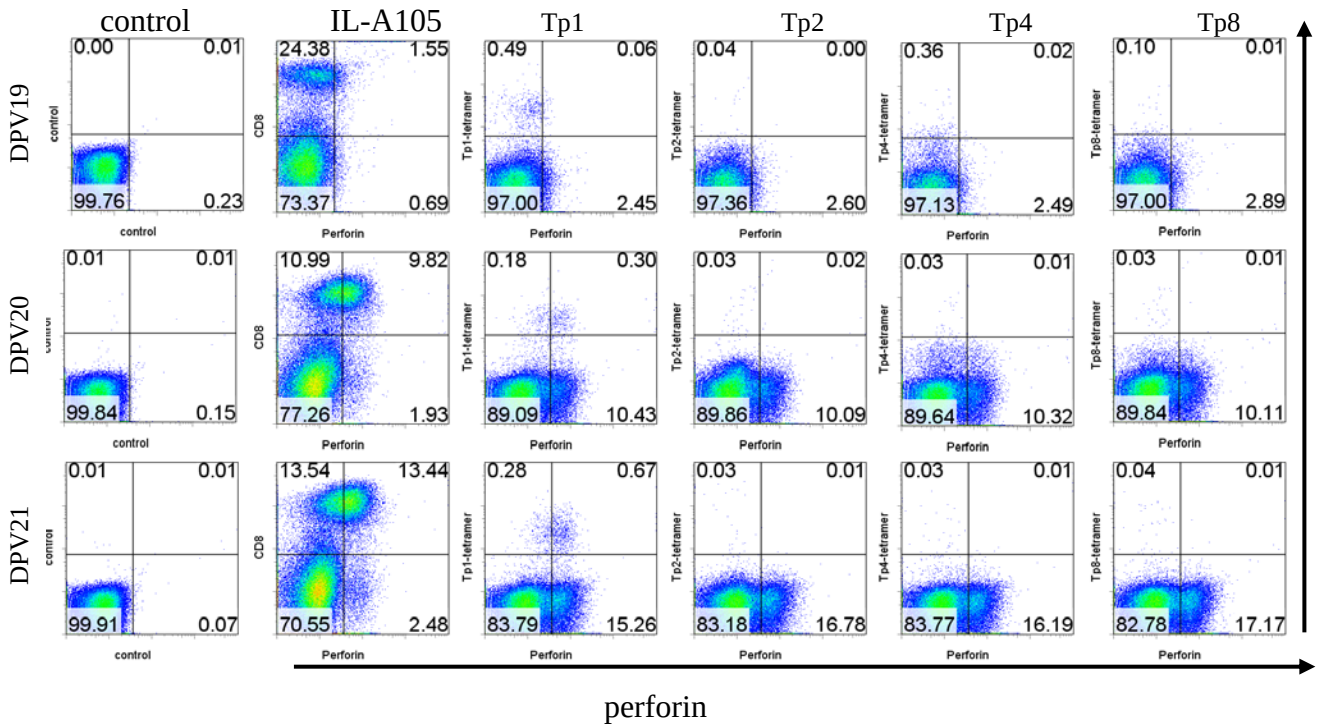
A: BB001



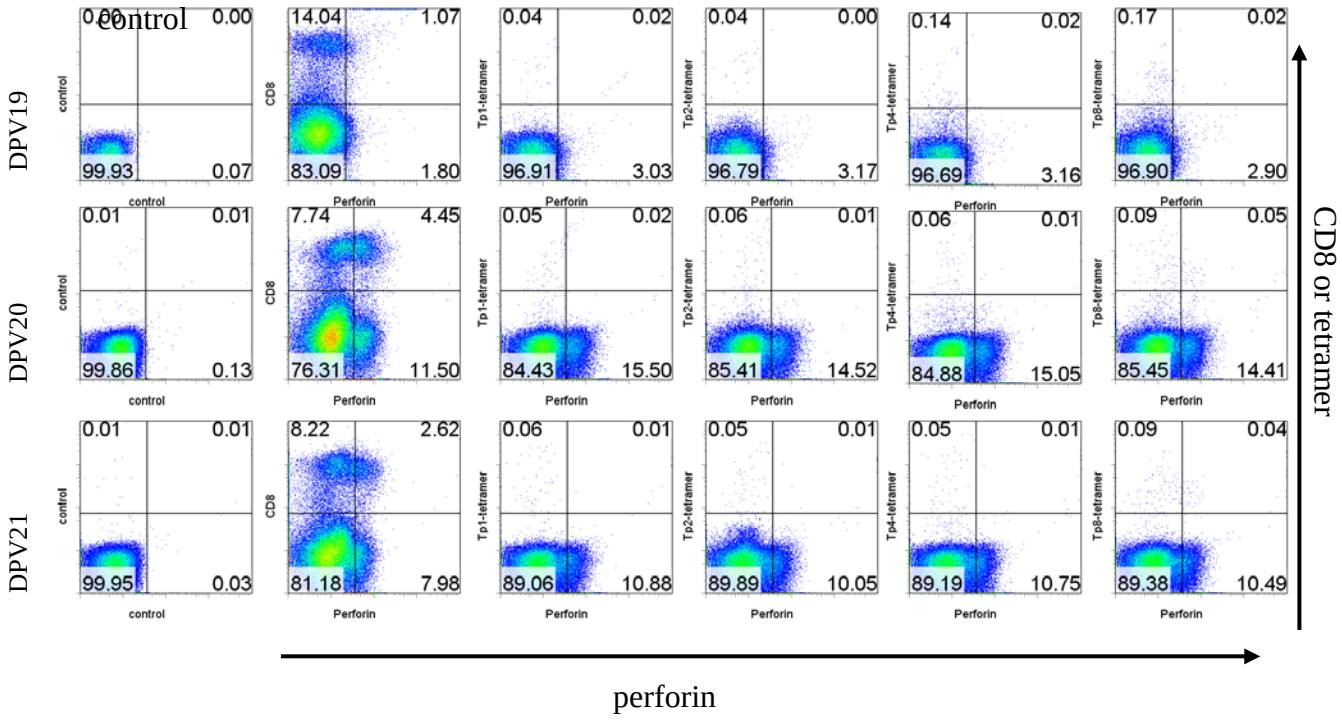
B: BB006



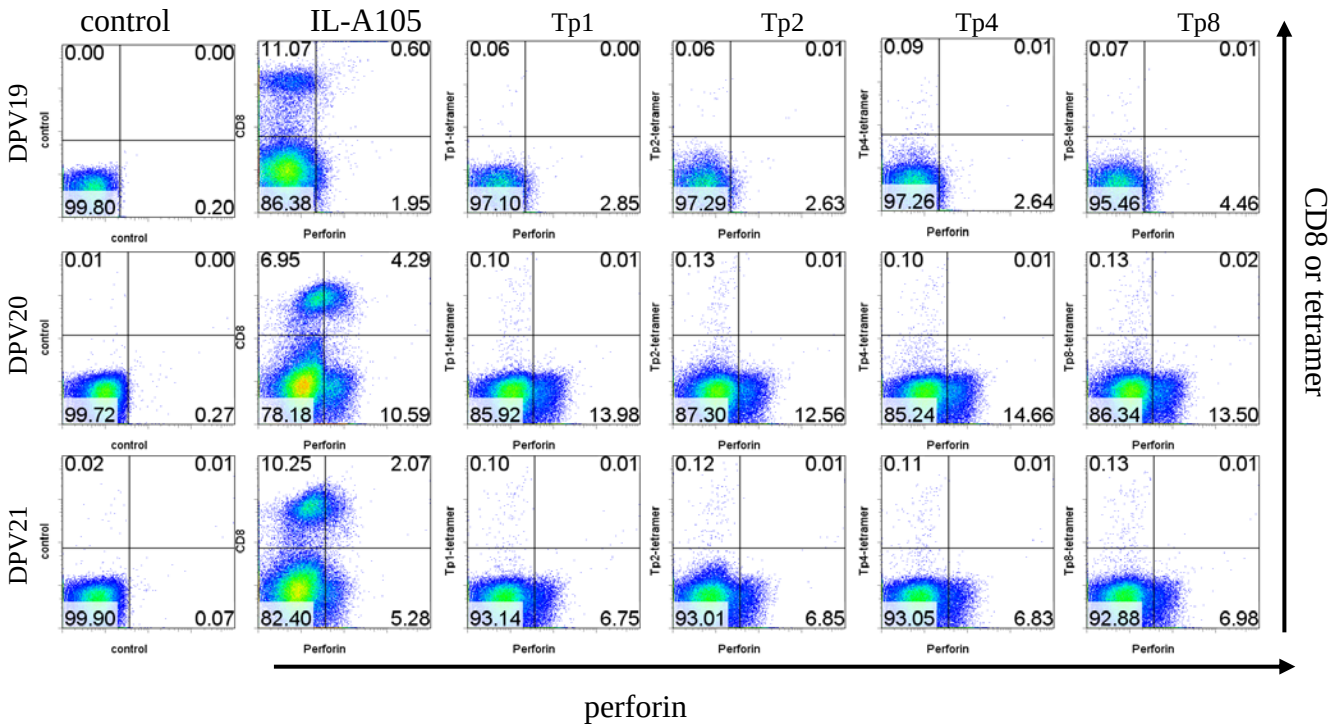
C: BB007



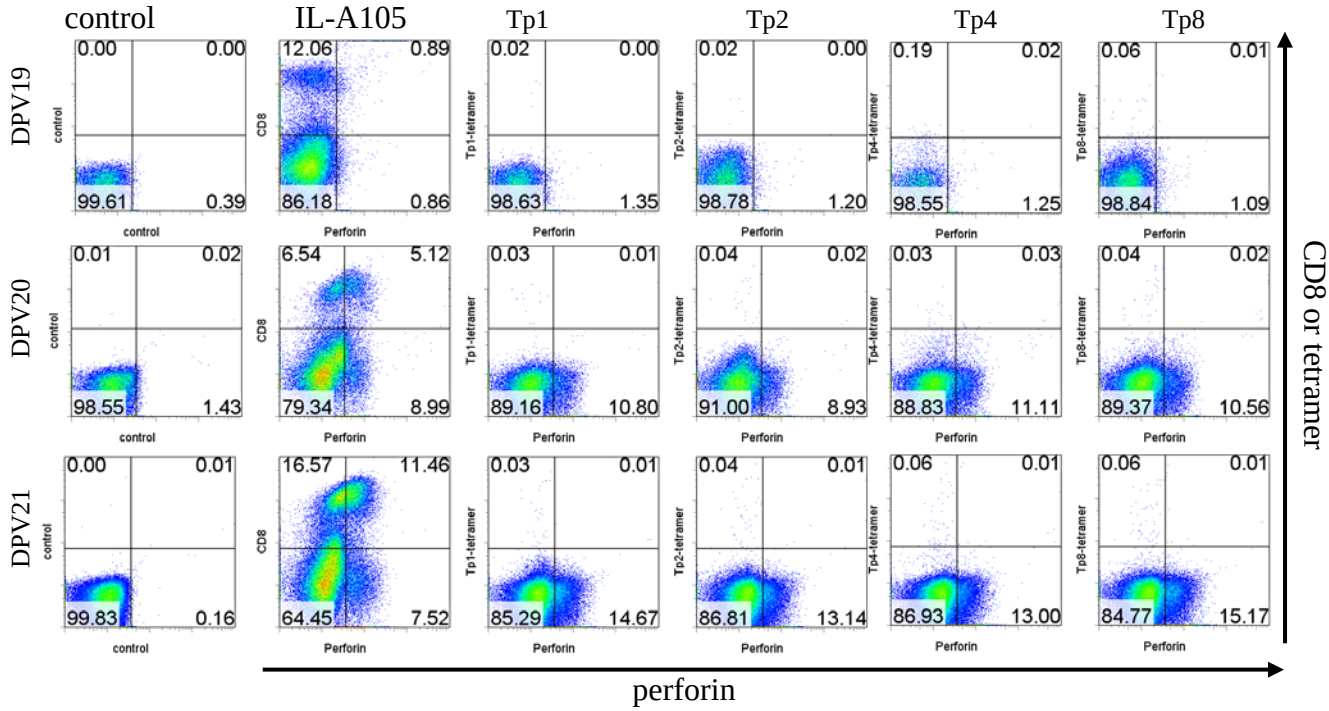
D: BB069



E: BB070



F: BB007



The functional relevance of tetramer$^+$ CD8$^+$ T cells was determined by antigen-specific CTL activity against peptide-pulsed lymphoblasts. Bulk cultures assayed for cytotoxic activity against autologous *T. parva* infected cells (TpM) and peptide-pulsed targets after one round of restimulation with TpM of PBMC harvested 12 days post-ITM and 14 days post-challenge. The data obtained is summarised in Table 14. Whereas TpM-specific killing activity was observed in PBMC post-ITM and challenge, cytotoxicity on peptide-pulsed targets was observed in all seven animals after the challenge infection. Only post-ITM PBMC from BB069 showed peptide-specific cytotoxic activity albeit at a low level (0.1%). Examination of FACS data obtained from staining the PBMC samples from the two time points (Appendix X) showed no or very low frequencies of tetramer staining cells on day 12 post-immunisation and a significant number of tetramer positive cells on day 14 post-challenge. Coupled with the low sensitivity associated with bulk cultures, it is likely that peptide-specific T-cells in PBMC sampled day 12 post-immunisation failed to proliferate sufficently relative to other cell types. Peptide-specific cytotoxic activity ranged from 1%-15% specific lysis and was observed in PBMC sampled on day 14 after challenge. Specifically, BoLA-N*01301 restricted Tp1 peptide specific cytotoxicity was detected at 15% specific lysis in BB007 (E: T ratio 40:1) whereas BoLA-N*00101 restricted Tp4 and Tp8 peptide specific cytolytic activity in the relevant animals was comparatively low at <6% specific lysis but nonetheless suggesting lytic activity from antigen-specific CTL in the bulk culture. BoLA-N*01201 restricted Tp2 peptide-specific cytolysis was observed to be at 13% E: T ratio 40:1. Given that Tp2 specific CD8$^+$ T cells were undetectable, it's likely that this cytotoxic activity may be due to non-specific killing of peptide-loaded targets. The bulk culture from BB006 showed a high level of non-specific killing of Con A activated lymphoblasts (9% E:T ratio 40:1) (Appendix XI). Similarly, peptide-specific cytotoxic activity in PBMC from BB070 (11%), a non-immunised animal, is likely due to non-specific killing of peptide-loaded targets. Cytotoxic activity of the bulk cultures is shown in Appendix IX.

4.2.3.4 Comparison of ELISpot assay and peptide-MHC tetrameric complexes in detection and enumeration of *T. parva* antigen-specific T-cells

Antigen-specific CD8$^+$ T cell-mediated IFN-γ responses were measured in PBMC obtained at selected time points in the course of the experiment. With the

Table 14: Cytotoxic activity of short-term (primary) T cell cultures and corresponding tetramer$^+$CD8$^+$ T cell frequencies after live vaccine immunisation and sporozoite challenge

BoLA class I restricting allele (associated serotype)	Animal	[c,d]Peptide-specific CD8$^+$ T cell cytotoxic activity after live vaccine immunisation (Frequency of tetramer$^+$CD8$^+$ T cells)				
		TpM	Tp1$_{214-224}$	Tp2$_{98-106}$	Tp4$_{328-336}$	Tp8$_{379-387}$
N*01301 (A18)	BB001	72% (2.6x10^5)	0%(ND)			
	BB007	85% (4.3x10^5)	0% (568)			
	BV115	ND	ND			
N*01201 (T2a)	BB006	90% (5.6x10^5)		0% (BDL)		
(A10)	BV115[a]	ND		ND		
N*00101 (A10-KN104)	BB001	72% (2.6x10^5)			0% (BDL)	0% (563)
	BB069	90% (3.7x10^5)			0% (2x10^3)	0.1% (8.2x10^3)
	BB070[b]	0% (2.7x10^5)			0% (BDL)	0% (BDL)
	BB169	0% (4.0x10^5)			0% (2x10^3)	0% (396)
	BX063[a]	3% (5.59x10^5)			0% (1.0x10^3)	0% (800)
		[c,e]Antigen-specific CD8$^+$ T cell cytotoxic activity after sporozoite challenge (Frequency of tetramer$^+$CD8$^+$ T cells)				
		TpM	Tp1$_{214-224}$	Tp2$_{98-106}$	Tp4$_{328-336}$	Tp8$_{379-387}$
N*01301 (A18)	BB001	21% (3.5x10^5)	1%(2.2x10^3)			
	BB007	40% (4.3x10^5)	15%(4.8x10^3)			
	BV115[a]	ND	ND			
N*01201 (T2a)	BB006	0% (5.1x10^5)		13% (BDL)		
(A10)	BV115	ND		ND		
N*00101 (A10-KN104)	BB001	72% (2.6x10^5)			5% (BDL)	0% (214)
	BB069	16% (4.1x10^5)			0% (922)	6% (565)
	BB070[b]	8% (9.6x10^4)			4% (BDL)	11% (BDL)
	BB169	14% (1.6x10^5)			6% (BDL)	1% (BDL)
	BX063[a]	73% (6.6x10^5)			5% (1.0x10^3)	1% (300)

[a] *T. parva* immune animals

[b] non-immunised control animal infected with sporozoites

[c] peptide-specific cytotoxic activity is determined from short-term (primary) bulk cultures expressed as % cytotoxic activity at effector:target ratio of 40:1. Frequencies of tetramer$^+$CD8$^+$ is shown in parentheses and expressed per 2x10^6 PBMC except when using TpM as the target where the frequencies correspond to CD8$^+$ T cells

[d] primary cultures were set up with PBMC harvested on day 12 post-immunisation

[e] primary cultures were set up with PBMC harvested on day 14 post challenge

TpM - *T. parva* (Muguga) schizont-infected cells

ND - not determined

BDL - below detection limit

Table 15a: Antigen-specific IFN-γ responses following live-vaccine immunisation and challenge

Class I BoLA restricting allele (associated serotype)	Animal	[d]IFN-γ responses to epitope							
		Post-immunisation				Post-challenge			
		$Tp1_{214-224}$	$Tp2_{98-106}$	$Tp4_{328-336}$	$Tp8_{379-387}$	$Tp1_{214-224}$	$Tp2_{98-106}$	$Tp4_{328-336}$	$Tp8_{379-387}$
N*01301 (A18)	BB001	+				+			
	BB007	+				+			
	BV115[a]	ND				ND			
N*01201 (T2a)	BB006		-				-		
(A10)	BV115[a]		ND				+		
N*00101 (A10-KN104)	BB001			+	+			+	+
	BB069			+	+			+	+
	BB070[b]			-	-			-	-
	BB169			+	+			+	+
	BX063[a,c]			+	+			+	+

[a] *T. parva* immune animals
[b] non-immunised control animal infected with sporozoites
[c] 'post-immunisation' data is based on pre-challenge data as animal was already *T. parva* immune
[d] IFN-γ response determined by ELISpot assay is shown as positive (+) or negative (-)
IFN- interferon
ND – not determined

exception of Tp2, responses to Tp1, Tp4, and Tp8 epitopes were detected before and after challenge as shown in Table 15a. Responses to Tp2 epitope appeared to be weak and below the assay detection limits. The Tp1-specific response was not detected until day 23 contrary to results obtained with tetramer staining which detected Tp1 specific T cells from around day 12 post immunisation. Tp4 and Tp8 specific T cells were detected by ELISpot on day 9/10 post-immunisation comparable to the results obtained by staining with Tp4 and Tp8 tetramers. Also in line with previous observations using tetramers, only the Tp1-specific response appeared to significantly increase from pre-challenge levels of 149 SFC/million PBMC to nearly 1000 SFC/million PBMC by day 15 after challenge while there appeared to be no significant change in the response to Tp4 and Tp8 epitopes over the 15 day period after challenge.

The frequencies of T cells reactive with Tp1, Tp4, and Tp8 epitopes were compared to the data obtained by parallel staining of the PBMC samples with the relevant tetrameric complex. As shown in Table 15b and c, ELISpot assays using *T. parva* antigen peptides indicated frequencies of Tp1-epitope specific T cells ranging from approximately 36-1400/10^6 PBMC, while Tp4 and Tp8 specific T cells ranged from 32-766/10^6 PBMC and 46-446/10^6 PBMC. Estimated frequencies of tetramer positive cells in corresponding samples were generally seen to be higher than those obtained by ELISpot assays. The frequency of BoLA N*01301 restricted Tp1 epitope ranged from 262-1986/10^6 PBMC while the frequencies of BoLA N*00101 restricted Tp4- and Tp8-epitopes ranged from 100-706/10^6 PBMC and 100-2500/10^6 PBMC, respectively. While the low frequencies obtained by the ELISpot may suggest sub-optimal performance of the assay in using PBMC as the responder population, it is also likely that the tetramers could be binding to T-cells that may not secrete IFN-γ upon stimulation.

4.2.4 Evaluation of responses induced by immunisation of cattle with *T. parva* antigens expressed individually in vaccine constructs

Access to cryopreserved PBMC from animals subjected to heterologous prime-boost immunisation regimes with three of the *T. parva* antigens (Graham *et al*., 2006, unpublished data) by using separate vaccine constructs for each antigen provided an opportunity to use tetramers to assess $CD8^+$ T cells induced in response

Table 15b: Frequencies of Tp1-, Tp2-, Tp4- and Tp8-antigen specific IFN-γ secreting T cells and tetramer^{+}CD8^{+} T cells after immunisation

Epitope	Class I restricting element (associated serotype)	Animal	Day post-immunisation					
			8	9	10	11	23	43
$Tp1_{214-224}$- VGYPKVKEEML	N*01301 (A18)	BB001	ND	ND	ND	ND	274 (ND)	250 (504)
		BB007	0 (BDL)	0 (BDL)	0 (BDL)	0 (BDL)	374 (8476)	48 (360)
		BV115	ND	ND	ND	ND	ND	ND
$Tp2_{98-106}$ - QSLVCVLMK	N*01201 (T2a) (A10)	BB006	BDL	BDL	BDL	BDL	BDL	BDL
		BV115	ND	ND	ND	ND	ND	ND
$Tp4_{328-336}$ - TGASIQTTL	N*00101 (A10-KN104)	BB001	28 (584)	196 (706)	22 (270)	0 (362)	0 (173)	66 (446)
		BB069	0 (BDL)	50 (BDL)	0 (BDL)	482 (553)	0 (BDL)	32 (BDL)
		BB169	8 (BDL)	0 (BDL)	2 (BDL)	480 (379)	0 (349)	6 (724)
		BX063	ND	ND	ND	ND	ND	6 (615)
$Tp8_{379-387}$ - CGAELNHFL	N*00101 (A10-KN104)	BB001	0 (BDL)	0 (BDL)	0 (BDL)	78 (214)	322 (2516)	50 (148)
		BB069	0 (BDL)	14 (292)	58 (239)	446 (239)	50 (632)	0 (BDL)
		BB169	0 (BDL)	2 (BDL)	292 (110)	158 (BDL)	4 (141)	0 (113)
		BX063	ND	ND	ND	ND	ND	2 (400)

Values from IFN-γ ELISpot and staining with tetramers (shown in parentheses) are given per 10^6 PBMC

ND – not determined

BDL – below detection limit

Table 15c: Frequencies of Tp1-, Tp2-, Tp4- and Tp8-antigen specific IFN-γ secreting T cells and tetramer^{+}CD8^{+} T cells after sporozoite challenge

Epitope	Class I restricting element (associated serotype)	Animal	Day post-challenge						
			5	6	7	8	9	13	15
$Tp1_{214-224}$- VGYPKVKEEML	N*01301 (A18)	BB001	314 (477)	14 (522)	568 (1161)	68 (1105)	60 (1105)	316 (1611)	476 (1986)
		BB007	70 (262)	70 (282)	72 (310)	0 (276)	36 (328)	1316 (5445)	1420 (5934)
		BV115	ND	ND	ND	ND	ND	ND	ND
$Tp2_{98-106}$ - QSLVCVLMK	N*01201 (T2a) (A10)	BB006	BDL	BDL	BDL	BDL	BDL	BDL	BDL
		BV115	ND	ND	ND	ND	ND	ND	ND
$Tp4_{328-336}$ - TGASIQTTL	N*00101 (A10-KN104)	BB001	152(BDL)	0 (388)	108 (BDL)	174 (246)	34 (BDL)	0 (BDL)	0 (BDL)
		BB069	162(BDL)	0 (BDL)	0 (BDL)	0 (BDL)	10 (BDL)	0 (BDL)	162 (B)
		BB169	0 (BDL)	362 (BDL)	208 (BDL)	34 (118)	28 (BDL)	766 (BDL)	88 (BDL)
		BX063	72 (115)	80 (137)	10 (BDL)	100 (110)	44 (49)	0 (222)	44 (183)
$Tp8_{379-387}$ - CGAELNHFL	N*00101 (A10-KN104)	BB001	286 (254)	0 (235)	138 (216)	248 (192)	56 (172)	100 (116)	100 (329)
		BB069	164 (134)	0 (132)	0 (BDL)	0 (BDL)	72 (109)	0 (202)	16 (122)
		BB169	0 (355)	0 (126)	74 (BDL)	16 (BDL)	0 (BDL)	30 (BDL)	180 (147)
		BX063	28 (1000)	118 (1100)	0 (100)	182 (500)	28 (300)	0 (500)	106 (700)

Values from IFN-γ ELISpot and staining with tetramers (shown in parentheses) are given per 10^6 PBMC

ND – not determined

BDL – below detection limit

to immunisation with defined antigens. Animals were immunised with recombinant canary pox viruses expressing Tp2, Tp4 and Tp8. After 4 weeks the animals received a booster dose of recombinant modified vaccinia virus (Ankara strain) (MVA) expressing the same antigens and were then challenged with *T. parva* sporozoites after 3 weeks (Graham *et al*., 2006, unpublished data). Specific CD8 T cell responses to the three antigens were examined every seven days by using peptides in an IFN-□ ELISpot and by CTL activity against peptide-pulsed lymphoblasts (Graham *et al*., unpublished data) and the summary are presented in Table 16a. Tetrameric complexes were used to stain cryopreserved PBMC after immunisation with MVA and challenge.

Results obtained using tetramers showed that with the exception of three animals (BA021, BA031 and BZ188-refer to Table 16a), 10 other animals generated responses to antigens for which the animal expressed the corresponding class I restriction genotype (Table 16b). Responses to Tp2 were generally poor with only three of six BoLA T2a$^+$ animals giving significant responses. T cells reactive to the BoLA-N*01201 Tp2 restricted epitope ranged from 0.02%-0.79% of circulating CD8$^+$ T cells corresponding to a frequency of 1:5108-1:126 lymphocytes. Frequencies of BoLA-N*00101 restricted Tp4 epitope restricted CD8 T cells were detected above threshold level in four of the seven A10-KN104$^+$ animals ranging from 0.02%-0.14% of circulating CD8$^+$ T cells (1:4900-1:733 lymphocytes) while significant BoLA N*00101 restricted Tp8 epitope restricted CD8 T cells were detected in all seven animals and ranged from 0.06%-0.09% of CD8 T cells (1:17000-1:1066 lymphocytes) above the cut-off value. This data is summarised in Table 16c. Following challenge infection, the majority of the animals (6/10) showed a clear recall of the responses with seven of the animals having higher frequencies of antigen-specific T cells than the frequencies observed after the MVA booster immunisation and two (BA091 and BA048) having comparable levels of antigen-specific T cells after MVA immunisation as well as challenge. Significant Tp4 and Tp8 specific responses could not be detected in BA064 and BA066 after challenge. The magnitude of responses varied between animals and antigens but it was observed that in animals that responded to Tp4 and Tp8 both restricted by BoLA N*00101, Tp8 responses appeared to dominate over Tp4 responses in most instances. It was however noted that in some animals (BA064, BA067, BA093-refer to table 16a) at time points where levels of Tp4-tetramer$^+$ T cells were above cut-off values, the magnitude of Tp4 specific T cells were significantly higher than Tp8 specific T cells. CD8$^+$ T cell

Table 16a: Summary details of cattle and their Tp2-, Tp4- and Tp8- antigen specific $CD8^+$ T cell responses

Animal	class I BoLA serotype	$CD8^+$ T cell IFN-γ responses		Peptide specific CTL activity		Status (outcome to challenge)
		post-immunisation	post-challenge	post-immunisation	post-challenge	
BA091	A10-KN104	+	+	+	+	non-survivor
BA063	A10-KN104	-	+	-	-	Survivor
BA064	A10-KN104	+	+	+	-	Survivor
BA066	A10-KN104	+	+	+	+	Survivor
BA067	A10-KN104	+	+	-	-	Survivor
BA075	A10-KN104	-	+	-	-	Survivor
BA093	A10-KN104	+	+	+	-	Survivor
BA021	A18/T2a	-	-	-	-	non-survivor
BA024	T2a	-	-	-	-	non-survivor
BA048	T2a	+	-	-	-	non-survivor
BZ188	T2a	-	+	-	-	non-survivor
BA031	T2a	-	-	-	-	Survivor
BZ015	T2a	-	-	-	-	Survivor

Animals were immunised with canary pox and modified vaccinia virus (Ankara strain) expressing Tp1, Tp2 and Tp8 antigens and challenged with sporozoites. Data is compiled from Graham *et al*., 2005, unpublished results.

Table 16b: Characterisation of the $CD8^+$ T cell response in animals after prime-boost immunisation with Tp2, Tp4 and Tp8

Class I restricting allele (associated serotype)	Animal	[a]Antigen specificity of $CD8^+$ T cells in PBMC		
		$Tp2_{98-106}$	$Tp4_{328-336}$	$Tp8_{379-387}$
N*00101 (A10-KN104)	BA091		+	+
	BA063		-	+
	BA064		+	+
	BA066		-	+
	BA067		+	+
	BA075		-	+
	BA093		+	+
N*01201 (T2a)	BA021	-		
	BA024	+		
	BA048	+		
	BZ188	-		
	BA031	-		
	BZ015	+		

[a]detection of antigen specific $CD8^+$ T cell response was determined by staining PBMC with Tp2- Tp4- or Tp8-specific tetramers.

Table 16c: Summary statistics of Tp2- Tp4- and Tp8- specific $CD8^+$ T cells in peripheral blood following vaccination with Tp2, Tp4 and Tp8

Tetramer	[a]Frequency	[b]Cut-off values
BoLA N*01201-$Tp2_{98-106}$	1:5108 – 1:126 0.02% - 0.79%	1:6600 (s.e. 0.156)
BoLA N*00101-$Tp4_{328-336}$	1:4900 – 1:733 0.02% - 0.14%	1:4998 (s.e. 0.19)
BoLA N*00101-$Tp8_{379-387}$	1:17000 – 1:1066 0.06% - 0.09%	1:18117 (s.e. 0.204)

[a]values are given as reciprocal frequencies and % $CD8^+$ T cell in PBMC and were determined by tetramer staining

[c]threshold value of individual tetramers is shown as reciprocal frequency and is calculated from the geometric mean + 2.6 standard deviation units of $tetramer^+CD8^+$ T cells from BoLA mis-matched PBMC samples. The standard error of means (s.e.) is shown in parentheses

responses in the animals are depicted in Figure 20 a and b.The identification of tetramer positive cells was correlated to cytotoxic activity detected in four of the vaccinates and the IFN-γ responses determined by ELISpot assay (Table 17a and b). The data obtained showed that with few exceptions, antigen-specific lytic activity paralleled the detection of peptide-specific $CD8^+$ T cells as shown by tetramer staining. Expression of perforin by $tetramer^+$ population was inconclusive as analysis of live gate events showed that the staining intensity of perforin was very low compared to the negative (unstained) control. In addition to high background levels, increased autofluorescence due to fixation and permeabilisation coupled with low frequencies of $tetramer^+$ population also presented a challenge in differentiating positive and negative events. There was good correlation between results obtained from ELISpot assays and those obtained by direct staining with tetrameric complexes. However it was noted that tetramer positive cells were on the average four-fold lower than ELISpot estimates.

Figure 20a: Tp4- and Tp8-specific $CD8^+$ T cell responses in A10-$KN104^+$ animals Animals were immunised with Tp2, Tp4 and Tp8 expressed individually in canary pox virus and modified vaccinia virus-Ankara strain. Cattle were primed with canary pox virus (data not shown) and boosted with MVA viruses expressing the same antigens after four weeks. Data shows $tetramer^+CD8^+$ T cell responses detected after the MVA booster immunisation following which they were challenged with *T. parva* sporozoites after three weeks on Day 21.

