Characterization of Antigen Specific Cytotoxic T-cells to *Theileria parva*: Evaluation of Biomarkers and Cytotoxicity Potential

Jerome Wendoh Milimu

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

Signature …….. Date……...

Jerome Wendoh Milimu

This thesis has been submitted for examination with our approval as University Supervisors.

Signature……. Date……

Prof. Rebecca Waihenya

JKUAT, Kenya

Signature……. Date……

Dr. Lucilla Steinaa

ILRI, Kenya

Signature……. Date……

Dr. Gerald M. Mkoji

KEMRI, Kenya

DEDICATION

I dedicate this thesis to my dear wife, Susan Wendoh, our parents, Mr. and Mrs. Austin and Margaret Manyonje together with Mr and Mrs. Ezekiel and Luciana Muhkaye. And not forgetting our siblings Maureen, Paulo and Franklin. Thank you all for your support.

"Family is not an important thing, it's everything"

Michael J. Fox

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LIST OF ABBREVIATIONS AND ACRONYMS

ACUC	Animal Care and Use Committee
BCIP	5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt
BoLA	Bovine Leukocyte Antigen
BSA	Bovine Serum Albumin
CD	Cluster of Differentiation
CTL	Cytotoxic T-cell
ECF	East Coast Fever
ELISPOT	Enzyme linked immunosorbent spot
ERC	Ethical Review Committee
FACS	Fluorescence Activated Cell Sorting
IFN	Interferon
ILRI	International Livestock Research Institute
ITM	Infection Treatment Method
ITROMID	Institute of Tropical Medicine and Infectious Diseases
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KEMRI	Kenya Medical Research Institute.
Kshs	Kenya Shillings
MA	Massachusetts
MAb	Monoclonal Antibody
MHC	Major Histocompatibility complex
MO	Missouri
NBT	nitro-blue tetrazolium chloride
NK	Natural Killer Cells
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
RPMI	Roswell Park Memorial Institute
SSC	Scientific Steering Committee.
TpM	*Theileria parva* Muguga Infected lymphocytes
TCR	T-cell Receptor
UK	United Kingdom.

ABSTRACT

Theileria parva is a protozoan parasite that causes the disease East Coast fever in cattle leading to major economic losses in Eastern and Southern Africa including Kenya. A live vaccine against the parasite exists normally administered by subcutaneously injecting live parasites simultaneously with long acting oxytetracycline antibiotic. Nevertheless, challenges such as requirement of cold chain storage, difficult production procedures and the live vaccine being potentially lethal, makes it vital to explore other vaccine alternatives including inactivated vaccines and recombinant subunit vaccines. This study seeks to provide scientific output that will help in continued effort to develop new vaccine strategies. The mainstay of this exploration is a much better understanding of the CD8+ T-cells which are believed to be the main line of defence in East Coast fever. Hence it is essential to generate a profile of the *T. parva* specific CD8+ killer cells. In this study the phenotypic and functional characterization of these *T. parva* specific CD8+ Cells was determined by; i) Expression of surface and intracellular effector biomarkers by FACS analysis, ii) Degree of cell activation by secretion of interferon γ by ELISPOT analysis, and iii) Cytotoxic potential of CD8+ T-cells against *T. parva* infected cells was done in comparison to γδ T-cells. Blood samples were taken from the A18 BoLA type experimental cattle immunized with the live sporozoite vaccine. Bulk CTL and purified CTL cell lines were then generated from the experimental animals. From this study various surface and intracellular biomarkers found on *T. parva* specific CD8+ T-cells were identified. These included; CD2, CD3, CD4, CD25, CD44, CD45RO, CD62L, CD71, WC1, γδTCR, Perforin, FasL. Two of these markers indicated the presence of another T cell subsets γδ T-cells (WC1 and γδ TCR). It was observed from three cell lines (BF076, BF077, and BF079) that the degree of activation of CD8+ T-cells to *T. parva* antigen $Tp1_{214\text{-}224}$ as compared to γδ T-cells was not significantly different ($P > 0.05$). The CD8+ T-cells exhibited significant cytotoxicity to *T. parva* infected lymphocytes while the γδ T-cells exhibited no measurable cytotoxicity. The study revealed that two of the activation markers, perforin and CD25 are possible surrogate markers for cytotoxicity, which is

known to correlate with immunity to ECF infection. This study provided additional tools for characterizing cell-mediated immunity to *T. parva* induced by recombinant parasite antigens.

CHAPTER ONE

INTRODUCTION

1.1. Introduction and background information

Over 20% of people in sub-Saharan Africa are classified as living below the poverty line and many of these people reside in rural areas where they are dependent on small scale livestock keeping for their livelihoods (De Leeuwav *et al*., 1995). Infectious diseases have a major impact on livestock production in these regions. One of the most important diseases of cattle is East Coast fever (ECF) caused by a tick-borne parasite *Theileria parva (*Mukhebi, 1992; Perry, 2009).

Theileria parva (Theiler, 1904) is an intracellular protozoan parasite of the Family *Theileriidae*, Subphylum *Apicomplexa* and Phylum *Myzozoa* (Morrison *et al*., 2006). The taxonomy of *T. parva* is yet to be fully resolved especially at the sub species level, but advances have been made in characterizing different strains. *T. parva* has a restricted host range and is the most pathogenic of all *Theileria* species. The disease is present in 16 countries in eastern and southern Africa and it is the most economically important in 11 of these countries (**Figure 1.1**). This distribution corresponds to the range of its main tick vector, *Rhipicephalus appendiculatus.*

Estimates of economic losses due to ECF exceed 300 million US dollars annually with about a million cattle dying annually *(*Mukhebi, 1992; Perry, 2009). The disease primarily adversely affects small scale farmers who do not have access to available vaccines. High cost of tick control precludes these farmers. The exotic cattle breeds which are increasingly being used to meet the demand for milk production are more susceptible than the indigenous breeds.

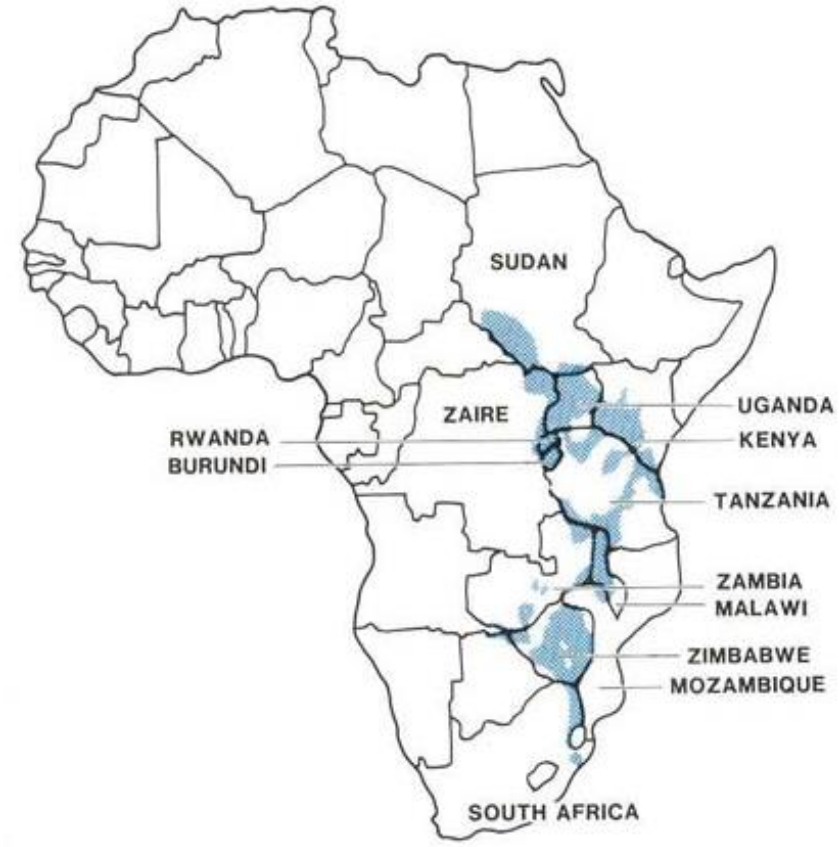

Figure 1.1: Distribution range of *T. parva* and the tick vector in Africa shown in blue (Perry & Young, 1992).

1.1.1 Life cycle of *T. parva*

In the life cycle of *T. parva* (**Figure 1.2**), ticks start by transmitting *T. parva* as infective sporozoites. The binding of the sporozoite to the lymphocyte is a passive ligand-receptor mediated process (Fawcett *et al.*, 1982). The developing schizont stage then induces lymphoproliferation of the infected cell. This schizont achieves this by associating with the mitotic spindle of the lymphocyte (Hulliger *et al*., 1964)

Among initial changes in host after infection is increase in lymphoblast population, making up 25% of all lymphocytes in the drainage lymph node (Emery *et al*.,

1981b). In addition, there is a cytokine storm, leading to rapid multiplication of the parasite (McKeever *et al*., 1997).

A proportion of schizonts further differentiate into the next invasive stage called merozoites. These then invade erythrocytes where they differentiate into piroplasms that are then taken up by ticks during feeding (Shaw & Tilney *et al*., 1992).

The piroplasms then further differentiate into the sexual stages (microgametocyte/ macrogamete) inside the erythrocyte after being ingested by ticks. When the erythrocytes are lysed in the tick gut, these are released (Schein *et al*., 1977). A zygote is the formed by the sygamy of micro- and macro- gamete.

What follows is the rapid differentiation to a kinete form (Mehlhorn *et al*., 1978). The kinete penetrates the salivary gland and develops into a sporoblast. Then sporozoites form and the cycle begin all over again.

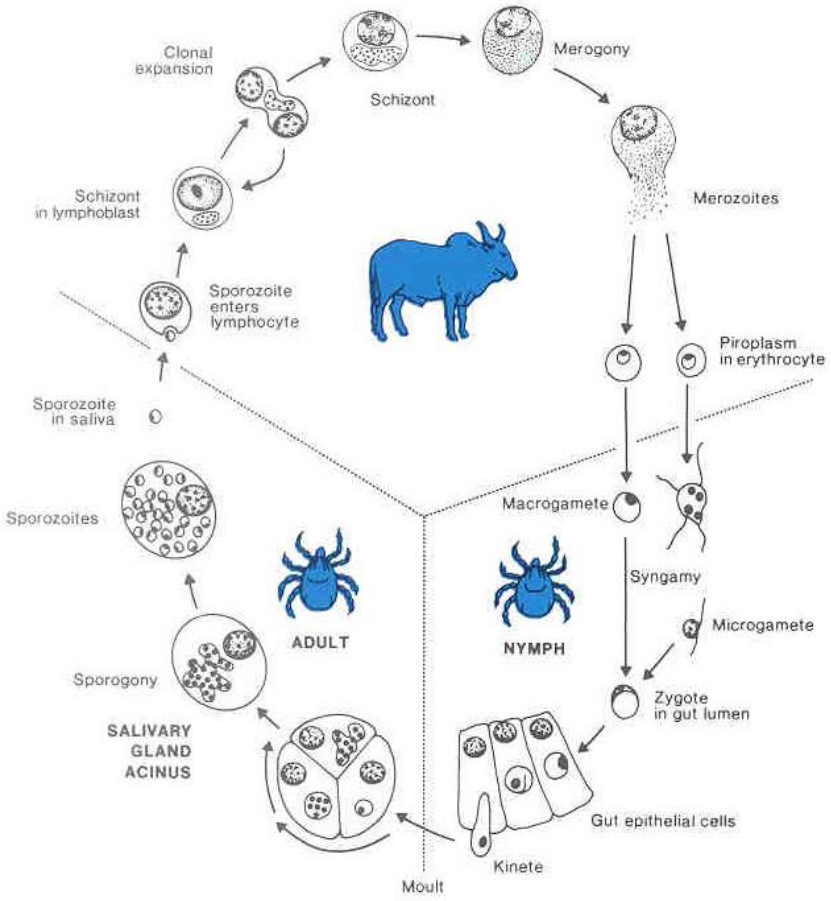

Figure 1.2: Life cycle of *T. parva* (Perry & Young, 1992).

1.1.2 Pathogenesis of East Coast fever

Cattle infection with *T. parva* usually leads to East Coast fever (ECF) which is an acute lymphoproliferative disease. Sporozoites are inoculated into the bovine host between one to three days after tick attachment. Disease is characterised in its initial stages by swelling of one of the parotid lymph nodes, the node draining the ear and eye surface which are the predilection feeding sites of the tick vector.

The incubation period is between 7 and 10 days following natural infection with fever (39.5°C or higher) from about day 10 (Geysen *et al*., 2000). At about 8 days post infection, schizont-infected cells are detectable in the lymph node which drains

the site of inoculation. Parasitized cells can be detected in other lymphoid tissues throughout the body 2 to 3 days after. The degree of parasitosis rises with the progress of infection, and after an additional 5 to 7 days over 30% of lymph node cells can be parasitized (Mckeever & Morrison, 1990).

In an acute infection, schizont infected cells disseminate to all lymph nodes, secondary lymph nodes and other organs. Schizonts are found in the interstitial tissues of the lungs, the gastro-intestinal tract, the kidneys and most other tissues. In the terminal stages of the disease, the temperature may fall below normal, severe pulmonary oedema may develop at the same time of a generalised non-specific lymphocytolysis, suggestive of severe immunopathological events (Visser *et al*., 1995), thereby often causing death of the host. In the course of 14 to 21 days of inoculation the majority of experimentally-infected animals die in the lack of treatment.

In post-mortem examination, vast numbers of parasitized cells are found throughout the lymphoid system. The likely cause of death commonly is the infiltration of other tissues, particularly the lungs and gastro-intestinal tract, with parasitized lymphoblasts, and severe pulmonary oedema and associated dyspnoea are commonly observed late in infection (Irvin *et al*., 1987). A notable feature of the histological appearance of lymphoid tissues at the time of death is a marked depletion of lymphocytes (Mckeever *et al*., 1990), ostensibly the result of the extensive lymphocytolysis which can be observed in the later stages of the disease and which is not restricted to parasitized cells (Irvin *et al*., 1987). It is probable that the invasion of haemopoietic tissues by parasitized cells during the course of the disease also adds to the reduction of cells in lymphoid organs.

The isolation of lymphocyte subsets using the flow cytometer and subpopulation-specific monoclonal antibodies (MAb) has allowed the identification of the cell types which the parasite is capable of infecting *in vitro*. It is clear that all subsets of lymphocytes including those expressing neither B nor T-cell surface antigens, are

susceptible to infection *in vitro*, while it is not possible to infect monocytes or granulocytes (Baldwin *et al*., 1988).

However, phenotypic studies of parasitized cells isolated from infected cattle suggest that very few arise from B cells, and while substantial numbers of infected CD8+ cells are observed. The majority of parasitized cells appear to be derived from the CD4 + subpopulation of T lymphocytes (Mckeever *et al*., 1990).
The substantial effect on the immune system which results from the predilection of the parasite for lymphocytes is of obvious significance to the potential of the infected animal to respond successfully to infection.
Parasitized cells in culture have been observed to induce T-cell growth factor (TCGF) activity. They are also known to stimulate the proliferation of naive lymphocytes *in vitro* (Mckeever *et al*., 1990). Such activity *in vivo* might be expected to result in the proliferation of non-infected lymphocytes. This could give rise to a cascade of lymphokine-mediated effects. In addition, when alloreactive T-cell clones are infected with *T. parva*, they have been observed to retain the potential to kill in an antigen-specific manner for 3-4 months (Geysen *et al*., 2000). .

1.2 Statement of the problem

Vaccination offers a sustainable means of controlling East Coast fever. Cattle can be immunized against the disease by infection with a pre-determined dose of a mixture of live parasites and simultaneous drug treatment with oxytetracycline, a long-acting antibiotic.

Although this infection treatment method (ITM) can be highly effective and can provide life-long immunity, it has some limitations: the live parasites need to be stored in liquid nitrogen; the cost to treat an animal is relatively high; expert veterinary supervision is required to administer ITM; and the process to produce the parasites for inoculation is very time consuming – currently its takes about 18 months to produce each batch (Morrison *et al*., 2009).

Furthermore the live parasites are potentially lethal. The vaccines also have the potential to confer a carrier status to the immunized animals. This means that the lethal parasite can be transmitted to an unimmunized animal by ticks and this can be deadly. Due to the above difficulties associated with the live vaccine, there is an urgent need to develop an alternative vaccine (Morrison *et al*., 2009).

The focus of the present study is to better understand the role of the CD8+ T-cells which are believed to be the main line of defence in East Coast fever infection.
Attempts in testing new Cytotoxic T Lymphocytes (CTL) vaccines and antigens will include measurement of the cytotoxic response. However, CTL cytotoxicity assays are time consuming, may require use of radioactivity. They also require several restimulations when blood is used as source of lymphocytes. Hence other correlates of CTL cytotoxicity are desirable.

1.3 Justification of the study

Understanding the immunochemistry of CD8+ T-cell is vital since it is believed to be the main protective mechanism for East Cost fever. This mechanism is related to the adoptive transfer of CD8+ cells from immunized animals that have been shown to protect naïve animals from challenge. Furthermore, it has also been shown that the time point of recovery correlates with a peak of CD8+ cells in the blood of the infected animals. Therefore understanding the immunological characteristics of *T. parva* specific CD8+ T-cells is vital in the search of improved vaccine.

Characterization of *T. parva* specific CD8+ cells has so far only been performed on bulk CTL cultures and *in vitro* generated clones. The phenotype of the specifically induced CTL against *T. parva* is largely unknown. Several *T. parva* antigens have been identified which are presented by specific Bovine Leukocyte Antigen (BoLA) to CD8+ T-cells. Apart from the CD8+ CTL it has been shown that there are other killer cells found among the γδ T-cells isolation of which the majority of cells are CD4 and CD8+ negative.

Use of tetramer staining of the specific T-cells subsets allows characterization of these antigen specific T-cells by surface and intracellular biomarkers they express by FACS analysis. This tetramer technology enables investigation of the cells of interest without much *in vitro* manipulation. The activation of cytotoxic potential of γδ T-cells can further be investigated by use of the Enzyme Linked Immunosorbent Spot (ELISPOT) and CTL assay techniques.

The CD8+ T-cell profiles generated in the course of the present study will provide further parameters for evaluating efficacy improved vaccine against ECF.

1.4 Research questions

1. Which cell surface biomarkers and intracellular biomarkers are expressed on the antigen specific CD8+ T-cells in *T. parva* infection?
2. How does immune cell activation signified by secretion of IFN-γ compare between CD8+ cells and γδ+ cells in T. parva infection?
3. What is the cytotoxicity potential of CD8+ cells in comparison to γδ+ T-cells in *T. parva* infection? Can the activation markers CD25 and perforin serve as correlates for cytotoxicity?

1.5 Research hypothesis

T. parva specific CD8+ cells express unique surface and intracellular biomarkers. The immune activation and cytotoxicity potential of these *T. parva* Specific CD8+ T-cells is significantly different from that of γδ+ T-cells.

1.6 Objectives

1.6.1 General objective

To characterize antigen specific cytotoxic T-cells by evaluation of their expression of surface and intracellular biomarkers. Additionally assessing their status of activation and cytotoxicity potential in *T. parva* infection.

1.6.2 Specific objectives

1. To determine variety of cell surface and intracellular biomarkers namely; activation, maturation and memory biomarkers in antigen specific CD8+ T-cells in *T. parva* infection by FACS analysis.
2. To compare the relative extent of immune cell activation signified by secretion of IFN-γ between CD8+ T-cells and γδ+ cells in *T. parva* infection by ELISPOT assay.
3. To determine the relative contribution in cytotoxicity between CD8+ T-cells and γδ+ cells and whether perforin and CD25 are correlates of cytotoxicity.

These two markers (perforin and CD25) stained very strongly in preliminary studies.

CHAPTER TWO

LITERATURE REVIEW

Cattle that recover from ECF develop long-term immunity, aquiring complete protection against homologous parasite stock challenge and many cross protect against several different parasite isolates (Mckeever *et al*., 1990).

In challenge experiments, especially when using multiple lethal doses, transient but significant numbers of schizonts can often be seen, but immune responses slow down multiplication and prevent pathology. This protection declines over time in the absence of challenge, indicating that the immunity induced is partially protective, not preventing infection but directed against the schizont stage (Morrison *et al*., 1995a). Several experiments have demonstrated that immunity against *T. parva* is cell mediated and primarily based on CTL responses mounted against the parasite infected lymphoblast stage (McKeever *et al*., 1994; Morrison *et al*., 1995b). Both antigen-specific and non-specific cytotoxic cells have been demonstrated in infected animals.

Although most immune cattle mount strong responses to the parasite, only non-specific CTLs are found during the latter stages of lethal infections with no indication of the presence of specific CTLs during primary infections. It has further been found that T-cells transformed by *T. parva* were resistant to natural killer (NK) cell mediated lysis and LAK (lymphocyte activated killing) activity was too variable to be considered as the main protective mechanism (Van de Putte *et al*., 1992). The fact that *T. parva* infects immune effector cells, which might outgrow the initial T-cell population, adds to the complexity of the responses.