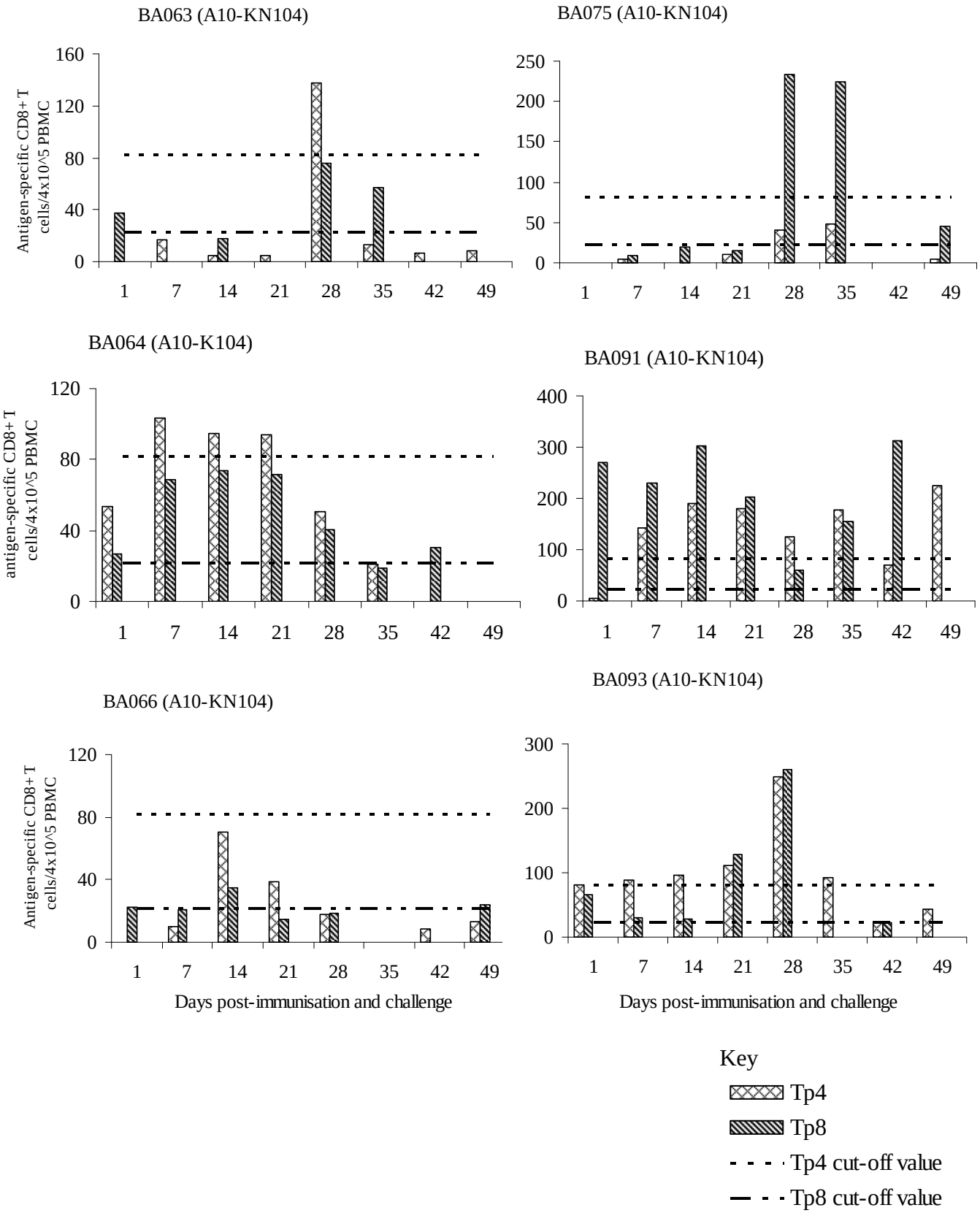

BA063 (A10-KN104)
BA075 (A10-KN104)
BA064 (A10-K104)
BA091 (A10-KN104)
BA066 (A10-KN104)
BA093 (A10-KN104)
Antigen-specific CD8+ T cells/4x10^5 PBMC
antigen-specific CD8+ T cells/4x10^5 PBMC
Days post-immunisation and challenge
Key
Tp4
Tp8
Tp4 cut-off value
Tp8 cut-off value

Figure 20b: Tp2-specific $CD8^+$ T cell responses in $T2a^+$ animals

Animals were immunised with Tp2, Tp4 and Tp8 expressed individually in canary pox virus and modified vaccinia virus-Ankara strain. Cattle were primed with canary pox virus (data not shown) and boosted with MVA viruses expressing the same antigens after four weeks. Data shows $tetramer^+CD8^+$ T cell responses detected after the MVA booster immunisation following which they were challenged with *T. parva* sporozoites after three weeks on Day 21 indicated by the arrow.

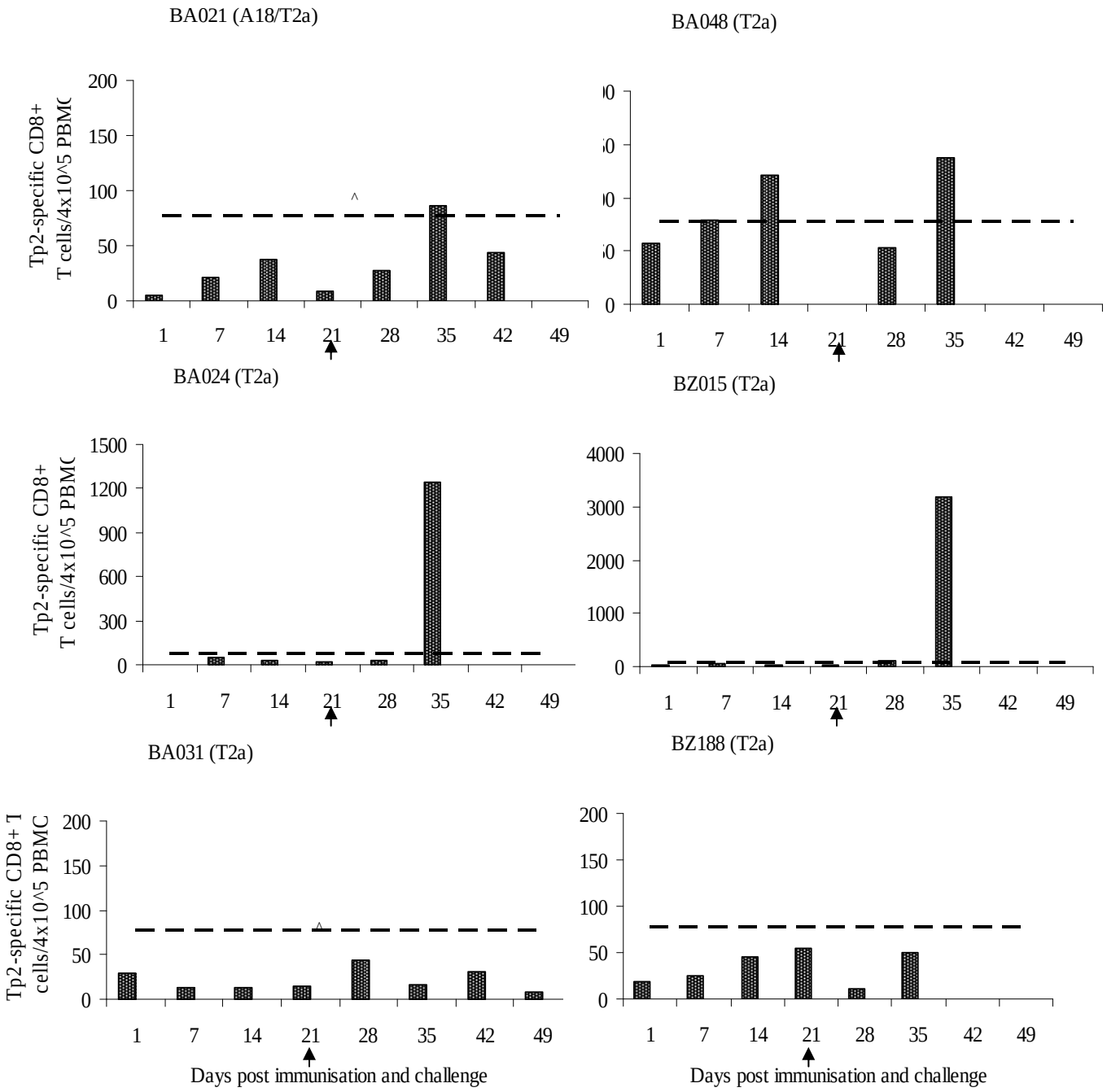

BA021 (A18/T2a)
BA048 (T2a)
BA024 (T2a)
BZ015 (T2a)
BA031 (T2a)
BZ188 (T2a)
Tp2-specific CD8+ T cells/4x10^5 PBMC
Days post immunisation and challenge
Key
Tp2
Tp2 cut-off value

Table 17a: Peptide-specific cytotoxic activity of short-term (primary) T cell cultures and corresponding frequencies of tetramer$^+$CD8$^+$ T cells in peripheral blood

Class I BoLA restricting allele (associated serotype)	Animal	[a]Peptide-specific CD8$^+$ T cell cytotoxic activity					
		post- immunisation			post-challenge		
		TpM	$Tp4_{328-336}$	$Tp8_{379-387}$	TpM	$Tp4_{328-336}$	$Tp8_{379-387}$
N*00101 (A10-KN104)	BA064	17% ($1.22x10^5$)	52% (236)	8% (183)	0% ($4.2x10^4$)	0% (52)	8% (46)
	BA066	3% ($1.24x10^5$)	34% (176)	6% (87)	4% ($6.9x10^4$)	41% (20)	2% (BDL)
	BA091	20% ($1.35x10^5$)	60% (448)	20% (759)	5% ($1.04x10^5$)	55% (562)	36% (780)
	BA093	0% ($1.28x10^5$)	41% (242)	3% (76)	0% ($8.82x10^4$)	0% (230)	0% (657)

[a]peptide-specific cytotoxic activity was determined from short-term (primary) T cell cultures and expressed as % cytotoxic activity at an effector ratio of 40:1. Frequencies of tetramer$^+$CD8$^+$ T cells in PBMC on the day that the primary culture was set up is shown in parentheses and expressed per $2x10^6$ PBMC except when using TpM as the target where the frequencies correspond to the CD8$^+$ T cell population. Cytotoxic activity data is obtained from a previous experiment by Graham *et al*., 2006, unpublished data.

BDL - below detection limit..

TpM - *T. parva* (Muguga) schizont-infected cell

Table 17b: Frequencies of Tp2-, Tp4- and Tp8-antigen specific IFN-γ secreting $CD8^+$ T cells and $tetramer^+CD8^+$ T cells in peripheral blood

Epitope	Class I restricting element (associated serotype)	Animal	[a]Frequencies of epitope-specific $CD8^+$ T cells	
			Post-immunisation	Post-challenge
$Tp2_{98-106}$ - QSLVCVLMK	N*01201 (T2a)	BA031	118 (BDL)	10 (BDL)
		BZ188	106 (102)	16 (BDL)
		BA021	0 (93)	BDL
		BZ015	22 (BDL)	40 (BDL)
		BA024	BDL	BDL
		BA048	10 (301)	0 (343)
$Tp4_{328-336}$ - TGASIQTTL	N*00101 (A10-KN104)	BA067	0 (507)	0 (101)
		BA091	1184 (448)	1454 (562)
		BA064	1778 (236)	1288 (BDL)
		BA093	1704 (242)	258 (230)
		BA075	10 (BDL)	BDL
		BA063	26 (BDL)	184 (BDL)
		BA066	BDL	BDL
$Tp8_{379-387}$ - CGAELNHFL	N*00101 (A10-KN104)	BA067	4 (197)	0 (31)
		BA091	0 (759)	0 (780)
		BA064	1942 (183)	1762 (46)
		BA093	2000 (76)	0 (652)
		BA075	0 (48)	0 (561)
		BA063	4 (45)	302 (143)
		BA066	0 (87)	12 (0)

[a]Values from IFN-γ ELISpot and staining with tetramers (shown in parentheses) are given per 10^6 PBMC harvested from animals after immunisation with Tp2, Tp4 and Tp8 antigens. ELISpot data is obtained from a previous experiment by Graham *et al*., 2006, unpublished data.
ND – not determined
BDL – below detection limit

4.3 Discussion

Bovine MHC class I molecules, N*01301, N*01201 and N*00101, β2-microglobulin and a peptide epitope derived from *T. parva* antigens Tp1, Tp2, and Tp4 or Tp8, (Graham *et al*., 2006, 2007, 2008) were used to construct soluble peptide-MHC complexes (tetramers) at LICR. These MHC class I tetramers were able to detect antigen specific T cells in cell lines expanded from PBMC *in vitro* and the specificity of the tetramers was also demonstrated. Tetramer staining of CTL lines and clones derived from PBMC from immune cattle identified what appeared to be two distinct sub-sets of $CD8^+$ T cells specific for $Tp1_{214-224}$, $Tp2_{98-106}$ and $Tp8_{379-387}$ epitopes. The differential binding of the MHC class I-peptide complexes to $CD8^+$ T cells resulting in $tetramer^{high}$ and $tetramer^{low}$ T cell populations, showed that a tetramer dilution assay provides a potential mechanism for analysis of TCR diversity within within a defined antigen-specific $CD8^+$ T cell pool. Few articles describe the construction of bovine tetramers (Norimine, Han and Brown, 2006; Han *et al*., 2008; Guzman, Birch and Ellis, 2010) and tetramers generated in this study would be the first successful construction of bovine MHC class I-peptide complexes that can be used to analyse $CD8^+$ T cell responses in cattle following immunisation or infection with *T. parva*. Some of these tetramers have since been used to study immunological dominance of the CD8 T cell response in cattle (MacHugh *et al*., 2009) as well as to define peptide binding to bovine MHC class I molecules (Macdonald *et al*., 2010). By directly binding the TCR the tetramers generated further presents reagents with a potential of being used as a tools for the analyses of TCR diversity.

Cattle can be immunised against *T. parva* by inoculation of live sporozoites and simultaneous treatment with a long-acting formulation of oxytetracycline (Radley *et al*., 1975a and b). One mechanism of protection induced by ITM is based on MHC class I-restricted cytotoxic $CD8^+$ T cell which target the schizont-infected lymphoblast (Morrison *et al*., 1987; McKeever *et al*., 1994). These previous studies demonstrated CTL in PBMC and efferent lymph from *T. parva*-immunised cattle following homologous or heterologous parasite challenge. In this study, MHC class I-peptide complexes were used to demonstrate primary *T. parva* antigen-specific T cell responses following infection during immunisation and the recall of memory T cells with subsequent challenge. Based on tetramer binding of $CD8^+$ T cells specific for epitopes on Tp1, Tp2 and Tp4 and Tp8 data obtained demonstrated a range of 0.75% -

8.76% of the activated CD8 T cells being specific for a single epitope at the peak of the primary response. As data obtained with Tp2 epitope- and Tp4 epitope-specific tetramers was statistically insignificant the functional relevance of the responses observed using these tetramers at specific time points is unclear nearly 50% of the animals survived and yet they did not exhibit a detectable CTL response as determined by cytotoxic activity. Several reasons may be advanced and this may include but not be limited to prior exposure to *T. parva*. Nonetheless, the detection of responses to Tp1 and Tp8 supports previous evidence that infection with *T. parva* induces a $CD8^+$ T cell response and that these antigens are immunodominant in line with previous observations (Graham *et al*., 2006, 2008).

The kinetics of the responses correlated well with those previously described for protective schizont-specific CTL in efferent lymph and peripheral blood after challenge of immune animals (Morrison *et al*., 1987; Taracha *et al*., 1992, 1995a; McKeever *et al*., 1994). Antigen specific $CD8^+$ T cell numbers dropped over time to below assay detection limits and upon challenge with *T. parva* sporozoites there was a substantial increase in the number of antigen-specific $CD8^+$ T cells from pre-challenge levels in most of the animals. Allowing for variation between epitopes and animal responses, and given that tetramer staining has not yet been tested vigorously, the frequency of antigen-specific $CD8^+$ T cells by tetramer staining were comparable to that obtained by limiting dilution analysis while Tp1 specific responses significantly increased from pre-challenge levels by day 9 after challenge as previously reported (Graham *et al*., 2006). Of significant interest however is the observation that the frequencies in the recall response were lower than post immunisation. The differences in the kinetics of the response may reflect different life spans of memory T cells, influences of immunodominance of restricting alleles or epitopes (other specificities), as well as being as a result of the biological function of the target proteins among others. Previous killing data in responding immune lymph showed a dramatic increase in frequency in the recall response over pre-challenge levels and as such use of a single epitope (tetramer) as a tracker may not reflect the true effector response in the animal unless the entire $CD8^+$ T cell response is directed against a single epitope.

The functional activity of tetramer positive cells was shown to be secretion of IFN-γ and *T. parva*-antigen specific cytotoxicity, possibly through perforin-mediated

cytolytic activity. By use of IFN-γ ELISpot assay and intracellular staining of PBMC for perforin, tetramer$^+$ T cells were shown to secrete IFN-γ and express perforin. Although *T. parva*-specific cytototoxic activity was demonstrated in PBMC obtained both post-immunisation and post-challenge, peptide-specific cytotoxic activity was observed in PBMC 14 days after challenge and this corelated with the demonstration of peptide-specific CD8$^+$ T cells in the total CD8 T cell population. Tetramer$^+$ cells in PBMC obtained post-immunisation were observed at significant levels on average after 15 days while the cytototoxicity assay was performed using PBMC from day 12 post-ITM. IFN-γ ELISpot assays with freshly isolated PBMC from donor animals in most instances detected effector CD8$^+$ T cells specific for Tp1, Tp2 and Tp8. In some samples the frequency obtained by tetramer analysis were in the same range as those determined by IFN-γ ELISpot. However it was also noted that in some samples, the estimates of T cell frequencies using tetramer staining was higher than those obtained by ELISpot assay. While the low frequencies obtained by the ELISpot may suggest sub-optimal performance of the assay in using PBMC as the responder population, it is also likely that the tetramers could be binding to T-cells that may not secrete IFN-γ upon stimulation accounting for differences observed between the two assays. Based on intracellular staining data, the expression of perforin in tetramer$^+$ populations in PBMC was significantly reduced as compared to staining CTL lines and clones possibly due to the release of pre-formed perforin through degranulation in response to activation through TCR recognition of its specific ligand on the infected cell, interaction of co-stimulatory molecules and other accesory activation signals. In determining cytotoxic activity of peptide-specific CD8$^+$ T cells, the assay required culturing PBMC with *T. parva* infected lymphoblasts for 7 days. At the end of the culture period the culture was tested for presence of peptide-specific cytotoxic T cells. Nagata and Golstein (1995) and Janssen *et al*., (2005) show that activated T cells are prone to apoptosis upon restimulation. At day 12 post-primary infection it is likely that a substantial fraction of effector CD8$^+$ T cells are prone to activation induced cell death or fail to divide to a sufficient extent to demonstrate detectable cytotoxic activity *in vitro* explaining why cytotoxic activity was not detected post-primary infection. It is also possible that not all of the antigen specific CD8$^+$ T cells differentiate into cytolytic CD8$^+$ T cells.

Immunodominance hierarchies are a substantial characteristic of CD8 T cell

mediated immunity (Yewdell and Bennick, 1999), however the underlying mechanisms are unclear. The size of both primary and secondary response is likely to be influenced by relative epitope levels and at least after secondary challenge, the magnitude is also determined by CD8 T cell precursor frequency. Recognising that the number of cattle used in this study is not sufficiently robust to draw a strong conclusion, comparison of frequencies of CD8 T cells directed against epitopes on Tp1, Tp2, Tp4 and Tp8 provided quantitative data on the extent to which these antigens dominate the response depending on their relative contribution to the total $CD8^+$ T cell response. Data obtained showed that the Tp1 response restricted by BoLA N*01301 dominated over the Tp2 and Tp4/Tp8 response restricted by BoLA N*01201 and BoLA N*00101 respectively. It thus appears that BoLA-N*01301 haplotype dominates as the restricting element in line with previous observations that the MHC influences the antigenic specificity of the *T. parva*-specific $CD8^+$ T cell response (Morrison, 1996; Machugh *et al.,* 2009). The pattern of immunodominance hierarchy was maintained during primary and secondary immune responses although the magnitude of $tetramer^+CD8^+$ T cells detected after secondary challenge was lower. The observation that Tp8 is consistently characterised by a poor recall response as compared to Tp1 further suggests that differential antigen presentation may regulate CD8 T cell immunodominance in primary and secondary *T. parva* infections. Studies in mice have shown that T-cell immunodominance can differ substantially between primary and secondary responses to infections (Selin *et al*., 1999; Belz *et al*., 2000; 2001). The specificity of the $CD8^+$ T cell responses has been shown to be due to due to differences in antigen presentation (Nugent *et al*., 1995; Butz and Bevan, 1998a; Crowe *et al*., 2003). With regard to the epitope on Tp4 and Tp8, the differential prominence of the CD8 T cell response suggests that the response is likely to be influenced by T cell receptor affinity/avidity and competition for presentation of these epitopes within antigen-presenting cells or these epitopes are restricted by closely related MHC class I elements. Immunodominance hierarchy is a well-described phenomenon for CD8 T-cell responses to viruses with several important features for the development of immunological hierarchies of T cells having been described (Chen *et al*., 2002, 2004; Yang *et al*., 2006; Kastenmuller *et al*., 2007; La Gruta *et al*., 2006, 2010).

With the identification of *T. parva* CTL target antigens, heterologous prime boost protocols involving injections of different vectors encoding the same

recombinant antigen have been used to focus the CTL response towards peptides with the recombinant antigen, which are the only CTL epitopes shared by the different agents. Studies by Graham *et al*., (2006) have demonstrated that priming with plasmid DNA or canary pox virus and boosting with recombinant modified vaccinia Ankara (MVA) generate significant levels of specific $CD8^+$ T cells. In these studies ELISpot and peptide-specific cytotoxic assays were used to measure animal responses to vaccination. Tetramer binding was used to determine the frequency of $CD8^+$ T cells in one such vaccine trial (Graham *et al*., unpublished data) in which animals were immunised with Tp2, Tp4 and Tp8. Using PBMC harvested after the MVA booster immunisation results obtained demonstrated a vaccine induced $CD8^+$ T cell response but no significant changes in the number of $tetramer^+CD8^+$ T cells specific for any of the epitopes was observed after parasite challenge. Due to low frequencies of epitope specific T-cells in the peripheral circulation, tetramer staining measured values largely at the lower limit of detection and the true meaning of these results obtained by tetramer staining cannot be appropriately evaluated working at the lower limit of the assay sensitivity. It will be necessary to re-evaluate the staining with improved protocols incorporating staining with monoclonal antibodies against CD4, CD14 or CD3 in combination with anti-CD8 and tetramer to help eliminate $CD4^+$, monocytes and CD3- cells which may be sources of non-specific binding of tetramer.

Comparison of tetramer binding and data obtained by ELISpot and cytotoxicity assays yielded results that varied considerably for the same sample. The ELISpot assay in most instances detected the highest numbers of peptide-specific $CD8^+$ T cells while there no significant concordance between the demonstration of $tetramer^+CD8^+$ and cytolytic activity. Both the IFN-γ ELISpot and peptide-specific cytotoxic assay have been evaluated extensively and performance described previously (Graham *et al*., 2006). The reproducibility of ELISpot is a function of the interassay variability and the necessity for enrichment of $CD8^+$ T cells by positive selection on immunobeads in the experimental format used in the vaccine trial. The cytotoxicity assay is on the other hand dependent on proliferation of antigen-specific T cells and differentiation into effector T cells following *in vitro* restimulation. Although requiring minimal manipulation, the reproducibility of the flow-cytometry based tetramer staining assay is governed by the operator's skills of setting the gates and excluding non-specific events, and is, therefore open to considerable subjectivity. It cannot however be assumed or expected that the three assay will yield comparable

results. On this basis, it can be hypothesised that the differences on frequencies of IFN-γ secreting $CD8^+$ T cells and $tetramer^+CD8^+$ T cells was probably due to down regulation of TCR following activation of T cell by MHC class I bound peptide antigen which consequently prevents tetramer binding. TCRs may be down regulated following stimulation (Appay *et al*., 2000) and thus some acutely stimulated T cells may not be detected using tetramers but will be detected by the IFN-γ ELISpot. The peptide specific $CD8^+$ T cells may also have failed to proliferate *in vitro* or are sensitive to activation induced cell death.

In the context of induction of immune responses and outcome to challenge, *T. parva* antigen vaccinated animals showed significantly lower frequencies of antigen specific $CD8^+$ T cells as compared to that observed in animals immunised using live vaccine. This finding can be extended to have implications for developing strategies of *T. parva* sub-unit vaccines in the choice of delivery systems and protocols. While the vaccination protocol did generate antigen-specific T cells, the generation of a strong and consequently effector response may be determined by the magnitude of the initial priming as seen in live vaccine immunised animals.

Chapter 5: General Discussion and Conclusion

An ideal vaccine should contain relevant antigens formulated in an appropriate vehicle and be capable of inducing desirable and measurable immune responses that result in durable protection of all members of an out-bred population. The success of developing an effective vaccine is therefore dependent on identifying appropriate antigens, availability of a suitable vaccine platform and capacity to monitor immunogenicity and efficacy of the vaccine candidate(s). Correlates of protective immunity are desirable for identifying protective antigens, demonstrating the immunogenicity of a vaccine candidate and its potential efficacy, and permitting optimization of the dose, vehicle, adjuvant, and schedule of immunisation. In addition, defining and understanding correlates of immunity would enable the targeting of vaccine candidates towards a particular response known to mediate protection, and in cases where such correlates are fully validated, help minimise the conduct of expensive challenge trials as well as being applied to monitor vaccine failures. Aside from lack of effective antigen delivery technologies for induction of $CD8^+$ T cell-based immunity, correlates of immunity as immunological endpoints aimed at predicting protection, which depend on availability of reliable assays, remain a major challenge in many vaccine development initiatives.

With regard to *T. parva*, findings from a preliminary vaccine trial of five schizont-derived $CD8^+$ T cell antigen-coding genes engineered in plasmid DNA and viral vectors demonstrated induction of antigen-specific IFN-γ and cytolytic $CD8^+$ T cell responses (Graham *et al*., 2006). Their potential as vaccine candidates will rely on the demonstration of their immunogenicity and efficacy following immunisation using an effective antigen delivery system that induces an appropriate memory CTL response. While the majority of animals that exhibited $CD8^+$ T cell secreting IFN-γ ELISpot responses succumbed to challenge infection cytolytic $CD8^+$ T cells were detected only after several rounds of re-stimulation *in vitro* suggesting low precursor frequencies that pose a major challenge for bulk culture cytotoxicity assays to detect. Subsequent characterisation of the epitopes recognised in the *T. parva* antigens together with their MHC class I restriction elements (Graham *et al*., 2008) allowed the development of the MHC class I tetramers evaluated in this study as tools for monitoring the responses. Four bovine MHC class I molecules loaded with the corresponding peptide epitopes on *T. parva* antigens Tp1, Tp2, Tp4 and Tp8 were successfully synthesised at the Ludwig Institute of Cancer Research, Brussels and

validated in this study using established $CD8^+$ T cell lines and clones. These four peptide-MHC complexes (tetramers) were used to define specific $CD8^+$ T cell populations in animals following live vaccine immunisation and subsequent challenge with live sporozoites. Results of this study showed that tetramers can be used for the detection and analysis of the kinetics and intensity of *T. parva* antigen-specific $CD8^+$ T cell responses more directly in PBMC populations. Previous studies of the kinetics and intensity of *T. parva*-specific $CD8^+$ T cell responses in cattle were based on assays of direct killing activity in PBMC and efferent lymph and the IFN-γ ELISpot assay (Morrison *et al*., 1987; Taracha *et al*., 1992; McKeever *et al*., 1994; Graham *et al*., 2006) all of which require *in vitro* stimulation and in some instances fractionation of the $CD8^+$ T cell population. In this study the data obtained demonstrated that *T. parva* antigens Tp1, Tp2, Tp4 and Tp8 are recognised by MHC class I restricted $CD8^+$ T cells with frequencies of antigen specific $CD8^+$ T cells as high as 1:36 for Tp1, 1:750 for Tp2, 1:280 for Tp4 and 1:68 for Tp8 at the peak of the response with $CD8^+$ T cell activity. Results obtained also indicated that Tp2, Tp4 and Tp8 are targets of $CD8^+$ T cell responses. The kinetics of *T. parva* antigen specific $CD8^+$ T cell responses have shown that activity appears between day 9 and 12 after live vaccine immunisation consistent with the appearance of schizont parasitosis in the draining lymph node. Immunised animals usually undergo an asymptomatic or mild ECF reaction (Brocklesby and Bailey 1965; Radley *et al*., 1975 a,b). The mechanism by which the oxytetracycline works to control infection and enhance immunity is not fully understood, but the drug seems to affect the degree of maturation of sporozoites to schizonts after infection of lymphocytes (Spooner, 1990). The mild transient infection thus allows for parasite molecules to access the host cell MHC class I antigen processing and presentation pathway. By use of peptide specific IFN-γ ELISpot assay, the results showed that Tp1, Tp2, Tp4 and Tp8 induce MHC class I restricted IFN-γ secreting $CD8^+$ T cells suggesting that the tetramer staining $CD8^+$ T cell population was functionally active. Additionally intracellular staining of $tetramer^+$ T cells for expression of perforin suggested lytic ability of the antigen specific $CD8^+$ T cells. Post-challenge analysis of the $CD8^+$ T cell response using tetramers, IFN-γ ELISpot and cytotoxic assays demonstrated that the four antigens are targets of MHC class I restricted IFN-γ secreting and lytic $CD8^+$ T cells further indicating that the live vaccine primes a $CD8^+$ T cell response against the *T. parva* antigens in line with previous observations (Graham *et al*., 2006). The response after challenge was of

lesser magnitude as compared to that obtained after immunisation suggesting a change in specificity of $CD8^+$ T cell responses between primary and secondary *T. parva* infections. Tetramer staining of T cell populations was used to examine the immunogenicity of Tp2, Tp4 and Tp8 in cattle following immunisation of cattle with a canary pox/MVA prime boost vaccination strategy (Graham *et al*., 2005, unpublished data). Responding $CD8^+$ T cells were identified in cryopreserved PBMC samples collected during the experiment using BoLA-N*01201 restricted $Tp2_{98\text{-}106}$ tetramer, and BoLA-N*00101-$Tp4_{328\text{-}387}$ tetramer or BoLA-N*00101-$Tp8_{379\text{-}387}$ tetramer. Generally the estimates of antigen-specific T cells correlated well with those obtained by IFN-γ ELISpot and demonstration of peptide-specific cytotoxic activity as reported but the intensity of the response appeared to be nearly 10 times lower than that observed in ITM immunised animals. The functional relevance of the induced CD8 T cell response however remained unclear as the animals were not protected following a challenge infection suggesting that the response did not develop into a sustained and efficient memory although it recognised schizont antigen. Although Tp2, Tp4 and Tp8 antigens may be characterised by low immunogenicity, a limitation of the vaccine delivery strategy in eliciting protective antigen specific $CD8^+$ cytotoxic T cells cannot be ruled out.

Research on the development of a *T. parva* sub-unit vaccine has focused on designing a vaccine that will stimulate the cellular arm of the immune system, in particular cytotoxic $CD8^+$ T cells. Among the scientific hurdles that need to be addressed include understanding of the mechanisms that will induce a $CD8^+$ T cell immune reponse that is of significant magnitude and appropriate functional differentiation and sustained memory, a choice of parasite antigens that will confer broad protection against *T. parva* strains by generating protective responses in animals of diverse MHC types and vaccine formulation. By use of Tp1- Tp2- Tp4- and Tp8-tetramers, this study identified antigen-specific, perforin expressing $CD8^+$ T cells. That *T. parva*-specific killing activity was demonstrated at the same time point (day 12 post-immunisation) when peptide-specific cytotoxic activity was not observed possibly due to the low frequency of appropriate cytotoxic CD8 T cells raises the question of the role of the Tp1, Tp2, Tp4 and Tp8 as potential vaccine candidates in induction and development of a protective immune response. Clearly other CD8 T cell specificities are present at early time points perhaps in the form of early schizont antigens that can be exploited to stimulate an early recall response. Taking into

consideration that *in vitro* restimulation of PBMC from immune cattle may create a bias in the specificites that are amplified and that a T cell response is directed to a few antigenic determinants tetramers can be used by negative selection to isolate CD8 T cells of unknown specificities from a responding PBMC population which can then be used to screen a *T. parva* antigen library to identify other potential CD8 T cell antigens.

In the context of the choice of antigens for potential sub-unit vaccines, tetramer staining revealed differences in the kinetics and frequencies of antigen-specific $CD8^+$ T cells suggesting a differences and a change in the specificity of responses between primary and secondary *T. parva* infections. This may be attributed to differential antigen processing and presentation which in turn may impact on the priming, induction and specificity of the CD8 T cell responses. Studies in the induction of CD8 T cell against *Plasmodium* in mice show that sporozoites need to be alive (attenuated or irradiated) and not heat killed, parasite replication is not essential and systemic or prior activation of dendritic cells (for cross-presentation) inhibits priming of the CD8 T cells (Jung *et al*., 2002). This is similar to observations done in earlier studies with *T. parva*. The hypothesis being dead parasites to not move and this mobility is essential for the parasites to get to the lymph nodes. In addition to this, during this migration parasites may shed particulate proteins which are then endocytosed by dendritic cells. Antigen processing, presentation and induction CTL responses in the mouse model is well characterised (Boscardin *et al*., 2006) unlike in the case of *T. parva* schizont infected cell which is a potential research area. To what role cross-presenting/priming plays in generation of CTL has not been demonstrated for ECF. Studies in *Anaplasma* suggest linked-recognition in the development of protective immunity (Morse *et al*., 2011). Whereas $CD4^+$ T cells have been isolated in the context of *T. parva*, the role of CD4 T cell response in driving the induction, maturation and generation of an effective CD8 memory is not determined. It is proposed that the relative processing efficiency of different epitopes determines the hierarchy of T cell responses following immunisation (Vijh *et al*., 1998), but this hypothesis has not been rigorously tested. In *T. parva* it is likely that although there is proposed a limited number of immunodominant antigens, it is also likely that they are processed differently with respect to the MHC expressed. With regard to sub-unit vaccines, then differential antigen presentation will impact on the choice of antigens and efficacy of peptide-based vaccination strategies. Antigens through direct or cross-

presentation should induce a CD8 T cell response against schizont antigen to stimulate a quick recall response. Currently there is no consistent and robust vaccination system to induce CTL in cattle. As mentioned previously, tetramer staining of PBMC samples from a *T. parva* vaccine trial that used an endogenous antigen delivery strategy using replication defective viruses showed priming of a $CD8^+$ T cell response but it was of low magnitude as compared to ITM vaccination and it was unclear if the response was capable of stimulating an efficient response against schizont antigen. However emerging technologies for inducing CD4 and CD8 T cell responses can now be explored using the available tetramers and *T. parva* antigens. This could include alternatives such as the use of particulate antigen as opposed to peptides and nano-bead based adjuvants in the induction of cellular immune responses. The identification of a vaccination strategy that induces CTL responses in cattle will be crucial in future efforts to develop a sub-unit vaccine. New approaches to cattle vaccination--parasite based vaccine vehicle for cattle pathogens have been described. *Trypanosoma theileri* has been tranformed to deliver a babesia antigen and it been shown to have generated specific immune responses (Mott *et al*., 2011) while genetically modifed babesia expressing protective tick antigen Bm86 for tick control (Suarez and Noh, 2011) have also been developed. Can a similar approach be used to deliver *T. parva* T cell antigens?

Previous investigations on the specificity of the cytotoxic T cell response of cattle to *T. parva* have shown that the $CD8^+$ T cell response is restricted to a limited number of immunodominant antigens with certain BoLA specifities dominating as restricting elements (Goodeeris *et al*., 1990; Taracha *et al*., 1995b Graham *et al*., 2008; MacHugh *et al*., 2009). Though assesed in a limited number of samples, animals heterozygous for A18 haplotype consistently responded to the Tp1 epitope presented by BoLA N*01301. This study with tetramers has extended the previous observation that Tp1, and Tp8 and to some extent Tp2 and Tp4 are dominant in animals with the corresponding MHC genotypes. Dominant $CD8^+$ T cell responses restricted by BoLA N*01301 allele have in addition been reported for respiratory syncytial virus (Morrison *et al*., 1999) and foot and mouth disease virus (Guzman *et al*., 2008). While differential antigen processing and presentation may regulate dominance hierarchies of CD8 T cell responses to infections (Crowe *et al*., 2003) of importance too is if immunodominance can be broken in vaccine strategies. Peptide immunisation is only useful in animals with appropriate BoLA molecules capable of

presenting that peptide. Due to the complexity of the host immune response against *T. parva* and the genetic restriction imposed by MHC molecules, an effective sub-unit vaccine containing multiple epitopes may be required to ensure a broad coverage of genetically heterogenous population similar to what has been attempted in malaria vaccine strategies (Joshi *et al*., 2001). A potential area for research would be the role of heat shock proteins in adaptive and innate immunity. Heat shock proteins have the ability to interact with a wide range of proteins and peptides - a property that is shared by major histocompatibility complex molecules- and this makes the HSPs uniquely suited to an important role in organismal survival by their participation in innate and adaptive immune responses (Srivastava, 2002a, b). The immunological properties of HSPs enable them to be used as antigen delivery vehicles with a potential of breaking MHC-restriction.

Although tetramer staining has been shown to be sensitive and specific in detection and quantitative analysis of *T. parva* antigen specific T cells, there are limitations inherent in this assay system. One, it is restricted to few epitopes and secondly, it is not applicable when the MHC class I and the T cell epitope is not known, therefore it may have limited application in out-bred animals. Another limitation is the inability to quantify functional T cells. In view of this latter disadvantage, concurrent with the development and evaluation of bovine tetramers was the cloning, expression and generation of antibodies to bovine perforin, granzymes A and B as potential markers of cytolytic lymphocytes. The significance of perforin and granzyme proteins *in vivo* through intracellular staining of T cell populations or detection of perforin secreting T cells by the perforin ELISpot or ELISA assays would support the idea that expression of the proteins is linked to cytotoxicity. Although a number of anti-perforin and anti-granzyme A and B antibodies were available when this study was initiated, only δG9 monoclonal antibody to human perforin (Hameed *et al*., 1992) showed recognition of the bovine native perforin molecule by immunofluorescence staining of bovine CTL. Further δG9 was only suitable for use in flow cytometry and consequently was used as a co-staining reagent to phenotype tetramer positive cells. Some of the constraints related to priming and assessment of CTL responses is inadequate tools and reagents. Most tools and methods being used in similar studies are optimised for the mouse system and this does not always work in larger mammals. There is thus a general need in the scientific community to develop tools and reagents for use in cattle models. The *T.*

parva/cattle ITM model provided such an opportunity to develop protocols that work in large animals with potential application to other diseases such as TB, HIV and malaria. There has been atempts to identify and characterise identification of a biologically active bovine homologue to granulysin - antimicrobial proteins found in the granules of human and swine cytotoxic lymphocytes. Intracellular flow cytometry and immunoblotting confirmed the presence of protein corresponding to the bovine granulysin homologue in activated bovine T lymphocytes and PBMC (Endsley *et al*., 2004). From this study the nucleotide sequence coding for bovine perforin, granzymes A and a granzyme B-like serine protease has been determined from cDNA generated from bovine CTL. The protein sequence derived from the 1665-bp bovine perforin cDNA is shown to contain 554 amino acid residues and displays, as expected, considerable homology with functional domains such as MACPF, EGF and Ca^{2+} binding domains, found in the family pore-forming proteins (Lichtenheld *et al*., 1988; Lowrey *et al*., 1989; Ishikawa *et al*., 1999). Two clones of cDNA of 303-bp and 756-bp, encoding two serine proteases, were also isolated. The deduced amino acid residues from the two cDNA possessed a high level of homology to sequences of human, rat and mouse granzymes, bovine duodenase and mast-cell proteases. Analysis of the enzyme structures allowed the identification of the catalytic amino acid triad and prediction of substrate specificity. Based on this homology data, the 303-bp cDNA, consisting of 101 amino acid residues DNA was concluded to be a member of granzyme A family, mapping to the N-terminus of human, mouse and rat granzyme A while the 756-bp cDNA consisting of 251 amino acid residues belonged to the bovine granzyme B family. Two overlapping fragments of bovine perforin and granzyme B and a fragment of granzyme A, were expressed in *Trichoplusia ni* insect cells using recombinant baculovirus as the expression of full length cDNA was not successful. The expressed fragments encoded the full-length coding sequence for bovine perforin, granzyme B-like serine protease and a partial coding sequence for bovine grazyme A. Analyses of the expressed proteins obtained showed that bovine perforin is recognised by δG9 whose epitope maps to the N-terminal region of the recombinant perforin while none of the human anti-granzyme A or B monoclonal antibodies tested in this study bound the respective bovine recombinant protein. Although generation of monoclonal antibody to recombinant perforin, granzymes A and B was not successful polyclonal antibodies to the recombinant proteins were generated in rats. However results obtained indicated that the immunogenic epitopes

were not accessible in the native form of the proteins reflected by lack of intracellular staining of bovine CTLs by rat polyclonal antibodies. Given this limitation, antibodies to 18-mer antigenic epitopes determined by Hopp and Woods (1981) algorithm from amino acid sequences of the recombinant proteins were generated in rabbits and mice. Antibodies generated showed variable intracellular staining of bovine CTL with four of five anti perforin monoclonal antibodies that were generated not binding to the native molecule. Given that these anti-perforin peptide monoclonal antibodies can be assumed to display similar epitope specificities it was concluded that these monoclonals were limited functionality in the 'capture-detection' based immuno-assays. Of the range of antibodies generated, only the perforin antibodies could be tested in capture-based perforin ELISA or ELISpot assay as they were raised against disparate perforin epitopes. However results obtained indicated that the assays were negative or unsuccessful.

The development and optimisation of capture-immunoassay for bovine perforin, granzymes A or B is still compelling as the use of perforin or granzyme ELISpot provides an attractive alternative to the use of radioactive-labelled target cells as a read out assay of cytotoxic $CD8^+$ T cells with the added advantage of eliminating the need of amplifying the frequency of precursor CTL prior to analysis. Monoclonal antibody 18.H3 (anti-bovine perforin) would be a potential antibody for further quality control because not only does it recognise bovine perforin, it also appears to have overlapping specifity with δG (anti-human perforin) and can be adapted for use in an ELISA based assay or an alternative 'capture-based' assays with appropriate polyclonal antibodies. With respect to granzyme A and B, monoclonal antibody GB36 also provides a potential reagent for development as it can be optimised for use in an ELISA assay as well as FACS staining. Similarly GA46 can be optimised for granzyme A specific assays. However the latter two antibodies will depend on verification of the expressed proteins.

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Appendices

Appendix I: GenBank sequences for perforin, granzyme A and B

Sequence alignments produced by ClustalW for perforin, granzyme A and B homologs identified by the TIGR database or Genbank accession numbers and primers used for cloning bovine cDNA

Perforin

Accession number	Size	Predicted function	Organism	Source
GI:190339	1668 bp	pore forming protein - perforin	*Homo sapiens*	GenBank
GI:34808711	1665 bp	pore forming protein - perforin	*Mus musculus*	GenBank
GI:59709458	1665 bp	pore forming protein - perforin	*Rattus norvegicus*	GenBank
GI:3559947	290 bp	pore forming protein - perforin	*Ovis aries*	GenBank
TC135848	1447 bp	EST showing significant similarity to perforin	*Bos taurus*	TIGRDB

ILRI oligo number	Primer sequence 5'-3'	Name	Primer binding site	Orientation
9229	CCA GGA CTG CTG TCC	Perf-Fwd2	486-500	forward
9338	CTG TTC CTC CTG GGC	Perf-Fwd1	13-27 (rat seq)	forward
9340	GGA CAG CAG TCC TGG	Perf-Rev1	486-500	reverse
12046	GCC TCT GCA CAT GCT TGT GGA GAG	BOV_PERF1	306-329	forward
12047	CCT TCA GAT AGG CGT CCG TGG CAG	BOV_PERF2	572-595	reverse
12067	GCA CGA GCC CTG GAG GGG CTC ACA	BOV_PERF3	1-24	forward
12068	CTG CCT GAG GGC CTC CCG CCG CGG	BOV_PERF4	337-360	reverse
12069	GGT CTG TGG GGA GAC TGG ATC ACA	BOV_PERF5	245-268 (sheep seq)	forward
12070	CTT TCC AGT TTG GCA CAG AGG TCC	BOV_PERF6	961-984	reverse
12071	CAT CAC ATC ACA TTT ATT GGT CAG	BOV_PERF8	1424-1447	reverse
12780	CGC **GGA TCC** GCC TCT GCA CAT GCT TG	BOV_PERF1BAMH1	similar to 12046	forward
12781	GCG **GTC GAC** CAT CAC ATC ACA TTT AT	BOV_PERF8SAL1	similar to 12071	reverse
12782	CGC **GGA TCC** ATG GCA GCC CGT CTG CT	BOV_PERF7BAMH1	1-17 (human seq)	forward

- Primer binding site is shown for bovine EST sequence or other sequences (indicated in parentheses) and marked in bold in the alignment file
-Restriction enzyme recognition sequences are shown in bold

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TC135848        ------------------------------------------------------------
GI3559947       ------------------------------------------------------------
GI190339        ATGGCAGCCCGTCTGCTCCTCCTGGGCATCCTTCTCCTGCTGCTGCCCCTGCCCGTCCCT 60
GI59709458      ATGGCC---GCGTACCTGTTCCTCCTGGGCCTTTTCCTGCTGCTGCCACGACCTGTCCCT 57
GI34808711      ATGGCC---ACGTGCCTGTTCCTCCTGGGCCTTTTCCTGCTGCTGCCACGACCTGTCCCT


TC135848        ------------------------------------------------------------
GI3559947       ------------------------------------------------------------
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GI59709458      GCTCCCTGCTACACTGCCACTCGGTCAGAATGCAAGCAGAAGCACAAATTCGTGCCTGGT
GI34808711      GCTCCCTGCTACACTGCCACTCGGTCAGAATGCAAGCAGAAGCACAAGTTCGTGCCAGGT


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GI59709458      GTGTGGGCGGCTGGGGAAGGTGTGGATGTGACCACCCTCCGCCGCTCCAGCTCCTTCCCA
GI34808711      GTATGGATGGCTGGGGAAGGCATGGATGTGACTACCCTCCGCCGCTCCGGCTCCTTCCCA


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GI59709458      GTGAACACAGGGAAGTTCCTGAGGCCTGACCGCACCTGCACCCTCTGTAAAAACGCCCTG
GI34808711      GTGAACACACAGAGGTTCCTGAGGCCTGACCGCACCTGCACCCTCTGTAAAAACTCCCTA


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GI59709458      ATGAATGACGCTATACAGCGCCTACCCGTGGCAATCGCCCACTGGCGGCCTCACGGCTCA
GI34808711      ATGAGAGACGCCACACAGCGCCTACCTGTGGCAATCACCCACTGGCGGCCTCACAGCTCA


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GI59709458      CACTGCCAGCGTAATGTGGCCACAACCAAGGTCAGCTCCACGGAGGGTGTGGCGCGGGAG
GI34808711      CACTGCCAGCGTAATGTGGCCGCAGCCAAGGTCCACTCCACGGAGGGTGTGGCCCGGGAG


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GI59709458      GCGGCTGCTAATATCAATAACGACTGGCGTGCGGGGCTGGATGTGAACCCTAAACCGGAA
GI34808711      GCAGCTGCTAATATCAATAACGACTGGCGTGTGGGGCTGGATGTGAACCCTAGGCCAGAG


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GI59709458      GCAAACGTGCATGTGTCCGTGGCTGGCTCCCATTCCAAGATAGCCAATTTTGCAGCCGAG
GI34808711      GCAAACATGCGCGCCTCCGTGGCTGGCTCCCACTCCAAGGTAGCCAATTTTGCAGCTGAG
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GI59709458      AAGGCCCATCAGGACCAATACAACTTTAATACTGACACAGTGGAGTGTCGCATGTACAGT
GI34808711      AAGACCTATCAGGACCAGTACAACTTTAATAGCGACACAGTAGAGTGTCGCATGTACAGT


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GI59709458      TTTCGCCTGGCACAAAAGCCTCCACTCCACCCTGACTTCAGAAAGGCACTCAAGAACCTT
GI34808711      TTTCGCCTGGTACAAAAACCTCCACTCCACCTTGACTTCAAAAAGGCGCTCAGAGCCCTC


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GI59709458      CCCCACAACTTTAACAGCTCCACAGAGCATGCTTACCGTAGACTCATCTCTTCCTATGGG
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GI59709458      ACTCATTTTATTACGGCTGTGGACCTAGGTGGCCGCGTCTCGGTCCTCACGGCTCTGCGT
GI34808711      ACGCACTTTATCACGGCTGTGGACCTCGCTGGCCGCATCTCGGTCCTTACAGCCCTGCGT


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GI59709458      ACCTGTCAGCTGACCCTGGATGGGCTCACAGCTGATGAGGTAGGAGACTGCTTGAGCGTG
GI34808711      ACCTGTCAGCTGACCCTGAATGGGCTCACAGCTGATGAGGTAGGAGACTGCCTGAACGTG


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GI59709458      GTCGAAGTGCTCGGTGGCCCCCTTGACTCCTCGAATGACCTGCTCTTCGGGAACCAAGCT
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                          .** ******.   *  **** ... .*.*****..***.***** **

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                **...***  * ** .* **  *.*  ..***.** ** ***....**** *.*.* **

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                ...* *****.************ ** ** ****.***. *****.*  **.:*** *

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                :: :**...**.  . . ********.****. : ***: * ** ** ** **  *.***

TC135848        **G**TCTTCTTCGCCGGCCAGGAGCAGAGGACCGCCACAGTATGGAACGATAACAACCCCACG 654
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GI34808711      CAGGTCTGGGATGCCGACTACGGCTGGGATGATGACCTTCTTGGTTCTTGTGACAGGTCT


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GI59709458      ------------------------------------------------------------
GI34808711      ------------------------------------------------------------


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GI190339        ------------------------------------------------------------
GI59709458      ------------------------------------------------------------
GI34808711      ------------------------------------------------------------


TC135848        TGGCTGGATTTACAGGCCAGCCCTCAGCCCTGTCCAAACTGCCTTAGCTCTTCAATATTC 1134
GI3559947       ------------------------------------------------------------
GI190339        ------------------------------------------------------------
GI59709458      ------------------------------------------------------------
GI34808711      ------------------------------------------------------------


TC135848        TCGAGCCCAGGAACGTAGGTGGAGCTGGGTCAGGCCCTGCAGCCAGTGCTTCACTGCCCT 1194
GI3559947       ------------------------------------------------------------
GI190339        ------------------------------------------------------------
GI59709458      ------------------------------------------------------------
GI34808711      ------------------------------------------------------------


TC135848        ACAGGTCAGGGACAGGGCAGAAAGCCCGGACGCTGTGCCCGCCAGGCTGCCTGGTGTCAC 1254
GI3559947       ------------------------------------------------------------
GI190339        ------------------------------------------------------------
GI59709458      ------------------------------------------------------------
GI34808711      ------------------------------------------------------------


TC135848        TTACACCATTCCCTCGCAGCCCCGCTTCTAGCGTGGTGAATTCTTCATCATATTAGACCT 1314
GI3559947       ------------------------------------------------------------
GI190339        ------------------------------------------------------------
GI59709458      ------------------------------------------------------------
GI34808711      ------------------------------------------------------------
```

```
TC135848        AACGTCTTTGGCCCCAGCTGCTCCGGTTACTCTGTTTTCTTGGCCCACCAAGCTTTGCCT 1374
GI3559947       ------------------------------------------------------------
GI190339        ------------------------------------------------------------
GI59709458      ------------------------------------------------------------
GI34808711      ------------------------------------------------------------

TC135848        CTCAGCCAAAATCCAGGACTGTCTTGACTTGTCTCATTGTGTACACAGTCTGACCAATAA 1434
GI3559947       ------------------------------------------------------------
GI190339        ------------------------------------------------------------
GI59709458      ------------------------------------------------------------
GI34808711      ------------------------------------------------------------

TC135848        ATGTGATGTGATG 1447
GI3559947       -------------
GI190339        -------------
GI59709458      -------------
GI34808711      -------------
```

Key:

Single letters nucleotides

*	identical nucleotides
:	conserved nucleotide substitutions
.	semi-conserved nucleotide substitutions

Granzyme A

Accession number	Size	Predicted function	Organism	Source
GI:6996012	878 bp	serine esterase 3 – granzyme A	*Homo sapiens*	GenBank
GI:6754101	783 bp	granzyme A	*Mus musculus*	GenBank
GI:23618882	786 bp	granzyme A	*Rattus norvegicus*	GenBank
TC399777	1002 bp	EST showing significant similarity to granzyme A	*Bos taurus*	TIGRDB

ILRI oligo number	Primer sequence 5'-3'	Name	primer binding site	Orientation
9341	TCC TCA TTC AAG ACC	grzA-FWD	146-160	forward
9342	TCC TGG TTT CAC ATC	grzA-REV	459-473	reverse
13767	CGC **GCC ATG** GTG CTA CTT GAT GGG	Bov_GRZA3-Nco1	See Appendix II pg 279	forward
13768	CGC CTA C**GT CGA C**GT TCC TGG TTT CAC	Bov_GRZA4-Sal1	See Appendix II pg 279	reverse

```
TC399777        --GAAACTAGTCTCCATATGTGAATAAC---AGGAGCCATGAA---------TATTCCTT
GI6996012       CAGATTTTCAGGTTGATTGATGTGGGACAGCAGCCACAATGAGGAACTCCTATAGATTTC 60
GI6754101       -------------------------------------ATGAGGAACGCCTCTGGTCCCC
GI23618882      -------------------------------------ATGAGGAACCCCTGTGCTCCCT
                                                     ****.         *. :

TC399777        TTCCTTTCTCTTTTCCTCCTGCCATTTGTCTCCTTCTAATTCCTGGAGTTTTTCCAGTAT
GI6996012       TGGCATCCTCTCTCTCAGTTGTCGTTTCTCTCCTGC---------TAATTCCTGAAGATG 111
GI6754101       GGGGGCCATCTCTTGCTACTCTCCTTTTTCTTCTGC---------TTATTCCTGAAGGAG
GI23618882      GGGTGTCCTCTCTTACTACTGTCATTTTTCTTCTGC---------TAATTCCTGAAGGTG
                       .*** *  *:  *  * *** *** ** *          :.**  * .** :

TC399777        CCTGCGAGGGAATTATAGGAGGAAATGAAGTGGCCCCTCACACAAGACGCTACATGGCTC
GI6996012       TCTGTGAAAAAATTATTGGAGGAAATGAAGTAAC**TCCTCATTCAAGACC**CTACATGGTCC 171
GI6754101       GCTGTGAAAGAATCATTGGAGGAGACACGGTTGTTCCTCACTCAAGACCGTATATGGCTC
GI23618882      GCTGTGAAAGAATCATTGGAGGAGACACAGTAGTTCCTCACTCAAGGCCATACATGGTTC
                 *** **...*** **:******.* ...** .  ***** :****.*  ** ****  *

TC399777        TAATCA------AAGGGCTGAAACTCTGTGCAGGGGCTTTAATCAAAGAAAACTGGGTGT
GI6996012       TACTTAGTCTTGACAGAAAAACCATCTGTGCTGGGGCTTTGATTGCAAAAGACTGGGTGT 231
GI6754101       TACTTAAACTTAGTTCAAATACCATCTGTGCTGGCGCTTTGATTGAAAAGAACTGGGTGT
GI23618882      TACTTAAACTTAAACCAGACAGCATCTGTGCTGGCGCTTTGATTGCAAAAAACTGGGTGT
                **.* *      .   . : * ..*******:** *****.** ..*.*..*********

TC399777        TGACAGCCGCTCATTGTGACCTGAAGGGCAATCCTCAAGTTATTCTTGGGGCCCACTCTA
GI6996012       TGACTGCAGCTCACTGTAACTTGAACAAAAGGTCCCAGGTCATTCTTGGGGCTCACTCAA 291
GI6754101       TGACTGCTGCCCACTGTAACGTGGGAAAGAGATCTAAGTTCATTCTTGGGGCTCACTCAA
GI23618882      TGACCGCTGCACATTGTATCCCGGGAAAGAAATCTGAGGTCATTCTTGGGGCTCACTCAA
                **** ** ** ** ***.:*  *.. .. *.  *  *. * ********** *****:*

TC399777        CATCCCATAAAGAGAAACTTGACCAAGTATTTTCCATTAAAAAGGCAATTCCCTACCCAT
GI6996012       TAACCAGGGAAGAGCCAACAAAACAGATAATGCTTGTTAAGAAAGAGTTTCCCTATCCAT 351
GI6754101       TCAATAAGG---AGCCAGAACAACAGATATTGACTGTTAAGAAAGCATTTCCCTATCCAT
GI23618882      TAAAAAAAG---AGCCGGAACAACAGATATTATCTGTTAAGAAAGCATATCCCTATCCTT
                 .:. .. .   **...  : *.**..**:*    .****.**.*..::****** **:*

TC399777        GCTTTGATCCACAGACATTTGAAGGGGATCTTCAACTACTTCAGCTGGAAGGTAAAGCAA
GI6996012       GCTATGACCCAGCCACACGCGAAGGTGACCTTAAACTTTTACAGCTGACGGAAAAAGCAA 411
GI6754101       GCTATGATGAATATACACGTGAGGGGGATCTACAACTTGTACGGCTAAAGAAAAAAGCAA
GI23618882      GCTTTGACAAACACACACATGAGGGTGATCTACAACTTCTACGGCTAAAGAAAAAAGCAA
                ***:***  .* . ***   **.** ** **:.****: *:*.***......:*******

TC399777        CTATGACCAAAGCTGTAGGAATACTTCAGCTACCAAGAACAGAAGACGATGTCAAACCCC
GI6996012       AAATTAACAAATATGTGACTATCCTTCATCTACCTAAAAAGGGGGAT**GATGTGAAACCAG 471**
GI6754101       CAGTTAACAGAAATGTGGCTATCCTTCACCTACCTAAAAAGGGAGATGATGTGAAACCAG
GI23618882      CACTTAACAAAAACGTGGCTATCCTTCATCTACCTAAAAAGGGGGATGATGTGAAACCAG
                .: * *.**.* . **.. :**.***** *****:*.**..*..** ***** *****.

TC399777        ACACCAAGTGTCATGTGGCAGGATGGGGAAGCACCAAAAAAGACGCATGTCAAATGTCTA
GI6996012       **GA**ACCATGTGCCAAGTTGCAGGGTGGGGGAGGACTCACAATAGTGCA---TCTTGGTCCG
GI6754101       GAACCAGATGCCGAGTAGCAGGATGGGGGAGATTTGGCAATAAGTCA---GCTCCCTCTG
GI23618882      GAACCAGATGTCATGTAGCAGGGTGGGGGCGTTTTCACAATAAGTCA---CCTCCCTCTG
                ..**** .** *.:** *****.*****..* :   ..**:..  **    .:   ** .

TC399777        ATGCCTTGAGAGAAGCCAACGTTACAGTGATAGATAGGAAAATATGCAATGATGCCCAGC
GI6996012       ATACTCTGAGAGAAGTCAATATCACCATCATAGACAGAAAAGTCTGCAATGATCGAAATC
GI6754101       AAACTCTGAGAGAAGTCAACATCACTGTCATAGACAGAAAAATCTGCAATGATGAAAAAC
GI23618882      ATACTCTGAGAGAAGTCAACATCACTGTCATAGACAGAAAAATCTGCAATGATGAAAAAC
                *:.*  ********* *** .* ** .* ***** **.***.*.*********  ..* *
```

```
TC399777        ACTATAATTTTAATCCAGTTATTGATCTCAGTATGATCTGTGCTGGTGGTAGAAAAGGTG
GI6996012       ACTATAATTTTAACCCTGTGATTGGAATGAATATGGTTTGTGCTGGAAGCCTCCGAGGTG
GI6754101       ACTATAATTTTCATCCTGTAATTGGACTAAACATGATTTGTGCAGGGGACCTCCGTGGTG
GI23618882      ACTATAATTTTAACCCTGTAATTGGACTAAATATGATTTGTGCCGGAAACCTCCGTGGTG
                ***********.* **:** ****.:.* *. ***.* ***** ** .. . ...:****

TC399777        AAGATGATTCATGTGAAGGGGATTCTGGAAGTCCTCTGATATGTGATAATGTTTTCAGAG
GI6996012       GAAGAGACTCGTGCAATGGAGATTCTGGAAGCCCTTTGTTGTGCGAGGGTGTTTTCCGAG
GI6754101       GAAAGGACTCCTGCAATGGGGATTCTGGCAGCCCTCTGCTATGTGATGGTATTTTGCGAG
GI23618882      GGAAGGACTCATGCTACGGAGATTCTGGAGGCCCTCTGCTATGTGAGGGTATTTTCCGAG
                .... ** ** **  * **.********..* *** ** *.** ** ..*.**** .***

TC399777        GTGTCACTTCCTTTGGCAAGTGTGGTA---------ATCCCCAGAAGCCTGGCATCTACA
GI6996012       GGGTCACTTCCTTTGGCCTTGAAAATAAATGCGGAGACCCTCGTGGGCCTGGTGTCTATA
GI6754101       GCATCACCTCTTTTGGTGGAGA---GAAGTGTGGAGATCGCCGATGGCCTGGTGTCTATA
GI23618882      GCATCACAGCTTTTGGCCTTGAGGGAAGGTGTGGAGACCCCAAAGGGCCTGGCATCTATA
                * .****  * *****     .    *         * *  ..  .****** .**** *

TC399777        TCCTCCTTACCAAAAAACACCTCAACTGGATAAAGAAAACCATTGCAGGAGCCATATAAC
GI6996012       TTCTTCTCTCAAAGAAACACCTCAACTGGATAATTATGACTATCAAGGGAGCAGTTTAAA
GI6754101       CTTTCCTCTCAGATAAACACCTCAATTGGATAAAGAAGATTATGAAGGGTTCTGTGTAA-
GI23618882      CTTTACTCTCAGACAAACACCTCGATTGGATAAGGAAGACTGCGAAGGGCGCTGTTTAA-
                   * ** :*..* *********.* *******  *:.*  .  ...**  * .* ***

TC399777        ATTTCTACTTCAAAGTAGAAAATCGAAGTAACCAAGTGAAAGGGTCTTAAACTTGACCTG
GI6996012       TAACCGTTTCCTTTCATTTACTGTG---------------GCTTCTTAATCTTTTCACA
GI6754101       ------------------------------------------------------------
GI23618882      ------------------------------------------------------------


TC399777        TCCGAAGAAGCATCTTGCAGCCCTTTGAACTTCACATACAAAATGCCGCCTGTCTAAAAT
GI6996012       AATAAA------------------------------------------------------
GI6754101       ------------------------------------------------------------
GI23618882      ------------------------------------------------------------


TC399777        AAATTCAGCAATGAAAGAAATGACCTCCTGCTGTCACTCTTCCCCGATCAGCAAAGACTG
GI6996012       ------------------------------------------------------------
GI6754101       ------------------------------------------------------------
GI23618882      ------------------------------------------------------------


TC399777        AGTCATCACAT
GI6996012       -----------
GI6754101       -----------
GI23618882      -----------
```

Key:

Single letters	nucleotides
*	identical nucleotides
:	conserved nucleotide substitutions
.	semi-conserved nucleotide substitutions

Granzyme B

Accession number	Size	Predicted function	Organism	Source
GI:20988817	744 bp	serine esterase 1 – granzyme B	*Homo sapiens*	GenBank
GI:7305122	744 bp	granzyme B	*Mus musculus*	GenBank
GI:31542925	747 bp	granzyme B	*Rattus norvegicus*	GenBank
TC134874	903 bp	EST showing significant similarity to granzyme B	*Bos taurus*	TIGRDB

ILRI Oligo Number	**Primer sequence 5'-3'**	**Name**	**primer binding position**	**Orientation**
9343	TCC CGC CCC TAC ATG GC	grzB-FWD	91-107 (human)	forward
9344	ACA CAC AAG GCC TCC AG	grzB-REV		
11848	CCG **GAA TTC** ATG GTC CTG CTC CTG CTC	Bov_GRAB1 EcoRI	47-64	forward
11849	CCG GCG GCC TCA CAC TGA TCC CTG GCG	Bov_GRAB2	785-802	reverse
11850	CTG GGA AGC TCA ATC AAT GTC ACC CTG	Bov_GRAB3	239-265	forward
11851	CTT GCT TGG ATC TCC AGC ACA TAT CTG	Bov_GRAB4	596-622	reverse

```
TC134874        GCATGAAAGTAGCAGCTGCTCAGATCTGGGCAGCTTTCCTGCAGAG**ATGGTCCTGCTCCT** **60**
GI20988817      ----------------------------------------ATGCAACCAATCCTGCT 17
GI7305122       ----------------------------------------ATGAAGATCCTCCTGCT
GI31542925      ----------------------------------------ATGAAGCTCCTCTTGCT
                                                         .:*.:.  ..*  * **

TC134874        **GCTC**CTGGTGGCCCTTCTGTCCCCTACTGGGGAGGCAGGGAAAATCATCGGGGGTCACGA 120
GI20988817      TCTGCTGGCCTTCCTCCTGCTGCCCAGGGCAGATGCAGGGGAGATCATCGGGGGACATGA 77
GI7305122       ACTGCTGACCTTGTCTCTGGCCTCCAGGACAAAGGCAGGGGAGATCATCGGGGGACATGA
GI31542925      CCTGCTGAGCTTCTCTCTGGCCCCCAAGACAGAGGCAGGGGAGATCATCGGGGGACATGA
                 ** ***.        ***    * *  . ..* ******.*.***********:** **

TC134874        GGCCAAGCCACACTCCCGTCCCTACATGGCGTTTCTTCTGTTCAAGACTTCAGGGAAATC 180
GI20988817      GGCCAAGCCCCAC**TCCCGCCCCTACATGGC**TTATCTTATGATCTGGGATCAGAAGTCTCT 137
GI7305122       AGTCAAGCCCCACTCTCGACCCTACATGGCCTTACTTTCGATCAAGGATCAGCAGCCTGA
GI31542925      AGCCAAGCCCCACTCTCGACCCTACATGGCCTATCTTCAGATCATGGATGAGTATTCTGG
                .* ******.***** ** ********** *::***  *:**: *..* .. .  .:

TC134874        T---CACATATGTGGGGGTTTCCTTGTGCGTGAGGACTTCGTGCTGACAGCAGCTCACTG 237
GI20988817      G---AAGAGGTGCGGTGGCTTCCTGATACAAGACGACTTCGTGCTGACAGCTGCTCACTG
GI7305122       G---GCGATATGTGGGGGCTTCCTTATTCGAGAGGACTTTGTGCTGACTGCTGCTCACTG
GI31542925      GAGTAAGAAGTGTGGCGGCTTCCTTATACGAGAAGACTTTGTGCTGACTGCTGCTCACTG
                     . * .** ** ** ***** .* *.:** ***** ********:**:********

TC134874        C**CTGGGAAGCTCAATCAATGTCACCCTG**GGGGCCCATAACATCATGGAACGAGAGAGGAC 297
GI20988817      TTGGGGAAGCTCCATAAATGTCACCTTGGGGGCCCACAATATCAAAGAACAGGAGCCGAC
GI7305122       TGAAGGAAGTATAATAAATGTCACTTTGGGGGCCCACAACATCAAAGAACAGGAGAAGAC
GI31542925      TTCGGGAAGCAAAATAAATGTCACGTTGGGGGCCCACAACATCAAAGAACAGGAGAAGAT
                   .***** : .**.********  ********** ** ****:.****..***. **

TC134874        CCAGCAGGTCATCCCAGTGAGAAGACCCATCCCCCACCCAGACTATAATGATGAGACTTT 357
GI20988817      CCAGCAGTTTATCCCTGTGAAAAGACCCATCCCCCATCCAGCCTATAATCCTAAGAACTT
GI7305122       CCAGCAAGTCATCCCTATGGTAAAATGCATTCCCCACCCAGACTATAATCCTAAGACATT
GI31542925      GCAACAAATCATCCCTGTGGTGAAAATCATTCCCCACCCAGCGTATAATTCTAAGACAAT
                 **.**. * *****:.**. .*.*  *** ***** ****. ****** .*.***. :*

TC134874        GGCCAACGACATCATGTTACTGAAGCTGACTAGGAAGGCTGACATTACGGATAAAGTGAG 417
GI20988817      CTCCAACGACATCATGCTACTGCAGCTGGAGAGAAAGGCCAAGCGGACCAGAGCTGTGCA
GI7305122       CTCCAATGACATCATGCTGCTAAAGCTGAAGAGTAAGGCCAAGAGGACTAGAGCTGTGAG
GI31542925      CTCCAATGACATCATGCTGTTAAAGCTGAAGAGTAAGGCCAAGAGGTCTAGCGCTGTGAA
                  **** ********* *. *..*****.. ** ***** .* .  :* .. ..:***..