2.1 Antibody-mediated immune responses against *T. parva*

Cattle infected with *T. parva* mount a strong antibody response. These humoral responses however decline over months in the absence of challenge. Humoral

responses towards sporozoite antigens, such as the sporozoite surface coat protein p67 (Musoke *et al*., 1982) and the microsphere secreted protein referred to as the polymorphic immunodominant molecule PIM (Toye *et al*., 1995), have been studied in detail. Both p67 and PIM have been suggested to be involved in the entry process of sporozoites into lymphocytes, although an indirect action through steric hindrance of ligands by one of them could not be ruled out (Toye *et al*., 1995).

Serum from immune animals which have been repeatedly challenged with infected ticks neutralises the infectivity of sporozoites for cattle (Musoke *et al*., 1982). This suggests that antibody directed at the sporozoite stage of the parasite can be of relevance to protection. Although antibody responses against the schizont stage of the parasite are detectable in recovering animals around the time of the elimination of infection, and indeed provide the most reliable evidence of exposure to the parasite, these mechanisms are not considered to play a major role in protection. This is supported by the observation that animals inoculated with heat-killed schizont-infected cells or semi-purified schizont antigens were not protected against challenge in spite of producing titres of anti-schizont antibodies which were similar to those of conventionally immunised animals (Emery *et al*., 1981c).

There are several aspects of immunity against *T. parva* which suggest that immune responses directed at the schizont-infected cell play a major role in p`rotection. These include the apparent requirement for the development of this stage of the parasite for the successful induction of immunity, the common occurrence of a low schizont parasitosis before the elimination of infection by immune animals, and the observation that animals immunised by infection and treatment are solidly immune to challenge with up to 5×10^8 schizont-infected cells (Eugui *et al*., 1981, Wilson *et al*., 1950). This evidence, coupled with the lack of correlation between the presence of anti-schizont antibody and protection, has led to the belief that immunity to *T. parva* in cattle is mediated predominantly by cellular mechanisms directed at the schizont-infected cell.

2.2 Cell-mediated immune responses against *T. parva*

The elucidation of cellular immune responses of cattle infected with *T. parva* has been greatly facilitated by the development of techniques whereby bovine lymphocytes can be infected with sporozoites *in vitro* (Brown *et al*., 1973). It was demonstrated that bovine peripheral blood mononuclear cells (PBMC) proliferated *in vitro* to irradiated autologous parasitized lymphocytes (Pearson *et al*., 1979).

They also observed that a population of the responding cells killed autologous infected cell lines, and to a lesser extent, parasitized allogeneic cells. These results suggested that infection of the lymphocyte with *T. parva* gave rise to an antigenic change on the cell surface which was responsible for the generation of cell-mediated responses, but the possibility that the responses were directed against non-parasite antigens acquired by the cells during long-term culture could not be excluded. This question was resolved when it was reported that schizont-infected cells prepared from various lymphoid organs of lethally infected cattle stimulated the proliferation of autologous PBMC's which had been cryopreserved prior to infection (Emery *et al*., 1980). These initial observations prompted several investigations of *in vivo* cytolytic responses to infection or immunisation. Further studies on the cytolytic activity during the terminal stages of lethal infection and found that PBMC contained cells which killed several allogeneic infected cell lines and atyt mouse tumour cell line, but not autologous parasitized lymphocytes (Emery *et al*., 1981).

These responses were in contrast to those in animals undergoing immunisation or challenge, where cytolytic activity restricted to the autologous infected cell lines was detectable in PBMC around the period of remission of infection. The restriction to autologous cell lines of the cytolytic activity detected in immunised animals was suggested that its induction might be the result of an association of parasite determinants with MHC molecules on the cell surface. Serological typing for class I MHC in conjunction with knowledge of parentage allows the identification of animals that are MHC haplo-identical or which share individual class I antigens.

In a study which involved the assessment of cytolytic activity in the PBMC of ten immune heterozygous animals after challenge with homologous sporozoites, it became clear that activity in individual animals was restricted to infected targets derived from animals which shared MHC class I specificities (Morrison *et al*., 1987a) The observation that a MAb specific for a monomorphic determinant on bovine class I molecules was capable of significantly inhibiting this activity, while two class II-specific MAb had no effect, confirmed that class I MHC products were indeed the restricting elements. Another feature of these results was that in most of the animals the restriction of the response was biased towards one or other of the haplotypes. This effect was interpreted as being a reflection of a hierarchy in dominance among the BoLA products which restrict the response. A subsequent study of the phenotype of the cells which mediate this class I MHC-restricted cytolytic activity (Goddeeris *et al*., 1986) has demonstrated that they are CD8+ T lymphocytes.

Analysis of the parasite strain specificities of cytotoxic T-cell clones derived from animals immunised with the Muguga stock of the parasite revealed that the heterogeneity of the response which was observed between individual cattle was reflected at the clonal level. Thus clones prepared from animals whose *in vivo* response was restricted to the Muguga stock were found to be specific for Muguga-infected target T-cells (Goddeeris *et al*., 1986a; Morison *et al*., 1986). Similarly, those animals whose PBMC were capable of cytotoxicity either stock yielded at least some clones which were cross-reactive. An additional finding was that the set of clones from each animal showed a clear bias towards one MHC specificity as the restricting element. Indeed, only one animal yielded clones restricted by specificities from both haplotypes. These results provide evidence that by selecting a particular parasite epitope, the restricting MHC molecules can influence the parasite specificity of the cytotoxic T-cell response. It is believed that the antigen receptor of cytotoxic T-cells restricted by class I MHC products recognises antigenic peptides in association with the restriction element (Townsend *et al*., 1986).

2.3 MHC class I restricted CD8+ T-cell responses in East Coast fever

Major histocompatibility complex (MHC) class I restricted CD8+ T-cell responses have been shown to play a critical role in immune response against several protozoan infections including *Theileria* species infections (Mckeever *et al.,* 1999). These CD8+ T-cells target the schizont-infected lymphocytes, the pathogenic stage of *T. parva.* These cells have been observed to operate in at least two mechanisms: one is cytotoxicity and the other is by production of cytokines such as Tumour Necrosis Factor alpha (TNFα) (Vassalli *et al.,* 1992), interleukins and interferon gamma (IFN-γ) (Boehm *et al.,* 1997).

For CTL cytotoxicity, two pathways have been described: the granule exocytosis pathway (Henkart, 1994; Kagi *et al.,* 1994) and the Fas-Fas ligand Mechanism (Nagata 1999; Rouvier *et al.,* 1993). The cytokine interferon gamma (IFN γ) that is secreted by activated CD8+ T-cell plays a critical role in immune response. It has exhibited immunoregulatory activity affecting mechanisms such as T-cell homeostasis, upregulation of Fas on target T-cells (Müllbacher *et al.*, 2002) and the class I and Class II antigen presentation pathways. (Sobek *et al.*, 2002)

Several *T. parva* CTL antigens have been identified which are presented by specific BoLA to CD8+ T-cells, such as Tp1 , Tp2, Tp4, Tp5, Tp7 and Tp8 (Graham *et al.,* 2008, 2006). The phenotype of the specifically induced CTL against *T. parva* is largely unknown. Use of tetramer staining of the specific T-cells allows characterization of these specific T-cells without much prior *in vitro* manipulation. By co-staining with tetramers and surface biomarkers such as activation and memory biomarkers will give us a clear profile of these specific CD8+ cells. Understanding the profile of CD8+ cell in protected animals (live vaccine immunized) is very important in order to have a guideline or a basis when developing a subunit vaccine. The aim of this project is to generate this knowledge which is a prerequisite for vaccine development.

2.4 Surface and intracellular biomarkers in CD8+Tcells during *T. parva* infection

2.4.1 CD2

CD2 is a specific marker for T-cells and NK cells. This cell adhesion molecule found on the surface of T-cells and natural killer (NK) cells has also been called T-cell surface antigen (Leong *et al*., 2003). It interacts with other adhesion molecules, such as lymphocyte function-associated antigen-3 (LFA-3/CD58) in humans, or CD48 in rodents, which are expressed on the surfaces of other cells (Wilkins *et al.,* 2003).
In addition to its adhesive properties, CD2 also acts as a co-stimulatory molecule on T and NK cells (Yang *et al.,* 2001).

2.4.2 CD3

CD3 is a specific T-cell marker and is at all stages of T-cell development. CD3 is a co-receptor protein complex that is composed of four distinct chains. In mammals, the complex contains a CD3γ chain, a CD3δ chain, and two CD3ε chains. These chains associate with the T-cell receptor (TCR) molecule and the ζ-chain to generate an activation signal in T lymphocytes. The TCR, ζ-chain, and CD3 molecules together comprise the TCR complex. The CD3γ, CD3δ, and CD3ε chains are highly related cell-surface proteins of the immunoglobulin superfamily containing a single extracellular immunoglobulin domain (Kuby *et al.,* 2007). CD3 is initially expressed in the cytoplasm of pro-thymocytes, the stem cells from which T-cells arise in the thymus. The pro-thymocytes differentiate into common thymocytes, and then into medullary thymocytes, and it is at this latter stage that CD3 antigen begins to migrate to the cell membrane. The antigen is found bound to the membranes of all mature T-cells, and in virtually no other cell type, although it does appear to be present in small amounts in Purkinje cells. This high specificity, combined with the presence of CD3, makes it a useful immunohistochemical marker for T-cells in tissue sections. (Leong *et al.,* 2003)

2.4.3 CD4

CD4 is a glycoprotein of the immunoglobulin superfamily and is found on the surface of immune cells such as T helper cells, monocytes, macrophages, and dendritic cells. (Bernard *et al*., 1984). In humans, the CD4 protein is encoded by the CD4 gene (Isobe *et al*., 1986) CD4+ T helper cells are white blood cells that are an essential part of the human immune system. They are often referred to as CD4 cells, T-helper cells or T4 cells. T-cells expressing CD4 molecules (and not CD8+) on their surface are MHC class II-restricted. That is, they are specific for antigens presented by MHC II and not by MHC class I.

2.4.4 CD25

CD25 is marker for activated T-cells and activated B cells. It is a transmembrane protein, the alpha chain of the IL-2 receptor. It associates with CD122 to form a heterodimer that can act as a high-affinity receptor for IL-2. Though CD25 has been used as a marker to identify CD4+FoxP3+ regulatory T-cells in mice, it has been found that a large proportion of resting memory T-cells constitutively express CD25 in humans (Triplett *et al*., 2012).

2.4.5 CD38

CD38 is a marker of cell activation and is located on the surface of many immune cells, including CD4+, CD8+, B lymphocytes and natural killer cells. CD38 also functions in cell adhesion, signal transduction and calcium signalling in humans (Nata *et al.,* 1997). The loss of CD38 function is associated with impaired immune responses, metabolic disturbances, and behavioural modifications. The CD38 produces an enzyme which regulates the release of oxytocin within the central nervous system (Higashida *et al.,* 2012).

2.4.6 CD44

CD44 is a denotative marker for effector-memory T-cells. . It is expressed in a large number of mammalian cell types. CD44 antigen is a cell-surface glycoprotein involved in cell–cell interactions, cell adhesion and migration. In humans, the CD44

antigen is encoded by the CD44 gene (Spring *et al*., 1988). Memory cell proliferation (activation) can also be assayed *in vitro* with CFSE chemical tagging. The standard isoform, designated CD44s, comprising exons 1–5 and 16–20 is expressed in most T-cell types. CD44 splice variants containing variable exons are designated CD44v. Some epithelial cells also express a larger isoform (CD44E), which includes exons v8–10 (Goodison *et al.*, 1999). This protein participates in a wide variety of cellular functions including lymphocyte activation, recirculation and homing, haematopoiesis, and tumour metastasis.

2.4.7 CD45R0

CD45R0 is one of the isoforms of the CD45 family and is located on memory T-cells. The CD4 5 antigen is a protein tyrosine phosphatase, receptor type, C also known as PTPRC. This is an enzyme that, in humans, is encoded by the PTPRC gene (Kaplan *et al*., 1990). The protein encoded by this gene is a member of the protein tyrosine phosphatase (PTP) family. PTPs are known to be signalling molecules that regulate a variety of cellular processes including cell growth, differentiation, mitotic cycle, and oncogenic transformation (Arendt *et al.*, 1997) The CD45 family consists of multiple members that are all products of a single complex gene.

2.4.8 CD62L

CD62L also identified as L-selectin is found on the surface of Central memory T-lymphocytes. This CD62L is a, cell adhesion molecule is not only found on lymphocytes but also on the preimplantation embryo. It belongs to the selectin family of proteins, which recognize sialylated carbohydrate groups. L-selectin acts as a "homing receptor" for lymphocytes to enter secondary lymphoid tissues via high endothelial venules. Ligands present on endothelial cells will bind to lymphocytes expressing L-selectin, slowing lymphocyte trafficking through the blood, and facilitating entry into a secondary lymphoid organ at that point (Robbins *et al*., 1998) Effector memory T-lymphocytes do not express L-selectin, as they circulate in the periphery and have immediate effector functions upon encountering antigen (James *et al*., 2012). High expression of L-selectin on human bone marrow progenitor cells

is an early sign of cells becoming committed to lymphoid differentiation (Kohn *et al*., 2012).

2.4.9 CD71

CD71 also known as Transferrin receptor protein 1 (TfR1) is an integral membrane protein that in humans is encoded by the TFRC gene (Rabin *et al.*, 1985). TfR1 is required for iron delivery from transferrin to cells (Aisen, 2004). Iron is critical in the proliferation of T-cells in immune response. The transferrin receptor is significantly expressed on placental syncytiophoblasts, myocytes, basal keratinocytes, hepatocytes, endocrine pancreas, spermatocytes, and erythroid precursors. The level of transferrin receptor expression is highest in early erythroid precursors. Maturation to erythrocytes results in loss of transferrin (Lesley *et al*., 1984).

2.4.10 Perforin

Perforin is an essential component of cytotoxic granules which directly contributes to T-cell mediated death via apoptosis or necrosis (Kagi., *et al*, 1994) by permiabilizing target T-cell membranes. Perforin inserts itself into the target T-cell's plasma membrane and facilitates endocytosis of itself and granzyme proteases. The subsequent translocation of pro-apoptotic granzymes into the cytoplasm (Pipkin *et al*., 2007) provides these proteases access to numerous protein substrates that promote apoptosis after cleavage. At higher concentrations, perforin also contributes to necrosis by facilitating osmotic disruption of the target T-cells. Perforin is highly conserved among mammals, expressed in lymphocytes and is essential to the T and NK lymphocyte-mediated cytotoxicity that controls infections by viruses and by intracellular bacteria (Bolitho *et al*., 2007).

2.4.11 FasL

Fas is a member of the superfamily of tumour necrosis factor and nerve-growth receptors and is expressed on many cells, whereas its ligand, FasL, is expressed on activated T-cells. Fas-induced apoptosis and the perforin pathway are the two main mechanisms by which cytotoxic T lymphocytes induce cell death in cells expressing

foreign antigens (Green *et al*., 2003). Activation-induced cell death (AICD) plays important role in maintaining normal immune function by modulating immune homeostasis and effectively eliminating antigen-reactive T-cells. Engagement of FasL leads to trimetization of Fas and clustering of its intracellular death domain, which in turn leads to the recruitment of the cytoplasmic Fas-associated death domain. The cascade of caspases is then set off and this ultimately leads to cell death (Pinkoski *et al*., 2005).

2.5 Gamma delta (γδ) T-cells

Gamma deltas (γδ) T-cells are one of two kinds of T-cells, distinguished by surface expression of either an αβ or a γδ T-cell receptor (TCR). These two types of cells develop independently as separate lineages in vertebrates (Goodier *et al*., 1992). They constitute the total pool of peripheral T-cells and are effectors of both cell-mediated immunity and T-cell help. The majority of mature αβ T-cells express either CD8+ or CD4 accessory molecules and recognize peptide antigens in association with class I or class II major histocompatibility complex (MHC) molecules, respectively. However, the potential of γδ T-cells to recognize diverse antigens and the restriction elements involved remain unclear (Goodier *et al.,* 1992).

The effector function of γδ T-cells in immune responses in general, and in infectious diseases in particular, is poorly understood, and no consensus has yet emerged about the overall role of these cells in immune systems of different species. γδ+ T-cells in birds, ruminants, humans, and rodents have been studied (Chien *et al.,* 1996)).

Some properties of γδ T-cells are remarkably conserved, whereas others differ greatly among species. These cells are very scarce in rodents and primates (~5% of blood lymphocytes) and are distributed preferentially at different mucosal surfaces (Goodier *et al.,* 1992). In contrast, more recent studies with artiodactyls, an order of animals that includes the ruminants and that diverged from the rodent primate evolutionary stream around 100 million years ago, show that γδ T-cells form a much larger proportion of the peripheral T-cell pool.

Although some γδ T-cells become localized at mucosal surfaces in these species, a large pool of cells recirculates among blood, tissue, and lymph and is widely disseminated throughout peripheral body compartments. The prominence of γδ T-cells in ruminants provides an opportunity for a detailed analysis of these lymphocytes (Hein *et al.*, 1991). Non-TCR lineage-specific biomarkers for bovine and ovine γδ T-cells that detect WC1, a 215-kDa Ag that belongs to the scavenger receptor cysteine-rich protein family, have been described. Although the function of the WC1 molecule is not clear, it has been proposed that it plays a role similar to that of CD4 and CD8+ and that it provides a mechanism for tissue specific homing (Hein *et al.*, 1991).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study site

The study was conducted at the International Livestock Research Institute (ILRI) headquarters in Nairobi, Kenya. ILRI is an international agricultural research institute founded in 1994 by the merging of the International Livestock Centre for Africa (ILRA) and the International Laboratory for Research on Animal Diseases (ILRAD). It is a member of the Consultative Group on International Agricultural Research (CGIAR) and focuses its research on building sustainable livestock systems and products for small-holder farmers in low-income countries.

Figure 3.1: Geographical map of the international livestock research institute

(Map from Google maps 2013: https: //www. maps.google.co.ke)

3.2 Study design

This was an experimental block design. Male cattle of known phenotype (BoLA type) were immunized with live sporozoites of known *T. parva* strain and thereafter monitored for cytolytic T cell immune responses. The blood samples were taken from the experimental animals and the PBMCs isolated. The PBMCs were then cultured to generate CD8+ cells and γδT cells. These cells were taken through FACS analysis, ELISPOT and CTL cytotoxicity assay.

3.3 Experimental animals

The experimental animals were male cattle Friesian and Ayrshire breeds and they carried the A18 MHC class I haplotype. The haplotype were determined by a combination of serological typing with class I-specific monoclonal antibodies (Ellis *et al.,* 2005) and class I allele-specific PCR assays (Ellis *et al.,* 1998). Previous studies have shown that of the identified *T. parva* antigens, A18 animals are most responsive to the $Tp1_{214\text{-}224}$ antigen (MacHugh *et al.,* 2009). All experiments were done in accordance with the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioural Research (National Research Council, 2003). The number of experimental animals was nine. The combination of factors that informed this sample size included; precedence from key past studies that used a similar number of animals (Graham *et al*., 2006, MacHugh *et al.,* 2009), The cost of purchase and maintenance of the animals at the ILRI animal unit for one year dictated the number of animals used, where the cost of purchase was Kshs 49,000 per animal and daily maintenance was Kshs 400 indoors (during immunization and sample collection) and Kshs 160 outdoors per animal. The other consideration was the application of the 3Rs (Replacement, refinement and reduction) concept for humane animal use in research (Russell, *et al*; 1959). Where the emphasis is to reduce the number of animals in research, reduce the suffering, refine the experiments and replace experimental animals where possible.

The animals were acquired from Nyeri in the highlands of Central Kenya when naïve to *T. parva* (Confirmed through serology). These animals were maintained at the International Livestock Research Institute's animal unit on a ration of hay and concentrates. They were monitored for any infections. The animals were given 6 months to acclimatize at the ILRI large animal unit. **Table 3.1** presents some bio data on the animals.

Table 3.1: Table of experimental animals

Animal Code	Breed	Date of birth	Date of immunization	Stabilate for immunisation
BB007	Friesian	24/1/06	22/8/12	*T. parva* Muguga
*BV115	Friesian	1995	7/8/10	*T. parva* Muguga
BF076	Friesian	10/2/10	28/8/12	*T. parva* Muguga
BF077	Ayrshire	1/11/09	28/8/12	*T. parva* Boleni
BF079	Ayrshire	24/12/09	28/8/12	*T. parva* Buffalo
BA219	Friesian	25/2/05	18/9/12	*T. parva* Muguga
BH054	Friesian	21/3/10	25/9/12	*T. parva* Muguga
BF091	Friesian	15/1/10	25/9/12	*T. parva* Muguga
BF092	Friesian	5/11/11	18/9/12	*T. parva* Muguga

*BV115 was immunized as part of an earlier study.

3.4 Experimental procedures

3.4.1 Treatment on experimental animals

After acclimatization, the animals were immunized via subcutaneous injection by infection with cryopreserved sporozoites and simultaneous administration of a long-acting formulation of oxytetracycline at 20 mg/kg (Radley *et al.*, 1975). A boost was then given five weeks after the initial immunization by inoculation of the vaccine without use of oxytetracyclins.

3.4.2 Sample collection and isolation of PBMC

Whole blood was collected in 60ml syringes under sterile conditions. 30ml blood was was sampled through jagular puncture into a syringe containing 30ml of Alsever's solution. The Alsever's solution is an anti-coagulant. 30ml of the jugular venous blood collected in Alsever's solution was overlaid on Ficoll-Paque of 20ml volume. These tubes containing blood overlaid on Ficoll-Paque were then centrifuged at 2500g for 25 minutes.

Following the density gradient centrifugation, the blood separated into distinct layers of plasma, leukocytes and erythrocytes. The buffy coat containing the leukocytes was then carefully aspirated into a sterile 50ml falcon tube. Leukocytes from every two tubes were aspirated into one falcon tube and topped up with PBS buffer. This was then spun as 1800g for 10 minutes.

The supernatant was poured out and Tris-ammonium Chloride added to the cell mixture to lyse any erythrocytes present. After exactly 3 minutes the tubes were topped up with PBS and washed twice at 1200g for 10 minutes. After the last wash the supernatant was discarded and the pellet broken. The cells were then resuspended in a known volume of complete RPMI media and was either used to set up a bulk culture or preceded to further fractionation of CD8+ T-cells and γδ cells for ELISPOT assay (**Appendix 2**).

3.4.3 Parasitized cell lines

Cell lines infected with *T. parva* were established by infection of PBMC *in vitro* with sporozoites as described by Baldwin, (Baldwin *et al*, 1988) and by culture of cells from PBMC of infected cattle (Hulliger *et al.*, 1964). The cryopreserved sporozoites that were used to establish these cell lines include; *T. parva* Muguga *T. parva* Boleni and *T. parva* buffalo.

3.4.4 Generation of CD8+ T-cell lines

CD8+ T-cell lines were generated from PBMCs isolated from the whole blood of the immunised animals. Bulk cultures were generated by stimulation of PBMC with irradiated autologous *T. parva* infected cells lines. Co-cultures of 1ml PBMC ($4x10^6$/well) and 1ml of infected cell lines ($2x10^5$/well) were plated in 24 well plates. These cultures were restimulated by irradiated autologous *T. parva* after every 7 days. CTL lines were generated and maintained in media (RPMI) 1640 (Sigma-Aldrich, St.Louis, MO, USA) supplemented with 10% FBS (Thermo Fisher Scientific, Waltham, MA, USA), 2mM L-Glutamine (Sigma-Aldrich, St. Louis, MO, USA), 50 μM 2-mercaptoethanol(Sigma-Aldrich, St.Louis, MO, USA), 100 IU of penicillin/ml (Sigma-Aldrich, St. Louis, MO, USA), 50 μg of gentamicin /ml, (Sigma-Aldrich, St. Louis, MO, USA). All this was carried out as described in the ILRI standard operating procedures (**Appendix 3**).

3.5 Data collection procedures

3.5.1 FACS Analysis for surface and intracellular biomarkers expressed by *T. parva* specific CD8+ T-cells

The cell membrane proteins that were analysed included the cell surface biomarkers and the bound bovine MHC class 1/*T. parva* tetramer (**Figure 3.1, Figure 3.2** and **Figure 3.3**). Linking several copies of the MHC/antigen complex improves the challenges of low MHC molecule for the CD8+ receptor. Phycoethyrin (PE)-labelled

bovine MHC class I / *T. parva* tetramers were generated by the Ludwig Institute for Cancer Research (Ludwig Institute for Cancer Research, Brussels, Belgium).

Co-staining with different surface biomarkers was performed (tetramer + other biomarkers). Cells were stained with red flourochrome coupled tetramers and specific antibodies differently coloured flourochromes such as green (Shapiro *et al*., 2003). In this way antigen specific CTL (tetramer+) showed red and green if they expressed a particular marker on the surface. Most of these antibodies were developed in house at the ILRI laboratories. The analysis was performed on a FACS CantoII (BD Biosciences).

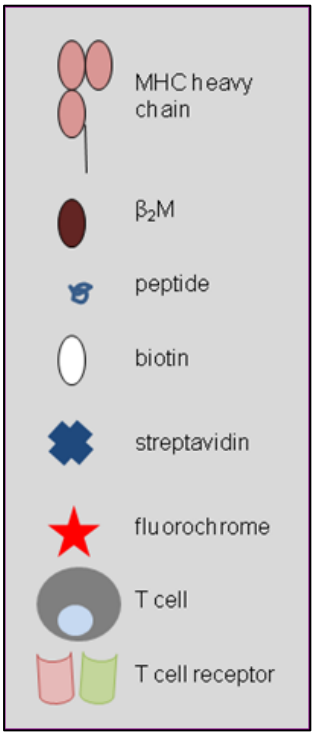

Figure 3.2: Components of the MHC tetramer complex (Horlock et al., 2007).

This panel shows a key for symbols used in all figures below.

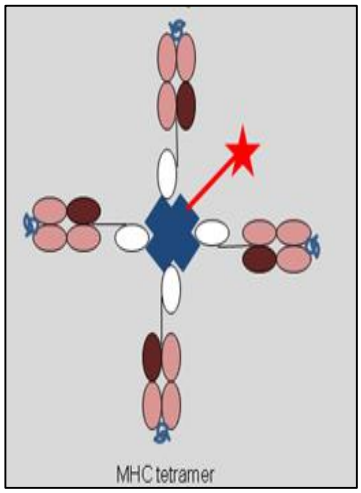

Figure 3.3: MHC tetramer complex (Horlock et al., 2007).

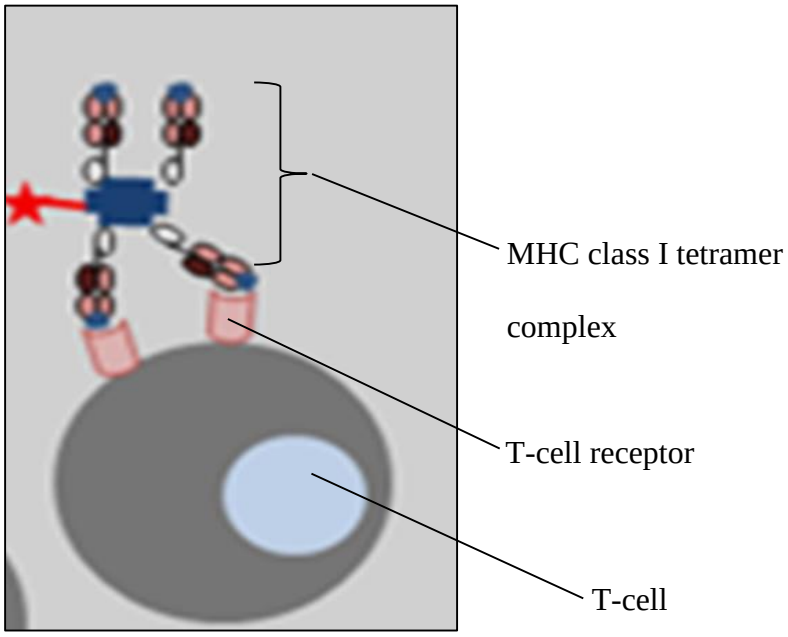

Figure 3.4: MHC Class I tetramer complex binding to a T-cell (Horlock *et al*., 2007).

3.5.2 Staining of cells with $Tp1_{214-224}$ tetramer, surface and intracellular biomarkers

Peripheral Blood Mononuclear Cells (PBMC) were harvested from culture plates into 50 ml or 20ml falcon tubes and centrifuged at 1600 g for 6 minutes. The pellet was then broken and resuspended in PBS-0.5% BSA and approximately $4x10^5$ cells were stained in BD FACS tubes by adding 25µl $Tp1_{214-224}$ (Kindly provided by Pierre van der Bruggen, Ludwig Institute for Cancer, Brussels Branch) to a final concentration of 40nM followed by incubation for 10 minutes in the dark at room temperature.

Then 25µl MAb (**Table 3.2**) for surface biomarkers (diluted 1/250 in PBS-0.5% BSA) was added followed by incubation for 30 minutes at 4ºC. Cells were then washed twice in 200µl PBS-0.5% BSA by centrifugation at 1600 g for 6 minutes. Then 25µ of the secondary antibodies (rat-anti-mouse) were added and incubated for 30 minutes at 4ºC followed by two washes as above. For the cells that proceeded to intracellular staining, a Fixation step followed. This involved addition of 200µl of 0.25% PFA7 to the cells per tube and incubating at room temperature in the dark for exactly 10 minutes.

The tubes were then spun in bench centrifuge, supernatant removed and vortexed to resuspend the pellet. The cells were then washed once with 200 µl of ice-cold D'PBS. What followed was a permeabilization step which involved addition to each tube 200 µl of PBS-Sap8 supplemented with 20% heat inactivated normal goat serum (Fc receptor blocking) and incubated at room temperature for 30' or overnight at 4ºC. The cells were then spun in the centrifuge and the supernatant removed. Staining with the Primary and secondary antibodies then proceeded as described above. All this was carried out as described in the ILRI standard operating procedures (**Appendices 4, 5** and **6**).

Table 3.2: Monoclonal antibodies used for primary labeling in FACS analysis

Antibody code	Isotype	Dilution	Specificity	Cell distribution.
IL-A43	IgG2a	1:250	CD2	T-cells, NK cells
MM1A	IgG1	1:250	CD3	T-cells
IL-A12	IgG2a	1:250	CD4	CD4+ T-cells
IL-A51	IgG1	1:250	CD8+α chain	CD8+ T-cells, subsets NK cells
IL-A105	IgG2a	1:250	CD8+	CD8+ T-cells, subset NK cells
IL-A111	IgG1	1:250	CD25	Activated T-cells and B-cells
*	IgG1	1:20	CD38	Activated T-cells andB-cells
IL-A108	IgG2a	1:250	CD44	Effector-memory T-cells
IL-A150	IgG3	1:250	CD45RO	Memory T-cells.
*	IgG1	1:250	CD62L	Central memory T-cells
IL-A77	IgM	1:250	CD71	Erythroid Precursors
*	IgG2a	1:10	Perforin	Cytotoxic T-cell activity
CC15	IgG2a	1:250	WC1	Peripheral γδ+ T-cells
GB21A	IgG2b	1:250	γδTCR	All γδ+ T-cells
*	IgG2a	1:10	FasL	Active T-cells

* Refers to antibodies that were not prepared in-house (in ILRI) but were sourced commercially hence don't have an ILRI code.

3.5.3 ELISPOT Assay comparing the activation of CD8+ T-cells and γδT-cells in *T. parva* infection

This procedure was carried out to detect presence of bovine IFN-γ. Whose presence in an immune cell population signifies immune activation. (Boehm *et al.*, 1997). This was carried out on CD8+ cell and γδ T-cells *ex vivo*. Interferon-γ release from purified (fractionated) CD8+ cells and γδ T-cells was measured on ELISPOT plates (Millipore, Billerica, MA,USA) precoated overnight with a monoclonal anti bovine IFN-γ antibody (Serotec, Oxford, UK).

Plates were blocked for 1h at 37^{o}C by adding 200µl complete RPMI media per well. CD8+ cells and γδ T-cells were purified from PBMC using magnetic beads as described in the Protocol for Mini Macs sorting (**Appendix 7**). CD8+ cells and γδ T-cells were added in two fold dilutions in complete RPMI media and the stimulating agents (*T. parva* infected Lymphocytes) were added in desired numbers as described in the ILRI standard operating procedures (**Appendix 8**).

The plates were incubated overnight (18h) at 37^{o}C then, plates were washed 4 times with commercial culture grade water and 4 times with PBS+0.05% Tween 20 (Sigma-Aldrich, St Louis, MO, USA) followed by addition of 50µl/well of rabbit anti-bovine IFNγ, 1:500 (ILRI produced, ammonium sulphate precipitated). It was incubated for 1h followed by 4 washes in PBS+0.05% Tween 20. Then the secondary antibody was added: 50µl/well of Alkaline phospahatase conjugated mouse-anti-rabbit IgG (1:2000) in PBS+0.05% Tween 20+0.1% BSA for 1h followed by 6 washes in PBS+0.05% Tween 20. Plates were developed by adding 50µl/well of substrate solution: 1 tablet of Sigma Fast (BCIP/NBT, Sigma-Aldrich, and St.Louis, MO, USA) in 10ml distilled water. These cell populations were measured for secretion of Interferon gamma (IFN-γ) by formation of spots in the wells. The number of spots corresponded to the number of antigen + cells. The spots were counted in an ELISPOT counter. All this was carried out as described in the ILRI standard operating procedures (**Appendices 7** and **8**).

3.5.4 Cytotoxicity potential of CD8+ T-cells and γδT-cells in *T. parva* infection

The cytotoxicity potential of these different subpopulations was determined in relation to their activation. The Effector cells prepared from the immunized animals by culturing of PBMC *in vitro* using *T. parva* infected cell lines as the stimulators as described in the ILRI standard operating procedures **(Appendix 3**).

After seven days these cells were harvested, centrifuged to form a pellet. Then resuspended in a known volume of media and counted under a microscope. The number of live cells would determine the number of stimulators needed for the subsequent subculture. Seven days after this second restimulation, the cells were harvested counted and purified for CD8+ and γδ-T-cells as described in **Appendix 7**. These purified cells were then subcultured for seven days using *T. parva* infected cell lines as the stimulators.

These cells were then harvested and used in the Cytotoxicity assay as described in **Appendix 9**. Standard 4h release assay using ^{51}Cr-labeled target T-cells (*T. parva* infected lymphocytes) were used to measure cytotoxicity. ^{51}Cr was obtained from American Radiolabelled Chemicals, Inc., St. Louis, MO, USA. Supernatants were counted using Lumaplates (PerkinElmer, Waltham, MA,USA) in a Top Counter (PerkinElmer, Waltham, MA, USA) The cytotoxicity was calculated as:

(Experimental release – Spontaneous release / Total release – Spontaneous release)

These CTLs were added in dilutions together with a fixed amount of Cr-51 labelled target T-cells in 96 well plates. The CTLs were allowed to kill the target T-cells for 4 hours and the supernatants (with released Cr-51) from the wells are harvested and added to a plate containing scintillation medium(Lumaplates) (Goddeeris *et al.*, 1988). The degree of cytotoxicity of the CTL is directly proportional to the Cr-51 there is released in the samples. All this was carried out as described in the ILRI standard operating procedures (**Appendices 7** and **9**).

3.6 Statistical analysis

The statistical analysis was performed using Ms Excel 2010 package (Microsoft®) and the program GenStat. VSN International (2011). GenStat for Windows 14th Edition. VSN International, Hemel Hempstead, UK. Web page: GenStat.co.uk. P-value of less than 0.05 was considered significant.

Paired Student-t test was used to analyse immune activation of CD8+ T cells and γδT cells.

Spearman's correlation analysis was used to analyse the correlation between expression of activation markers and cytotoxic capacity.

CHAPTER FOUR

RESULTS

4. 1 FACS analysis: surface and intracellular biomarkers

4. 1.1 Preliminary FACS experiments

It was essential to conduct the preliminary experiments to determine the ideal reagents and antibodies for use and also to determine the optimal volumes of these reagents and antibodies. This was done to ensure that the results obtained in the long run were reproducible. The preliminary FACS analysis involved determining the optimal antibody volumes for the anti-CD8+ secondary antibody. It was necessary to use a secondary antibody because the primary antibody was not conjugated to any flourochrome. The secondary antibody was conjugated to Percp (Figure 4.1).

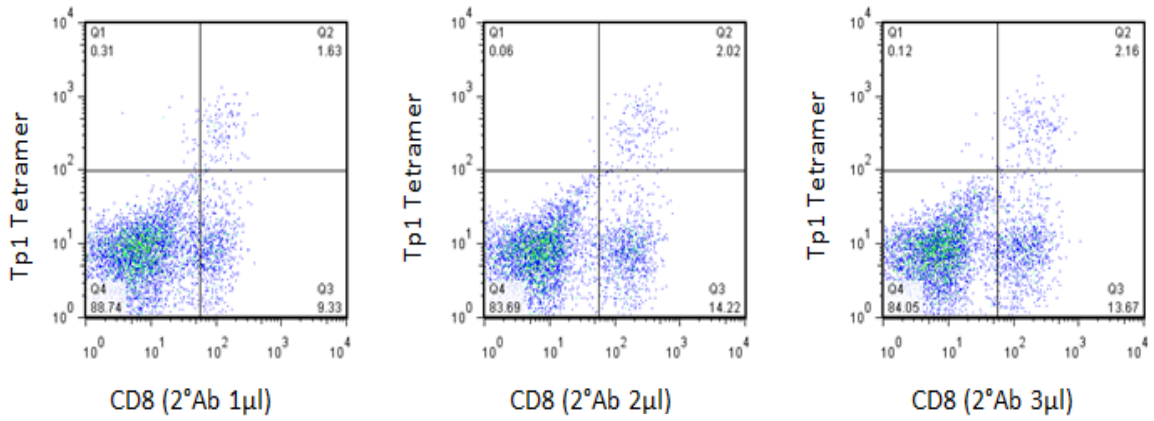

Figure 4.1: FACS staining results for anti CD8+ secondary antibody volume optimization

From this experiment, it was observed that with 2µl and 3 µl volumes of the anti-CD8+ secondary antibody, there was a much better separation of the populations stained (in the Quadrants Q2 and Q3) as compared to using 1µl volume of the antibody. It was also noted that there was no difference between using 2µl and 3 µl.

Therefore it was concluded that 2µl of the Percp-conjugated AntiCD8+ antibody would be used.

Two different isotypes of the in-house antiCD8+ antibody were also compared for optimization as part of the preliminary experiments. The two antibodies were IL A51, an IgG1 isotype and IL A105, an IgG2a isotype (Figure 4.2).

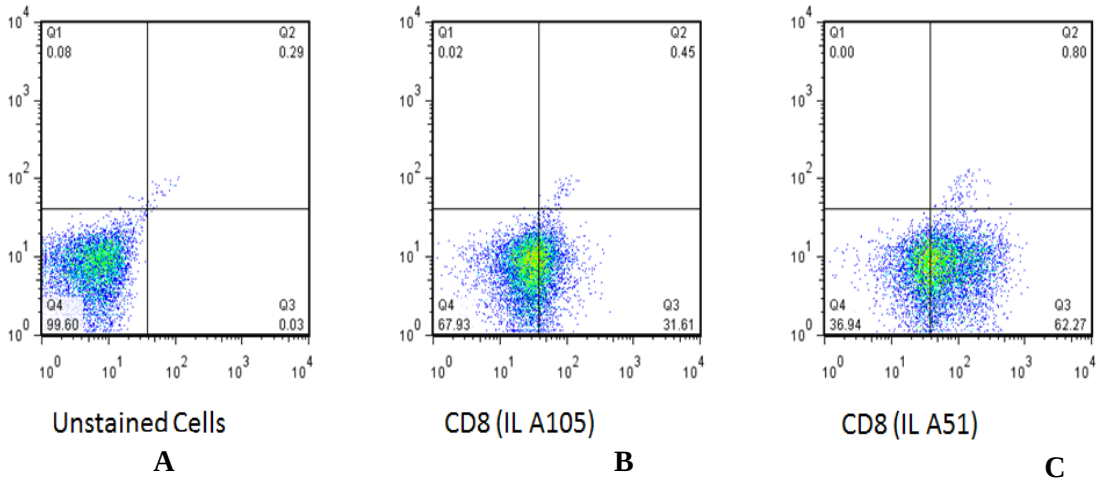

Figure 4.2: Comparison of two different isotypes of the anti-CD8+ antibodies for optimization: Where **A** was unstained cells, **B** was stained with the IL A105 isotype of the anti-CD8 antibody and **C** was stained with the IL A51 isotype of the anti-CD8antibody.

4. 1.2 Surface and intracellular markers expressed on *T. parva* antigen specific CD8+ T-cells

The CD8+ cell surface and intracellular biomarkers profiles from the A18+ animals exhibited various expression levels for the different biomarkers. The *T. parva* specific T-cells from both the CD8+ cell lines, and PBMC bulk cultures expressed high levels of CD44, CD 62L and Perforin. Moderate levels of CD 45R0 were expressed and very little or no levels of CD 71 and Fas ligand.

4.1.3 Surface markers

CD2: For the cell lines BB007 CTL, BV115 CTL and BB007 bulk, the percentages that stained for $Tp1_{214\text{-}224}$ Tetramer are, 42.9%, 8.65% and 4.45% respectively (Table 4.1). CD2 being a specific marker for T-cells and NK cells, the CD2+

$Tp1_{214-224}$ Tetramer- cells were most likely; the *T. parva* non-specific CD8+ T-cells, CD4+ T-cells, NK cells and γδ T-cells. The Unstained cells (CD2- cells) were likely to include; monocytes, macrophages Basophils, Neutrophils, Eosinophils and Dendritic cells.