TC134874        CCCCATCAATCTGCCCAGGAGCTTGGCGGAGGTGAAGCCAGGGATGATGTGCAGTGTGGC 477
GI20988817      GCCCCTCAGGCTACCTAGCAACAAGGCCCAGGTGAAGCCAGGGCAGACATGCAGTGTGGC
GI7305122       GCCCCTCAACCTGCCCAGGCGCAATGTCAATGTGAAGCCAGGAGATGTGTGCTATGTGGC
GI31542925      GCCTCTCAATCTGCCCAGACGCAACGTCAAAGTGAAACCAGGAGATGTGTGCTATGTGGC
                 ** .***. **.** ** ..*:: *   * *****.*****. : . .***:.******

TC134874        CGGCTGGGGGCGACTGGGGGTAAATATGCCCTCTACAGACAAACTACAGGAGGTAGATCT 537
GI20988817      CGGCTGGGGGCAGACGGCCCCCCTGGGAAAACACTCACACACACTACAAGAGGTGAAGAT
GI7305122       TGGTTGGGGAAGGATGGCCCCAATGGGCAAATACTCAAACACGCTACAAGAGGTTGAGCT
GI31542925      TGGTTGGGGAAAGCTGGGCCCAATGGGCAAATACTCAGACACACTACAAGAGGTTGAGCT
                 ** *****..... **    ..: .  ... . :** ***..*****.***** .* .*

TC134874        TGAAGTCCAAAGTGAGGAGAAATGTATCGCTCGCTTCAAAAAC---TACATCCCCTTCAC 594
GI20988817      GACAGTGCAGGAAGATCGAAAGTGCGAATCTGACTTACGCCATTATTACGACAGTACCAT
GI7305122       GACAGTACAGAAGGATCGGGAGTGTGAGTCCTACTTTAAAAATCGTTACAACAAAACCAA
GI31542925      AACAGTACAGGAGGATCAGAAGTGTGAGTCCTACTTAAAAAATTATTTCGACAAAGCCAA
                 ..*** **... **  ...*.** .:  *  .*** ....*    *:*.:*.    **
```

```
TC134874        A**CAGATATGTGCTGGAGATCCAAGCAAG**AGGAAGAATTCTTTCTCGGGTGACTCTGGGGG 654
GI20988817      TGAGTTGTGCGTGGGGGACCCAGAGATTAAAAAGACTTCCTTTAAGGGGGACTCTGGAGG
GI7305122       TCAGATATGTGCGGGGGACCCAAAGACCAAACGTGCTTCCTTTCGGGGGGATTCTGGAGG
GI31542925      TGAGATATGTGCAGGGGACCCAAAGATCAAACGTGCTTCCTTTCGGGGGGACTCTGGAGG
                : **:*.** *  **.** ***.. *  *.... ..*** **   *** ** *****.**

TC134874        CCCGCTTGTGTGTAATGGTGTGGCCCAGGGCATTGTGTCCTATGGAAGAAATGATGGGAC 714
GI20988817      CCCTCTTGTGTGTAACAAGGTGGCCCAGGGCATTGTCTCCTATGGACGAAACAATGGCAT
GI7305122       CCCGCTTGTGTGTAAAAAAGTGGCTGCAGGCATAGTTTCCTATGGATATAAGGATGGTTC
GI31542925      GCCTCTTGTGTGTAAAAAAGTGGCCGCGGGCATCGTCTCCTATGGACAAAATGATGGTTC
                 ** ********** .. *****  ..***** ** ********* .:** .**** :

TC134874        AACTCCAGATGTCTACACCAGAATCTCCAGCTTTCTGTCCTGGATCCATTCAACAATGAG 774
GI20988817      GCCTCCACGAGCCTGCACCAAAGTCTCAAGCTTTGTACACTGGATAAAGAAAACCATGAA
GI7305122       ACCTCCACGTGCTTTCACCAAAGTCTCGAGTTTCTTATCCTGGATAAAGAAAACAATGAA
GI31542925      AACTCCACGGGCATTCACCAAAGTCTCGACTTTCCTATCATGGATAAAGAAAACTATGAA
                ..***** . *  * *****.*.**** *  **  *. ..*****..* :.*** ****.

TC134874        ACGGTACAAA**CGCCAGGGATCAGTGTGA**TGTGTGCTCAAGGTGGACCCCTCCATGTTCCC 834
GI20988817      ACGCCACTAA----------------------------------------------------
GI7305122       AAGCAGCTAA----------------------------------------------------
GI31542925      AAAGAGCTAA----------------------------------------------------
                *..  .*:**

TC134874        TGGGATTGGAAGCATTGATCAAAGTGTGTGAAGGAAGGTTGCCTGGAACTTAATAAACAT 894
GI20988817      ------------------------------------------------------------
GI7305122       ------------------------------------------------------------
GI31542925      ------------------------------------------------------------


TC134874        TCATCTCTT 903
GI20988817      ---------
GI7305122       ---------
GI31542925      ---------
```

Key:

Single letters	nucleotides
*	identical nucleotides
:	conserved nucleotide substitutions
.	semi-conserved nucleotide substitutions

Appendix II: Gene sequences for cloned bovine perforin, granzymes A and B

Bovine perforin, granzymes A and B was cloned from TpM stimulated CTL as described in materials and methods. The cloned sequences were subcloned into bacterial and insect cell vectors for protein expression. Primer binding sites are shown on the specific sequences

DEFINITION: Perforin 2188 bp linear
ORF: +1 10-1674 length 1665 bp
ACCESSION: FJ176744
SOURCE: CDNA
ORGANISM: BOVINE
BASE COUNT 434 a 701 c 633 g 417 t 3 others
ORIGIN

```
        1 cgcggatcca tggcagcccg tctgctcctc cttggcatcc tcctgcttct gcccacacct
       61 gcccctgccc cctgctacac agccgcgcgc tctgagtgcc agcgcgccct caagtttgtg
      121 ccaggctcct ggctggcagg ggagggcgtg gatgtgacca gcctccagcg ctcaggctcg
      181 ttcccagtgg acacacagcg tttcctgcgg cccgacggca cttgcaccct ctgccgcaat
      241 gccctgcaga aggatgtcct ccagcgcctg cccctggcaa tcaccgactg gcgtgcccac
      301 ggagcgggct gcaagcgcag ggtggtcaag ctagagggcc gctccaccga ggatgtggct
      361 ggggaggcgg ccaacgggat ccgcaacgac tggcaggtgg ggctggacgt gtctcccaag
      421 ccaaatgcta atgtccgtgt gacagtggcg ggctcccact ccgaggatgc caacttcgcc
      481 gcccagaaga ctcaccagga caactaccgc ttcggcatgg acttagtgga gtgtcgcttt
      541 tacagttttc acctggtgca cactccccca gtacaccctg agttcaagag ggccctcaag
      601 acactgcccc cccacttcaa cacctccacc aagcccgact accacaggct catctccagc
      661 tacggaaccc acttcatccg gtccatggag ctgggcggnc gcatctcggc cctcaccgnc
      721 ctgcgnacct gcgagctggc cctggagggg ctcacagcca gcgaggtcga ggactgcctg
      781 gctgtcgagg ctgaggtcag cataagcgac agggccagtg cctcgccatc gttcaaggca
      841 tgtgaggaga agaagaagaa ccacaaggtg gggacctcct tccaccaggc ctaccgggag
      901 cgccattcca atgtcgatgg tggccaccac tcaaccatgc atgacctgct cttcgggagc
      961 caggctgggc ccgagcagtt ctcagcctgg gtggcctcac tgcaggacag ccctggcctg
     1021 gtggactaca cgctggagcc tctgcacatg cttggtggaga gccaggacc gcggcgggag
     1081 gccctcaggc aggccgtgag caagtacgtg actgacaggg cacgctggag ggactgcaac
     1141 cgcccgtgcc ccccggggca acacaagaac ccgaagaacc catgccagtg catgtgtcct
     1201 ggttcagcag ccaccaccca ggactgctgt ccccggcaga agggactggc ccacctggag
     1261 gtcatgaact tcaaggcatc aggtctgtgg ggagactgga tcactgccac ggacgcctat
     1321 ctgaaggttt ttttcgccgg ccaggagcag aggaccgcca cagtatggaa cgataacaac
     1381 cccaggtgga tgacgcggct ggacttcggg gatgtgctcc tggccaccgg gggccccctg
     1441 agggtgcagg tctgggatgc agactttggc tgggacgatg accttcttgg cacttgtgac
     1501 cgcaccccaa agtctggctc acatgaggtg tcatgcccca tggaccacgg tctcttgaaa
     1561 ttctcctacc aggtcaaatg cttgcctcac ctgacggggg agaggtgcct gcagtatgcc
     1621 ccccaagggc ttctggggaa tcctccagga aaccggagtg ggccagtgtg gtgagagaag
     1681 tgggtcctga gaggacctct gtgccaaact ggaaaggaac ccagtgcagg aacccagagg
     1741 gcctatatct ccatatctcc attagacaga cagaaaactg agacttcttt ttttcttcca
     1801 atgaggtggc tggatttaca ggccagccct cagccctgtc caaactgcct tagctcttca
     1861 atattctcga gcccaggaac gtaggtggag ctgggtcagg ccctgcagcc agtgcttcac
     1921 tgccctacag gtcagggaca gggcagaaag cccggacgct gtgcccgcca ggctgcctgg
     1981 tgtcacttac accattccct cgcagccccg cttctagcgt ggtgaattct tcatcatatt
     2041 agacctaacg tctttggccc cagctgctcc ggttactctg ttttcttggc ccaccaagct
     2101 ttgcctctca gccaaaatcc aggactgtct tgacttgtct cattgtgtac acagtctgac
     2161 caataaatgt gatgtgatgg tcgaccgc
```

ILRI oligo number	Primers for cloning perforin into pFASTBac HTB Primer sequence 5' → 3' end	Restriction enzyme and primer binding site	Primer orientation
13841	CGC G**CC ATG G**TG CCC TGC TAC ACA	NcoI (70-81)	forward
13842	CGC GCT C**GT CGA C**GT GGT TCC GTA GCT	SalI (658 -669)	reverse
13843	CGC G**CC ATG G**AC TTA GTG GAG TGT	NcoI (521 -534)	forward
13844	CGC GCT C**GT CGA C**GT GTC AGT CAC GTA	SalI (1105-1117)	reverse
13845	CGC G**CC ATG G**TG GAG AGC CAG GAC	NcoI (1055-1068)	forward
13846	CGC GCT C**GT CGA C**GT CCA CAC TGG CCC	SalI (1660-1671)	reverse

```
DEFINITION      Granzyme A              322 bp          linear
ORF             -2                      1-303           length 303
ACCESSION       FJ176746
SOURCE          CDNA
ORGANISM        BOVINE

FEATURES
BASE COUNT       73 a     70 c     75 g    104 t
ORIGIN
        1 tcctggtttc acatcatcgc cctcttttgg gagctgaagg atagccacgt ttttattaag
       61 tgttgctttt ttgttcagct ttagaagttt aagatcgcct tcatgtgtgt ccgggtcata
      121 gcatggatag ggaaactctt tcttaacaaa cataatctgt ttttcaggct cttccttgtt
      181 tcttgagtgg gccccaagaa tgatctggga tttctggttc agggaacagt gagctgcagt
      241 caatacccag tcttcggcaa tcaaagctcc ggcacagatg tttcccccat caagtagcac
      301 catgtagggt cttgaatgag ga
```

ILRI oligo number	Primers for cloning granzyme A in to pFASTBac HTB Primer sequence 5' → 3' end	Restriction enzyme and primer binding site	Primer orientation
13768	CGC CTA C**GT CGA C**GT TCC TGG TTT CAC	SalI (1-12)	reverse
13767	CGC G**CC ATG G**TG CTA CTT GAT GGG	NcoI (286-299)	forward

Forward primer is derived from the reverse complement of the DNA sequence because the open reading frame is on the reverse strand

```
DEFINITION      Granzyme B              774 bp          linear
ORF             +1                      10-765          length 756
ACCESSION       FJ176745
SOURCE          CDNA
ORGANISM        BOVINE

FEATURES
BASE COUNT      200 a    196 c    216 g    162 t
ORIGIN
        1 ccggaattca tggtcctgct cctgctcctg gtggcccttc tgtcccctac cagggaggca
       61 gggaaaatca tcgggggcca cgaggccaag ccacactccc gtccctacat ggcgtttctt
      121 caggtcaaga cttcagggaa atctcacaac tgtgggggtt tcctcgtgcg tgaggacttc
      181 gtgctgacag cagctcactg cctgggaagc tcaatcagtg tcaccctggg ggcccacaac
      241 atcaaacaac gagagacgac ccagcaggtc atcccagtga gaagacccat cccccaccca
      301 gactataatg atgagacttt ggccaacgac atcatgttac tgaagctgac taggaaggct
      361 gacattacgg ataaagtgag ccccatcaat ctgcccagga gcttggcgaa ggtgaagaca
      421 gggatgatgt gcagtgtggc cggctggggg cgactggggg taaatatgcc ctctacagac
      481 aaactacagg aggtagatct tgaagtccaa aataagaaga aatgtaagga tcgcttccaa
      541 gattacaatg cctccataca gatatgtgct ggagatccaa gcaagaggaa gagttttttt
      601 ttgggtgatt ctgggggccc gcttgtgtgt aatggtgtgg cccagggcat tgtgtcctat
      661 ggaagagatg atgggacacc tccaaatgtc tacaccagaa tctccagctt tctgtcctgg
      721 atccagacaa caatgagacg gtacaaacgc cagggatcag tgtgaggccg ccgg
```

ILRI oligo number	Primers for cloning granzyme B into pGEX 3T. Primer sequence 5' → 3' end	Restriction enzyme and primer binding site	Primer orientation
647597	CGC **GGA TCC** ATG GGG AAA ATC ATC GGG	BamHI (61-75)	forward
647598	GCG **GTC GAC** TCA CAC TGA TCC CTG GCG	SalI (748-765)	reverse

ILRI oligo number	Primers for cloning granzyme B into pFASTBac HTB. Primer sequence 5' → 3' end	Restriction enzyme and primer binding site	Primer orientation
13847	CGC G**CC ATG G**CG TTT CTT CAG GTC	NcoI (112-126)	forward
13848	GCG GCT C**GT CGA C**GT TAT GGA GGC ATT	SalI (547-558)	reverse
13849	CGC G**CC ATG G**GC TGG GGG CGA CTG	NcoI (443-456)	forward
13850	GCG GCT C**GT CGA C**GT CAC TGA TCC CTG	SalI (751-762)	reverse

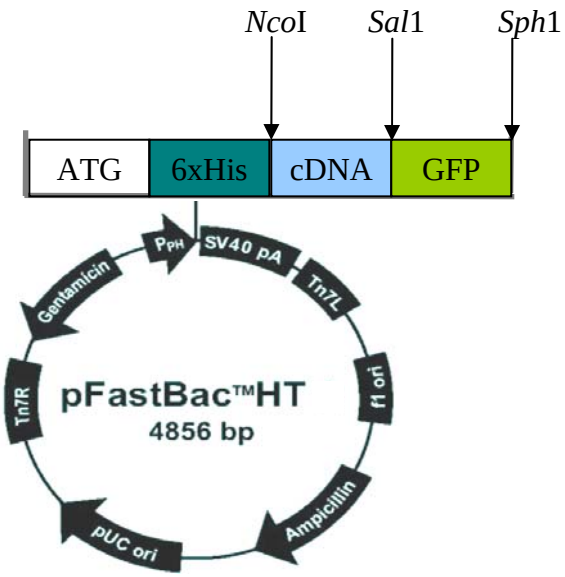
```
DEFINITION      enhanced GFP            729 bp          linear
ACCESSION
FEATURES
ORF             +1                      1-720           720bp
BASE COUNT      175 a    243 c    207 g    104 t
ORIGIN
        1 atggtgagca aggcgagga gctgttcacc ggggtggtgc ccatcctggt cgagctggac
       61 ggcgacgtaa acggccacaa gttcagcgtg tccggcgagg gcgagggcga tgccacctac
      121 ggcaagctga ccctgaagtt catctgcacc accggcaagc tgcccgtgcc ctggcccacc
      181 ctcgtgacca ccctgaccta cggcgtgcag tgcttcagcc gctaccccga ccacatgaag
      241 cagcacgact tcttcaagtc cgccatgccc gaaggctacg tccaggagcg caccatcttc
      301 ttcaaggacg acggcaacta caagacccgc gccgaggtga agttcgaggg cgacaccctg
      361 gtgaaccgca tcgagctgaa gggcatcgac ttcaaggagg acggcaacat cctggggcac
      421 aagctggagt acaactacaa cagccacaac gtctatatca tggccgacaa gcagaagaac
      481 ggcatcaagg tgaacttcaa gatccgccac aacatcgagg acggcagcgt gcagctcgcc
      541 gaccactacc agcagaacac ccccatcggc gacggccccg tgctgctgcc cgacaaccac
      601 tacctgagca cccagtccgc cctgagcaaa gaccccaacg agaagcgcga tcacatggtc
      661 ctgctggagt tcgtgaccgc cgccgggatc actctcggca tggacgagct gtacaagtaa
      721 ggatccgcg
```

ILRI oligo number	Primers for cloning GFP into pFASTBac HTB Primer sequence 5' → 3' end	Restriction enzyme and primer binding site	Primer orientation
13769	CGC AC**G TCG AC**G ATG GTG AGC AAG	SalI (1-12)	forward
13770	CGC **GCA TGC** TTA CTT GTA CAG CTC GTC	SphI (703-720)	reverse

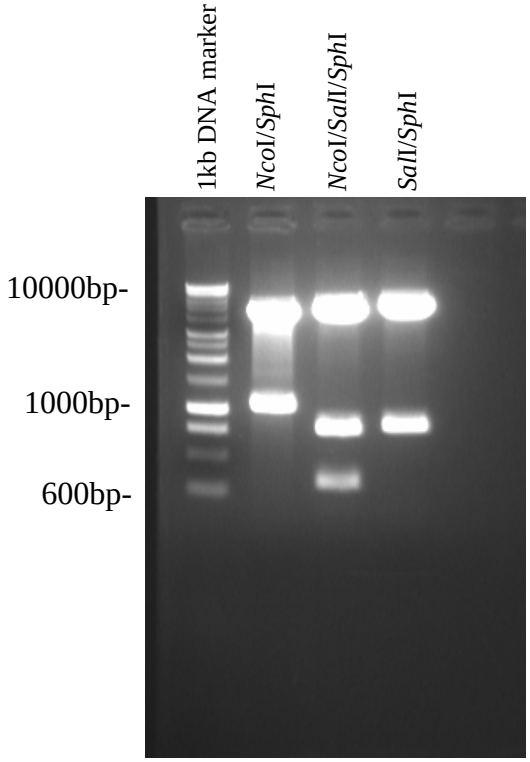
Appendix III: Cloning bovine perforin, granzymes A and B cDNA in pFASTBac

Gene fragments were cloned in-frame to the initiating ATG and N-terminal His-tag. GFP was clone fused to the C-terminal of the cDNA as shown in panel A. Cloning was verified by restriction digest of the recombinant pFastBacHT as shown in panel B – restriction digest of perforin (fragment I)-GFP construct.

A


NcoI
Sal1
Sph1
ATG
6xHis
cDNA
GFP
PpH
SV40 pA
Tn7L
f1 ori
Ampicillin
pUC ori
Tn7R
Gentamicin
pFastBac™HT
4856 bp

B


1kb DNA marker
NcoI/SphI
NcoI/SalI/SphI
SalI/SphI
10000bp-
1000bp-
600bp-

Appendix IV: Buffers and solutions

Buffers and solutions for DNA analysis

50x TAE buffer

242 g Tris base, 57.1 ml glacial acetic acid, 100ml EDTA (0.5M, pH 8.0) dissolved in a total volume of 1000ml

Ethidium bromide (10mg/ml)

1g Ethidium bromide dissolved in 100ml distilled water

Bacteria growth medium

2xYT medium

16g bacto-trypton, 5g bacto-yeast extract, 10g NaCl dissolved in 1L of distilled water

NZYCM medium for plates (per litre)

10g NZ amine, 5g NaCl, 1g casamino acids, 5g bacto-yeast extract, 2g $MgSO_4$, 15g agar dissolved in 1L of distilled water. Sterilise by autoclaving and cool to 55°C before adding ampicillin to a final concentration of 50μg/ml. Pour solution onto plastic petridishes. Driyplates at 37°C and store the plates at 4°C

Reagents for SDS-PAGE and western blotting

30 % acrylamide/ 0.8 % bisacrylamide

30.0 g acrylamide and 0.8 g N, N'-methylene-bisacrylamide dissolved in a total volume of 100 ml deionised distilled water. Filter solution through a 0.45-μm filter and store at 4˚C in the dark

0.5 M Tris-HCl/SDS, pH 6.8

6.05 g Tris base in 40 ml dissolved in distilled and deionised water. Adjust pH to 6.8 with 1N HCl. Add deionised distilled water to 100 ml total volume. Filter the solution through a 0.45-μm filter, added 0.4 g SDS and store at 4˚C

1.5 M Tris-HCl/SDS, pH 8.8

91g Tris base in 300 ml dissolved distilled and deionised water. Adjust pH to 8.8 with 1N HCl. Add deionised distilled water to 500 ml total volume. Filter the solution through a 0.45-μm filter, add 2g SDS and store at 4˚C

Preparation of polyacrylamide separating and stacking gels

12.5% separating gel

5.0 ml 30% acrylamide/bis, 3.0 ml 1.5 M Tris-cl, pH 8.8, 5.0 ml water, 45 μl 10% APS, 10 μl TEMED

5% stacking gel

700 µl 30% acrylamide/bis, 1.0 ml 0.5 M Tris-cl, pH 6.8, 2.3 ml water, 30 µl 10% APS, 10 µl TEMED

10X electrophoresis buffer

30 g Tris base, 144.1 glycine, 10 g SDS dissolved in a total volume of 1000 ml deionised distilled water. To make 1X running buffer dilute 1:10 in distilled and deionised water

6x SDS sample buffer

7 ml 0.5 M Tris-HCl/SDS, pH 6.8, 3.6 ml glycerol, 0.93 g DTT, 1g SDS, 1.2 mg Bromophenol blue. Store in 0.5-ml aliquots at -70˚C

10 % SDS

10 g SDS in total volume of 100 ml dissolved in deionised distilled water

1X protein transfer buffer (20mM Tris/150mM glycine, pH 8.0)

9.7 g Tris base, 44.7 g glycine in 3000 ml dissolved in deionised distilled water, adjuste pH to 8.0 with 6N HCl, add 800 ml methanol, 4 ml 10% and deionised distilled water to make a total volume of 4 liters. Store in the cold room

Lysis buffer A (100 mM NaH_2PO_4, 10 mM Tris-Cl, 6 M GuHCl, pH 8.0)

13.8 g NaH_2PO_4, 1.2 g Tris base, 573 g guanidine hydrochloride dissolved in 1000 ml distilled water. Adjust pH to 8.0 using NaOH

Lysis buffer B* (100 mM NaH_2PO_4, 10 mM Tris-Cl, 8 M Urea, pH 8.0)

13.8 g NaH_2PO_4, 1.2 g Tris base, 480.5 g Urea dissolved in 1000 ml distilled water. Adjust pH to 8.0 using NaOH

Wash buffer C* (100 mM NaH_2PO_4, 10 mM Tris-Cl, 8 M Urea, pH 6.3)

13.8 g NaH_2PO_4, 1.2 g Tris base, 480.5 g Urea dissolved in 1000 ml distilled water. Adjust pH to 6.3 using HCl

Elution buffer E* (100 mM NaH_2PO_4, 10 mM Tris-Cl, 8 M Urea, pH 4.5)

13.8 g NaH_2PO_4, 1.2 g Tris base, 480.5 g Urea dissolved in 1000 ml distilled water. Adjust pH to 4.5 using HCl

1 M Tris-Cl, pH 8.0

121.1 g Tris base dissolved in 800 ml of distilled water. Adjust pH to 8.0 by adding 42 ml concentrated HCl. Adjust volume of the solution to 1 L with distilled water

*Due to dissociation of urea, the pH of buffers B, C, E were adjusted immediately prior to use

Buffers for ELISA

Bicarbonate buffer pH 9.6

STOCK A: 0.2 M Na_2CO_3

21.2g Na_2CO_3 dissolved in 1L distilled water

STOCK B: 0.2 M NaHCO3

16.8 g NaHCO3 dissolved in I L distilled water

For bicarbonate coating buffer

Mix 80 ml A + 170 ml B + 250 ml distilled water

Appendix V: Amino acid residues

Amino Acid	Short abbreviation	
Alanine	A	Ala
Cysteine	C	Cys
Aspartic acid	D	Asp
Glutamic acid	E	Glu
Phenylalanine	F	Phe
Glycine	G	Gly
Histidine	H	His
Isoleucine	I	Ile
Lysine	K	Lys
Leucine	L	Leu
Methionine	M	Met
Asparagine	N	Asn
Pyrrolysine	O	Pyl
Proline	P	Pro
Glutamine	Q	Gln
Arginine	R	Arg
Serine	S	Ser
Threonine	T	Thr
Selenocysteine	U	Sec
Valine	V	Val
Tryptophan	W	Trp
Tyrosine	Y	Tyr

Appendix VI: Peptides derived from predicted amino-acid sequences of perforin, granzymes A and B

Perforin

```
perf_mouse      240 TCQLTLNGLTADEVGDCLNVEAQVSIGAQASVSSEYKACEEKKKQHKMATSFHQTYRERHVEVLGGPLDSTHDLLFGNQATPEQFSTWTASLPSNPGLVDYSLEPLHTLLEEQNPKREAL
perf_rat        240 TCQLTLDGLTADEVGDCLSVEAQVSIGAQASVSSEYKACEEKKKQHKIATSFHQTYRERHVEVLGGPLDSSNDLLFGNQATPEHFSTWIASLPTRPDVVDYSLEPLHILLEDSDPKREAL
Bovine_cDNA     240 TCELALEGLTASEVEDCLAVEAEVSISDRASASPSFKACEEKKKNHKVGTSFHQAYRERHSNVDGGHHSTMHDLLFGSQAGPEQFSAWVASLQDSPGLVDYTLEPLHMLVESQDPRREAL
perf_human      241 TCELALEGLTDNEVEDCLTVEAQVNIGIHGSISAEAKACEEKKKKHKMTASFHQTYRERHSEVVGGHHTSINDLLFGIQAGPEQYSAWVNSLPGSPGLVDYTLEPLHVLLDSQDPRREAL

perf_mouse      360 RQAISHYIMSRARWQNCSRPCRSGQHKSSHDSCQCECQDSKVTNQDCCPRQRGLAHLVVSNFRAEHLWGDYTTATDAYLKVFFGGQEFRTGVVWNNNNPRWTDKMDFENVLLSTGGPLRV
perf_rat        360 RQAISHYVMSRARWRDCNRPCRAGQHKSSRDSCQCVCQDSNVTNQDCCPRQRGLAKLMVRNFQAKGLWGDYITSTDAYLKVFFGGQEIRTGVVWNNNHPSWSDKMDFGNVLLSTGGPLRV
Bovine_cDNA     360 RQAVSKYVTDRARWRDCNRPCPPGQHKNPKNPCQCMCPGSAATTQDCCPRQKGLAHLEVMNFKASGLWGDWITATDAYLKVFFAGQEQRTATVWNDNNPRWMTRLDFGDVLLATGGPLRV
perf_human      361 RRALSQYLTDRARWRDCSRPCPPGRQKSPRDPCQCVCHGSAVTTQDCCPRQRGLAQLEVTFIQAWGLWGDWFTATDAYVKLFFGGQELRTSTVWDNNNPIWSVRLDFGDVLLATGGPLRL
```

Granzyme A

```
GrzA_mouse        1 MRNASGPRGPSLATLLFLLLIPEGGCERIIGGDTVVPHSRPYMALLKLSSNTICAGALIEKNWVLTAAHCNVGKRSKFILGAHSINKE-PEQQILTVKKAFPYPCYDEYTREGDLQLVRL
GrzA_rat          1 MRNPCAPWVSSLTTVIFLLLIPEGGCERIIGGDTVVPHSRPYMVLLKLKPDSICAGALIAKNWVLTAAHCIPGKKSEVILGAHSIKKE-PEQQILSVKKAYPYPCFDKHTHEGDLQLLRL
Bovine_cDNA       1 ----------------------------------------MVLL--DGGNICAGALIAEDWVLTAAHCSLNQKSQIILGAHSRNKEEPEKQIMFVKKEFPYPCYDPDTHEGDLKLLKL
GrzA_human        1 MRNSYRFLASSLSVVVSLLLIPEDVCEKIIGGNEVTPHSRPYMVLLSLDRKTICAGALIAKDWVLTAAHCNLNKRSQVILGAHSITREEPTKQIMLVKKEFPYPCYDPATREGDLKLLQL