CD3: For the cell lines BB007 CTL, BV115 CTL and BB007 bulk, the percentages that stained for $Tp1_{214-224}$ Tetramer are, 35.3%, 1.63% and 2.75% respectively (Table 4.1). The CD3+ $Tp1_{214-224}$ Tetramer negative cells here were likely to include; the *T. parva* non-specific CD8+ T-cells, CD4+ T-cells and γδ T-cells. The Unstained cells CD3- and $Tp1_{214-224}$ 1 Tetramer negative cells were likely to include; NK cells, monocytes, macrophages Basophils, Neutrophils, Eosinophils and Dendritic cells (Appendix 1).

CD4: For the cell lines BB007 CTL, BV115 CTL and BB007 bulk, the percentages that stained for $Tp1_{214-224}$ Tetramer are, 0.94%, 0.26% and 1.36% respectively (Table 4.1).

CD25: For the purified CTL cell lines BB007 CTL and BV115 and the PBMC bulk lines BB007, BA219, BF091, BF092, the proportion of The CD25+ $Tp1_{214-224}$ Tetramer+ cells were; between 0.46% and 4.81% (Table 4.1). The CD25+ $Tp1_{214-224}$ Tetramer- cells were likely to include activated *T. parva* non-specific CD8+ cells while the former are activated *T. parva* specific CD8+ cells.

CD38: For the purified CTL cell lines BB007 CTL and BV115 and the PBMC bulk lines BB007, BA219, BF091, BF092, the proportion of The CD38+ $Tp1_{214-224}$ Tetramer+ cells were; between 0.003% and 0.58% (Table 4.1).

CD44: For the purified CTL cell lines BB007 CTL and BV115 and the PBMC bulk lines BB007, BA219, BF091, BF092, the proportion of The CD25+ $Tp1_{214-224}$ Tetramer+ cells were; between 5.52% and 39.1% (Table 4.1).

CD45RO: For the purified CTL cell lines BB007 CTL and BV115 and the PBMC bulk lines BB007, BA219, BF091, BF092, the proportion of The CD25+ $Tp1_{214-224}$ Tetramer+ cells were; between 1.29% and 5.03% (Table 4.1).

CD62L: For the purified CTL cell lines BB007 CTL and BV115 and the PBMC bulk lines BB007, BA219, BF091, BF092, the proportion of The CD25+ $Tp1_{214-224}$ Tetramer+ cells were; between 1.09% and 7.04% (Table 4.1).

CD71: For the purified CTL cell lines BB007 CTL and BV115 and the PBMC bulk lines BB007, BA219, BF091, BF092, the proportion of The CD25+ $Tp1_{214-224}$ Tetramer+ cells were; between 0.01% and 0.64% (Table 4.1).

WC1: For the purified CTL cell lines BB007 CTL and BV115 and the PBMC bulk lines BB007, BA219, BF091, BF092, the proportion of The CD25+ $Tp1_{214-224}$ Tetramer+ cells were; between 0.63% and 3.79% (Table 4.1).

FasL: For the purified CTL cell lines BB007 CTL and BV115 and the PBMC bulk lines BB007, BA219, BF091, BF092, the proportion of The CD25+ $Tp1_{214-224}$ Tetramer+ cells were; between 0.001% and 0.48% (Table 4.1).

4. 1.4 Intracellular markers

Perforin: For the purified CTL cell lines BB007 CTL and BV115 and the PBMC bulk lines BB007, BA219, BF091, BF092, the proportion of The CD25+ $Tp1_{214-224}$ Tetramer+ cells were; between 0.58% and 43.6% (Table 4.1).

Table 4.1: Surface and intracellular markers expressed by antigen specific CD8+ T cells

Marker	Percentage of markers and mean fluorescence intensity (MFI) for cell line as gated on $Tp1_{214-224}$ Tetramer.						Antibody code	Antibody isotype
	BB007 CTL	BV115	BB007 BULK	BA219	BF091	BF092		
CD2	42.9%	8.65%	4.45%	-	-	-	IL-A43	IgG2a
	4233	3605	11100					
CD3	35.3%	1.63%	2.75%	-	-	-	MM1A	IgG1
	1370	3694	10900					
CD4	0.94%	0.26%	1.36%	-	-	-	IL-A12	IgG2a
	200	2099	10600					
CD25	1.84%	4.43%	4.81%	0.46 %	2.86 %	1.95%	IL-A111	IgG1
	533	5790	1529	5466	1369	1145		
CD38	0.58%	0.01%	0.01%	0.003 %	0.00 3%	0.004%	*	
	97.0	1382	6163	3529	5243	4020		
CD44	39.1%	7.77%	5.52%	-	-	-	IL-A108	IgG2a
	1798	4715	5058					
CD45RO	5.03%	1.29%	4.72%	-	-	-	IL-A150	IgG3
	372	3653	1354					
CD62L	7.04%	1.09%	4.31%	-	-	-	*	IgG1
	660	1838	2151					
CD71	0.64%	0.05%	0.01%	-	-	-	IL-A77	IgM
	107	1988	5455					
Perforin	43.6%	9.15%	7.96%	0.58 %	3.28 %	3.17%	*	IgG2a
	5673	4119	7118	3775	3137	5306		
WC1	3.79%	0.63%	1.07%	-	-	-	CC15	IgG2a
	370	2353	12200					
FasL	0.48%	0.06%	0.001%	0.002 %	0.00 4%	0.004%	*	IgG2a
	97.3	1669	7108	3228	5373	4201%		

* Refers to antibodies that were not prepared in-house (in ILRI) but were sourced commercially hence don't have an ILRI code.

- Refers to markers which were not tested on the animals BA219, BF091 and BF092 where the emphasis was activation markers.

4.1.5 Cell cultures from *T. parva* immunized cattle show avidity differences for the $Tp1_{214-224}$ CTL epitope

Four PBMC bulk cell lines (BB007, BA219, BF091, and BF092) and two purified CD8 CTL lines (BB007 and BV115) from *T. parva* immunized cattle were used for correlation of FACS data and cytotoxic potential in CTL assays.

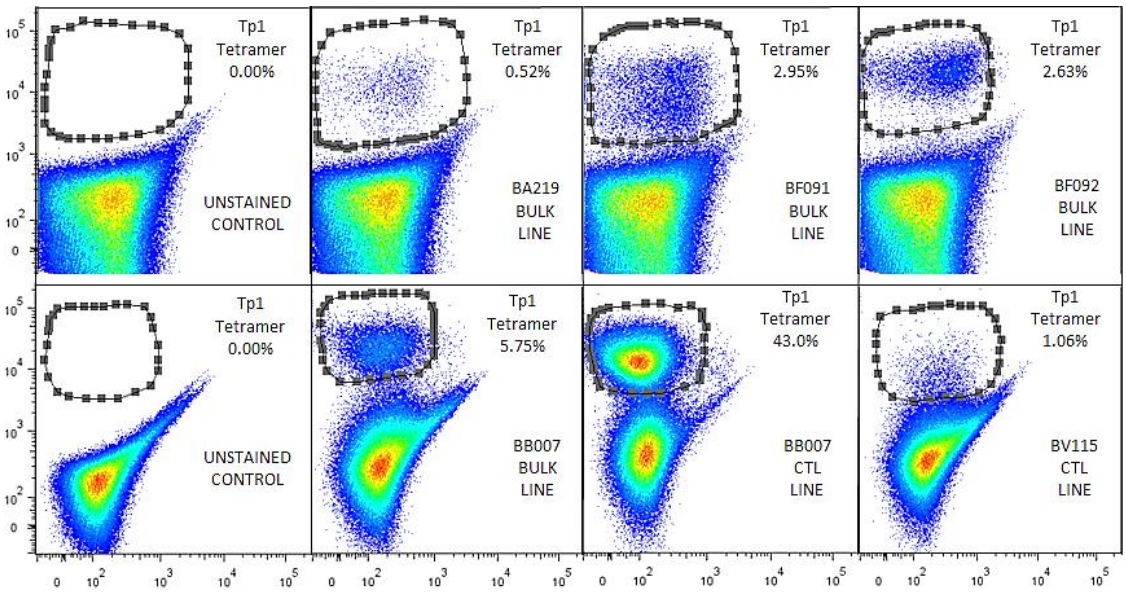

Figure 4.3: $Tp1_{214-224}$ tetramer + cells in the six cell lines

Percentages of tetramer + cell in each of the six cell lines (four bulk lines and two purified CD8 cell lines) are indicated in each diagram. Tetramers were PE-labelled.

Table 4.2: Percentages of tetramer + cells, CD25+ and perforin+ cells and their mean fluorescence intensities

Cell line	Tp1 tetramer + cells (%)		CD8+ cells out of total cells (%)	Net MFI of Tp1 Tet+ cells	Perf+ cells (%)	Net MFI of Perf+ cells	CD25 + cells (%)	Net MFI of CD25+ cells
	of total cells	of CD8 cells						
BA219 Bulk	0.52	2.17	24.00	14072	0.51	3309	0.25	3035
BF091 Bulk	2.95	24.38	12.1	11550	2.90	2920	1.52	611
BF092 Bulk	2.63	23.27	11.3	23881	2.57	4324	1.82	782
BB007 Bulk	5.75	19.76	29.1	22850	5.62	6733	2.53	386
BB007 CTL	43.00	43.57	98.7	15742	42.9	5542.6	29.20	288.8
BV115 CTL	1.06	1.11	95.2	8547	0.81	11384	0.15	2432

Percentages of cells stained with tetramer, α-CD25 and α-perforin in the PBMC bulk cultures and in the purified cell lines. These populations of cells are gated on the Tp1 tetramer + (Tp1 tet+) cells. The net mean fluorescent intensities (MFI) for the tetramer staining, the CD25+ staining and the perforin (perf+) staining are also shown. The net MFI's are calculated by subtracting the MFI for the negative control.

4. 1.6 Tetramer specific cells from cell cultures express perforin and CD25 at different levels

The expression of perforin and CD25 on the Tp1 tetramer + cells was also examined (Fig. 4.3). Staining for perforin revealed that essentially all tetramer + cells were positive for perforin in BB007 bulk, BB007 CTL line and for BV115 CTL line. A very small overlap of the perforin control and the perforin staining were present for BA219 and BF092, showing that the majority of cells were + for perforin in these cell lines. In BF091 a slightly bigger overlap was evident suggesting that some Tp1 specific cells were negative for perforin. The percentage of perforin+ cells are shown in column 5 in Table 4.2.

All cell lines stained + for CD25, although the staining did not result in discrete populations, indicating that some of the tetramer + cells were low or negative in CD25 expression. The BA219 bulk line and the BV115 CTL line had the highest expression of CD25. The tetramer populations for these two cell lines had an average fluorescence of approximately 10^4 for CD25 before subtraction of the MFIs for the controls whereas the MFIs were approximately 500 on average for the rest of the cell lines. The net MFIs for perforin and CD25 are shown in column 6 and 7 in Table 4.2.

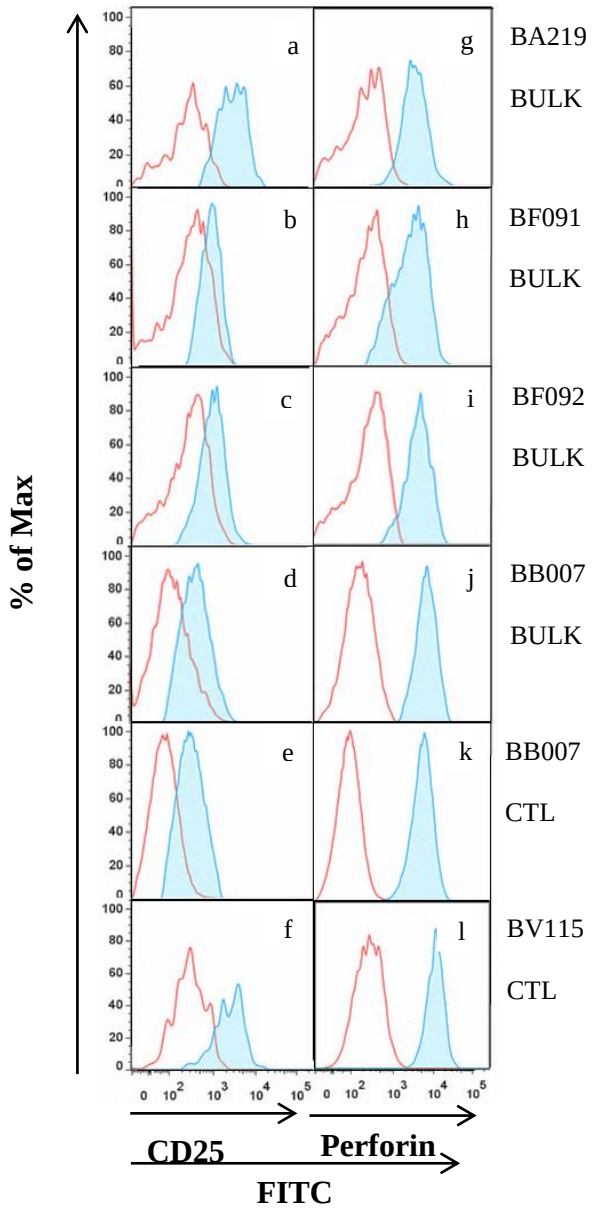

Figure 4.4: Staining of perforin and CD25+ tetramer + CTL

Unshaded (red) – negative controls

Shaded (blue) – stained positively for either perforin or CD25

Overlay histogram of gated tetramer + cells and their expression of either perforin or CD25 versus the negative controls. CD25 + tetramer specific cells are represented by a, b, c, d, e and f referring to the six different T-cell lines as indicated on the figure. Red line represents the negative control and blue line represents the CD25 staining. Perforin + tetramer specific cells are represented by g, h, i, j, k and l referring to the six cell lines. Red line representing the negative control antibody and blue line representing perforin stained cells.

4. 2 Activation potential of CD8+ T-cells and γδ T-cells.

The activation of the CD8+ T-cells and γδ T-cells by interferon gamma (IFN-γ) response was analysed using an ELISPOT assay. Purified CD8+ T-cells were used to ensure that the *T. parva* specific IFN – γ release was limited to CD8+ T-cells. Fractionation of both the CD8+ cells and the γδ cells was done using MACS magnetic beads. From the animals analysed (BF076, BF077, BF079) CD8+ cells T-cells produced IFN-γ in response to the autologous cell line infected with the *T. parva* Muguga. The γδ (gamma delta) cells also showed high degree of activation to the *T. parva* Muguga infected cell lines albeit the activation of these cells was lower than that of the CD8+ cell for the three animals (Table 4.3)

Table 4.3: Number of spot forming cells per well for different titres of CD8+ cells and γδ T-cells in the animals BF076, BF077 and BF079

T-Cell Titration	**Animal BF076** n = 2				**Animal BF077** n = 2				**Animal BF079** n = 2			
	CD8+		**γδ**		**CD8+**		**γδ**		**CD8+**		**γδ**	
$5x10^5$	705	487	341	568	17	28	10	3	169	388	103	247
$2.5x10^5$	263	193	22	367	21	15	14	8	369	266	81	228
$1.25x10^5$	274	76	236	86	20	13	10	8	89	201	41	212
$6.25x10^4$	155	206	21	50	16	26	1	3	3	102	68	161
$3.125x10^4$	62	81	129	124	4	2	0	2	166	228	56	20
$1.563\ x10^4$	115	94	107	155	0	2	5	1	215	209	64	89
Cells only	2	6	0	3	2	0	0	3	86	95	2	1
TpM only	134	47	90	26	3	1	0	2	180	116	135	217

To determine whether there was any significant difference in the means of the number of spot forming cells per well for the two cell types (CD8+ and γδ T-cells) for each animal, the student t test was applied. These means (for the double determinations) were considered at three T-cell titrations namely; $5x10^5$, $2.5x10^5$ and

1.25x10^5. P values less than 0.05 were considered significant. The P values for the means of spot forming cells per well for the experimental animals are indicated in Figure 4.5. Therefore the means of spot forming units per well for CD8+ and γδ T-cells were statistically insignificant for all the experimental animals for the three T-cell concentrations examined, since in all cases the p values were larger than 0.05 (Figure 4.5).

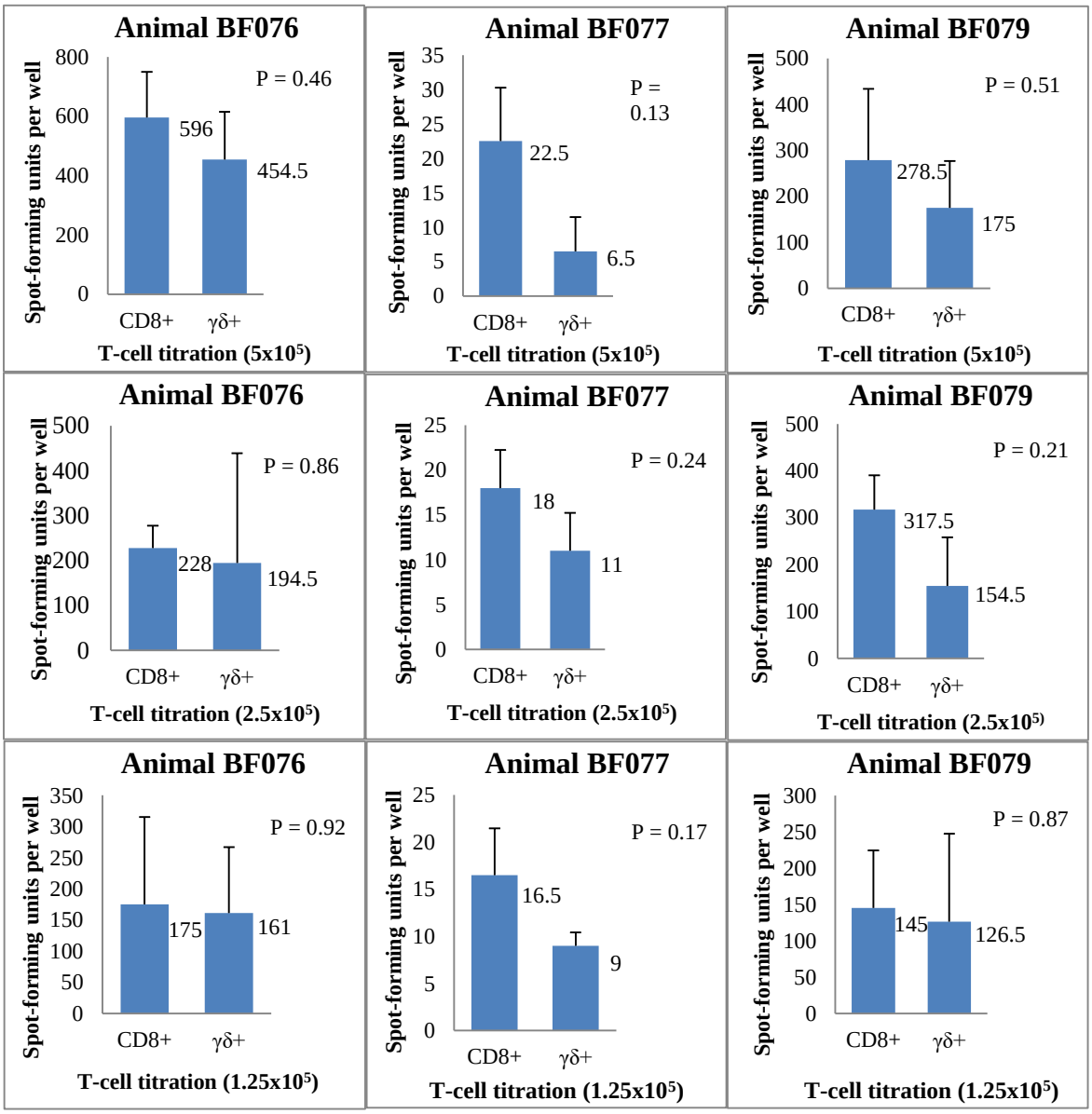

Figure 4.5: CD8+ and γδ cells from the animals BF076, BF077 and BF079 for three T-cell titrations. 5x10^5, 2.5x10^5 and 1.25x10^5 ·These means were analyzed

using the Student –t-test. Statistical significance tested using a T-test where, ($P < 0.05$) and indicated on the figure with an asterisk (*).

4. 3 Cytotoxic potential of CD8+ T-cells and γδ T-cells

The $Tp1_{214-224}$ peptide specific CTL cells were tested for their cytotoxic potential against the *T. parva* -infected homologous cell line that they had been stimulated with in culture and a heterologous (MHC-mismatched) line. To this end the two cell types under study (CD8+ and γδ cells) was isolated from growing tertiary PBMC bulk cultures from the experimental animals. This was done using MACs magnetic beads. The CTL cells from the animals analysed (BF076, BF077, BF079, BF91, BF054, BF92) exhibited cytotoxicity against the *T. parva*-infected homologous cell lines. For the γδ cells there was zero cytotoxicity in all the animals against the homologous *T. parva*-infected cell line (Figures 4.6 to 4.9).

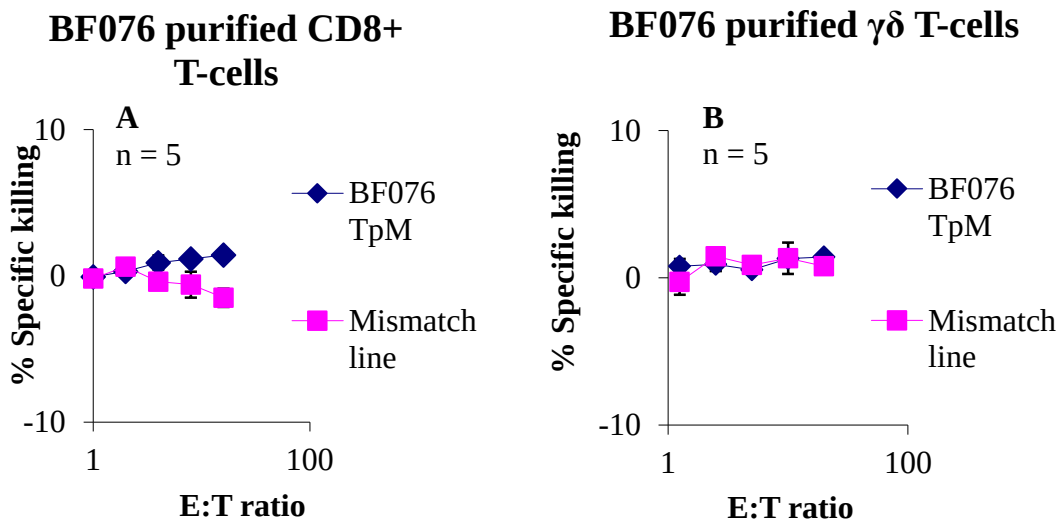

Figure 4.6: CTL cytotoxicity assay on cells from the animal BF076. **A** shows CD8+ T-cells and **B** shows γδ+ T-cells. Both T. parva-specific CD8+ cells and the γδ T-cells were tested against homologous *T. parva* infected cell lines (◆) and *T. parva* infected heterologous cell lines (■).

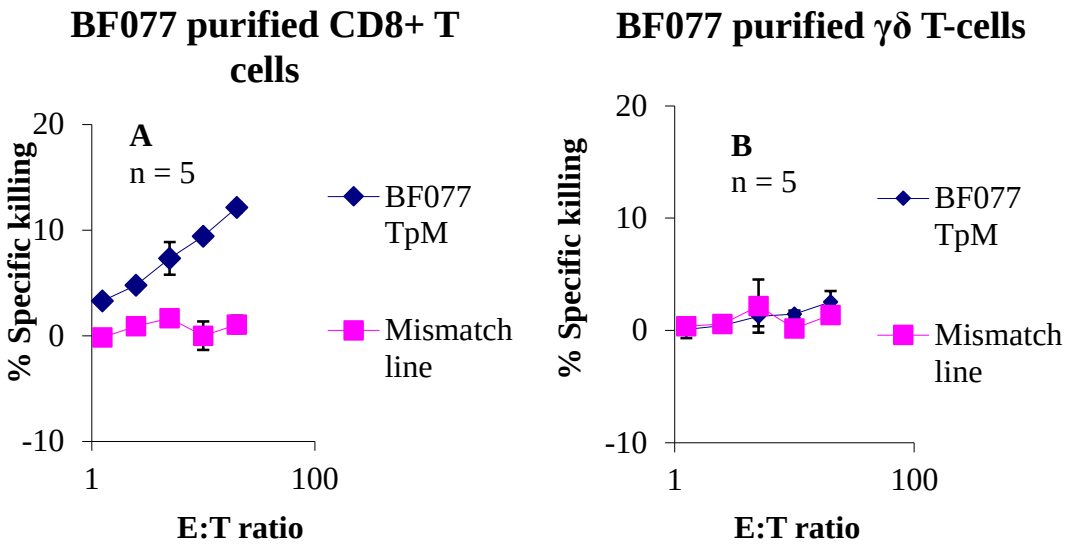

Figure 4.7: CTL cytotoxicity assay results for the animal BF077A shows CD8+ T-cells and B **shows γδ T-cells.** Both *T. parva*-specific CD8+ cells and the γδ T-cells were tested against homologous *T. parva* infected cell lines **(◆)** and *T. parva* infected heterologous cell lines **(■)**.

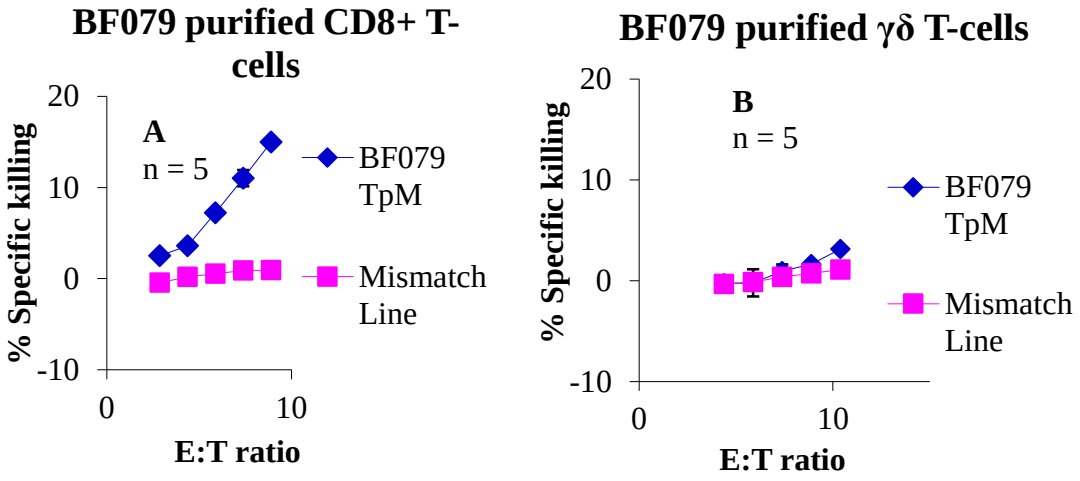

Figure 4.8: CTL cytotoxicity assay results for the animal BF079. A **shows CD8+ T-cells and** B **shows γδ T-cells.**

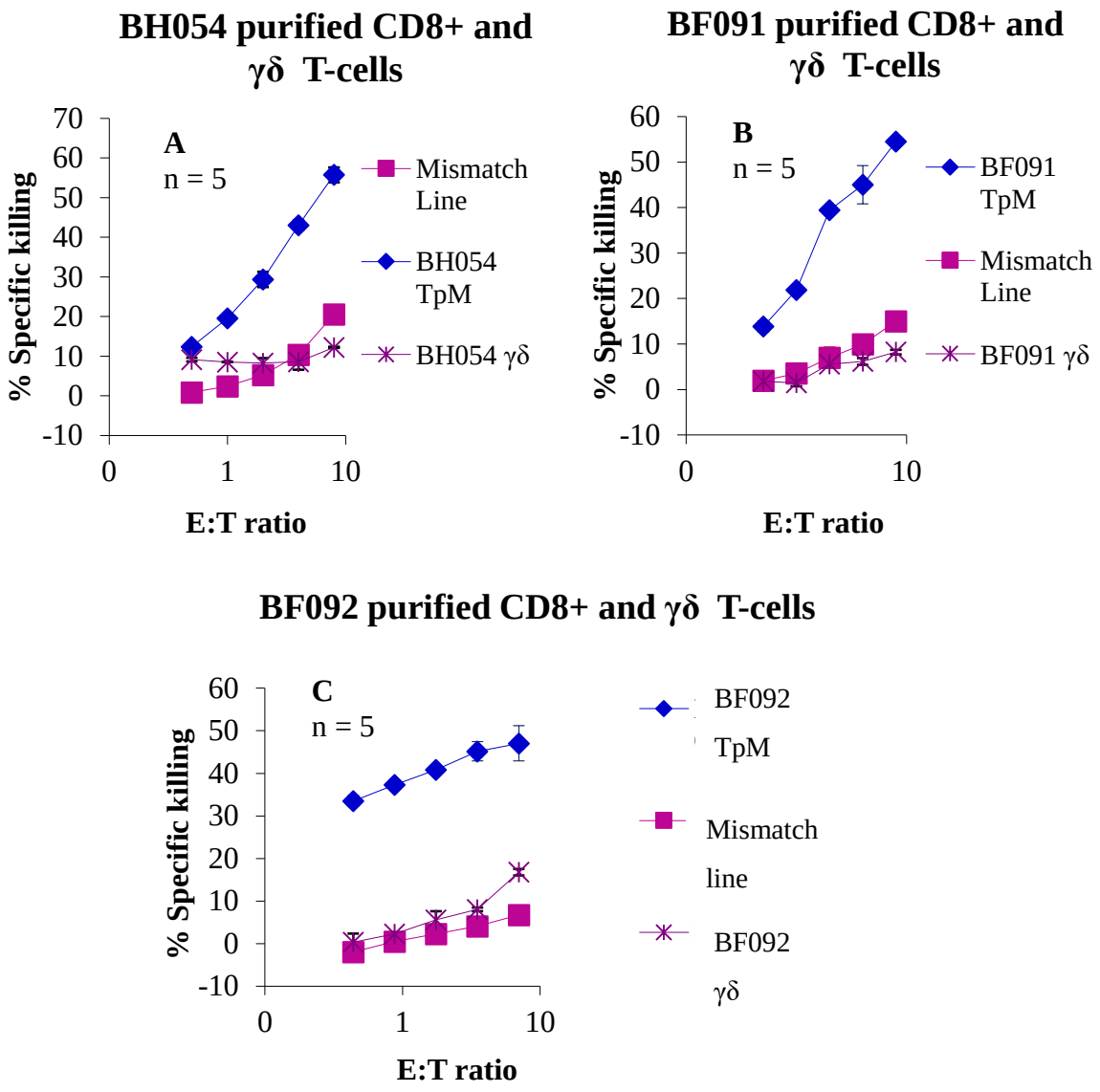

Figure 4.9: CTL assay results from animals; BH054, BF091 and BF092 A - C shows CD8+ and γδ T-cells for the animals BH054, BF091 and BF092. Both *T. parva*-specific CD8+ cells and the γδ T-cells were tested against homologous *T. parva* infected cell lines (♦), (ж) and *T. parva* infected heterologous cell lines (■).

4.3.1 Cytotoxic potential of cell cultures correlates with tetramer+ cells and expression of perforin and CD25

In order to correlate the presence of tetramer specific cells and the expression of perforin and CD25 with the cytotoxicity, a CTL assay was performed using the same bulks and cell lines as for the FACS analysis (**Figure 4.4**). *T. parva* specific PBMC bulks and cell lines were tested for the cytotoxic potential using their respective autologous Muguga infected cell line (TpM) as target as well as an MHC mismatched control cell line, $Tp1_{214\text{-}224}$ pulsed PBMC and PBMC control cells. The highest level of cytotoxicity were obtained using the TpMs except for CTL originating from BB007 that had a very high level of cytotoxicity using $Tp1_{214\text{-}224}$ pulsed PBMC. A higher level of cytotoxicity using TpM than $Tp1_{214\text{-}224}$ pulsed PBMC indicates presence of other CTL specificities. The net cytotoxicity for the autologous TpM and the pulsed PBMCs at three different effector to target ratios (7.5, 10 and 20) are listed in Table 4.4. These values were used for correlation with the number of stained cells, the MFIs or the product of these two parameters.

Spearman Rank correlation was used for statistical evaluation of the correlation between FACS parameters and the cytotoxicity. Table 4.5 shows the parameters that correlated significant with the cytotoxicity. For simplicity, only the statistics for an effector: target ratio of 20 is shown, since this was representative for the two other ratios. The number of $Tp1_{214\text{-}224}$ tetramer + cells correlated with the cytotoxicity for PBMC pulsed with the peptide. The correlation was better if the cytotoxicity was correlated with the product of the number of $Tp1_{214\text{-}224}$ tetramer + cells and the net MFI, reflecting the importance of both the number of cells and the avidity of interaction. The number of perforin + (gated tetramer + cells) and the MFIs for perforin also correlated with the cytotoxicity.

Almost all of the tetramer + cells were perforin +. Again a perfect correlation was seen when the cytotoxicity was correlated with the product of the number of perforin + cells and the net MFI. The CD25 staining generated overlapping populations with the negative control. Therefore, it was not possible to obtain a number of + cells.

When cytotoxicity using TpM was correlated with the FACS data, no significant relationships were detected. This was expected since FACS data were obtained on single tetramer specificity and not on the whole CD8+ T-cell population

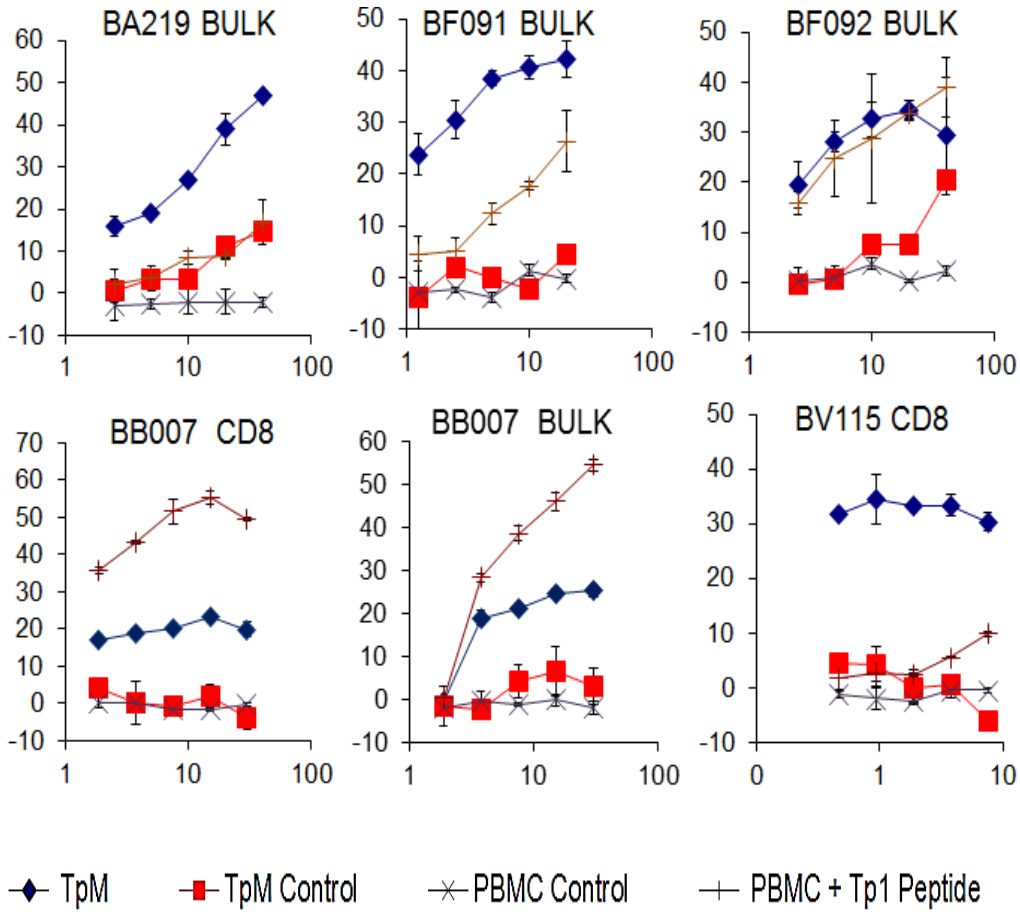

Figure 4.10: Cytotoxicity assay for the cell lines BA219 bulk, BF091 bulk, BF092 bulk, BB007 purified CTL, BB007 bulk and BV115 purified CTL.

Table 4.4: Cytotoxicity of $Tp1_{214-224}$ pulsed PBMC and TpM

Cell Line	Effector : target ratio	TpM cytotoxicity (%)	$Tp1_{214-224}$ pulsed PBMC cytotoxicity (%)
BA219 BULK	7.50	20.14	8.28
	15.0	24.7	10.71
	20.00	27.81	10.98
BF091 BULK	7.50	36.62	15.89
	15.0	40.65	22.22
	20.00	35.75	26.58
BF092 BULK	7.50	24.81	24.13
	15.0	26.89	30.58
	20.00	26.96	33.64
BB007 BULK	7.50	16.96	38.77
	15.0	18.32	46.18
	20.0	19.82	46.96
BB007 CD8	7.50	20.89	53.05
	15.0	21.33	56.82
	20.0	23.13	53.86
BV115 CD8	7.50	36.31	10.34

Cytotoxicity of the PBMC bulk and purified CD8 cell lines was examined using a ^{51}Cr-release assay at the indicated effector: target ratios (E: T ratio). Target T-cells consisted of autologous PBMCs pulsed with the $Tp1_{214-224}$ peptide and autologous Muguga-infected lymphocytes (TpM). Unpulsed PBMC and heterologous infected cell lines are used as controls. The net cytotoxicity for the autologous Tp1 peptide pulsed PBMCs and TpM were calculated by subtracting the respective controls.

Table 4. 5: Correlation of cytotoxicity and biomarker parameters

Parameters correlated with cytotoxicity of $Tp1_{214-224}$ pulsed PBMC at effector: target ratio of 20	**Spearman's correlation coefficient**	**P-Value**
% Total Tp1 tetramer + cells	0.9	0.004
% Tp1 tetramer + cells times MFI	1	<0.001
% perforin + cells (gated on Tp1 tetramer + cells)	0.9	0.004
MFI for perforin (gated on Tp1 tetramer + cells)	0.8	0.021
% perforin + cells times MFI for perforin (gated on tetramer + cells)	1	<0.001
MFI for CD25 (gated on Tp1 tetramer + cells)	-0.9	0.004

Parameters used for correlation with cytotoxicity at E:T ratio of 20 are listed. Spearman Rank correlation was used. Correlation coefficients and P-values are

CHAPTER FIVE

DISCUSSION

5.1 Cell surface and intracellular biomarkers expressed on the antigen specific CD8+ cells in *T. parva* infection

In the endeavour to understand the dynamics of CD8+ T-cell response to *T. parva* in East Coast fever infection, it was essential to phenotypically and functionally profile these *T. parva* specific CD8+ T-cells. The use of Tetramers of MHC class I molecules containing the antigenic peptide provided a great way for quantitating antigen – specific CD8+ T-cells. This method not only permits direct visualization of *T. parva* -specific CD8+ T-cells, but also the analysis can be carried out without extensive *in vitro* manipulation (Altman *et al.,* 1996).

With regard to the expression profiles of the three cell types, it was interesting to note that there was significantly high expression of the effector memory T-cell marker CD44, the central memory T-cell marker CD62L and the cytotoxicity marker Perforin. This observation corresponded to previous studies where CD44 has been described as a ubiquitous transmembrane glycoprotein protein, multi-structural and multifunctional adhesion molecule, involved in cell–cell and cell–matrix interactions in a broad range of tissues (Alstergren, *et al*., 2005).

Perforin (pore forming protein) expression is a key marker for cytotoxic potential. Cytotoxic CD8+ T-cells primarily act through secretion of cytotoxic granules containing perforin and several granule associated proteases, including granzymes (Cellerai *et al*., 2010).

Recently, a mechanism of perforin-dependent granule independent CTL cytotoxicity has also been shown (Makedonas *et al*., 2009). All three cell types under study expressed perforin albeit in differing degrees. The CD8+ purified cell lines showed higher expression of perforin than the CD8+ bulk. This expression corresponded with the cytotoxic potential of the cells.

There was also considerable expression of the memory T-cell marker CD45R0 and relatively low levels of the alpha chain IL-2 receptor marker CD25. CD45 isotypes are often used to characterize memory and naive T-cell populations in humans. There is conversion from a (CD45RA+, CD45RO-) phenotype for naive cells to the (CD45RA-, CD45RO+) phenotype characteristic of memory cells activation by antigen. There have also been observed some reversion from the memory to the naive phenotype (Burns *et al*., 2001). Therefore the relatively low numbers of CD45R0+ cells may have been as a result of low activated memory cells or possible reversion from memory to naïve cells. The experimental animals also expressed very little or no levels of the iron delivery protein marker CD71 and FAS ligand. The level of transferrin receptor expression is highest in early erythroid precursors. (Lesley *et al*., 1984). Fas is expressed on many cells, whereas its ligand, FasL, is expressed on active T-cells. (Green *et al*., 2003). However, it has been shown that the FAS-ligand pathway is not important with regards to *T. parva* infection (Küenzi *et al*., 2003).

5.2 IFN-γ linked Immune cell activation of CD8+ cells and γδ+ T-cells in *T. parva* infection

With regard to CD8+ T-cell activation, the activated cells secrete IFN-γ in response to antigen recognition. *In vivo* the IFN-γ leads to macrophage activation, MHC and co stimulator activation among other functions (Kindt, *et al.,* 2006). The γδ T-cells have been associated with infections caused by intracellular pathogens. This has led to the suggestion that γδ T-cells constitute an innate surveillance system that acts as a first line of defence against infectious diseases (Daubenberger *et al*., 1999). Therefore, it was endeavoured to find out whether bovine γδ T-cells response to cells infected with *T. parva* differed significantly from CD8+ cells**.**

In this study, the patterns of the IFN-γ responses in the two different T-cell types (CD8+ and γδ T-cells) were not significantly different in two out of three animals.