GRAA_mouse      120 KKKATVNRNVAILHLPKKGDDVKPGTRCRVAGWGRFGNKSAPSETLREVNITVIDRKICNDEKHYNFHPVIGLNMICAGDLRGGKDSCNGDSGSPLLCDGILRGITSFGGE-KCGDRRWP
GrzA_rat        120 KKKATLNKNVAILHLPKKGDDVKPGTRCHVAGWGRFHNKSPPSDTLREVNITVIDRKICNDEKHYNFNPVIGLNMICAGNLRGGKDSCYGDSGGPLLCEGIFRGITAFGLEGRCGDPKGP
Bovine_cDNA      77 NKKATLNKNVAILQLPKEGDDVKPG----------------------------------------------------------------------------------------------
GRAA_human      121 TEKAKINKYVTILHLPKKGDDVKPGTMCQVAGWGRTHNSASWSDTLREVNITIIDRKVCNDRNHYNFNPVIGMNMVCAGSLRGGRDSCNGDSGSPLLCEGVFRGVTSFGLENKCGDPRGP
```

Granzyme B

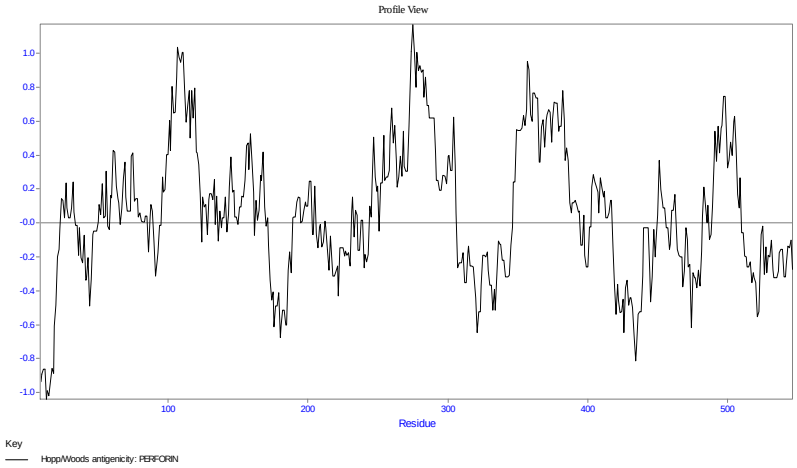
```
Bovineduodenase       100 NDETLANDIMLLKLTRKADITDKVSPINLPRSLAEVKPGMMCSVAGWGRLGVNMPSTDNLQEVDLEVQSEEKCIARFKNYIP-FTQICAGDPSKRKNSFS
sheepMCP-3            100 NDETCANDIMLLQLTRKAEMTDAVSLINLPRSLEKVKPGMMCSVAGWGQLGVNMPSADKLQEVDLEVQREEKCIARFKDYIP-VTQICAGDPSKRKDSFL
Bovine_cDNA           100 NDETLANDIMLLKLTRKADITDKVSPINLPRSLAKVKTGMMCSVAGWGRLGVNMPSTDKLQEVDLEVQNKKKCKDRFQDYNA-SIQICAGDPSKRKSSFL
GrzB_human            101 NPKNFSNDIMLLQLERKAKRTRAVQPLRLPSNKAQVKPGQTCSVAGWGQTAPLGKHSHTLQEVKMTVQEDRKCESDLRHYYDSTIELCVGDPEIKKTSFK
```

Regions selected using by Hopp and Woods (1981) algorithm for antigenicity. Peptides are highlighted as follows; Yellow, perforin peptide 264 and 260; Blue, granzyme A peptides 258 and 263; Red, granzyme B peptide 265 and 259

Appendix VII: Hopp and Woods (1981) antigenicity plots of bovine perforin granzyme A and granzyme B

Regions of antigenicity are identified by assigning each amino acid in an amino acid sequence an antigenicity index based on hydrophilicity and accessibility to solvent. The higher the antigenicity the more likely that antibody will bind to the region. Antigenic regions are identified as those regions above a set threshold shown by the dotted line

A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0.8
0.6
0.4
0.2
-0.0
-0.2
-0.4
-0.6
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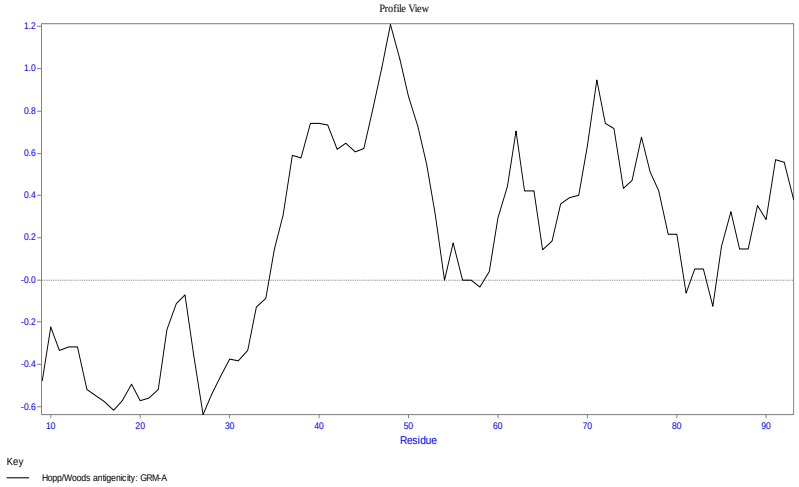
-1.0
100
200
300
400
500
Residue
Key
Hopp/Woods antigenicity: PERFORIN

B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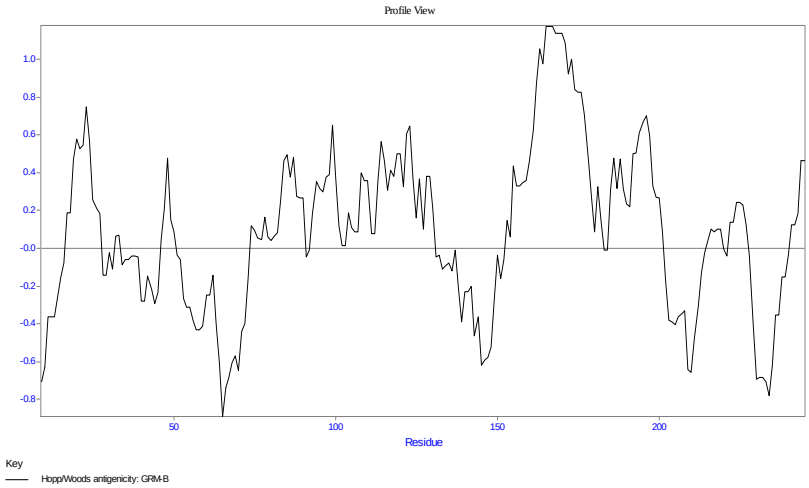
-0.4
-0.6
10
20
30
40
50
60
70
80
90
Residue
Key
Hopp/Woods antigenicity: GRM-A

C


Profile View
1.0
0.8
0.6
0.4
0.2
-0.0
-0.2
-0.4
-0.6
-0.8
50
100
150
200
Residue
Key
Hopp/Woods antigenicity: GRM-B

Appendix VIII: Class I BoLA specific monoclonal antibodies matched with their respective specificities

Monoclonal antibody	Isotype	BoLA class I specificity	Reference
P3	IgG_{2a}	Class I KN18 and others	Lalor *et al.*, 1986
FJ101	-	Class I Subgroups of w6	Taracha *et al.*, 1995b
B4/18	IgM	Class I Polymorphic (w6,2)	Goddeeris and Morrison, 1988
IL-A4	IgM	Class I KN104	Toye *et al.*, 1990
IL-A7	IgG_{2a}	Class I w10 and others	Bensaid *et al.*, 1988
IL-A9	IgM	Class I w4 ,w7 and others	-
IL-A10	IgM	Class I w10 and T7 associated	Toye *et al.*, 1990
IL-A13	IgG_1	Class I w4 and w7	-
IL-A31	IgG_3	Class I w10 - associated	-
IL-A34	IgG_{2a}	Class I w10 - associated	Toye *et al.*, 1990