Despite the fact that the purified CD8+ lines had higher response values than the γδ cells. Upon statistical analysis, this apparent difference was not statistically significant. The fact that there was considerable activation of these γδ T-cells, this was consistent with the findings of previous studies which indicate that γδ cells have a significant role in parasitic immune response (Daubenberger *et al*., 1999).

The exact role of γδ T-cells in infectious and parasitic disease is still unresolved. In humans infected with malaria there is evidence that a proportion of γδ T-cells are increased in both peripheral blood and spleen (Perera *et al*., 1994). In cattle, it has also been observed that γδ T-cells expand in cell lines derived from Babesia bovis-immune cattle by stimulation with merozoite fractions (Brown *et al*., 1994).

The phenotypic characterization of these γδ T-cell lines showed that a subpopulation of cells expressed CD2. However, it is not known whether these cells coexpress CD2 with WC1. Furthermore, since the γδ T-cells are not MHC restricted in their response, it was unclear which antigens triggered their response. This is because previous studies have shown that they can react to a variety of antigens including, altered self-proteins, stress proteins, or other autoantigens (Brown *et al*., 1994).

5.3 Cytotoxic capacities of CD8+ cells and γδ+ T-cells in *T. parva* infection

This study found that there was a significant number of γδ T-cells that were activated and secreted IFN γ, however, their cytotoxicity in this study was negligible in all the six experimental animals. This seems to indicate that the role of γδ cells in immunity to *T. parva* may not be the direct cytotoxicity of the infected cells. The low cytotoxicity of the γδ T-cells was possibly due to much lower sensitivity to the mismatch line than the autologous line by which the CD8+ T-cell lines had been stimulated with in culture.

There is strong evidence that the protective immune response to *T. parva* is based on elimination of infected cells by parasite-specific class I MHC-restricted CTL. In addition, class II MHC-restricted CD4+ T-cells with helper and cytolytic activity can

be detected after immunization of cattle with *T. parva* (Mckeever *et al*., 1994). The exact mechanism of bovine γδ T-cells activity in *T. parva* infection remains unclear. Hence requires further investigation.

5.3.1 Perforin and CD25 expression in T. parva specific CTL correlate with cytotoxicity

Investigation of new biomarkers for prediction of disease outcome is an increasingly Important topic including the finding of correlates with protection after vaccination. In this series of experiments the correlation of tetramer + cells, perforin and CD25 expression the cytotoxicity in six cell lines originating from immunized cattle was investigated.

It was found that the percentage of tetramer + cells correlated with the cytotoxicity when the target T-cells were pulsed with the peptide used in the tetramer. An even better correlate for cytotoxicity was the product of the percentage of tetramer + cells and the net MFI of the staining. The MFI is a result of the number of TCRs per cell and their affinity for the tetramer, which obviously is of importance during the interaction of the CTL and the target T-cell.

This suggests that tetramer staining (the product of the percentage of + cells X the mean staining intensity of the tetramer population) can be used to predict the level of cytotoxicity, which in the case of *T. parva* correlates with protection. Such correlations may be elaborated further to include a mix of tetramers if several specificities are known or investigated at the same time. The tetramer staining did not correlate with the cytotoxicity to the *T. parva* infected target T-cells, indicating that there are other CTL specificities in the cell lines which contribute to the cytotoxicity.

Cytotoxic CD8 T-cells primarily act through secretion of cytotoxic granules containing perforin and several granule associated proteases, including granzymes (Trapani *et al.*, 2002) A mechanism of perforin-dependent granule independent CTL

cytotoxicity has also been shown (Makedonas *et al*., 2009). In all cell lines under study, the *T. parva* specific CD8 T-cells expressed high levels of perforin. The CD8 enriched cell lines showed higher expression of perforin than the CD8 bulk consistent with the fact that they were almost pure CD8 cells. These CD8 enriched cell line were made six months prior, hence there may be a selection for high avidity clones in these, explaining the higher MFI. Both the number of perforin + cells and the MFI correlated with the cytotoxicity independently. The product of the percentages of perforin and tetramer double + cells and the net mean staining intensity of perforin showed a perfect correlation with the cytotoxicity of the peptide pulsed PBMC (McElhaney *et al*., 2009). Perforin has previously been suggested as a marker for protection in tuberculosis based on the presence of perforin in immune cells compared to non-immune cells after restimulation (Bolitho *et al*., 2007). Other potential cytotoxicity markers could be molecules such as e.g. granzyme B and granulysin. Granzyme B is also a mediator of cytotoxicity and has been shown to correlate with protection and enhanced CTL responses in virus-stimulated peripheral blood mononuclear cells after influenza vaccination (McElhaney *et al*., 2009). Granulysin has been proposed as a prospective candidate biomarker for protection after immunization against *Mycobacterium bovis* (Capinos *et al*., 2009)

The MFI for CD25 correlated inversely with the cytotoxicity which may relate to the life cycle of the T-cell. CD25 is up-regulated on actively proliferating cells and the current results may reflect a down regulation due to the mature state of the effector CTL. This inverse correlation has not, to the authors' knowledge, been described and CD25 has not previously been proposed as a marker for cytotoxicity but its upregulation is a known marker for activated/proliferating T-cells. A low CD25 expression could be used as a potential maker for the level of cytotoxicity.

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CHAPTER SIX

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

6.1 Summary

The aim of this study was to help the development of an alternative ECF vaccine to the current live vaccine. This will be achieved through a much better understanding of the CD8+ T-cells. These are believed to be the main line of defense in East Coast fever. Hence it is essential to generate a profile of the *T. parva* specific CD8+ killer cells.

In this study the phenotypic and functional characterization of these *T. parva* specific CD8+ Cells was performed. This was done on the basis of expression of surface and intracellular effector biomarkers degree of cell activation by secretion of interferon γ, and cytotoxic potential against *T. parva* infected cells was done in comparison to γδ T-cells.

From this study it was possible to elucidate the various surface and intracellular biomarkers found on *T. parva* specific CD8+ T-cells. These include; CD2, CD3, CD4, CD25, CD44, CD45RO, CD62L, CD71, WC1, γδTCR, Perforin, FasL. It was also observed from three cell lines the degree of activation of CD8+ T-cells to *T. parva* antigen peptide Tp1214-224 as compared to γδ T-cells was not significantly different. The study revealed that two of the activation markers, perforin and CD25 as possible surrogate markers for cytotoxicity, which is known to correlate with protection against *T. parva* infection. The mean staining intensity of CD25 positive tetramer positive cells correlated negatively with the cytotoxicity. These results suggest that perforin and CD25 could be possible biomarkers for the cytotoxicity to *T. parva* immunizations.

6.2 Conclusions

The following conclusions can be made from the study:

1. Using Tetramer technology, on these antigen specific CD8+ cells, it was possible to detect lineage markers; CD2 and CD3, activation markers; perforin and CD25 and memory markers; CD44, CD45RO and CD62L.

2. A significant outcome of this study was the observation that both bovine γδ T-cells and CD8+ T-cells were activated in the presence of *T. parva*-infected cells. This activation was measured by IFNγ secretion.

3. In the presence of *T. parva*-infected cells, CD8+ T-cells exhibited cytotoxicity while for γδ+ T-cells the level of cytotoxicity was not detectable. The levels of perforin and CD25 are potential biomarkers for cytotoxicity.

6.3 Limitations

1. The funding was a limitation as to how many animals were to be used in the study. The cost of purchase for the animals (Kshs. 49,000) and the cost of maintenance per day (Kshs. 400) especially during immunization and the rest of the time for 18 months was prohibiting.

2. Tetramer technology that was used for FACS analysis had limited application prior in bovine research and was also very expensive.

6.4 Recommendations

1. Use Tetramer technology can further be used to carry out in depth correlation studies between the various effector biomarkers such as perforin, CD25, FasL, CD38 and cytotoxicity. This will provide surrogate markers for cytotoxicity and avoid the tedious and potentially harmful cytotoxicity assays that make use of radiation.

2. Even though this study reports a clear activation of γδ+ T-cells but negligible cytotoxicity, this population of T-cells clearly has an important role in immunity especially considering they can make up to 50% of all peripheral T-cells in young cattle. Hence studies geared towards a clear understanding of γδ+ T-cells effector mechanism will prove very beneficial in the search of a recombinant subunit vaccine East coast fever vaccine.

3. Further studies on other important and potentially important players in *T. parva* infection immunology such as CD4+ T cells and cytokine regulation should be explored in the endeavor to develop an alternative vaccine to the current live vaccine.

REFERENCES

Aisen, P. (2004). Transferrin receptor 1. Int. J. Biochem. *Cell Biology*. **36 (11),** 2137–43.

Alstergren, P., Zhu, B., Glogauer, M., Mak, T. W., Ellen, R. P., and Sodek, J. (2005). Erratum to Polarization and directed migration of murine neutrophils is dependent on cell-surface expression of CD44. *Cellular Immunology*, **234(1),** 76.

Altman, J. D., Moss P. A., Goulder, P. J., Barouch, D. H., McHeyzer-Williams, M. G., Bell J. I., and Davis M. M. (1996). Phenotypic analysis of antigen-specific T lymphocytes. *Science*, **274(5284),** 94-96.

Arendt, C. W., and Ostergaard, H. L. (1997). Identification of the CD45-associated 116-kDa and 80-kDa proteins as the alpha- and beta-subunits of alpha-glucosidase II. *Journal of Biological Chemistry,* **272 (20),** 13117–25.

Baldwin, C. L., Black, S. J., Brown, W. C., Conrad, P. A., Goddeeris, B. M., Kinuthia, S. W., Lalor, P. A… and Newson, J. (1988). Bovine T-cells, B cells and null cells are transformed by the protozoan parasite *T.parva*. *Infection and Immunity*, **56,** 462 – 467.

Bernard, A. (1984). Leucocyte typing: human leucocyte differentiation antigens detected by monoclonal antibodies: specification, classification, nomenclature: [report on the first international references workshop sponsored by INSERM, WHO and IUIS]. Berlin: *Springer*. 45–48.

Boehm, U., Klamp, T., Groot, M. and Howard, J. C. (1997). Cellular responses to interferon-c. *Annual Review in Immunology*, **15,** 749–795.

Bolitho, P., Voskoboinik, I., Trapani, J. A., and Smyth, M. J., (2007) Apoptosis induced by the lymphocyte effector molecule perforin, *Current Opinion in Immunology*, **19**, 339–347.

Brown, W. C., Davis, W. C., Choi, S. H., Dobbelaere, D. A. E., and Splitter, G. A. (1994). Functional and phenotypic characterization of WC11 g/d T-cells isolated from Babesia bovis-stimulated T-cell lines. *Cellular Immunology*, **153**, 9–27.

Brown, C. G. D., Stagg, D. A., Purnell, R. E., Kanhai, G. K. and Payne, R. C. (1973). Infection and transformation of bovine lymphoid cells *in vitro* by infective particles of *T.parva*. *Nature*, **245,** 101-103.

Burns, J. B., Bartholomew, B. D., and Lobo, S. T. (2001). Isolation of CD45RO< sup>+</sup>, Memory T-cells Recognizing Proteolipid Protein from Neurologically Normal Subjects. *Cellular immunology*, **212(1),** 44-50.

Capinos, S. C. F., Endsley, J. J., De Aguiar, J. B., Jacobs, Jr W. R., Larsen, M. H., Palmer, M., and Mark, Estes D. (2009). Evaluation of granulysin and perforin as candidate biomarkers for protection following vaccination with mycobacterium bovis BCG or M. bovisΔRD1. *Transboundary and emerging diseases*, **56(6-7),** 228-239.

Cellerai, C., Perreau, M., Rozot, V., Enders, F. B., Pantaleo, G., and Harari, A. (2010) Proliferation potential and cytotoxic activity are mediated by functionally and phenotypically distinct virus-specific CD8+ T-cells defined by interleukin-7rα CD127) and perforin expression. *Journal of Virology*, **84(8),** 3868–3878.

Chien, Y. H., Jores, R., and Crowley, M. P. (1996). Recognition by γδ T-cells. *Annual, Review in Immunology*, **14**,511–532.

Daubenberger, C. A., Taracha, E. L., Gaidulis, L., Davis W. C., and McKeever, D. J. (1999). Bovine gammadelta T-cell responses to the intracellular protozoan parasite *Theileria parva*. *Infection and Immunity*, **67(5)**, 2241-9.

De Leeuwav, P. N. (1995) Monitoring of livestock health and production in sub-Saharan Africa Preventive Veterinary Medicine **25,** 195-212

Ellis, S. A., Morrison, W. I., MacHugh, N. D., Birch, J., Burrells, A., and Stear, M. J. (2005) Serological and molecular diversity in the cattle MHC class I region. *European Journal of Immunogenetics*, **57**, 601-606

Ellis, S. A., Staines K. A., Stear M. J., Henson E. J., and Morrison W. I. (1998) DNA typing for BoLA class I using sequence specific primers (PCR-SSP). *European Journal of Immunogenetics*, .**25**, 365-370

Emery, D. L., Eugui, E. M., Nelson, R. T., and Tenywa, T. (1981). Cell-mediated immune responses to *Theileria parva* (East Coast fever) during immunisation and lethal infections in cattle. *Immunology*, **43,** 323-335.

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

Emery, D.L. (1981c). — Adoptive transfer of immunity to infection with *Theileria parva* (East Coast fever) between cattle twins. *Research in Veterinary Science*, **30,** 364-367.

Emery, D. L., and Morrison, W.I. (1980). - Generation of autologous mixed leucocyte reactions during the course of infection with *Theileria parva* (East Coast fever) in cattle. *Immunology,* **40,** 229-237.

Eugui, E. M., and Emery, D. L., (1981). Genetically restricted cell-mediated cytotoxicity in cattle immune to *Theileria parva*. *Nature*, **290,** 251-254

Fawcett, D. W., Doxsey, S., and Büscher, G. (1982). Salivary gland of the tick vector of East Coast fever. III. Ultrastructure of sporogony in *Theileria parva. Tissue and Cell* **14,** 183-206.

Geysen, D. (2000). The application of Molecular Biology techniques to analyse diversity in *Theileria parva* populations in Zambia. Department of Biology and Biochemistry, London: *Brunel University*.

Goddeeris, B. M., and Morrison, W. I., (1988) Techniques for the generation, cloning and characterization of bovine cytotoxic T-cells specific for the protozoan *Theileria parva. Journal of Tissue Culture Methods,* **11**, 101-110

Goddeeris, B. M., Morrison, W. I., and Teale, A. J. **(1986).** Generation of bovine cytotoxic cell lines specific for cells infected with the protozoan parasite *Theileria parva* and restricted by products of the major histocompatibility complex. *European Journal of Immunology,* **16,** 1243-1249.

Goddeeris, B. M., Morrison, W. I., Teale, A. J., Bensaid, A., and Baldwin, C. L. **(1986a).** Bovine cytotoxic T-cell clones specific for cells infected with the protozoan parasite *Theileria parva:* specificity and class I major histocompatibility complex restriction. *Proceedings of the National Academy of Sciences, USA,* **83,** 5238-5242.

Goodier, M., Fey P., Eichmann, K., and Langhorne, J. (1992). Human peripheral blood γδ T-cells respond to antigens of *Plasmodium falciparum*. *Infection and Immunity,* **4**, 33–41.

Goodison, S., Urquidi, V., and Tarin, D. (1999). CD44 cell adhesion molecules. *MP, Molecular Pathology,* **52 (4),** 189–96.

Green, D. R., Droin, N., and Pinkoski, M. (2003). Activation-induced cell death in T-cells, *Immunology Reveiws,* **193,** 70–81.

Graham, S. P., Pelle R., Honda, Y., Mwangi, D. M., Tonukari, N. J., Yamage, M., and Glew, E. J. (2006). *Theileria parva* candidate vaccine antigens recognized by immune bovine cytotoxic T lymphocytes. *Proceedings of the National Academy of Sciences, USA* **103(9),** 3286-91.

Graham, S. P., Pellé, R., Yamage, M., Mwangi, D. M., Honda, Y., Mwakubambanya, R., and De Villiers, E., (2008). Characterization of the fine specificity of bovine CD8+ T-cell responses to defined antigens from the protozoan parasite *Theileria parva. Infection and Immunity*, **76(2)**, 685-94.

Hein, W. R., and Mackay, C. R. (1991). Prominence of γδ T-cells in the ruminant immune system. *Immunology Today,* **12**, 30–34.

Henkart, P. A. (1994). Lymphocyte-mediated cytotoxicity: two pathways and multiple effector molecules. *Immunity*, **1(5),** 343-346.

Higashida, H., Yokoyama, S., Huang, J. J., Liu, L., Ma, W. J., Akther, S., Higashida, C., Kikuchi, M., Minabe, Y., Munesue, T. (2012). Social memory, amnesia, and autism: brain oxytocin secretion is regulated by NAD+ metabolites and single nucleotide polymorphisms of CD38. *Neurochemistry International,* **61 (6),** 828–38.

Hulliger, L. Wilde, J. K. H., Brown, C. G. D. and Turner, *L*. (1964). Mode of Multiplication of *Theileria parva* in Cultures of Bovine Lymphocytic Cells. *Nature,* **203**, 728-30.

Irvin, A. D., and Morrison, W. I., (1987). Immunopathology, immunology and imunoprophylaxis of *Theileria* infections. In immune responses in parasitic infections: Immunopathology, immunology and imunoprophylaxis. *Florida: CRC press Boca Raton,* **3,** 223 – 274.

Isobe, M., Huebner, K., Maddon, P. J., Littman, D. R., Axel, R., and Croce, C. M., (1986). The gene encoding the T-cell surface protein T4 is located on human chromosome 12. *Proceedings of the National Academy of Sciences, U.S.A.* **83 (12),** 4399–402.

James, J. L., Carter, A. M., Chamley, L. W., (2012). Human placentation from nidation to 5 weeks of gestation. Part I: What do we know about formative placental development following implantation? *Placenta* **33 (5),** 327–34.

Kagi, D. F., Vignaux, B., Ledermann, K., Burki, V., Depraetere, S., Nagata, H., and Hengartner, P. G., (1994). Fas and perforin pathways as major mechanisms of T-cell-mediated cytotoxicity, *Science,* **265,** 528–530.

Kaplan, R., Morse, B., and Huebner, K., (1990). Cloning of three human tyrosine phosphatases reveals a multigene family of receptor-linked protein-tyrosine-phosphatases expressed in brain. *Proceedings of the National Academy of Sciences, U.S.A.* **87 (18),** 7000–4.

Kindt, J. T., Osborne, A. B., and Goldsby, A. R. (2006) Kuby Immunology, Sixth Edition. San Francisco: W H Freeman and Company. 351 – 358.

Kohn, L. A., Hao, Q. L., Sasidharan, R., Parekh, C., Zhu, Y., Mikkola, H. K., and Crooks G. M., (2012). Lymphoid priming in human bone marrow begins before expression of CD10 with upregulation of L-selectin. *Nature Immunology,* **13 (10),** 963–71.

Kuby, J., Kindt, T. J., Goldsby, R. A., and Osborne, B. A. **(2007).** *Kuby immunology*. San Francisco: W.H. Freeman.

Küenzi, P., Schneider, P., and Dobbelaere, D.A.E., (2003). *Theileria parva-Transformed T-cells Show Enhanced Resistance to Fas/Fas Ligand-Induced Apoptosis 1*. *The Journal of Immunology,* **171 (3),** 1224-1231

Leong, A. S. Y., Cooper, K., Leong, F., and Joel, W. M. (2003). *Manual of Diagnostic Cytology* (2 ed.). London**:** Greenwich Medical Media, Ltd.

Lesley, J., Hyman R., and Schulte, R., (1984). Expression of transferrin receptor on murine hematopoietic progenitors. *Cellular Immunology,* **83,** 14-25.

MacHugh, N. D., Connelley, T., Graham, S. P., Pelle, R., Formisano, P., Taracha, E. L., Ellis, S. A… and Morrison, W. I. (2009). CD8+ T-cell responses to *Theileria parva* are preferentially directed to a single dominant antigen: Implications for parasite strain-specific immunity. *European Journal of Immunology,* **39(9),** 2459-69.

Makedonas, G., Banerjee, P. P., Pandey, R., Hersperger, R. A., Sanborn, B. K., Hardy, A. D. G., Orange, S. J., and Betts, R. M., (2009). Rapid Up-Regulation and Granule-Independent Transport of Perforin to the Immunological Synapse Define a Novel Mechanism of Antigen-Specific CD8+ T-cell Cytotoxic Activity. *Journal of Immunology,*. **182(9)**, 5560-5569.

McElhaney, J. E., Ewen, C., Zhou, X., Kane, K. P., Xie, D., and Hager, W.D. (2009) Granzyme B: correlates with protection and enhanced CTL response to influenza vaccination in older adults. *Vaccine*; **27(24),** 18–25.