IL-A35	IgG_{2a}	Class I w10 - associated	Toye *et al.*, 1990
IL-A36	IgM	Class I KN18-associated	-
IL-A38	IgG_{2a}	Class I w10 - associated	Toye *et al.*, 1990
IL-A88	IgG_{2a}	Monomorphic class I	Toye *et al.*, 1990
IL-A89	IgG_{2a}	Class I w25	Gwakisa, 1992
IL-A120	IgM	Polymorphic w1	-
IL-A121	IgG_3	Polymorphic KN8	-

Appendix IX: Experimental animal records

Cattle were immunised by the Infection and Treatment method (ITM). One ml of stabilate diluted 1:20 was administered sub-cutaneously below the right ear using cryopreserved viable *T. parva* (Muguga) sporozoites ILRI stabilate No. 4133 and a slow-release formulation of antibiotic, Alamycine 300 LA, at 20 mg/kg body weight. Homologous challenge infections were achieved by a lethal dose of 1 ml (1:20 dilution) of stabilate No. 4133. Following inoculation with sporozoites, cattle were monitored clinically for temperature and parasitosis by microscopic examination of giemsa-stained smears of needle aspirates of local lymph nodes and observations scored as H for hyperplasia or Ma for macroshizonts. Examination for parasitosis and measurement of rectal temperature were performed daily starting 5 days after inoculation with sporozoites until recovery. A rectal temperature of 39.5ºC or higher, associated with schizont parasitosis, was considered a pyrexic response and treatment done using an anti-theileriacidal drug Butalex® (Coopers Animal health).

ILRI EXPERIMENTAL ANIMAL RECORD

DATE 2/7/07 ITM EXPERIMENT No. TETRAMER

PREVIOUS RECORD Nawa ANIMAL NUMBER & DESCRIPTION BB001

DATE	2/7	3/7	4/7	5/7	6/7	7/7	8/7	9/7	10/7	11/7	12/7	13/7	14/7	15/7	16/7	17/7	18/7	19/7	20/7	21/7	22/7	23/7	24/7	25/7	26/7	27/7	28/7	29/7
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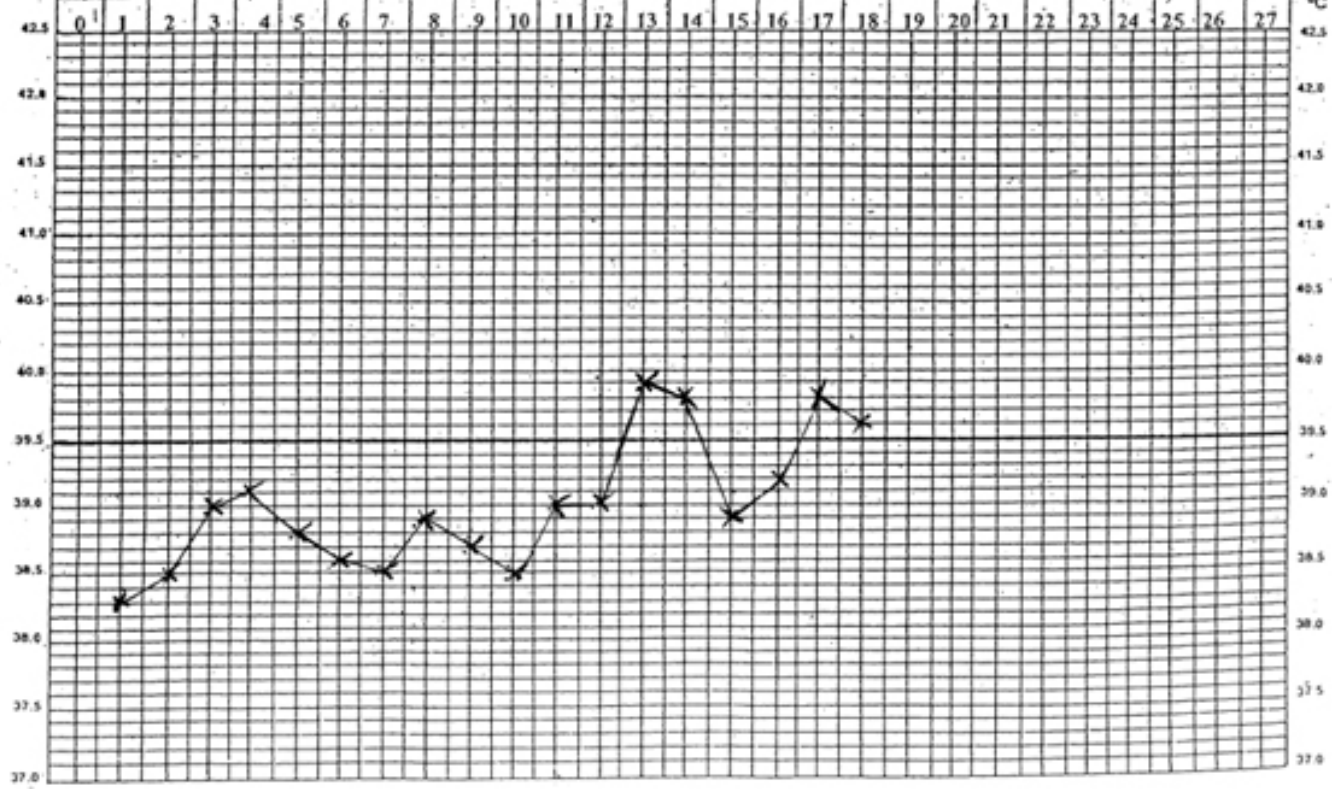
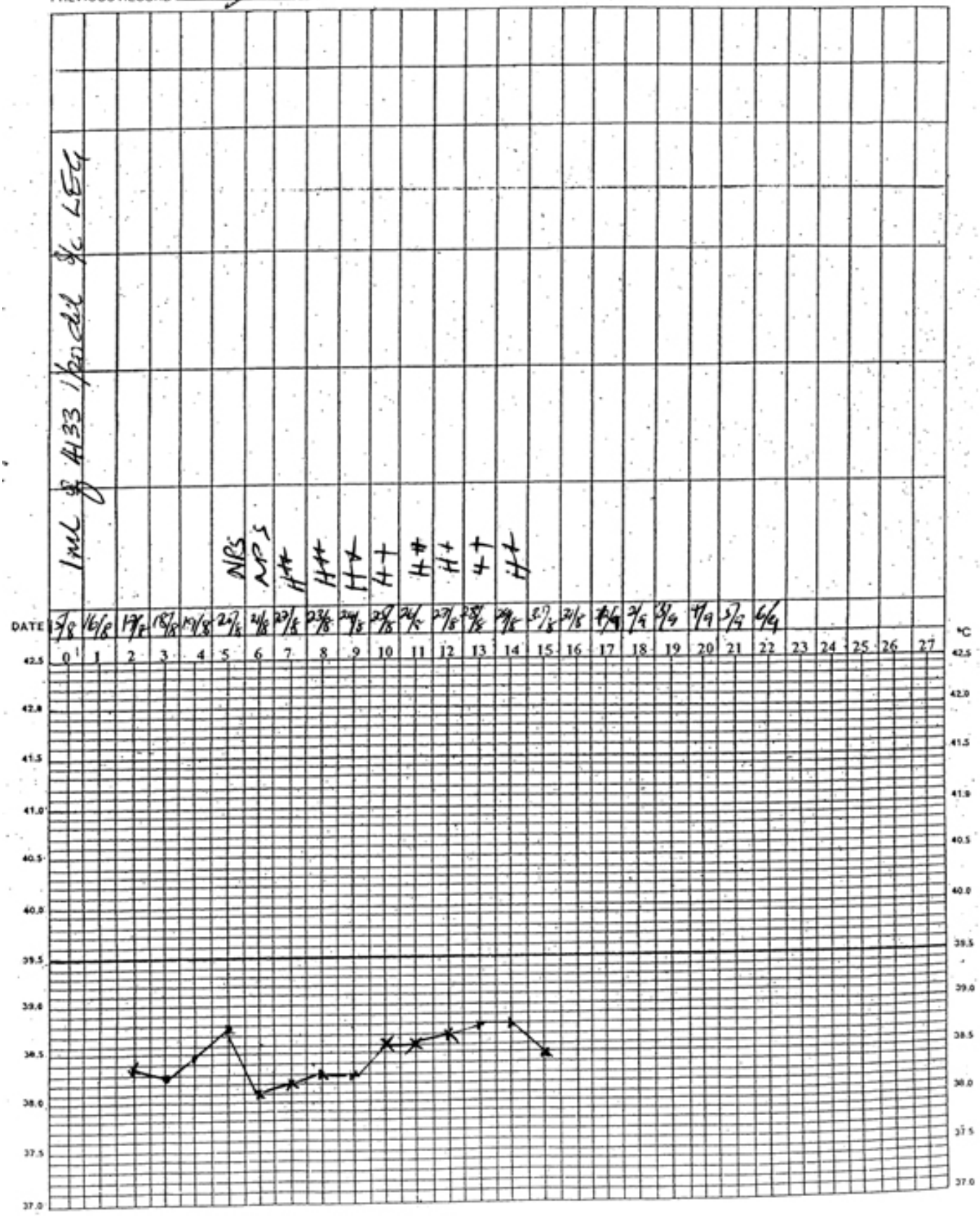
Inf of						++	++	+	+	+	+	Mg+	Mg ++	Mg ++	Mg ++	Mg+	Mg+	Mg+	++	++	++	+							
1:20 Stab 4133													++	++	Mg+	++	++	++	++	+	+	+							
S/c RED + TAA															IGMB Pentalex TM	NPS						STOP							



ILRI EXPERIMENTAL ANIMAL RECORD

DATE 15/8/07 EXPERIMENT No.

PREVIOUS RECORD I Im ANIMAL NUMBER & DESCRIPTION BB001

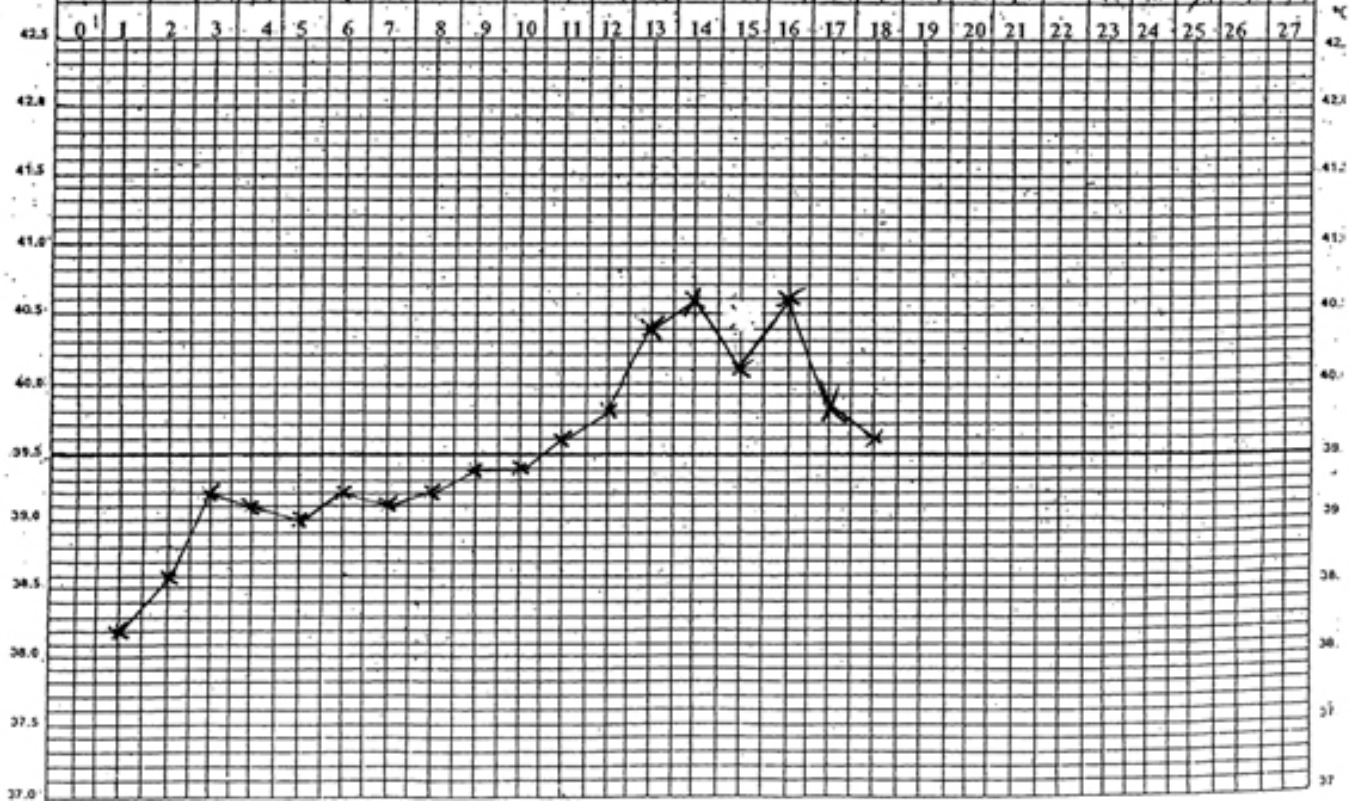


ILRI EXPERIMENTAL ANIMAL RECORD

DATE 2/7/07 ITM EXPERIMENT No. TETRAMER

PREVIOUS RECORD Nawe ANIMAL NUMBER & DESCRIPTION BB006

DATE	2/7	3/7	4/7	5/7	6/7	7/7	8/7	9/7	10/7	11/7	12/7	13/7	14/7	15/7	16/7	17/7	18/7	19/7	20/7	21/7	22/7	23/7	24/7	25/7	26/7	27/7	28/7	29/7
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
Inil of						H+	H+	H+	H+	H#	H#	Ma+	Ma+	Ma#	Mg#	Ma#	Ma#	Ma+	H##	H##	H##	H+						
1:20 Stab 4133													H+	H+	Ma+	Ma+	Ma+	H+	H+	H+	H+	H+						
S/C REA + TLA													NPS	NPS	NPS 12Mls Pentelox IM	NPS	NPS 12mls Butalex IM.	NPS.	NPS			STOP						



ILRI EXPERIMENTAL ANIMAL RECORD

DATE 15/8/07 EXPERIMENT No. Tetramer

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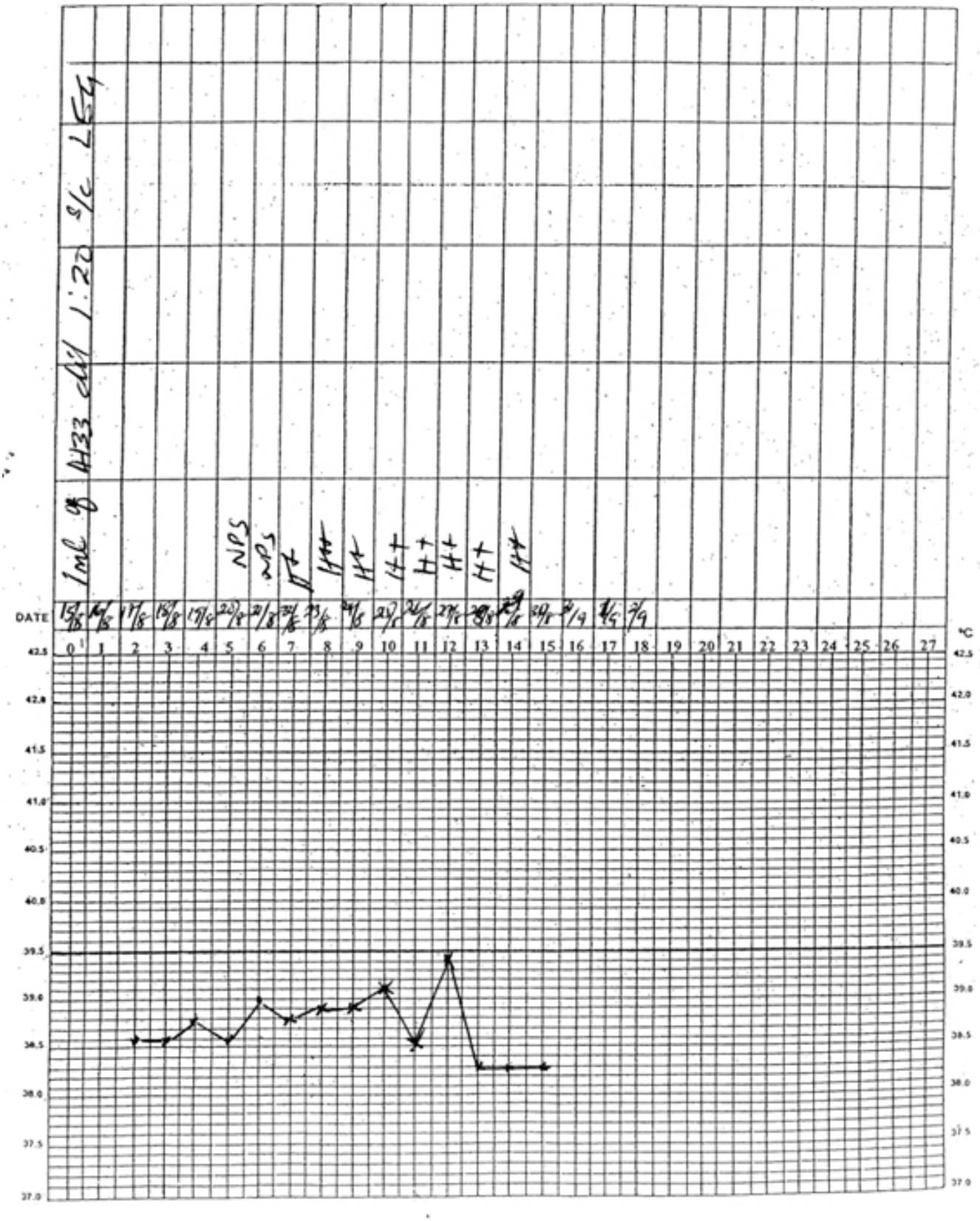

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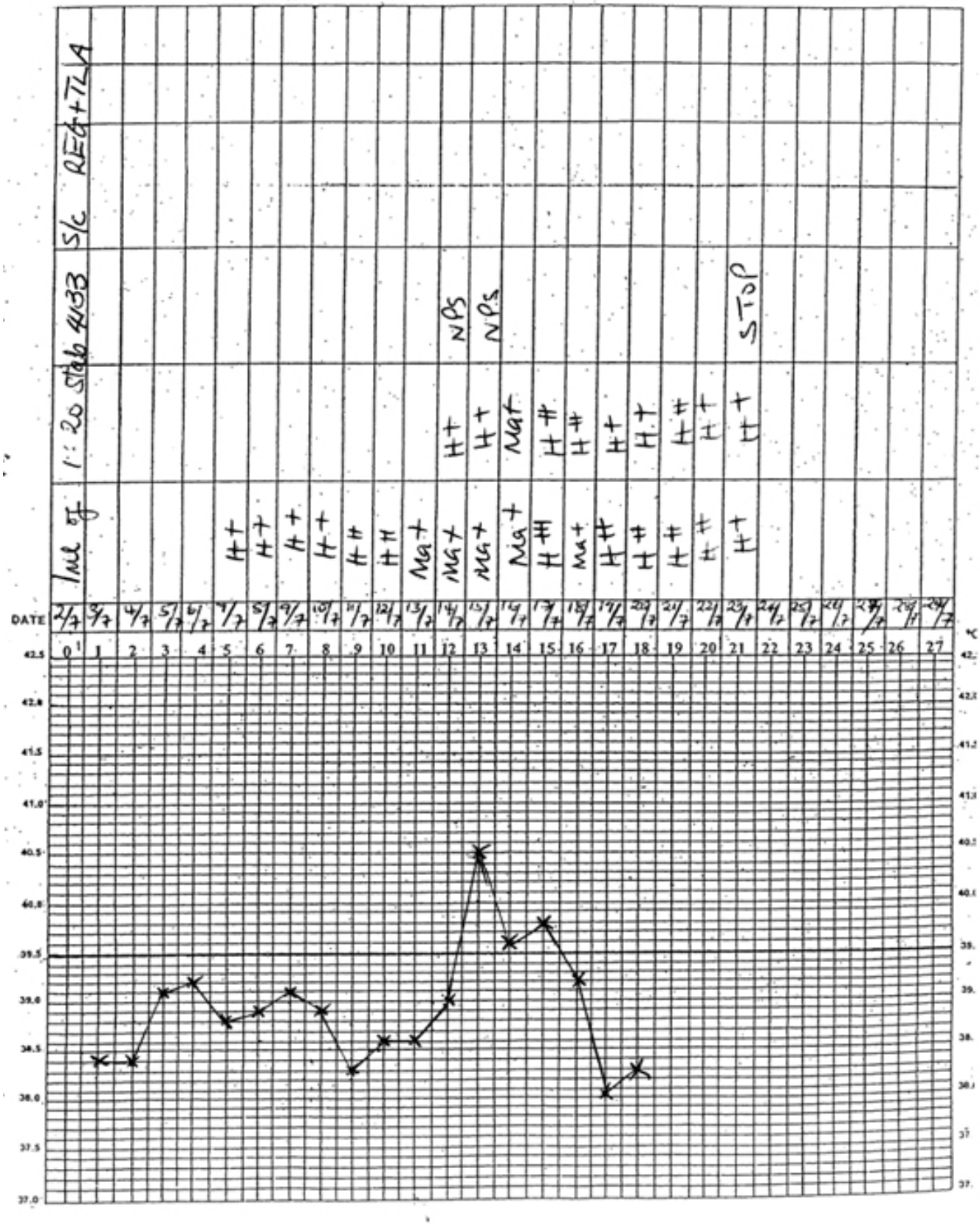
DATE 2/7/07 ITM EXPERIMENT No. TETRAMER

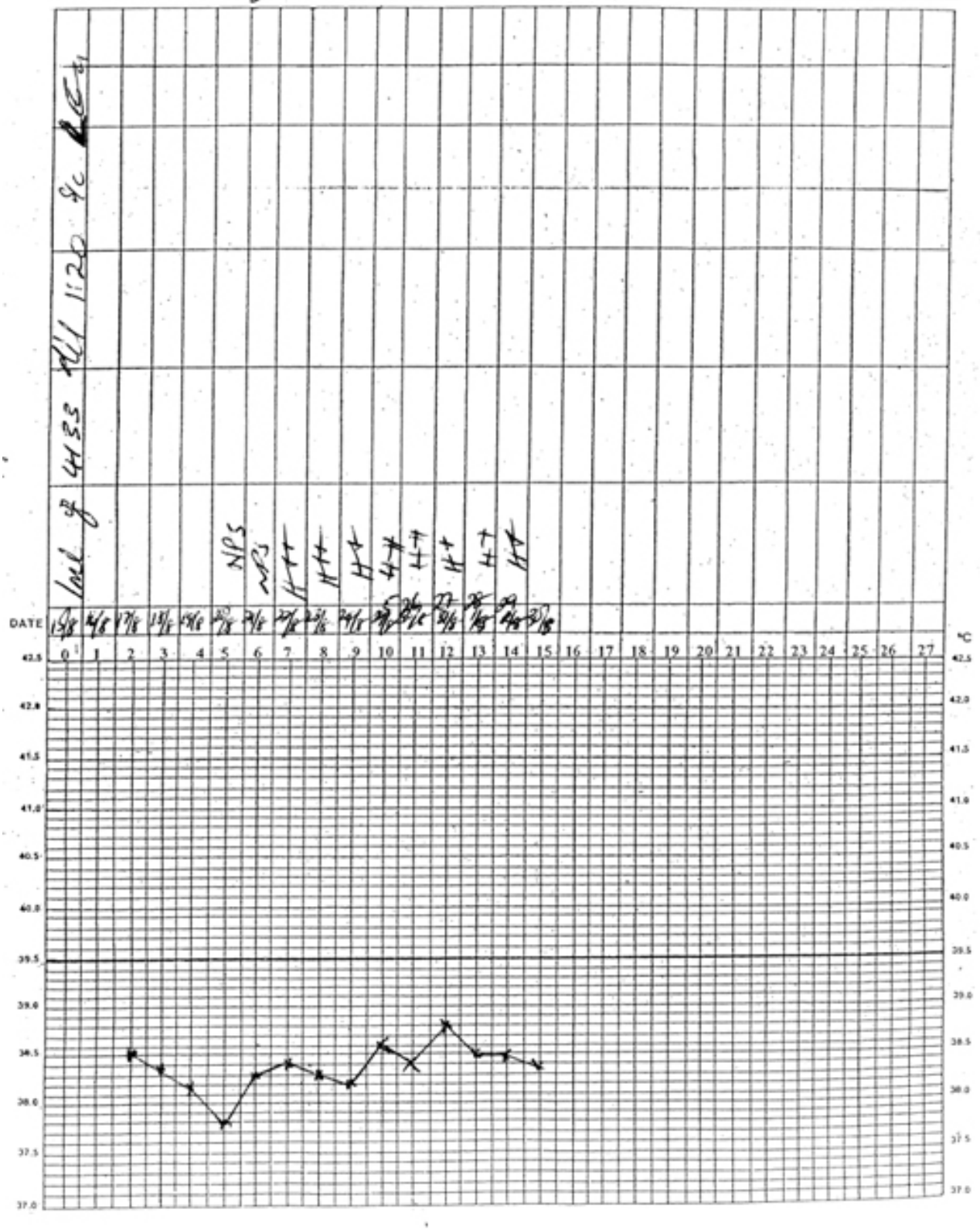
PREVIOUS RECORD Nawe ANIMAL NUMBER & DESCRIPTION BB007



ILRI EXPERIMENTAL ANIMAL RECORD

DATE 15/8/07 EXPERIMENT No. Tetramer

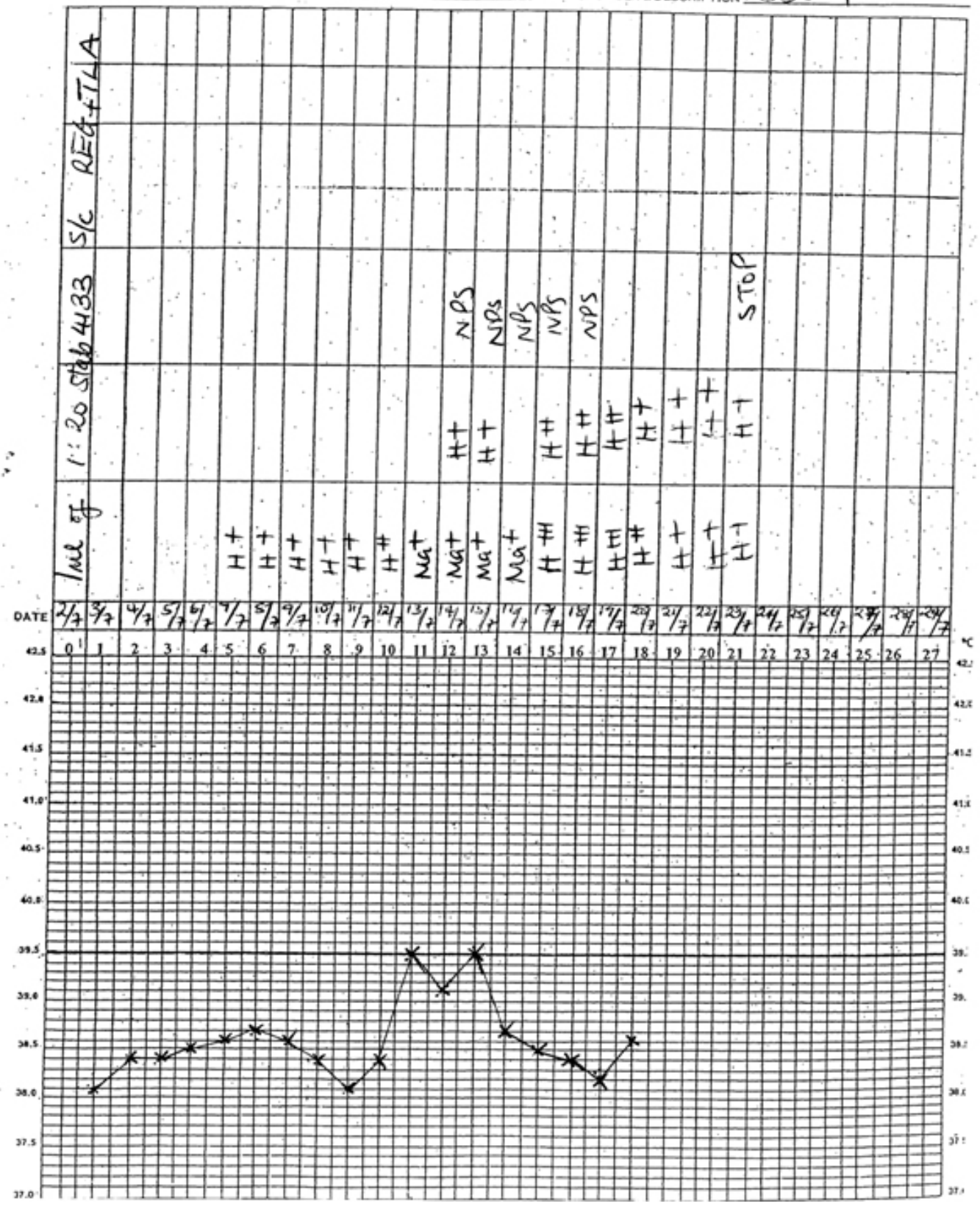
PREVIOUS RECORD I TM ANIMAL NUMBER & DESCRIPTION BB007



ILRI EXPERIMENTAL ANIMAL RECORD

DATE 2/7/07 ITM EXPERIMENT No. TETRAMER

PREVIOUS RECORD Nawe ANIMAL NUMBER & DESCRIPTION BB069



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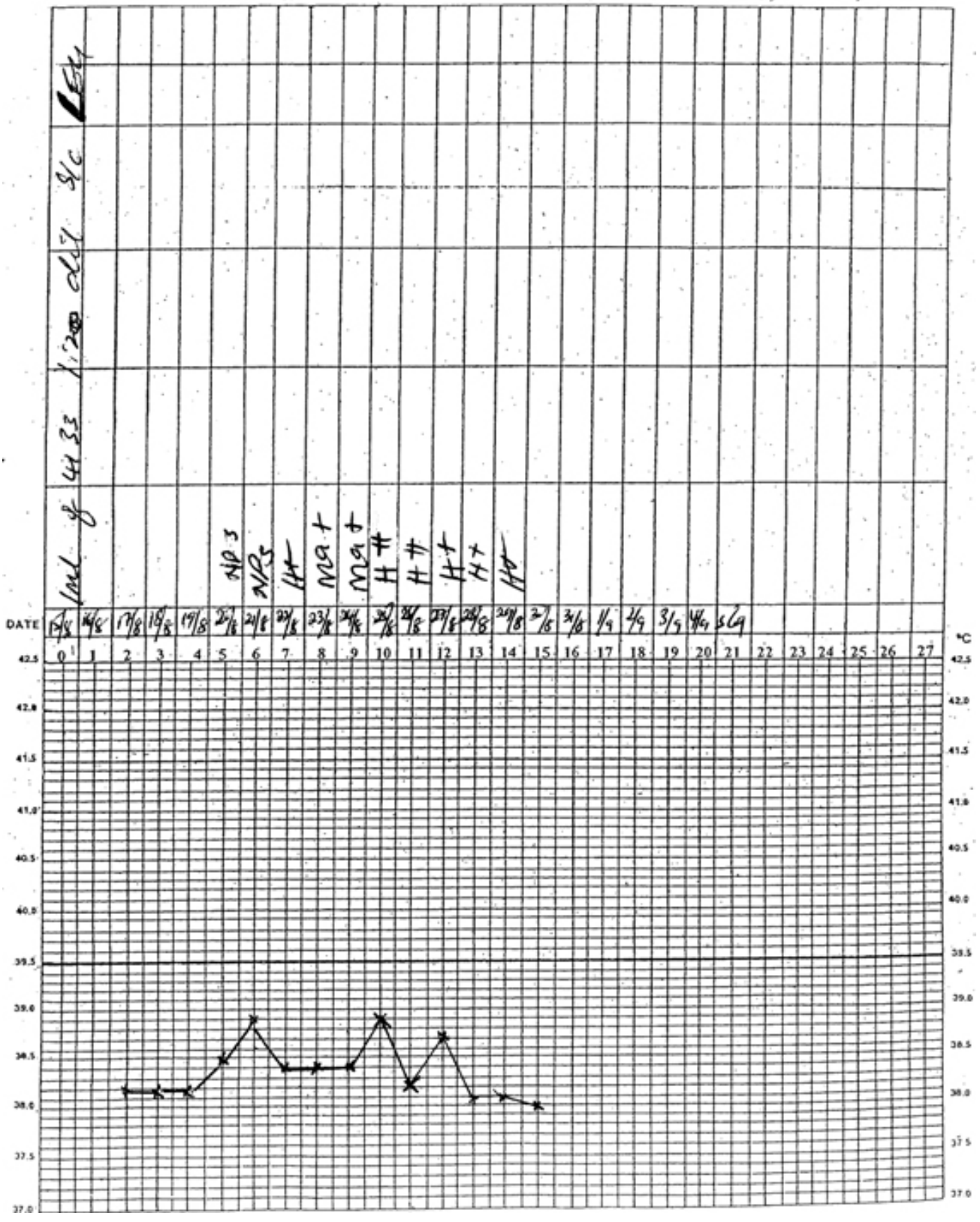
DATE 15/8/07 EXPERIMENT No. Tetramer

PREVIOUS RECORD I TM ANIMAL NUMBER & DESCRIPTION BB069



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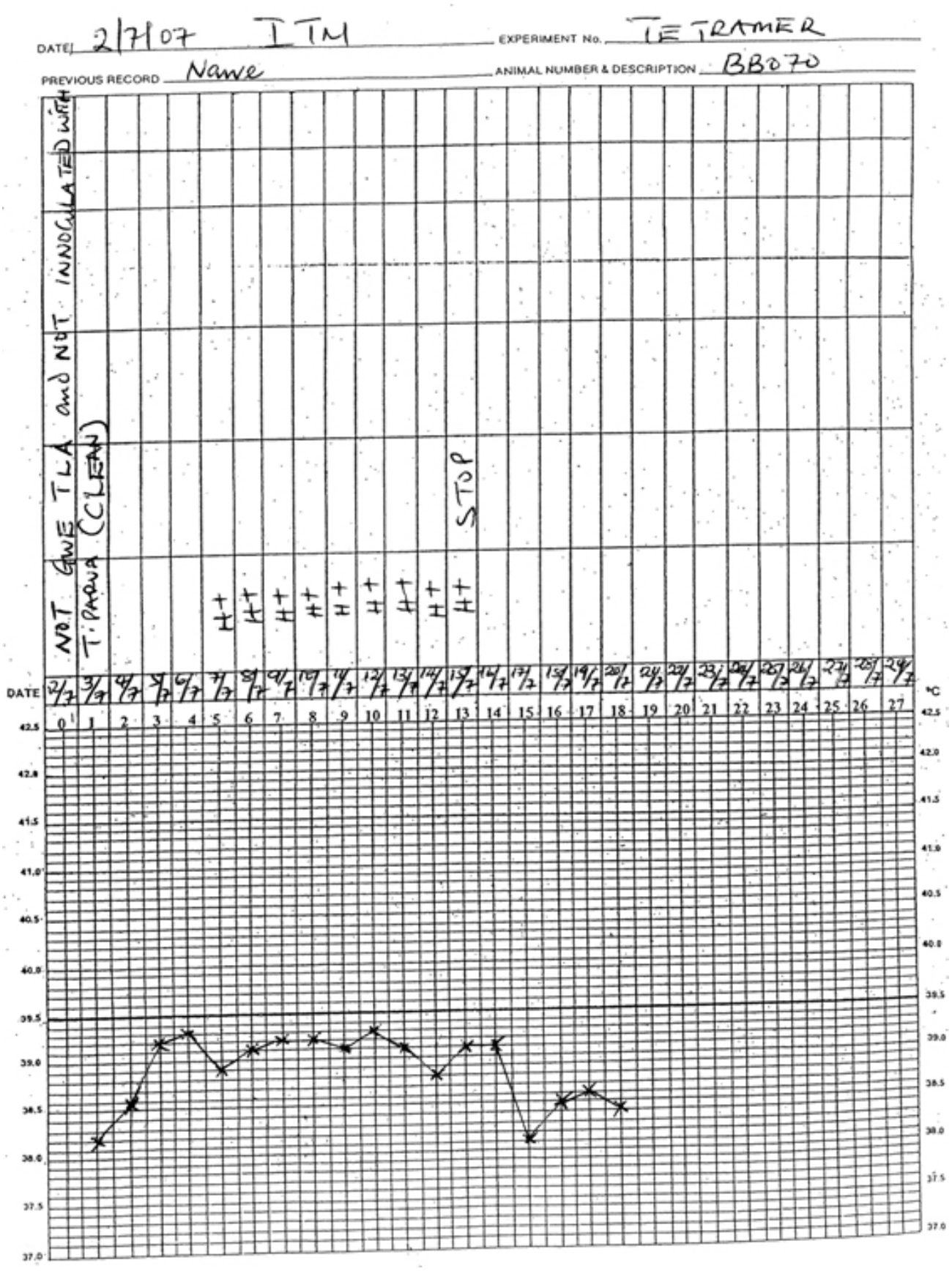
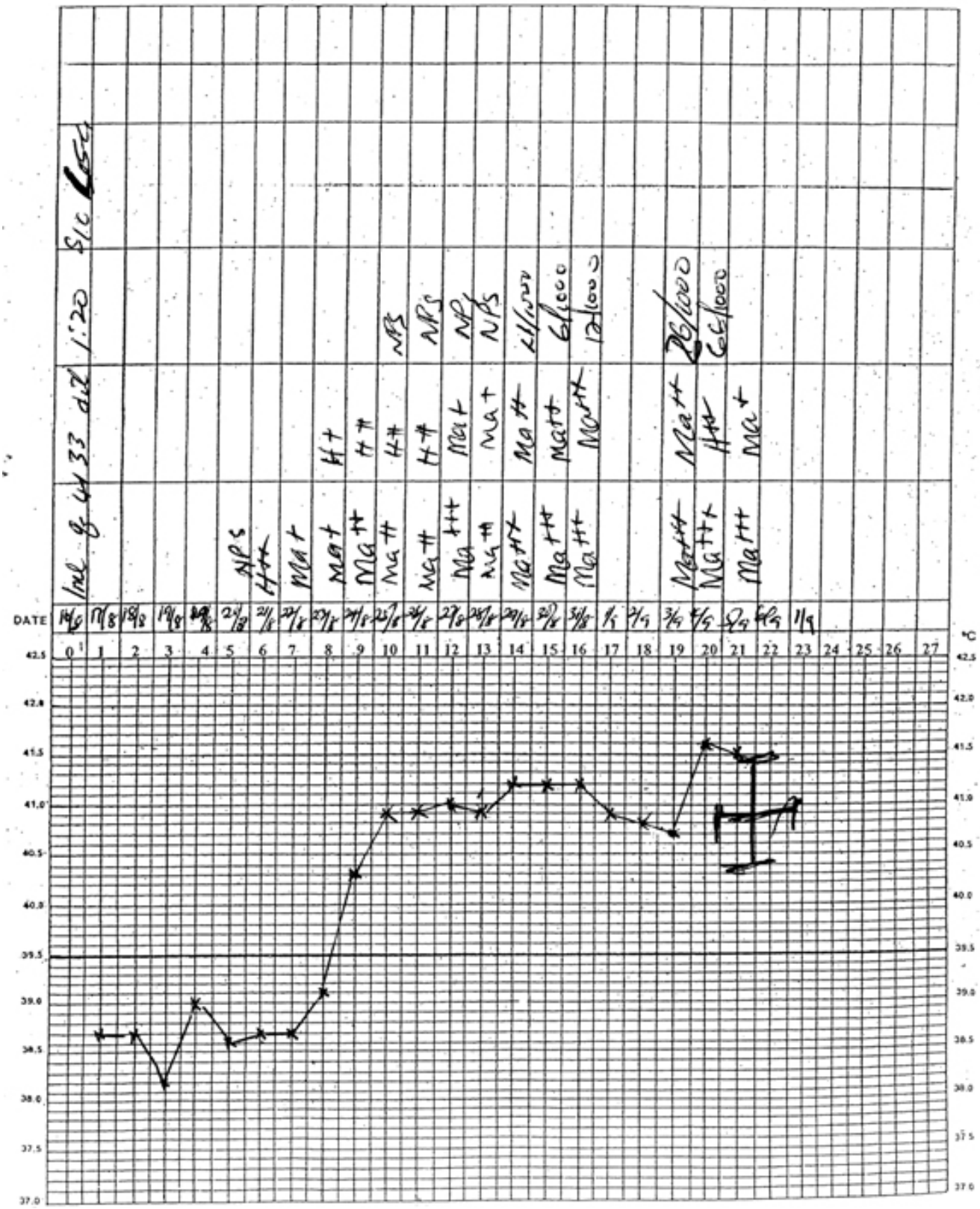


ILRI EXPERIMENTAL ANIMAL RECORD

DATE 15/8/07 EXPERIMENT No. Tetramer

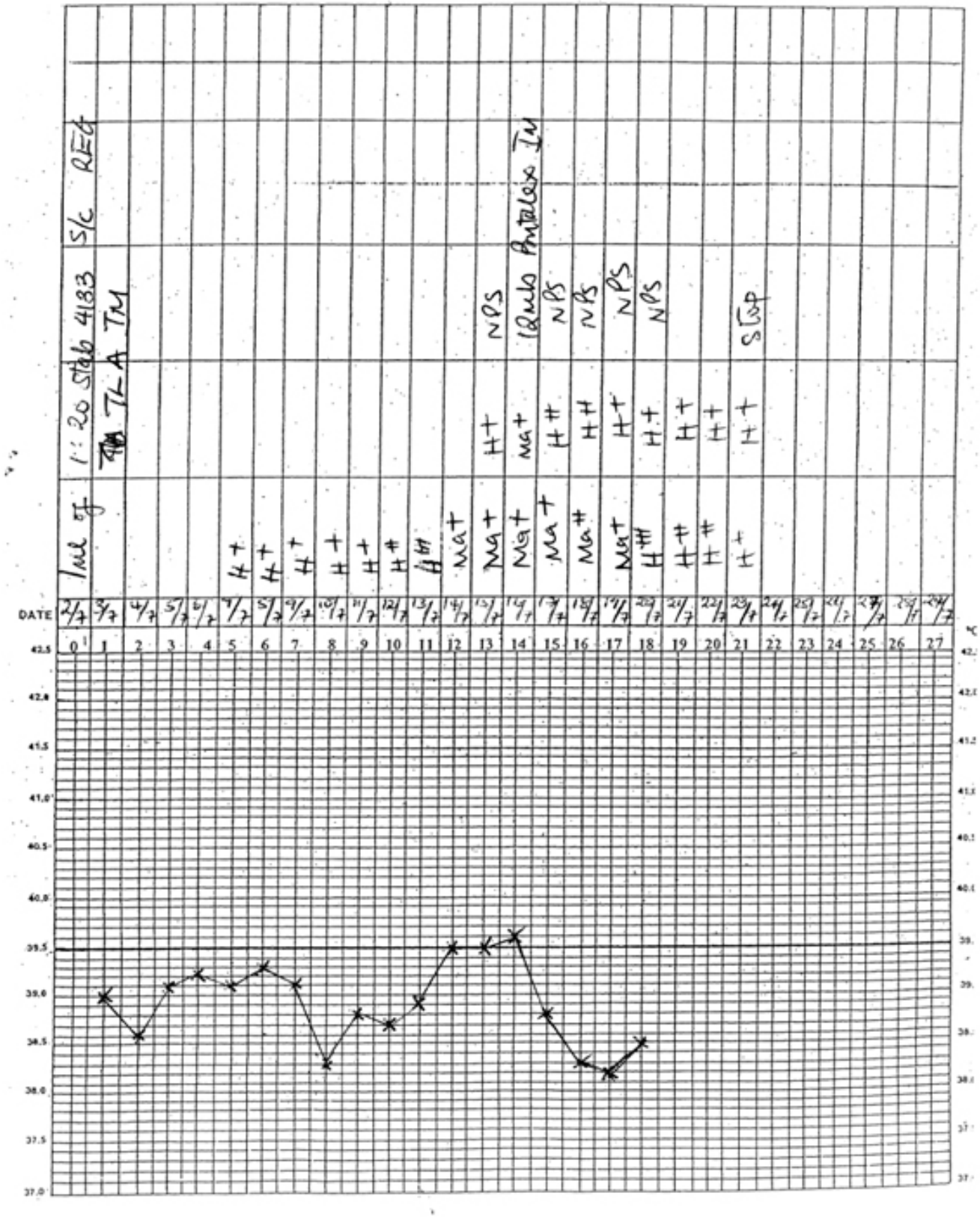
PREVIOUS RECORD I Im ANIMAL NUMBER & DESCRIPTION BB070



ILRI EXPERIMENTAL ANIMAL RECORD

DATE 2/7/07 ITM EXPERIMENT No. TETRAMER

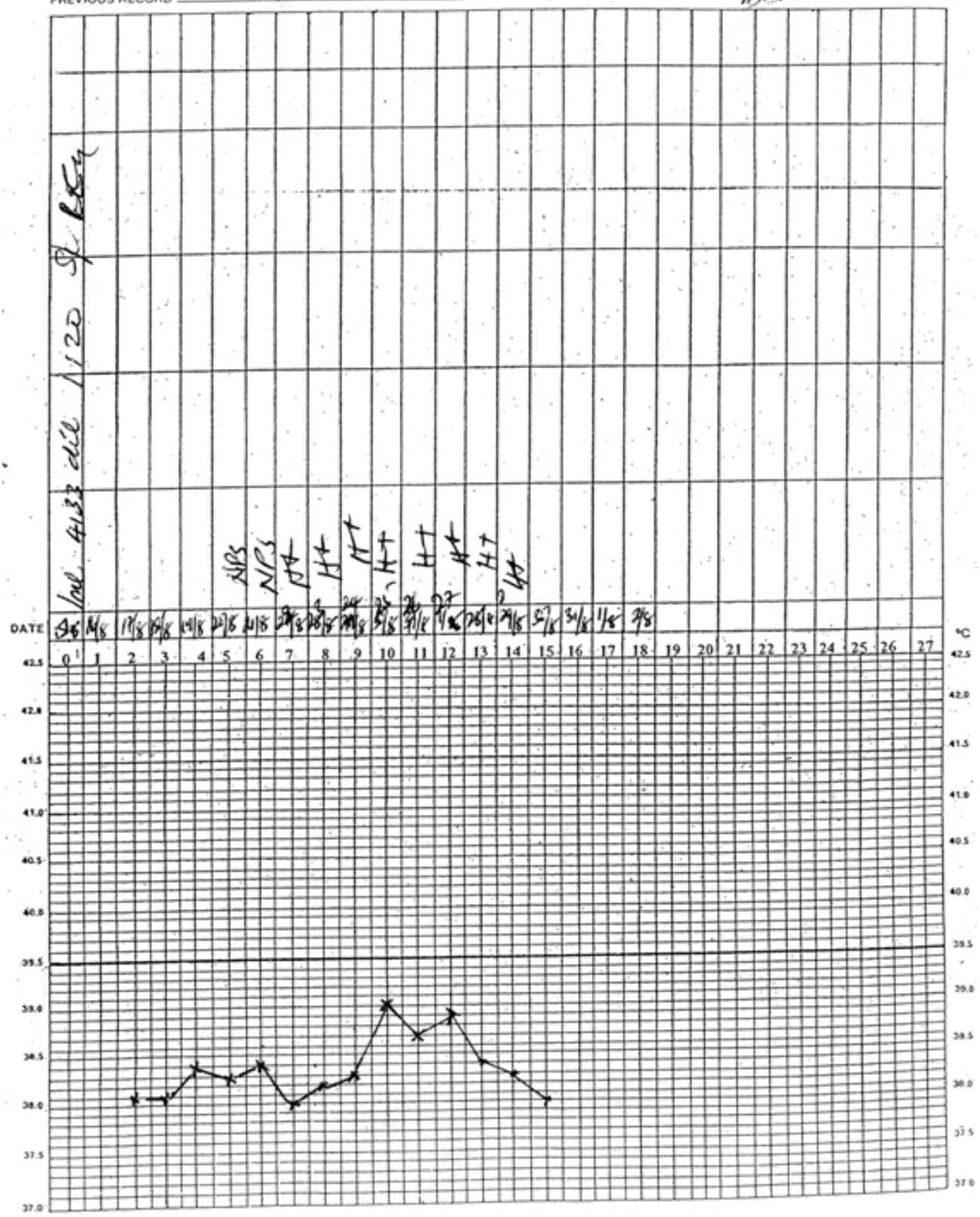
PREVIOUS RECORD NAWR ANIMAL NUMBER & DESCRIPTION BB169



ILRI EXPERIMENTAL ANIMAL RECORD

DATE 15/8/07 EXPERIMENT No. Tetramer

PREVIOUS RECORD ITM ANIMAL NUMBER & DESCRIPTION BB 169



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DATE 15/8/07 EXPERIMENT No. Tetramer

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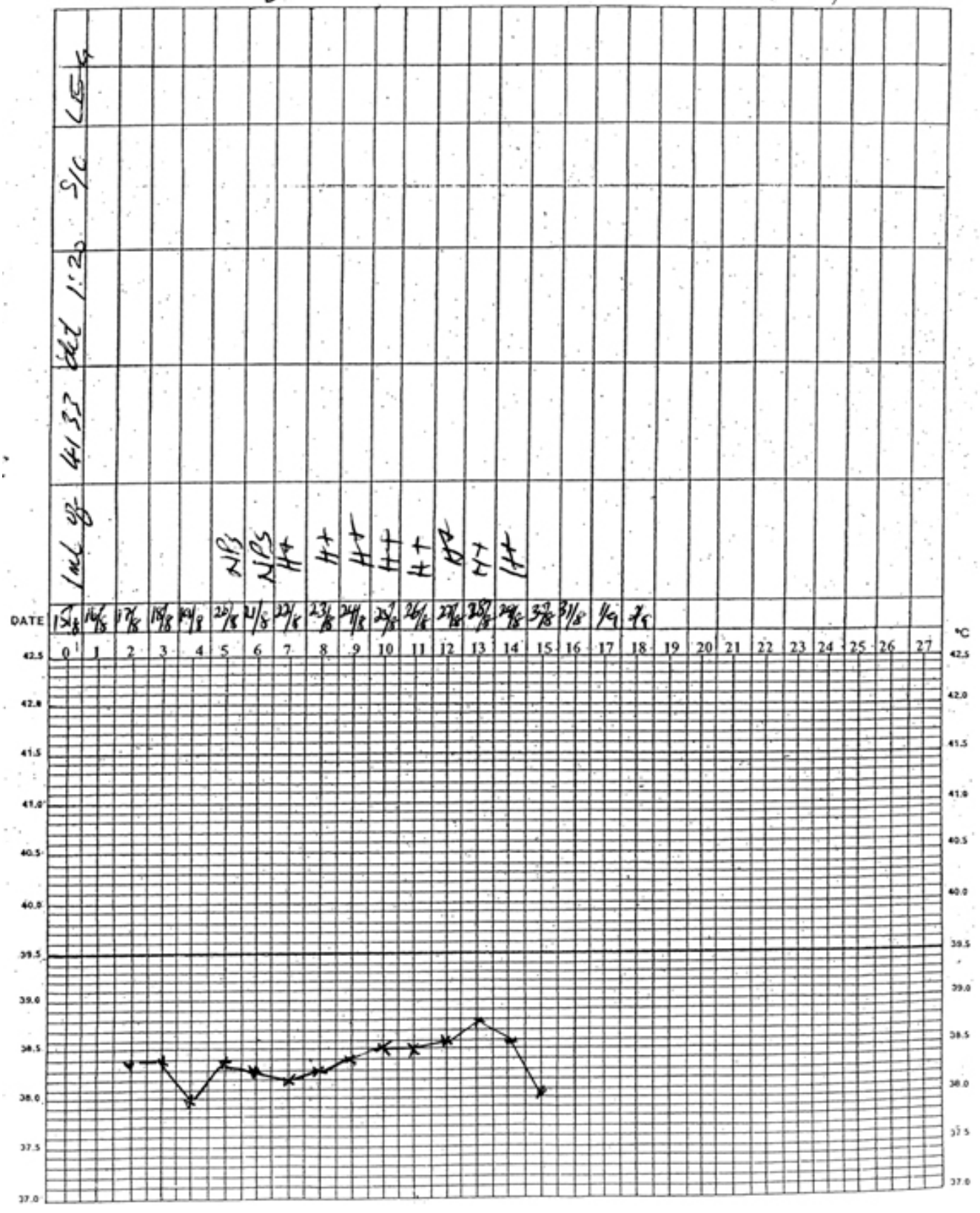
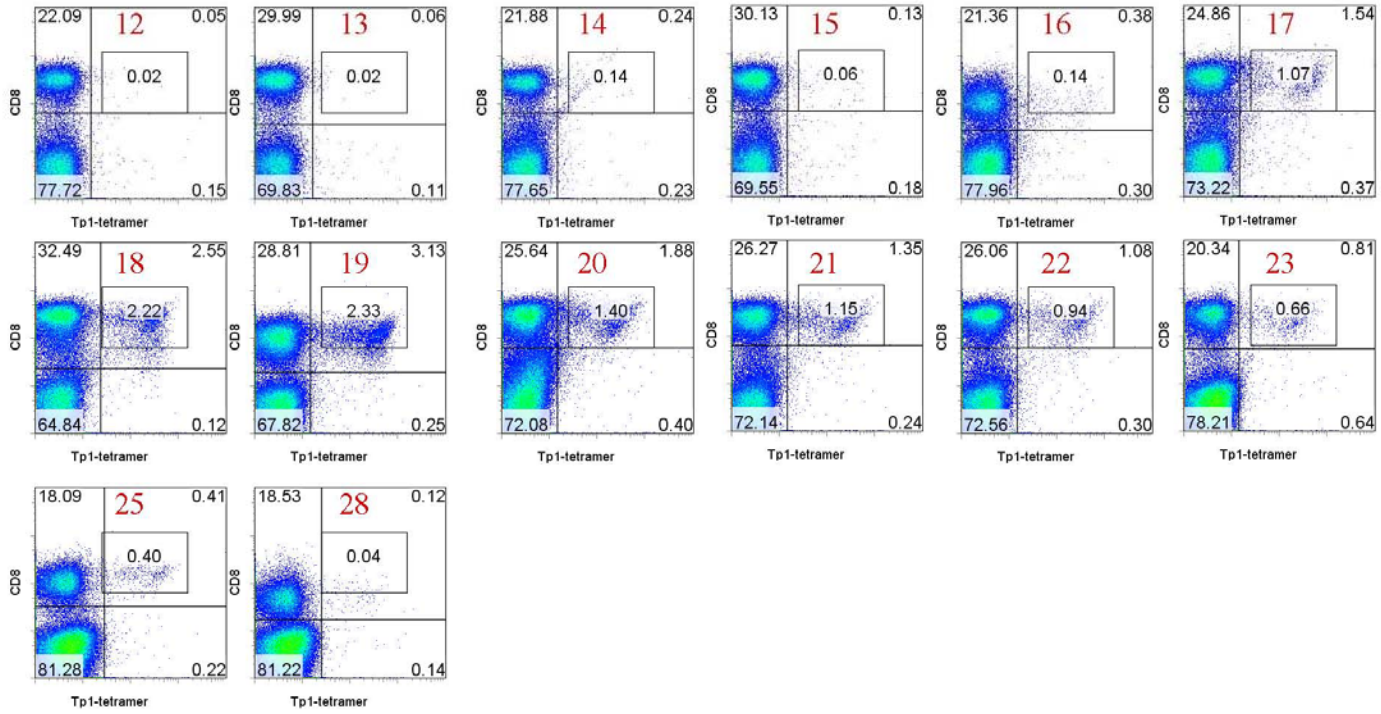


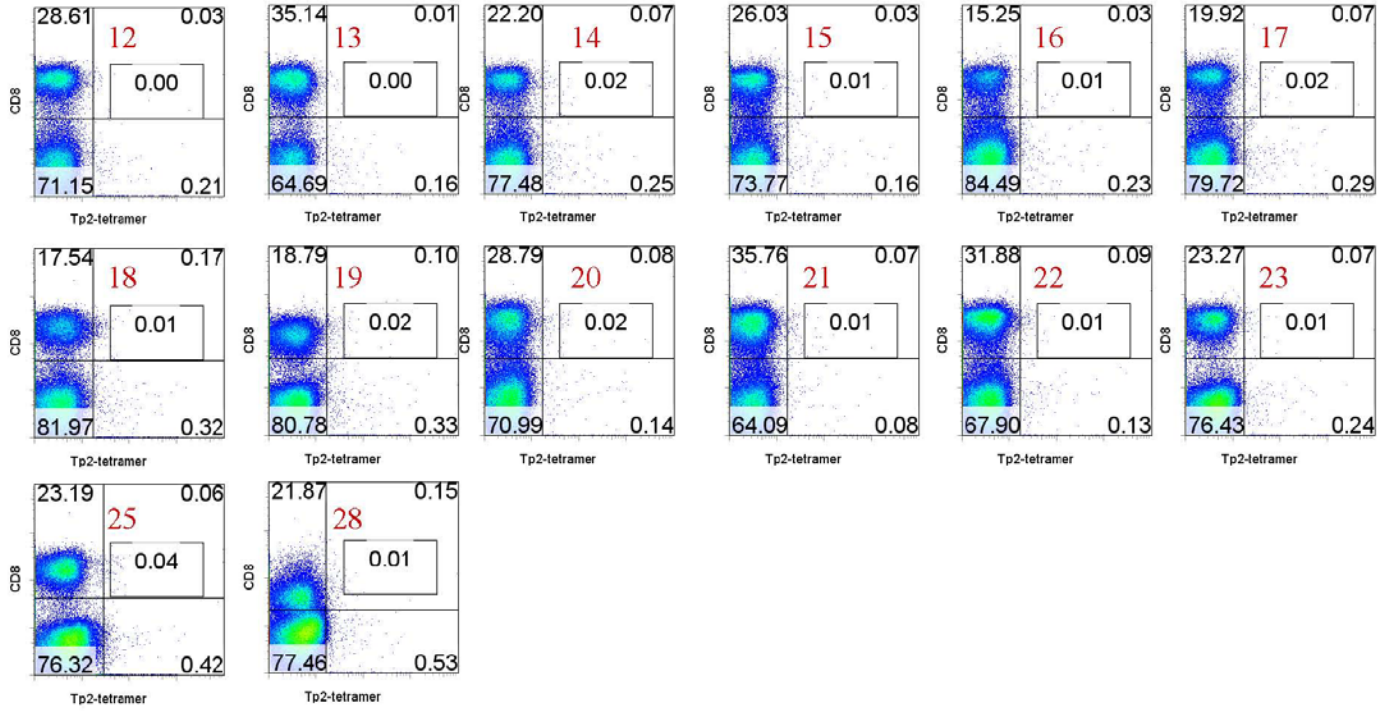
Appendix X: Tetramer^{+}CD8^{+} T cells in animals immunised by ITM and challenged with *T. parva* sporozoites

Peripheral blood mononucleated cells were stained with corresponding tetramer followed by anti CD8-antibody. Dot plot profile shows log fluorescence intensity of CD8 staining (FL1) versus Tetramer staining (FL3) on specific days indicated (in red) after immunisation and challenge

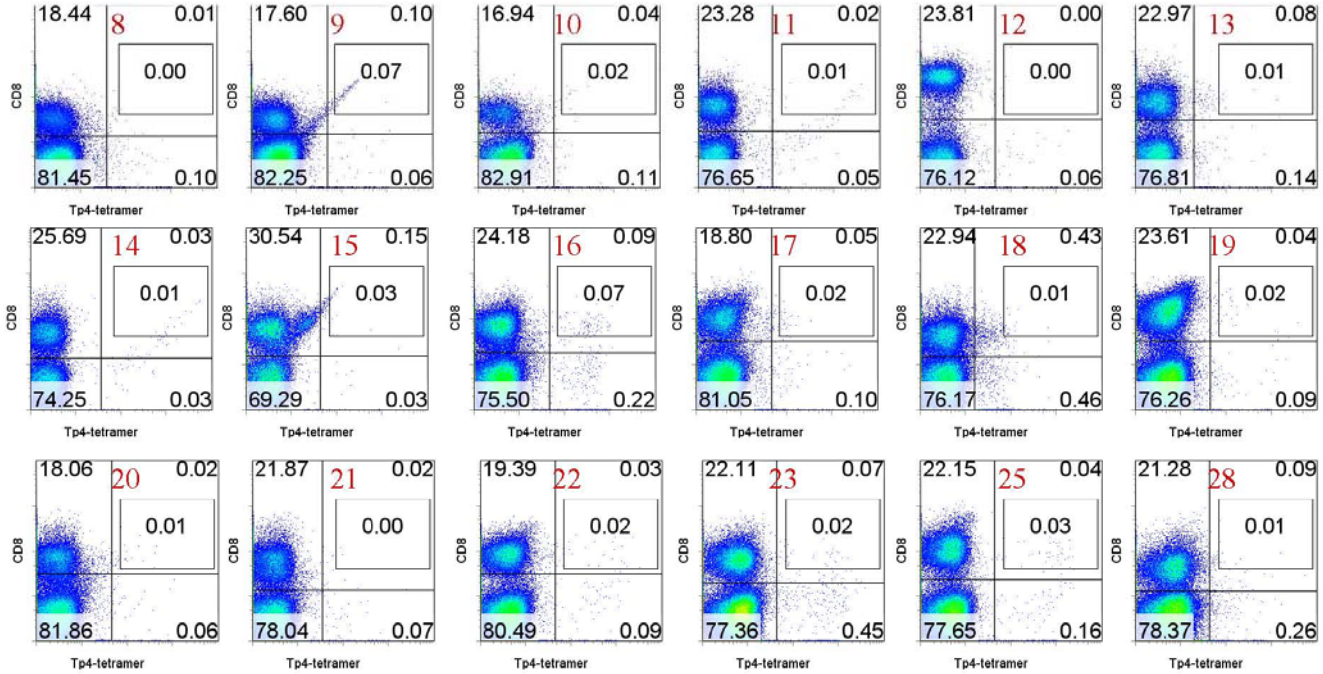
BB007 Tp1-tetramer^{+}CD8^{+} T cells (post immunisation)



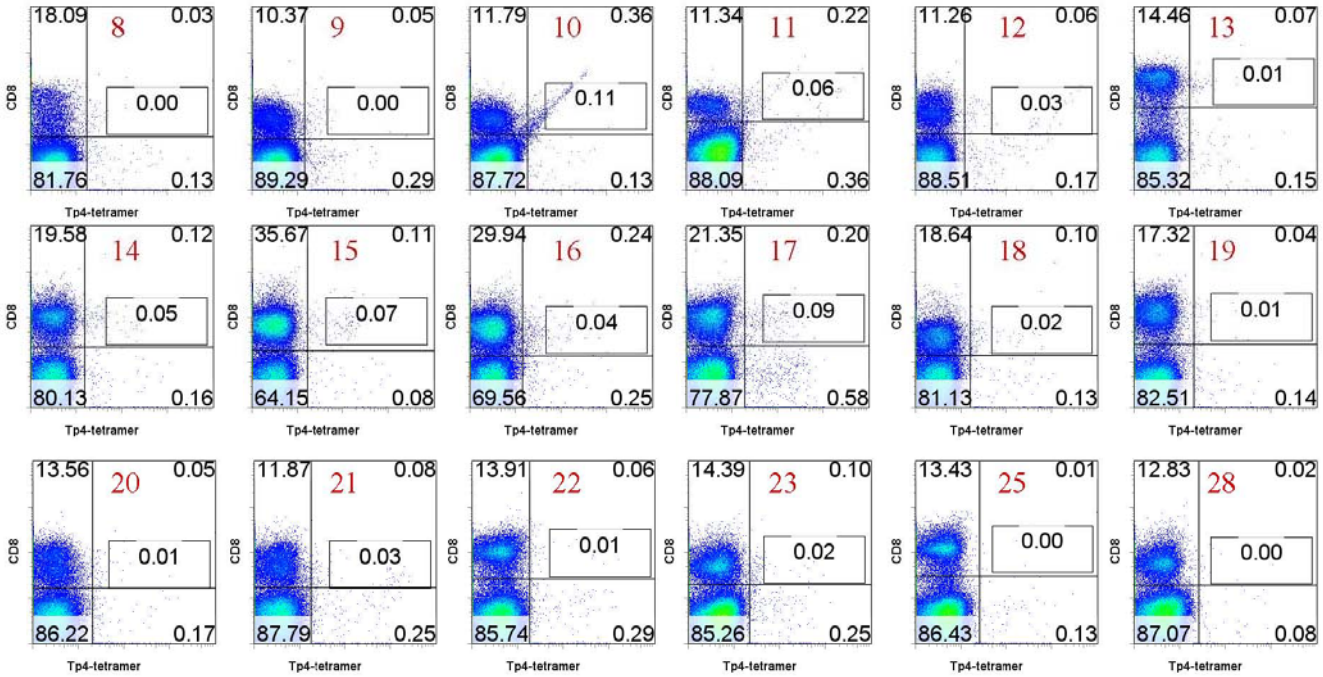
BB006 Tp2-tetramer^{+} CD8^{+} T cells (post immunisation)



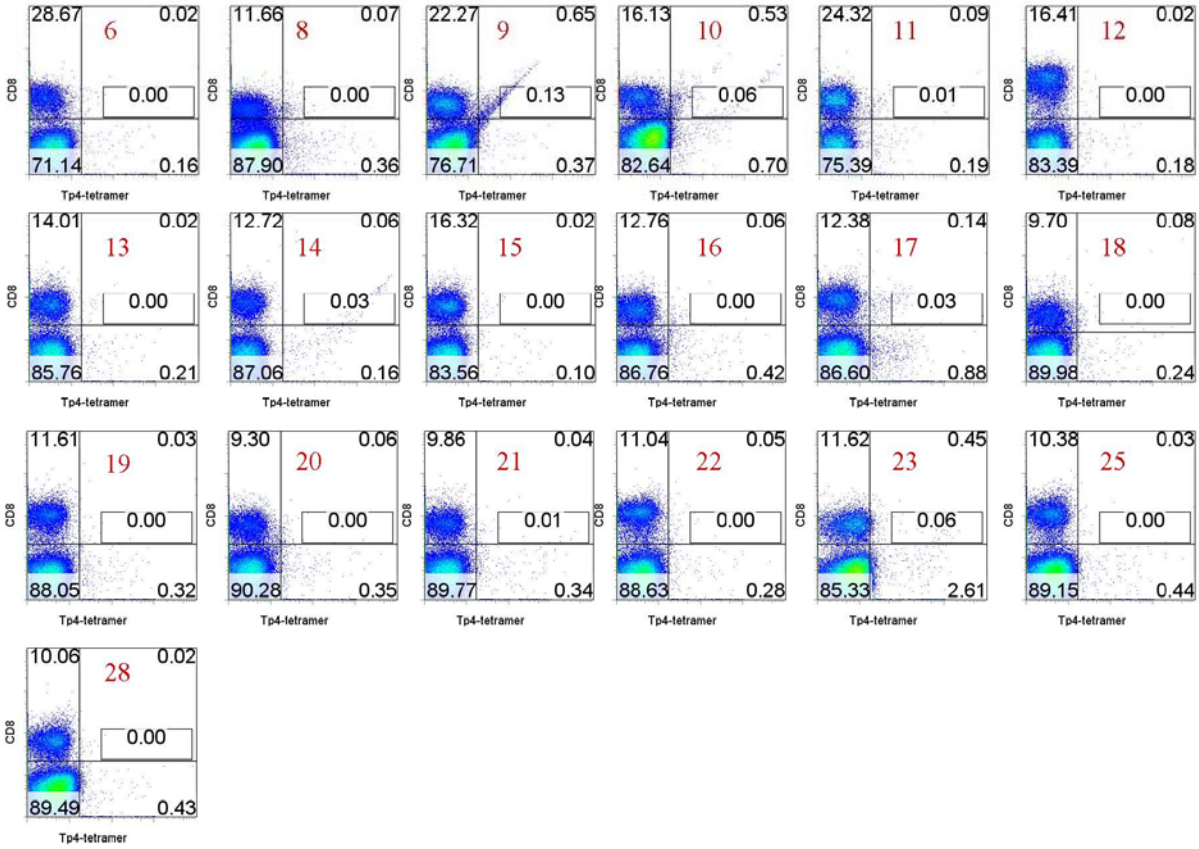
BB001 Tp4-tetramer^{+} CD8^{+} T cells (post immunisation)



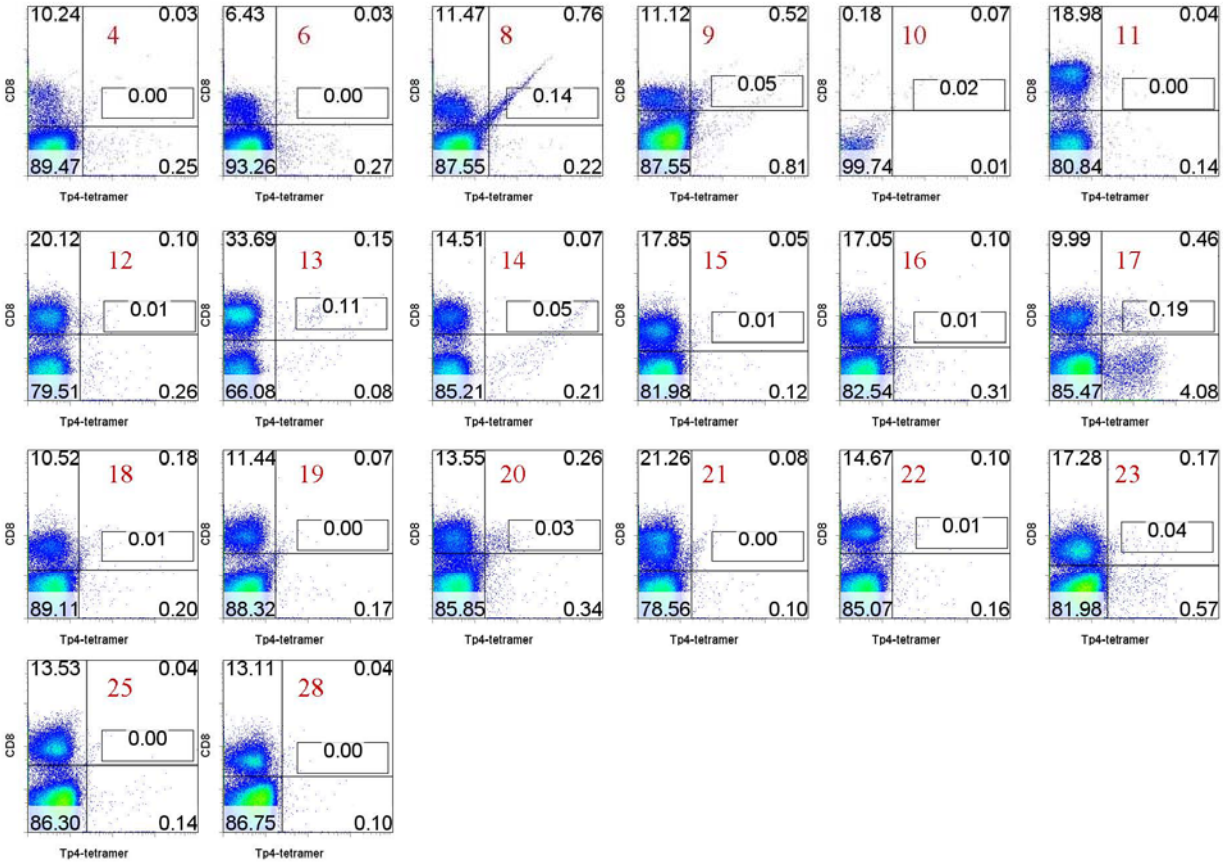
BB0069 Tp4-tetramer^{+} CD8^{+} T cells (post immunisation)



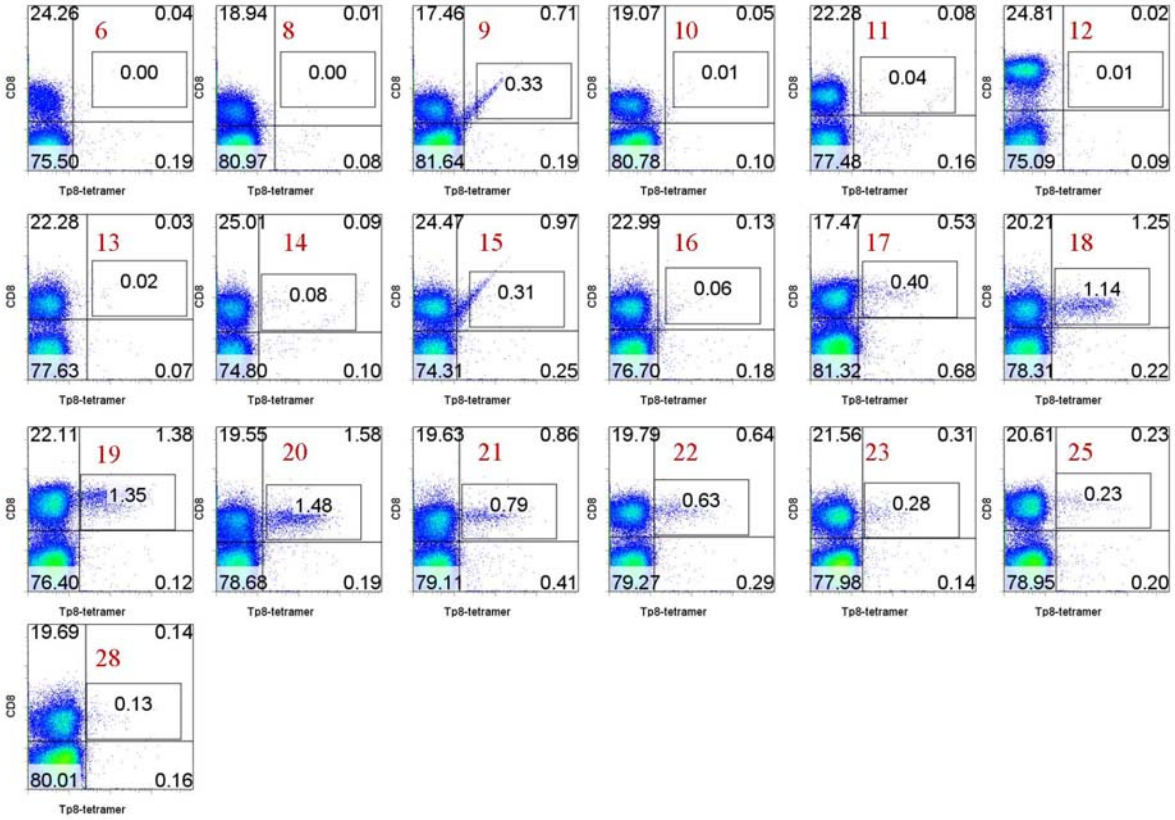
BB0070 Tp4-tetramer^{+} CD8^{+} T cells (post immunisation)



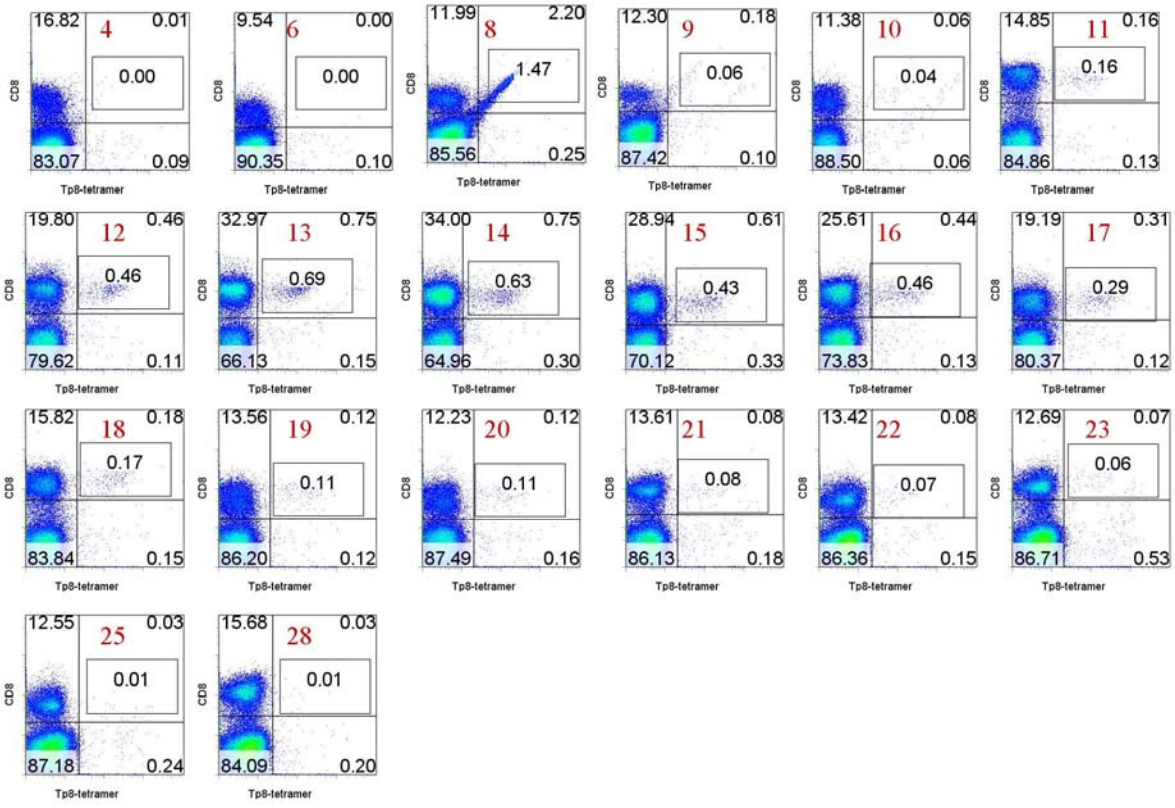
BB169 Tp4-tetramer^{+} CD8^{+} T cells (post immunisation)



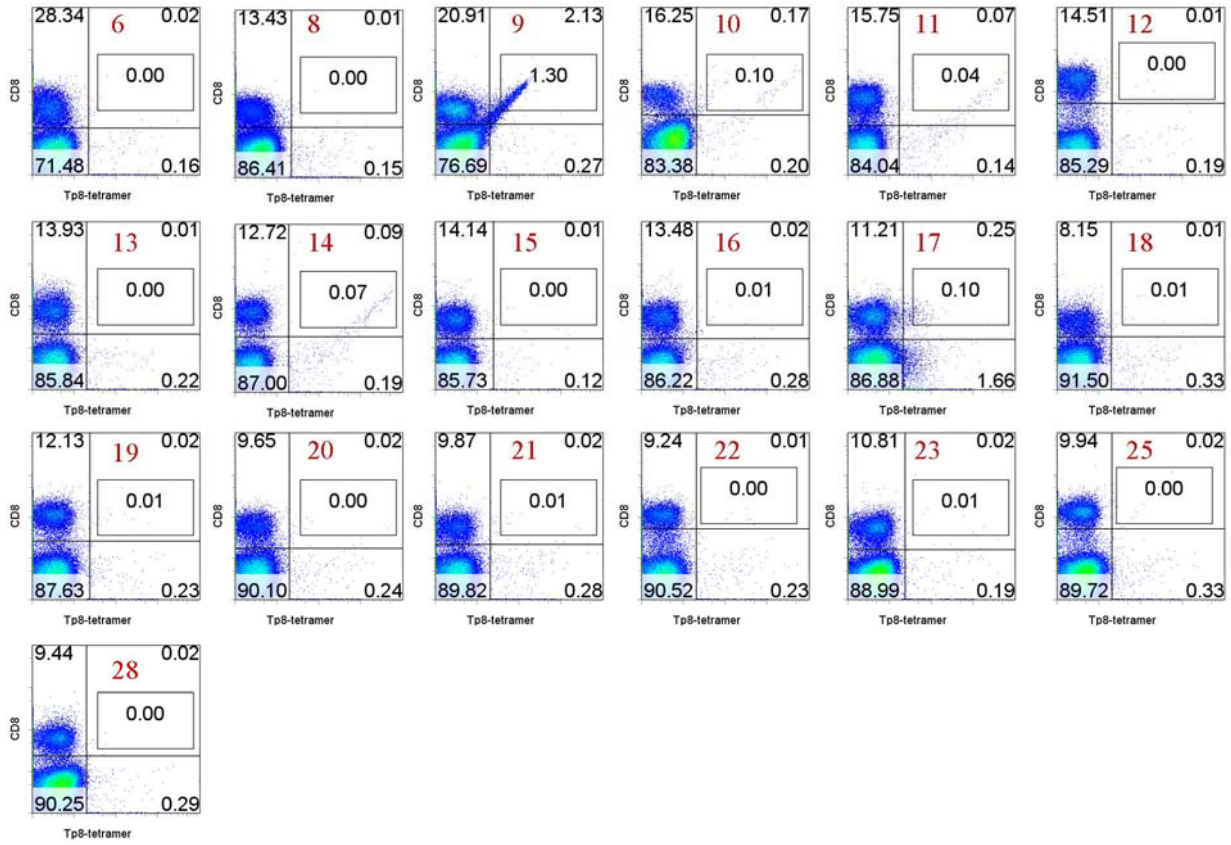
BB001 Tp8-tetramer^{+} CD8^{+} T cells (post immunisation)



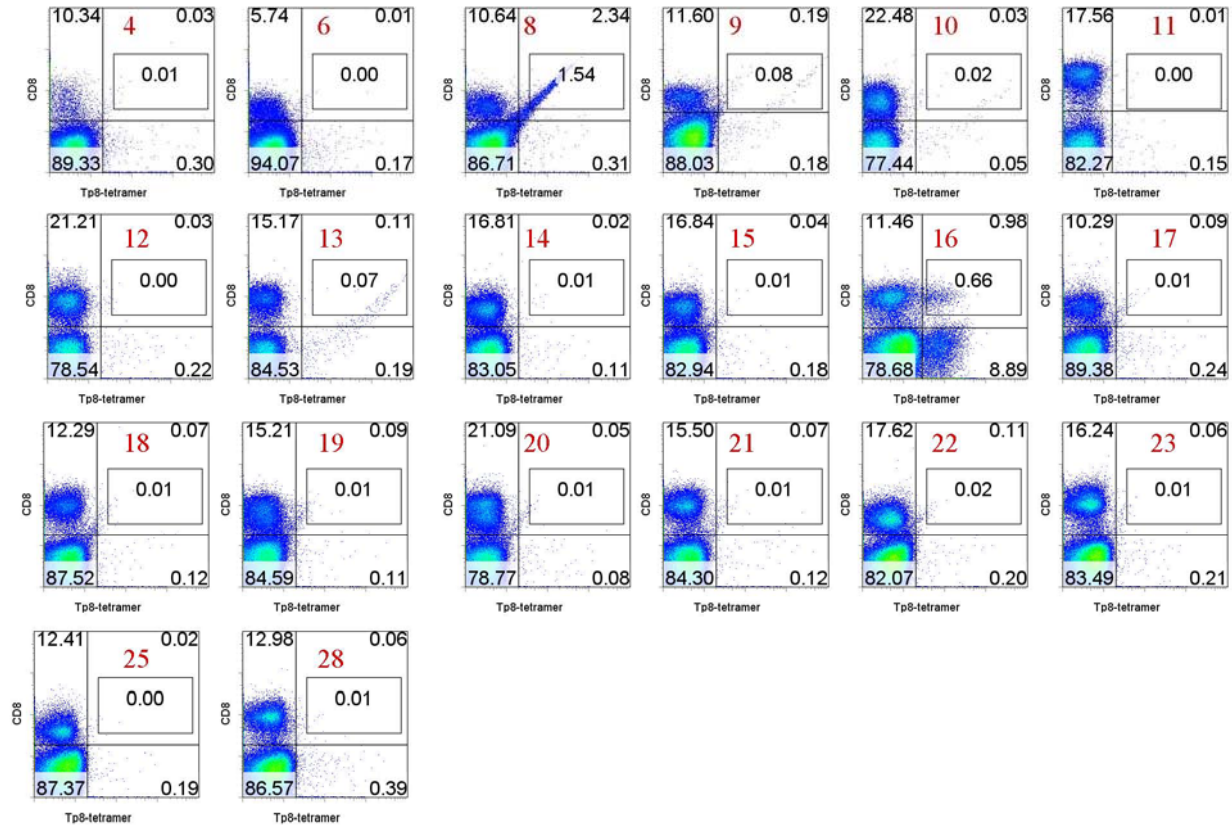
BB069 Tp8-tetramer^{+} CD8^{+} T cells (post immunisation)



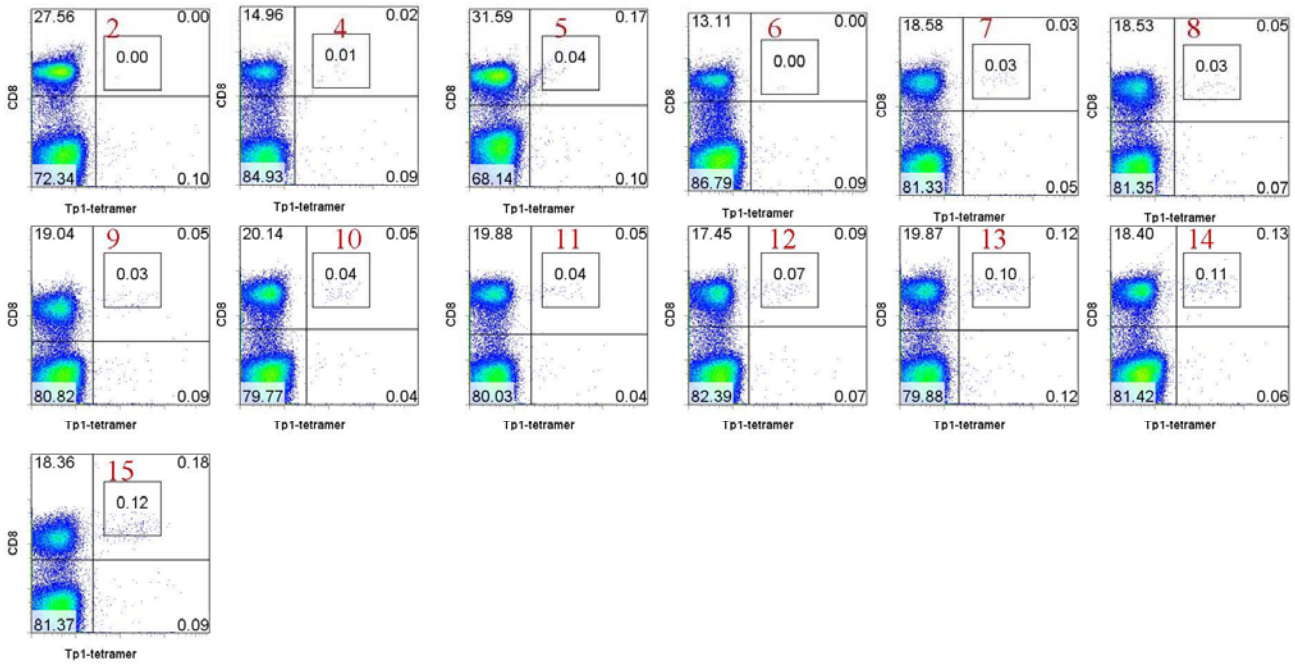
BB070 Tp8-tetramer^{+} CD8^{+} T cells (post immunisation)



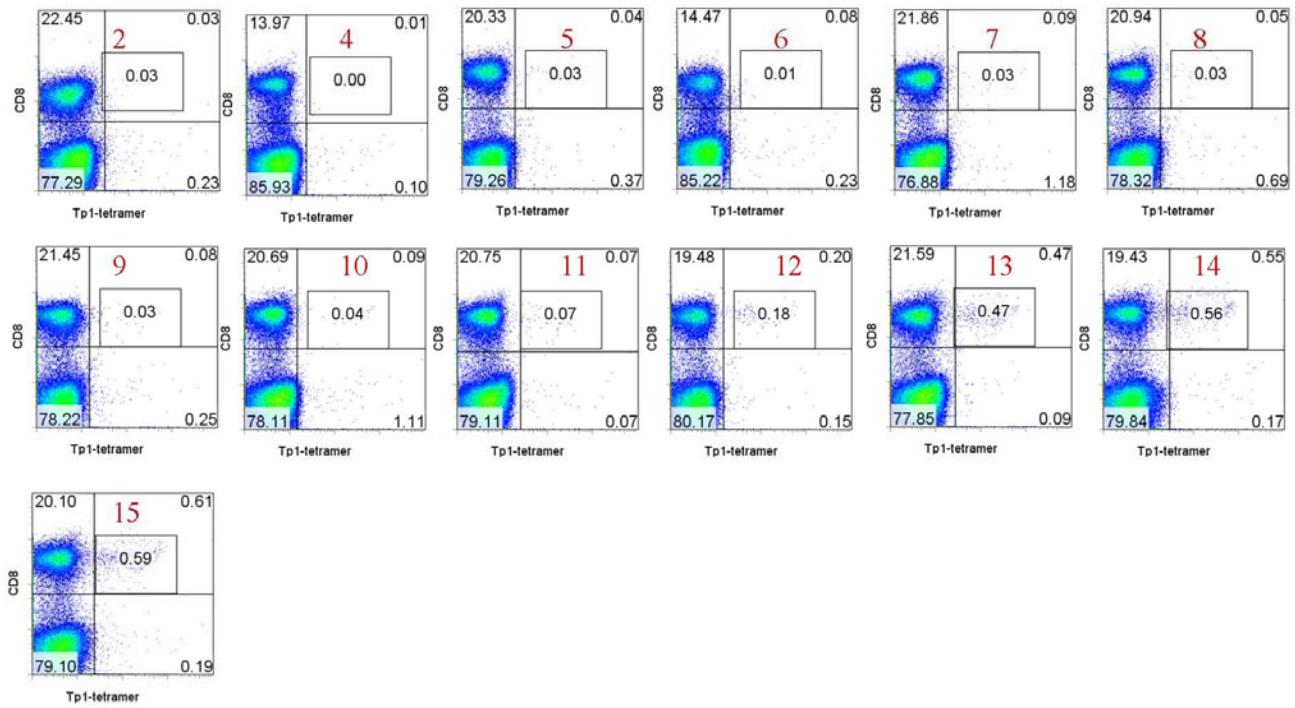
BB169 Tp8-tetramer^{+} CD8^{+} T cells (post immunisation)



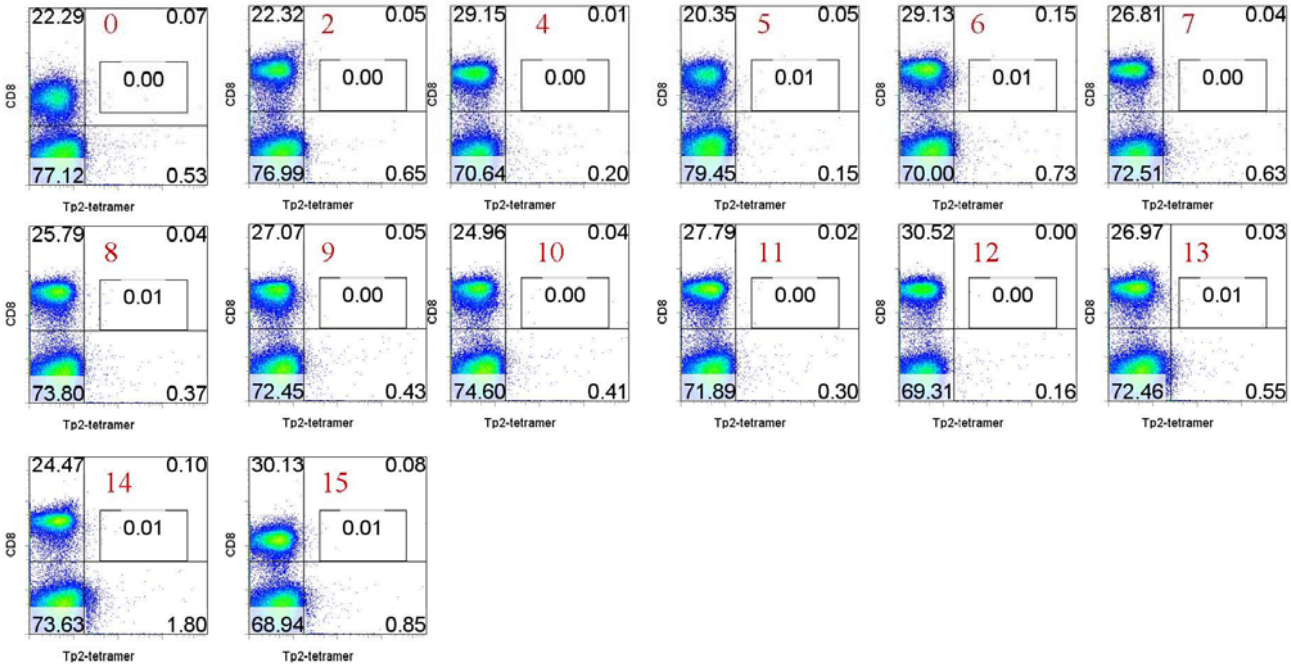
BB001 Tp1-tetramer^{+} CD8^{+} T cells (post challenge)



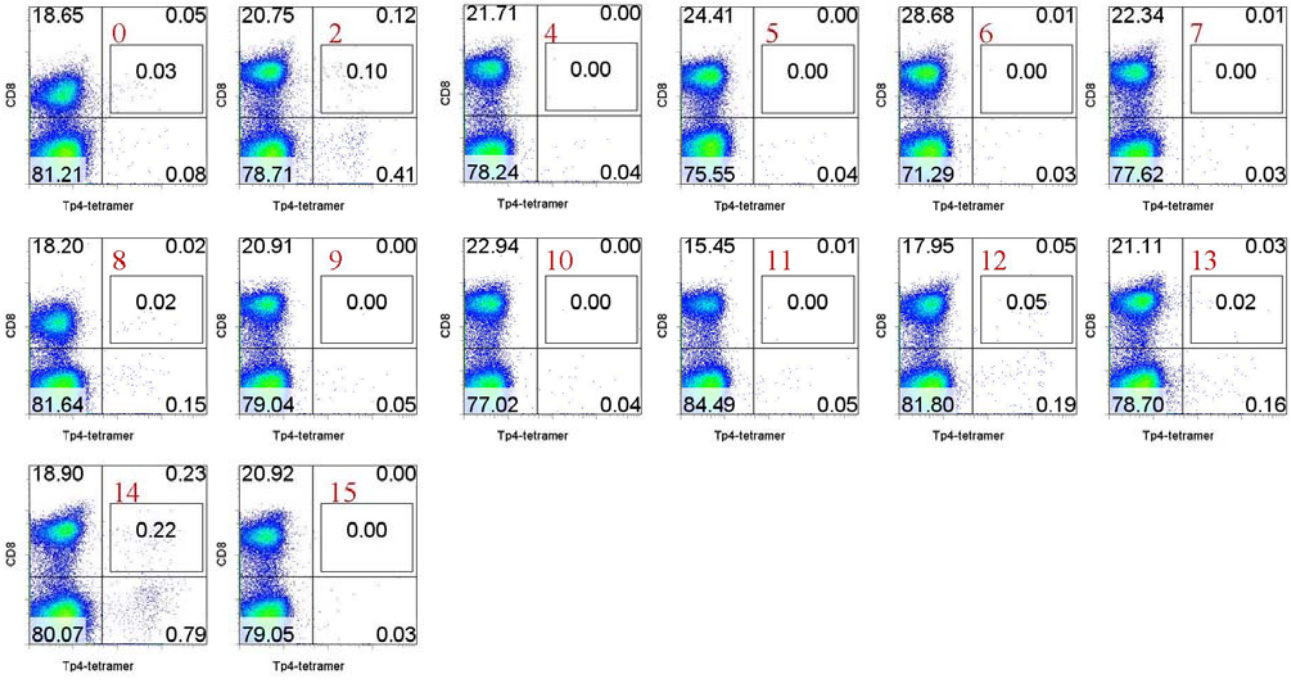
BB007 Tp1-tetramer^{+} CD8^{+} T cells (post challenge)