McKeever, D. J., Taracha, E. L. N., Morrison, W. I., Musoke, A. J., and Morzaria, S. P. (1999). Protective Immune Mechanisms against< i> *Theileria parva*</i>: Evolution of Vaccine Development Strategies. *Parasitology Today,* **15(7),** 263-267.

McKeever, D. J., Nyanjui, J. K., and Ballingall, K. T. (1997). *In vitro* infection with *Theileria parva* is associated with IL10 expression in all bovine lymphocyte lineages. *Parasite immunology,* **19(7),** 319-324.

McKeever, D. J., Taracha, E. L., Innes, E. L., MacHugh, N. D., Awino, E., Goddeeris, B. M., and Morrison, W. I. (1994). Adoptive transfer of immunity to *Theileria parva* in the CD8+ fraction of responding efferent lymph. *Proceedings of the National Academy of Sciences of the United States of America*, **91(5),** 1959–63.

McKeever, D. J., and Morrison, W. I. (1990). *Theileria parva*: the nature of the immune response and its significance for immunoprophylaxis. *Revue Scientifique ET Technique de L` Office International Des Epizooties,* **9 (2),** 405-421

Mehlhorn, H., Schein, E., and Warnecke, M. (1978). Electron microscopic studies on the development of kinetes of *Theileria parva* in the gut of the vector ticks *Rhipicephalus appendiculatus. Acta Tropica,* **35,** 123-136.

Morrison, W. I., Goddeeris, B. M., Teale, A. J., Baldwin, C. L., Bensaid, A. and Ellis, J. (1986). Cell mediated immune responses of cattle to *Theileria parva*. *Immunology Today,* **7,** 211-216.

Morrison, W. I., Goddeeris, B. M., Teale, A. J., Groocock, C. M., Kemp, S. J. and Stagg, D. A. (1987a). Cytotoxic T-cells elicited in cattle challenged with *Theileria parva* (Muguga): evidence for restriction by class I MHC determinants and parasite strain specificity. *Parasite Immunology,* **9,** 563-578.

Morrison, W. I. (1987). Host effector mechanisms against parasites. *Veterinary Parasitology,* **25 (2)**, 163-76.

Morrison, W. I., Taracha, E. L. N., and Mckeever, D. J. (1995a) *Theileriosis*: progress towards vaccine development through understanding immune responses to the parasite. *Veterinary Parasitology,* **57,**177-187

Morrison, W. I., Taracha, E. L. N., and Mckeever, D. J. (1995b). Contribution of T-cell responses to immunity and pathogenesis in infections with *Theileria parva*. *Parasitology Today,* **11, (1),**14-18.

Morrison, W. I. (1996). Influence of host and parasite genotypes on immunological control of *Theileria* parasites. *Parasitology*. **112**, S53–S66.

Morrison, W. I. (2006). Current status of vaccine development against *Theileria* parasites. *Veterinary Parasitology,* **133,** 169-197

Morrison, W. I. (2009). Progress towards understanding the immunobiology of *Theileria* parasites. *Veterinary Parasitology,* **130 (2)**, 1-12

Mukhebi, A. W., (1992). Economic impact of *Theileriosis* and its control in Africa. In the Epidemiology of *Theileriosis* in Africa, eds Norval RA, Perry BD, Young AS. *London: Academic press*: 379-403.

Müllbacher, A., Lobigs, M., Hla, R. T., Tran, T., Stehle, T., and Simon, M. M. (2002). Antigen-dependent release of IFN-γ by cytotoxic T-cells up-regulates Fas on target T-cells and facilitates exocytosis-independent specific target T-cell lysis. *Journal of Immunology,* **169,** 145–150.

Musoke, A. J., Nantulya, V. M., Buscher, G., Masake, R. A., and Otim, B. (1982) Bovine immune response to *Theileria parva*: neutralizing antibodies to sporozoites. *Immunology,* **45,** 663-668.

Nagata, S. (1999). Fas ligand-induced apoptosis. *Annual review of genetics*, **33(1),** 29-55.

Nata, K. T., Karasawa, T., Kumagai, T., Hashioka, T., Tohgo, W., Yonekura, A., Takasawa, H., Nakamura, S., and Okamoto, S. H., (1997). Human gene encoding CD38 (ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase): organization, nucleotide sequence and alternative splicing. *Gene* **186 (2),** 285–92.

Norval, R. A. I., Perry, B. D., and Young, A. S. (1992). The epidemiology of *Theileriosis* in Africa. *London: Academic Press*.

Pearson, T. W., Lundin, L. B., Dolan, T. T., and Stagg, D. A. (1979). - Cell-mediated immunity to *Theileria*-transformed cell lines. *Nature*, **281,** 678-680.

Perera, M. K., Carter R., Goonewardene, R., and Mendis, K. N. (1994). Transient increase in circulating gamma/delta T-cells during Plasmodium vivax malarial paroxysms. *The Journal of experimental medicine*, **179(1),** 311-315.

Pinkoski, M. J., and Green, D. R. (2005). Apoptosis in the regulation of immune responses, *Journal of Rheumatology Supplement,* **74,** 19–25.

Pipkin, M. E., and Lieberman, J. (2007). Delivering the kiss of death: progress on understanding how perforin works, *Current Opinion in Immunology,* **19,** 301–308.

Rabin, M., McClelland, A., Kühn, L., and Ruddle, F. H. (1985). Regional localization of the human transferrin receptor gene to 3q26.2---qter. *American Journal of Human Genetics,* **37 (6),** 1112–6.

Radley, D. E., Brown, C. G., Cunningham, M. P., Kimber, C. D., Musisi, F. L., Purnell R. E., and Stagg, S. M. (1975). East Coast Fever: challenge of immunized cattle by prolonged exposure to infected ticks. *Vet Rec,* **96 (24),** 525-7.

Robbins, S. L., Cotran, R. S., Kumar, V., and Collins, T. (1998). Robbins Pathologic Basis of Disease. Philadelphia: *W.B Saunders Company***.**

Rouvier, E., Luciani, M. F., and Golstein, P. (1993). Fas involvement in Ca2+-independent T-cell-mediated cytotoxicity. *Journal of Experimental Medicine,* **177,** 195-200.

Shapiro, H. M. (2003). Practical Flow Cytometry. New York: *Wiley Liss*.

Shaw, M. K., and Tilney, L. G. (1992). How individual cells develop from a syncytium: merogony in *Theileria parva* (Apicomplexa). *Journal of cell science,* **101(1),** 109-123.

Schein, E. Mehlhorn, H. and Warnecke, M. (1977). The development of *Theileria parva* (Theiler, 1904) in the gut of *Rhipicephalus appendiculatus*. *Parasitology,* **75,** 309-316.

Sobek, V., Balkow, S., Körner, H., and Simon, M. M., (2002). Antigen-induced cell death of T effector cells *in vitro* proceeds via the Fas pathway requires endogenous interferon-γ and is independent of perforin and granzymes. *European journal of immunology,* **32(9),** 2490-2499.

Spring, F. A., Dalchau, R., Daniels, G. L., Mallinson, G., Judson, P. A., Parsons, S. F., Fabre, J. W., and Anstee, D. J. (1988). The Ina and Inb blood group antigens are located on a glycoprotein of 80,000 MW (the CDw44 glycoprotein) whose expression is influenced by the In (Lu) gene. *Immunology,* **64 (1),** 37–43.

Theiler, A. (1904). Spirillosis of cattle. *Journal of Comparative Pathology and Therapeutics,* **17**, 45 – 55.

Townsend, A. R. M Rothbard, J., Gotch F. M., Bahadur, G., Wraith, D., and Mcmichael, A. J. (1986). - The epitopes of influenza nucleoprotein recognised by cytotoxic T lymphocytes can be defined with short synthetic peptides. *Cell,* **44,** 959-968.

Toye, P. G., Metzelaar, M. J., Wijngaard, P. J. L., Nene, V., lams, K., Roose, J., Nyanjui, J. K., Gobright, E., , A. J. and Clevers, H. C. (1995) Characterisation of the gene encoding the polymorphic immunodominant molecule, a neutralizing antigen of *Theileria parva*. *Journal of Immunology,* **155,** 1370- 1381.

Russell, W. M. S., Burch, R. L., and Hume, C. W. (1959). The principles of humane experimental technique. London: Methuen

Triplett, T. A., Curti, B. D., Bonafede, P. R., Miller, W. L., Walker, E. B., and Weinberg, A. D. (2012). Defining a functionally distinct subset of human memory CD4+ T-cells that are CD25POS and FOXP3NEG. *European journal of immunology,* **42(7),** 1893-1905.

Van de Putte, T. (1992) Investigation of neonatal immunity of calves against *Theileria parva*. Internal research report, Nairobi: ILRI

Vassalli, P. (1992). The pathophysiology of tumor necrosis factor. Anna. *Reviews in Immunology,* **10**, 411-452.

Visser, A. E., Abraham, A., Sakyi, L. J. B., Brown, C. G. D., and Preston, P. M. (1995). Nitric oxide inhibits establishment of macroschizont-infected cell lines and is produced by macrophages of calves undergoing bovine tropical *Theileriosis* or East Coast fever. *Parasite immunology,* **17,** 91-102.

Wilkins, A., Yang, W., and Yang, J. (2003). Structural biology of the cell adhesion protein CD2: from molecular recognition to protein folding and design. *Current Protein and Peptide Science,* **4 (5),** 367–73

Wilson, S.G. (1950). An experimental study of East Coast fever in Uganda. I. A study of the type of East Coast fever reactions produced when the number of ticks is controlled. *Parasitology,* **40,** 195-209.

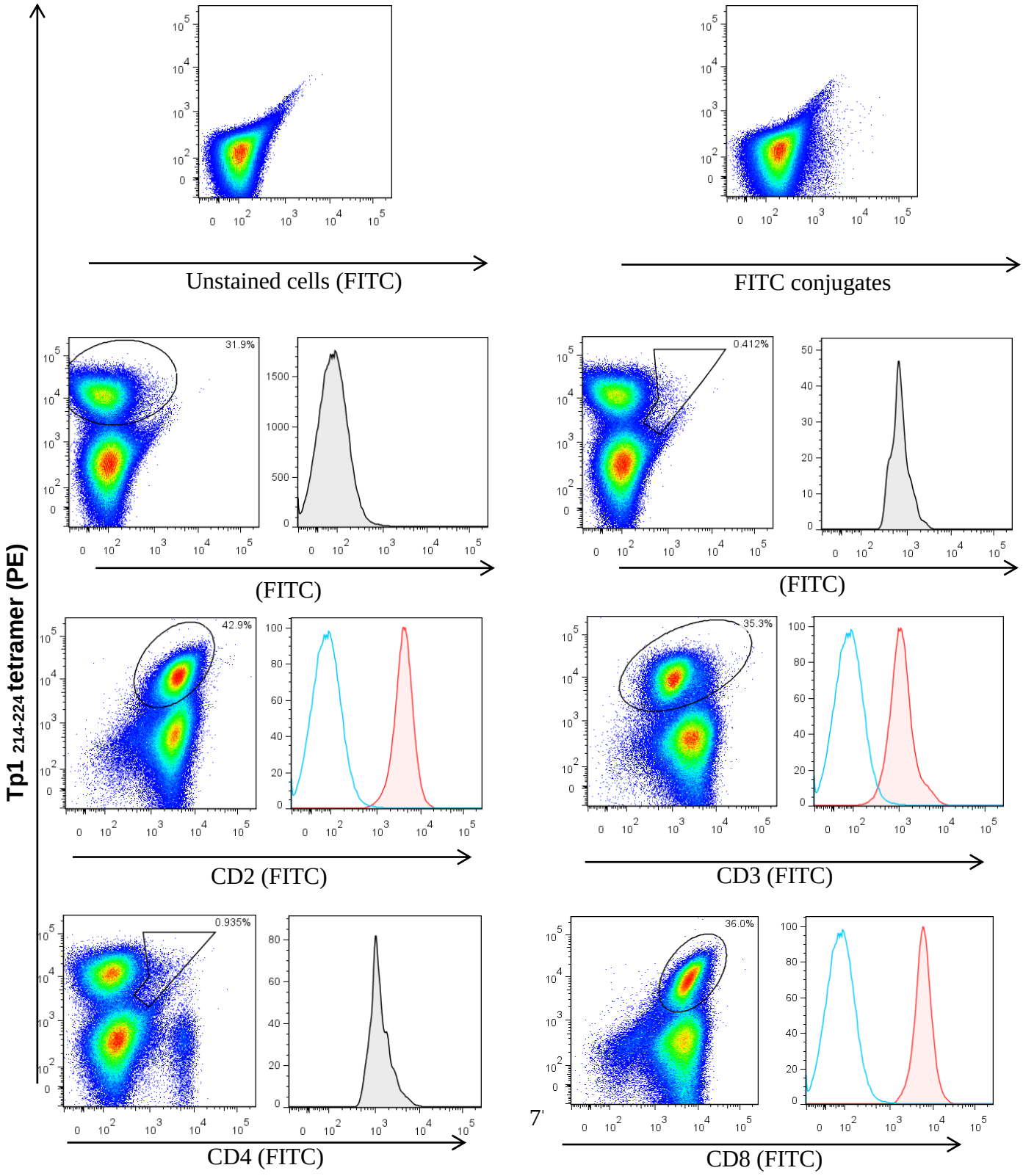
Yamane, T. (1967). Elementary sampling theory. New Jersey: Prentice-hall inc.

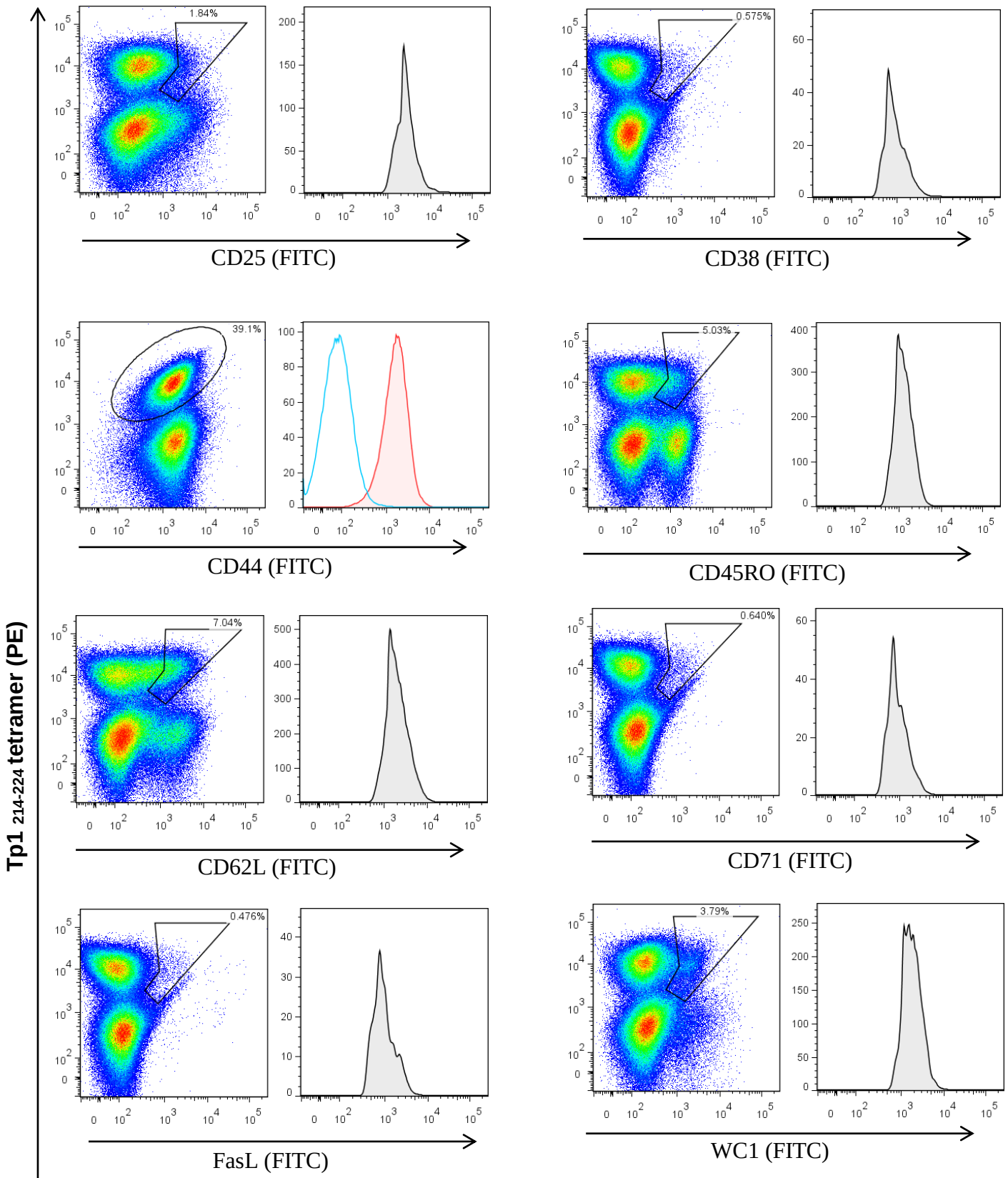
Yang, J. J., Ye, Y., Carroll, A., Yang, W., and Lee, H.W. (2001). Structural Biology of the Cell Adhesion Protein CD2 Alternatively Folded States and Structure-function Relation. *Current Protein and Peptide Science*, **2(1),** 1-17.

APPENDICES

Appendix 1: Surface and intracellular markers analyzed.

Figure 6. 1 (a-v): Animal BB007 CTL cell lines' FACS data




Tp1 214-224 tetramer (PE)
1.84%
CD25 (FITC)
0.575%
CD38 (FITC)
39.1%
CD44 (FITC)
5.03%
CD45RO (FITC)
7.04%
CD62L (FITC)
0.640%
CD71 (FITC)
0.476%
FasL (FITC)
3.79%
WC1 (FITC)