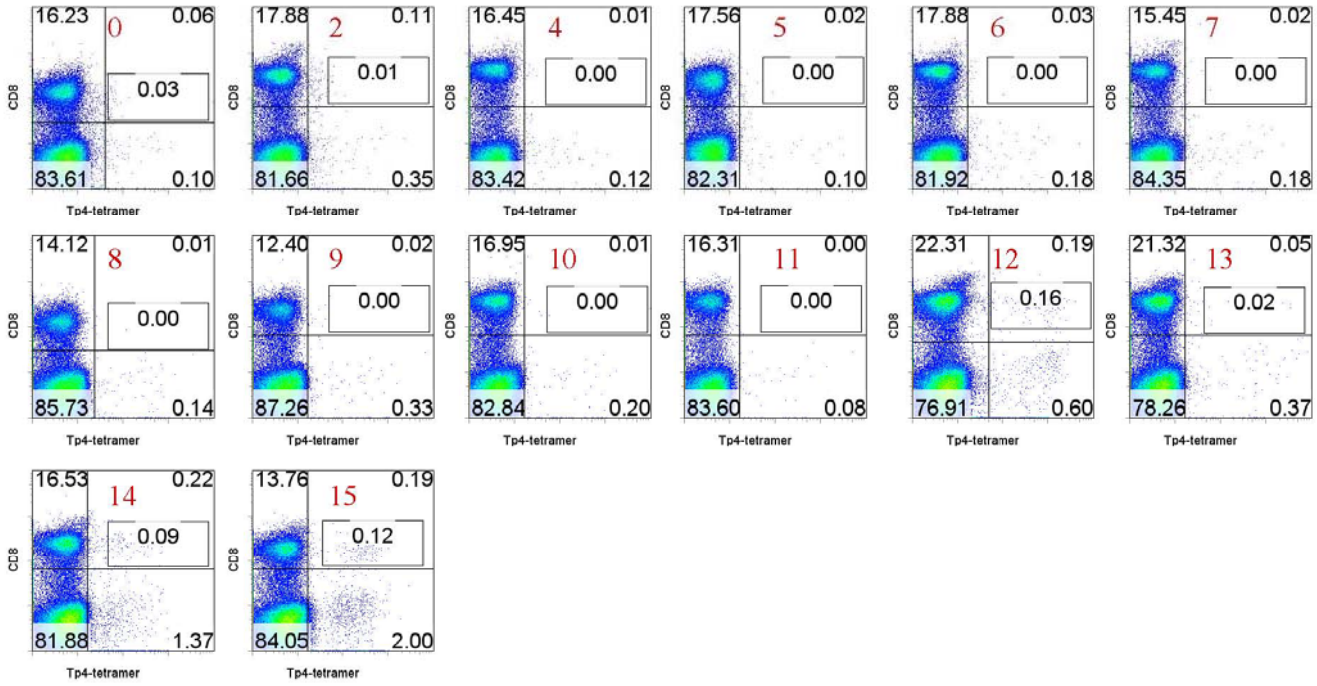
BB006 Tp2-tetramer^{+} CD8^{+} T cells (post challenge)



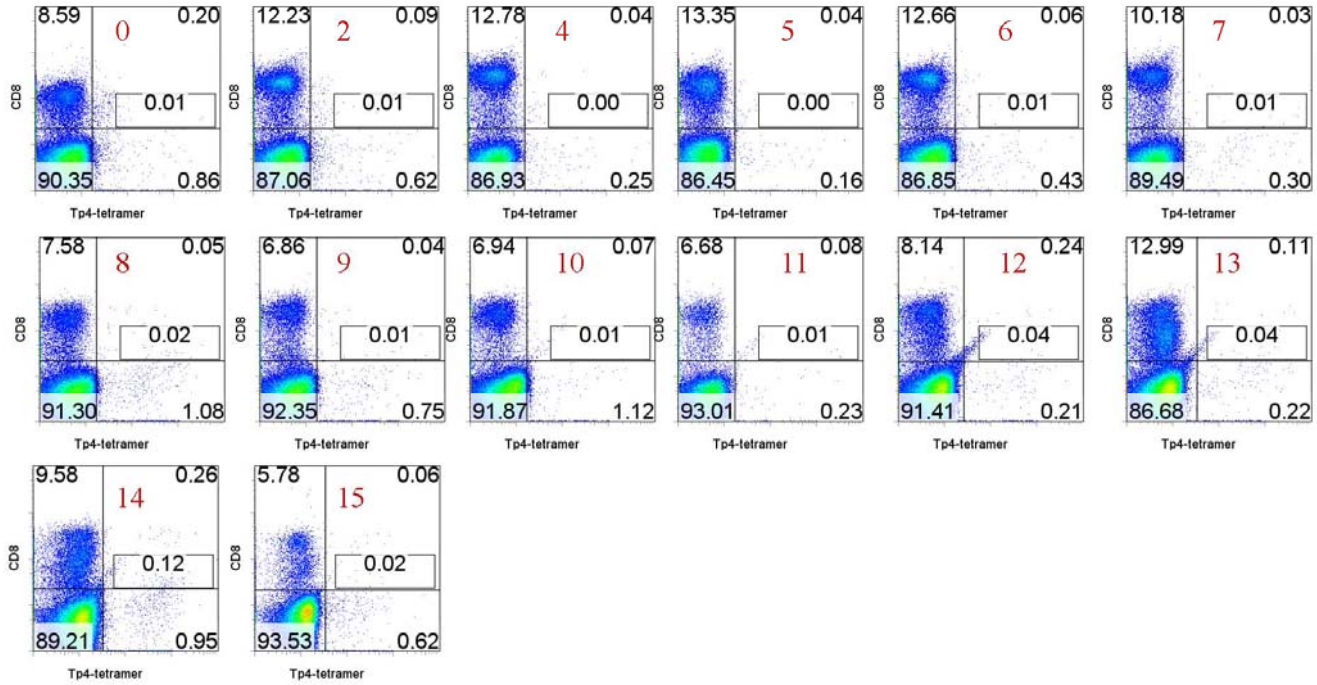
BB001 Tp4-tetramer^{+} CD8^{+} T cells (post challenge)



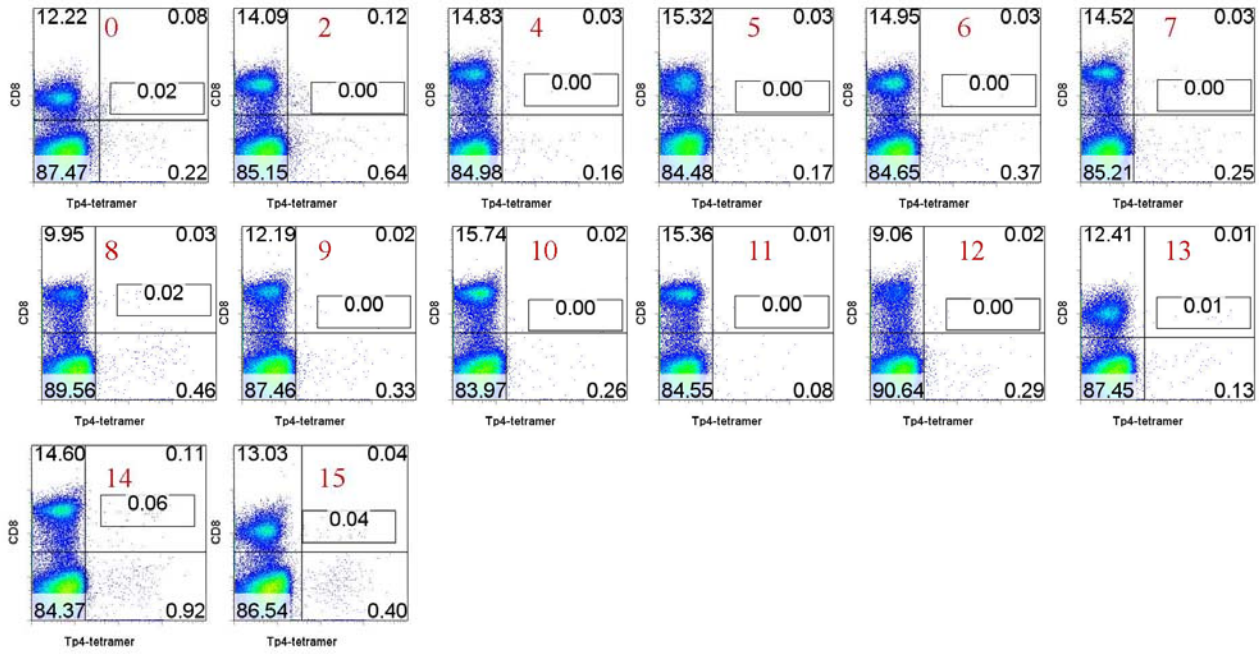
BB069 Tp4-tetramer^{+} CD8^{+} T cells (post challenge)



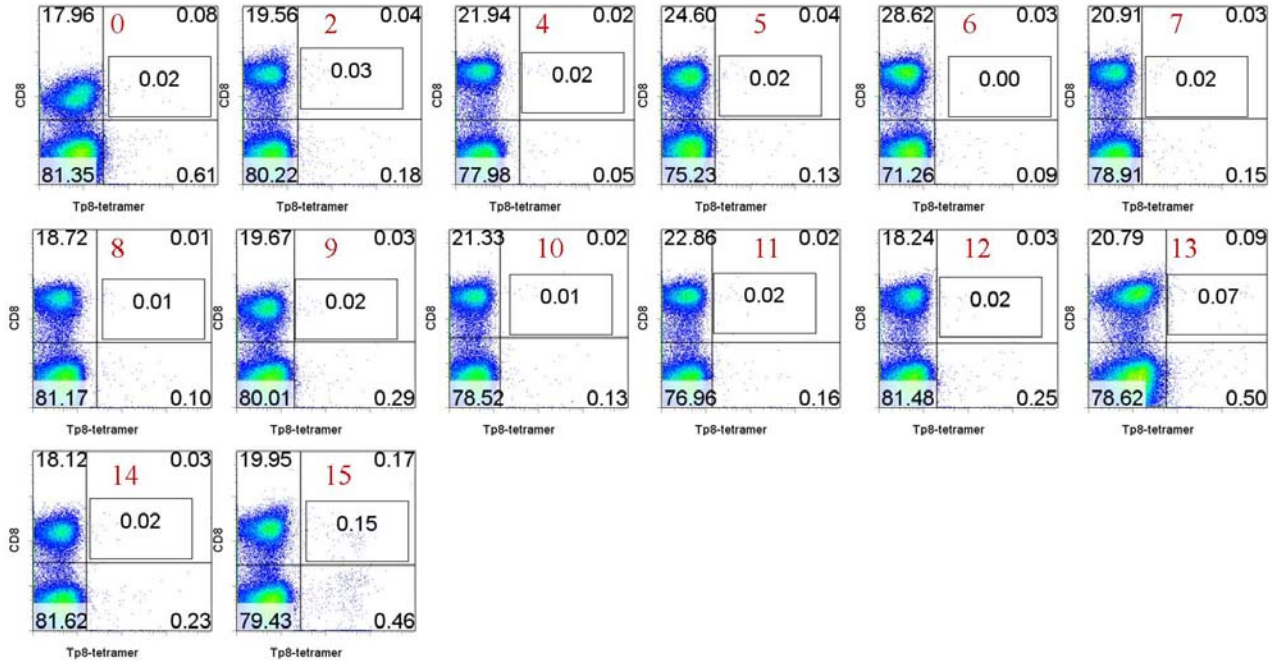
BB070 Tp4-tetramer^{+} CD8^{+} T cells (post challenge)



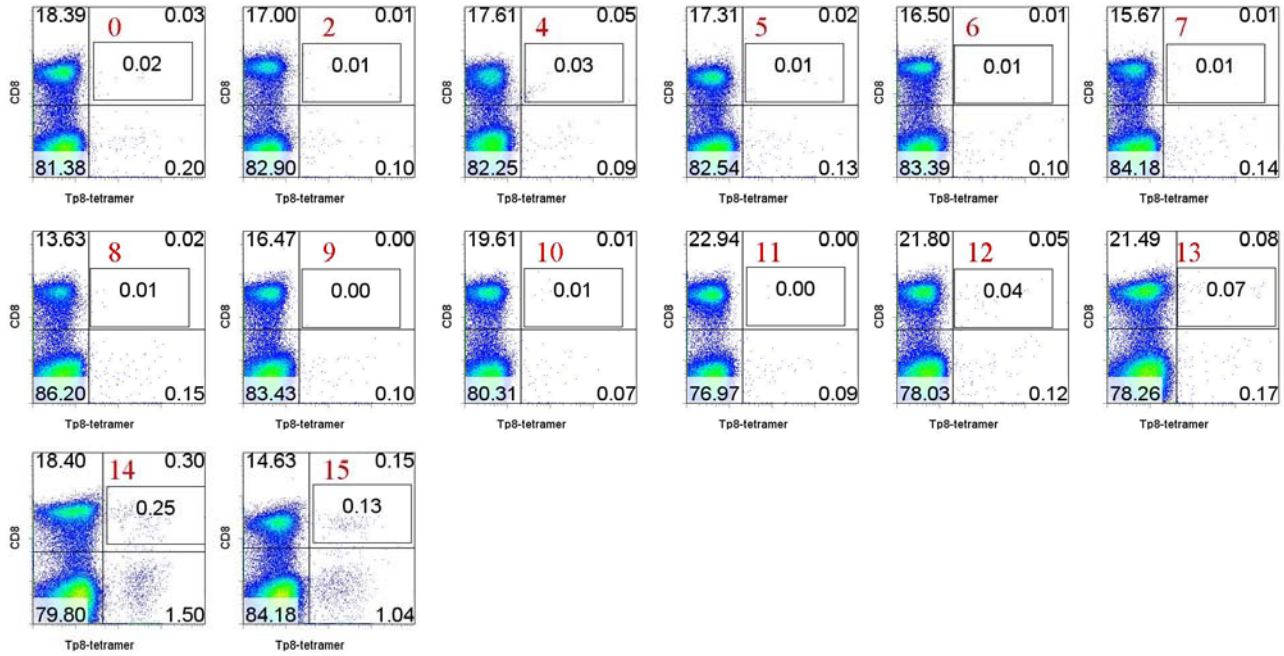
BB0169 Tp4-tetramer^{+} CD8^{+} T cells (post challenge)



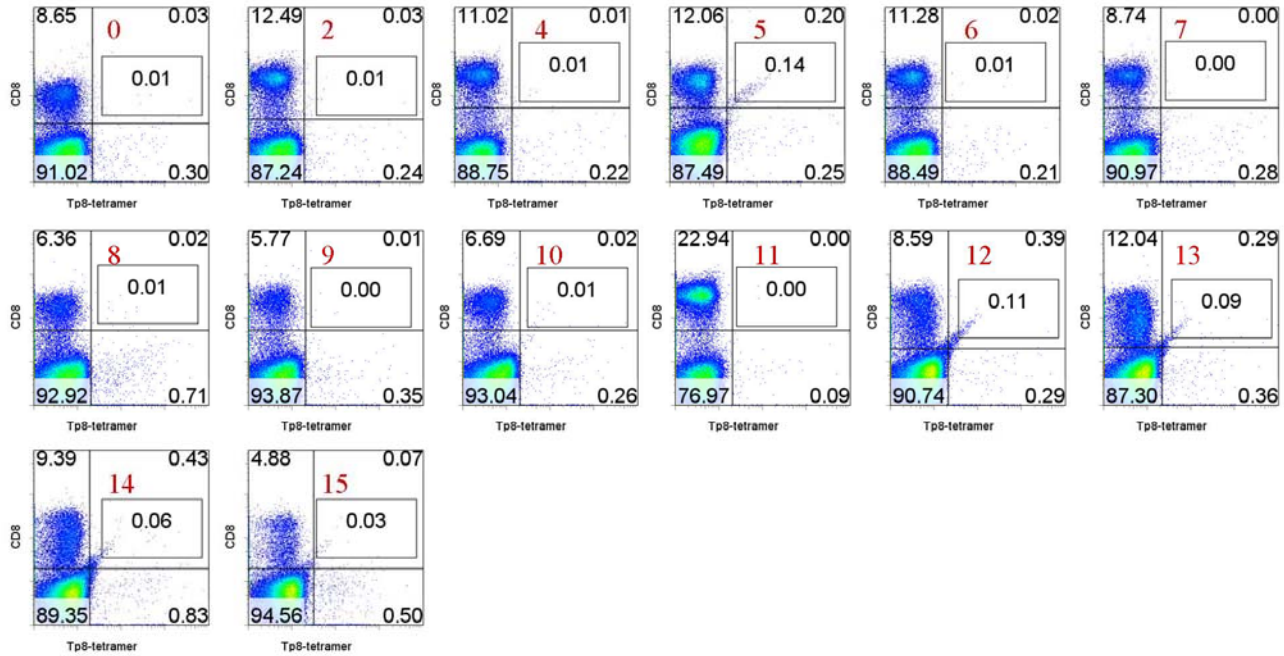
BB001 Tp8-tetramer^{+} CD8^{+} T cells (post challenge)



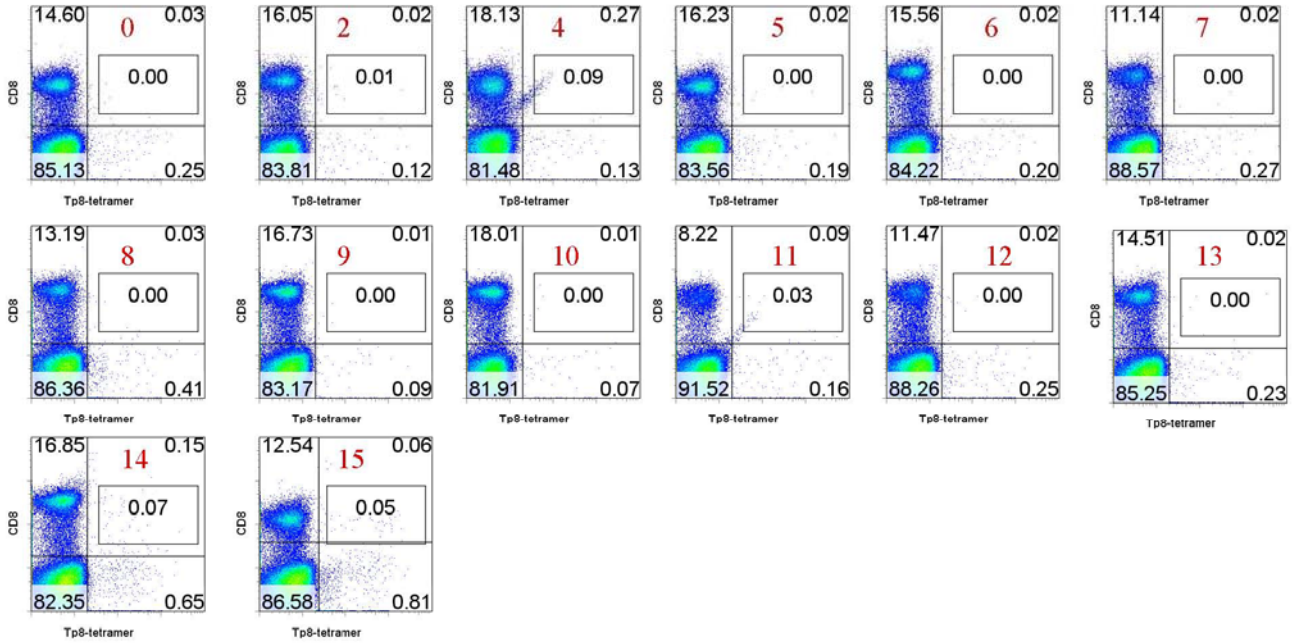
BB069 Tp8-tetramer^{+} CD8^{+} T cells (post challenge)



BB070 Tp8-tetramer^{+} CD8^{+} T cells (post challenge)



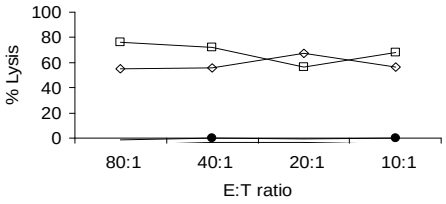
BB169 Tp8-tetramer^{+} CD8^{+} T cells (post challenge)



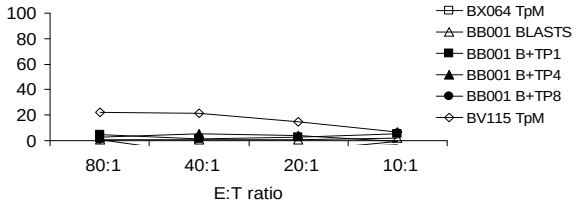
Appendix XI: Cytotoxic activity against *Theileria parva* infected cells and peptide-pulsed lymphoblasts following immunisation and challenge

Cytotoxic activity was determined in primary bulk cultures derived from PBMC harvested on day 12 post-immunisation and day 14 post-challenge. No lytic activity was detected in PBMC cultures of BB169 hence data is not shown. BB070 is *T. parva* non-immune and data was based on PBMC collected post-challenge infection. BX064 is *T. parva* immune and was used as a post-challenge positive control.

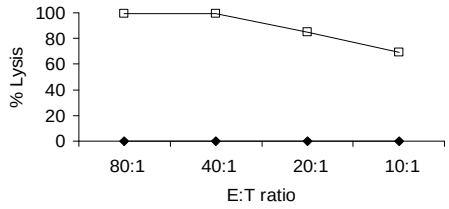
BB001: Cytotoxic activity against *Theileria parva* infected cells and peptide pulsed lymphoblasts post-immunisation



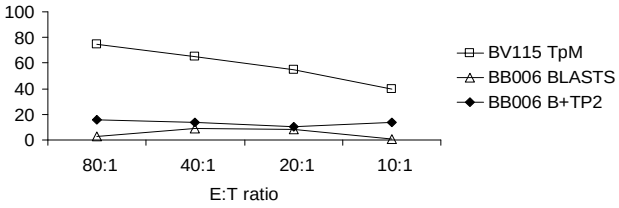
BB001: Cytotoxic activity against *Theileria parva* infected cells and peptide pulsed lymphoblasts post-challenge



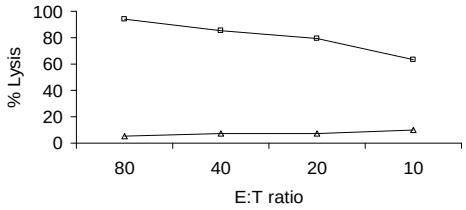
BB006: Cytotoxic activity against *Theileria parva* infected cells and peptide pulsed lymphoblasts post-immunisation



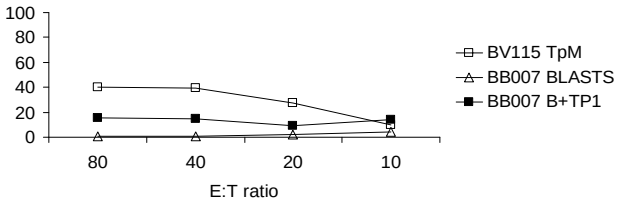
BB006: Cytotoxic activity against *Theileria parva* infected cells and peptide pulsed lymphoblasts post-challenge



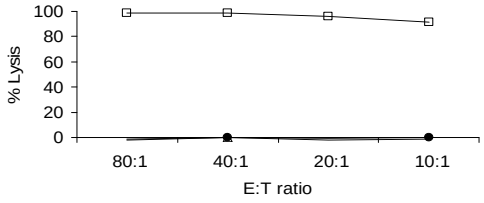
BB007: Cytotoxic activity against *Theileria parva* infected cells and peptide pulsed lymphoblasts post-immunisation



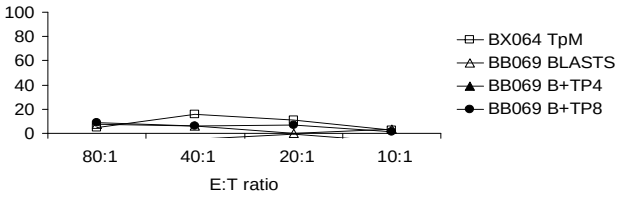
BB007: Cytotoxic activity against *Theileria parva* infected cells and peptide pulsed lymphoblasts post-challenge



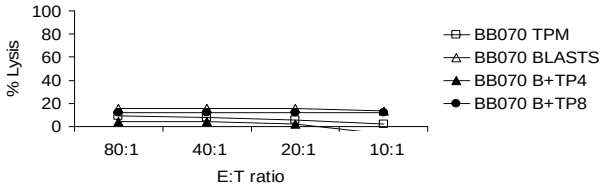
BB069: Cytotoxic activity against *Theileria parva* infected cells and peptide pulsed lymphoblasts post-immunisation



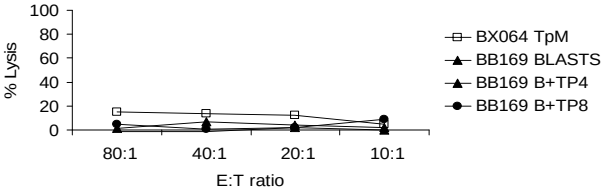
BB069: Cytotoxic activity against *Theileria parva* infected cells and peptide pulsed lymphoblasts post-challenge



BB070: Cytotoxic activity against *Theileria parva* infected cells and peptide pulsed lymphoblasts post-challenge



BB169: Cytotoxic activity against *Theileria parva* infected cells and peptide pulsed lymphoblasts post-challenge



BX064: Cytotoxic activity against *Theileria parva* infected cells and peptide pulsed lymphoblasts post-challenge