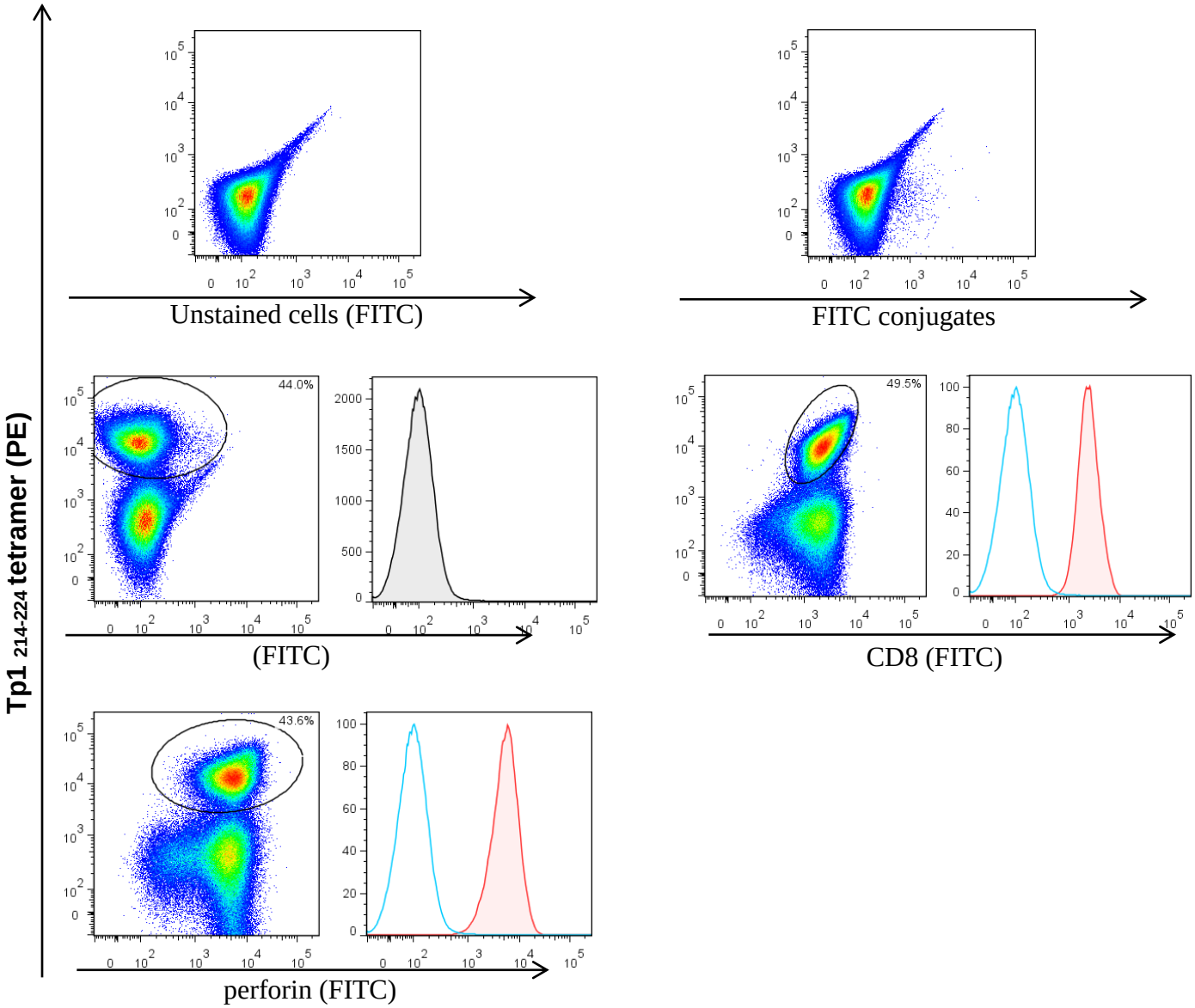
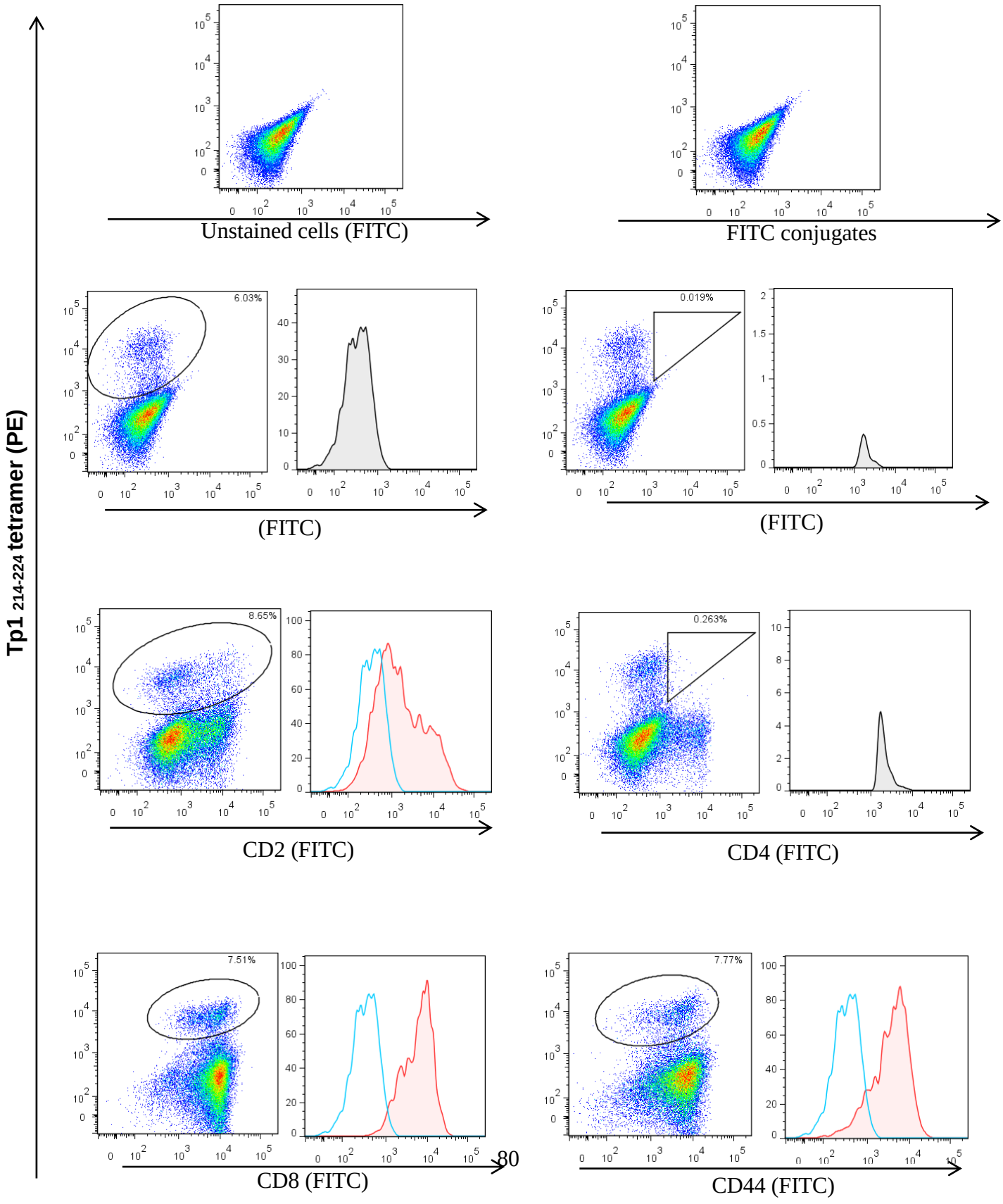
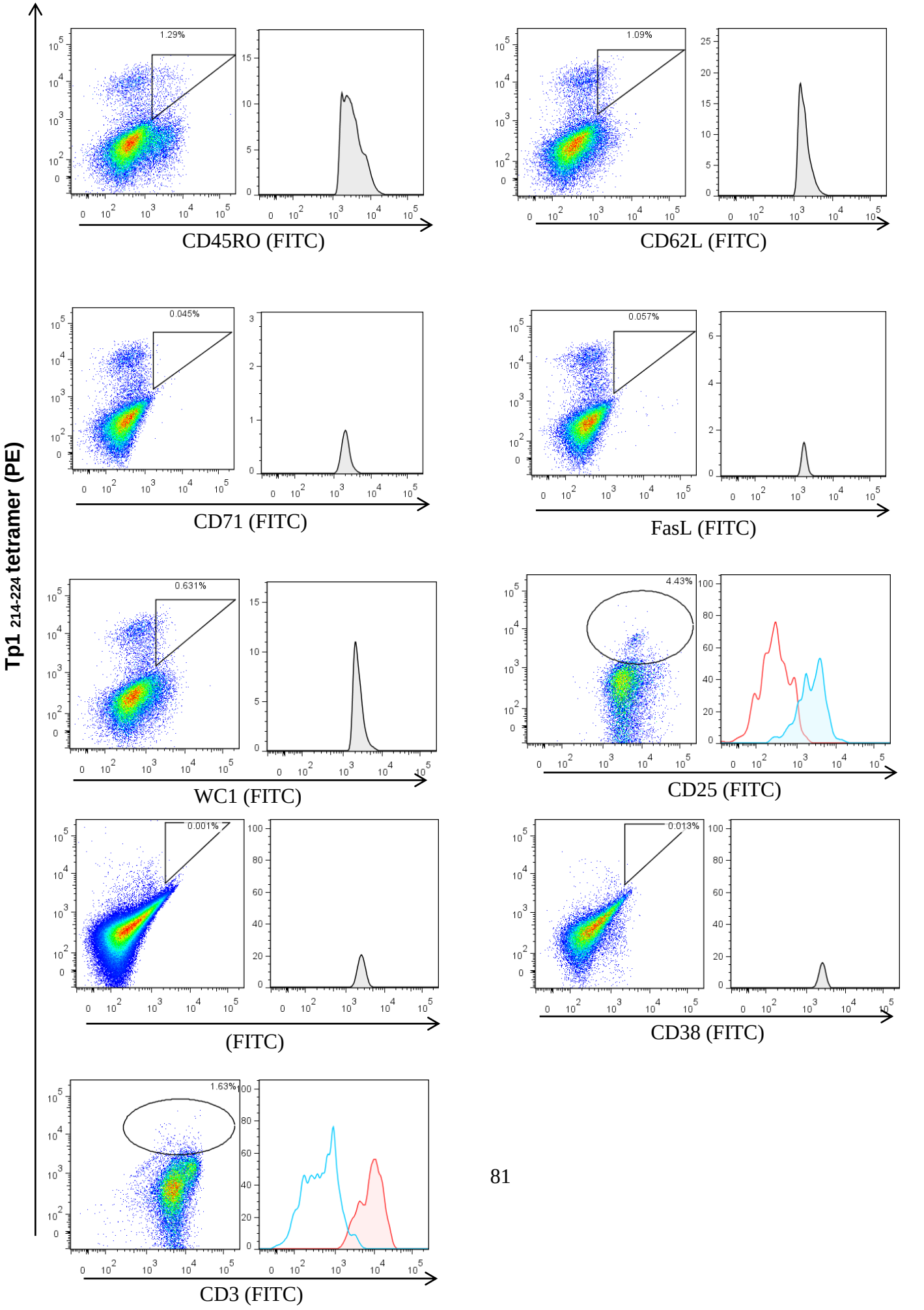


Figure 6.1 (a-v): FACS Staining for surface and intracellular biomarkers for BB007 CTL line.

In this experiment, purified CTL cell lines in culture were harvested and stained to determine the surface and intracellular marker expression profiles of *T. parva* antigen specific CD+ cells.

Figure 6. 2 (a-v) Animal BV115 CTL Cell Lines' FACS Data




Tp1 214-224 tetramer (PE)
1.29%
CD45RO (FITC)
1.09%
CD62L (FITC)
0.045%
CD71 (FITC)
0.057%
FasL (FITC)
0.631%
WC1 (FITC)
4.43%
CD25 (FITC)
0.001%
(FITC)
0.013%
CD38 (FITC)
1.63%
CD3 (FITC)

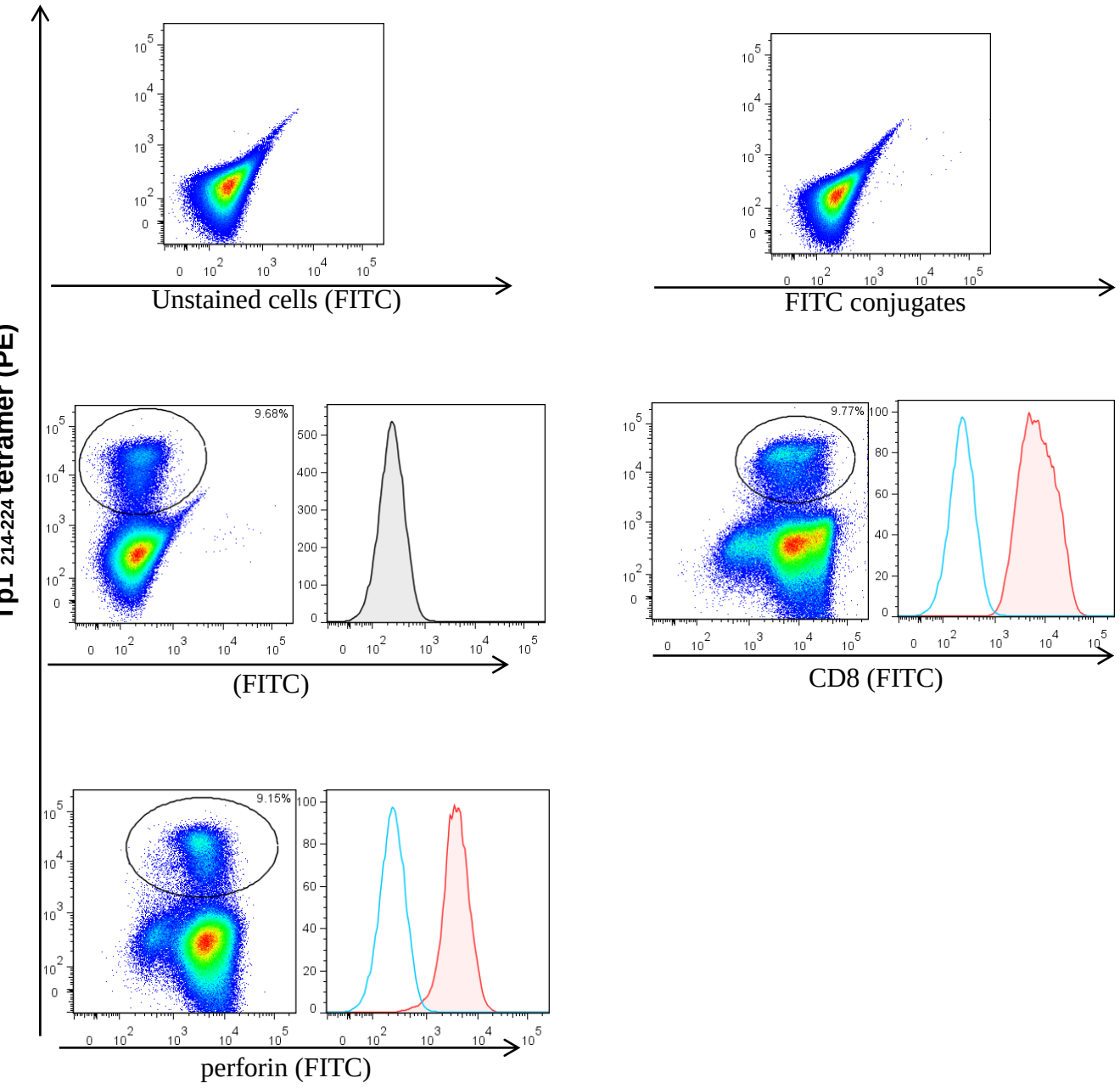
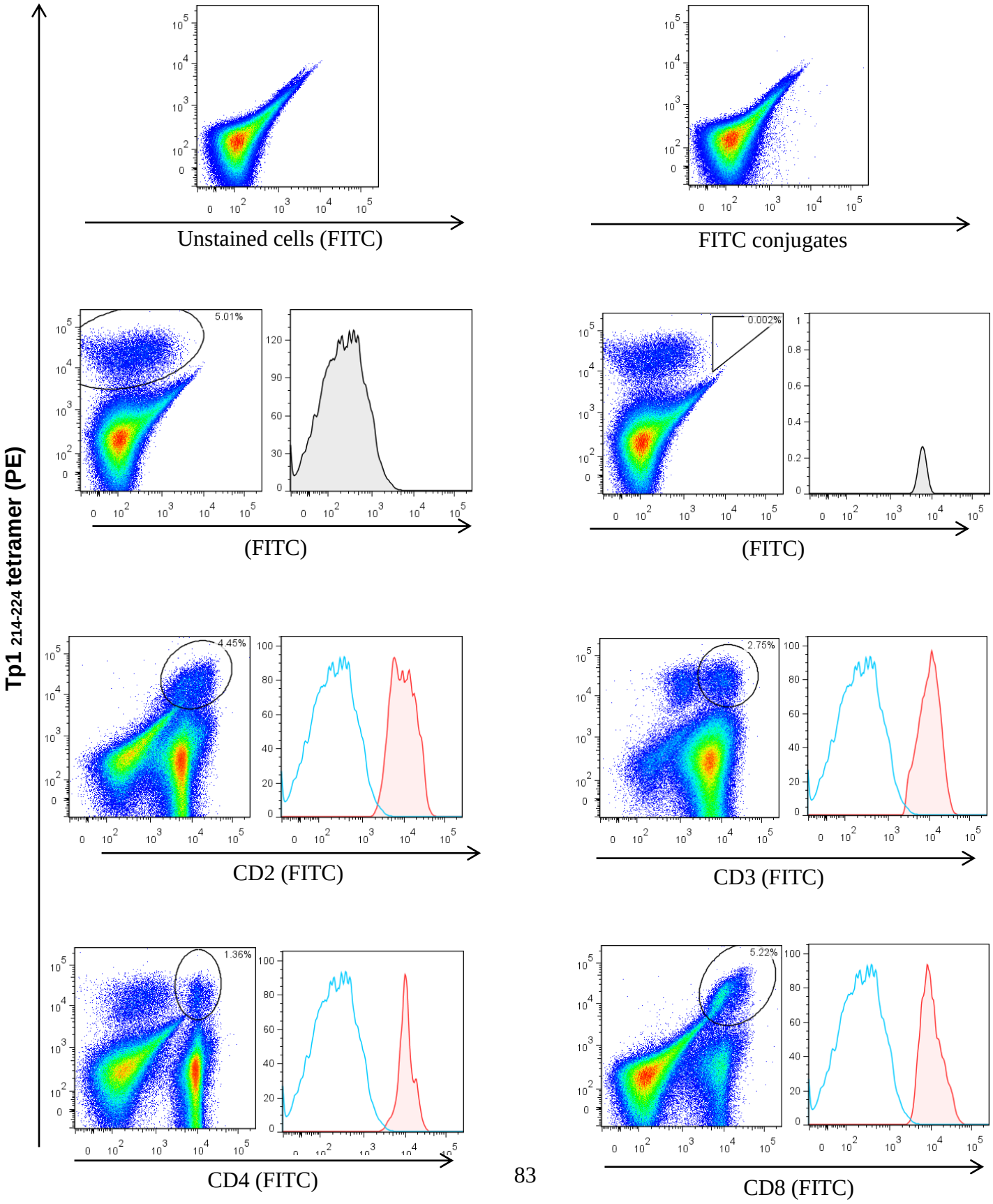
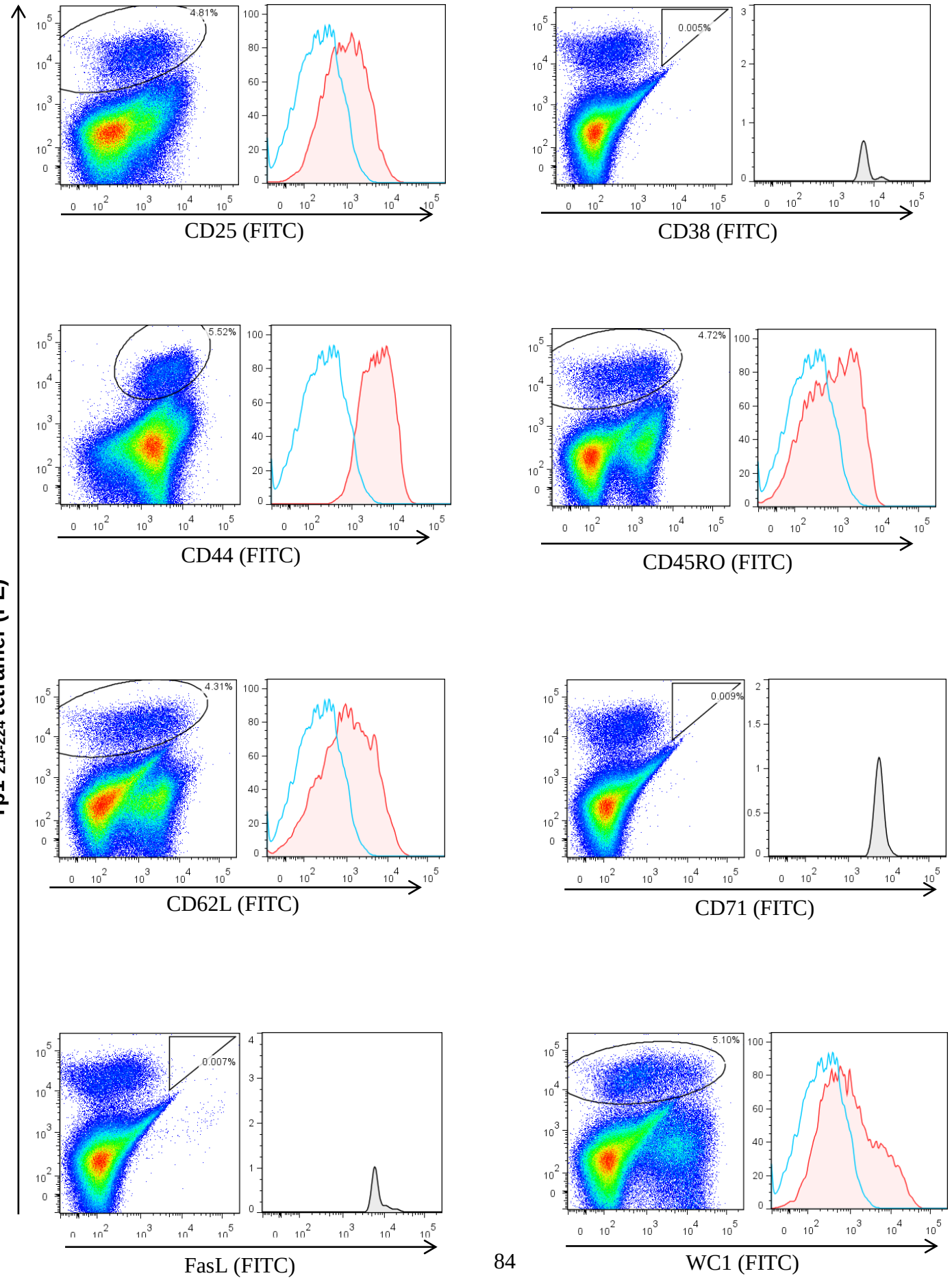


Figure 6.2 (a-v): FACS Staining for surface and intracellular biomarkers for BV115 CTL line.

In this experiment, purified CTL cell lines in culture were harvested and used to determine the surface and intracellular marker expression profiles of *T. parva* antigen specific CD+ cells.

Figure 6. 3 (a-v): Animal BB007 PBMC Bulk Cell Lines' FACS Data




Tpl 214-224 tetramer (PE)
4.81%
CD25 (FITC)
0.005%
CD38 (FITC)
5.52%
CD44 (FITC)
4.72%
CD45RO (FITC)
4.31%
CD62L (FITC)
0.009%
CD71 (FITC)
0.007%
FasL (FITC)
5.10%
WC1 (FITC)

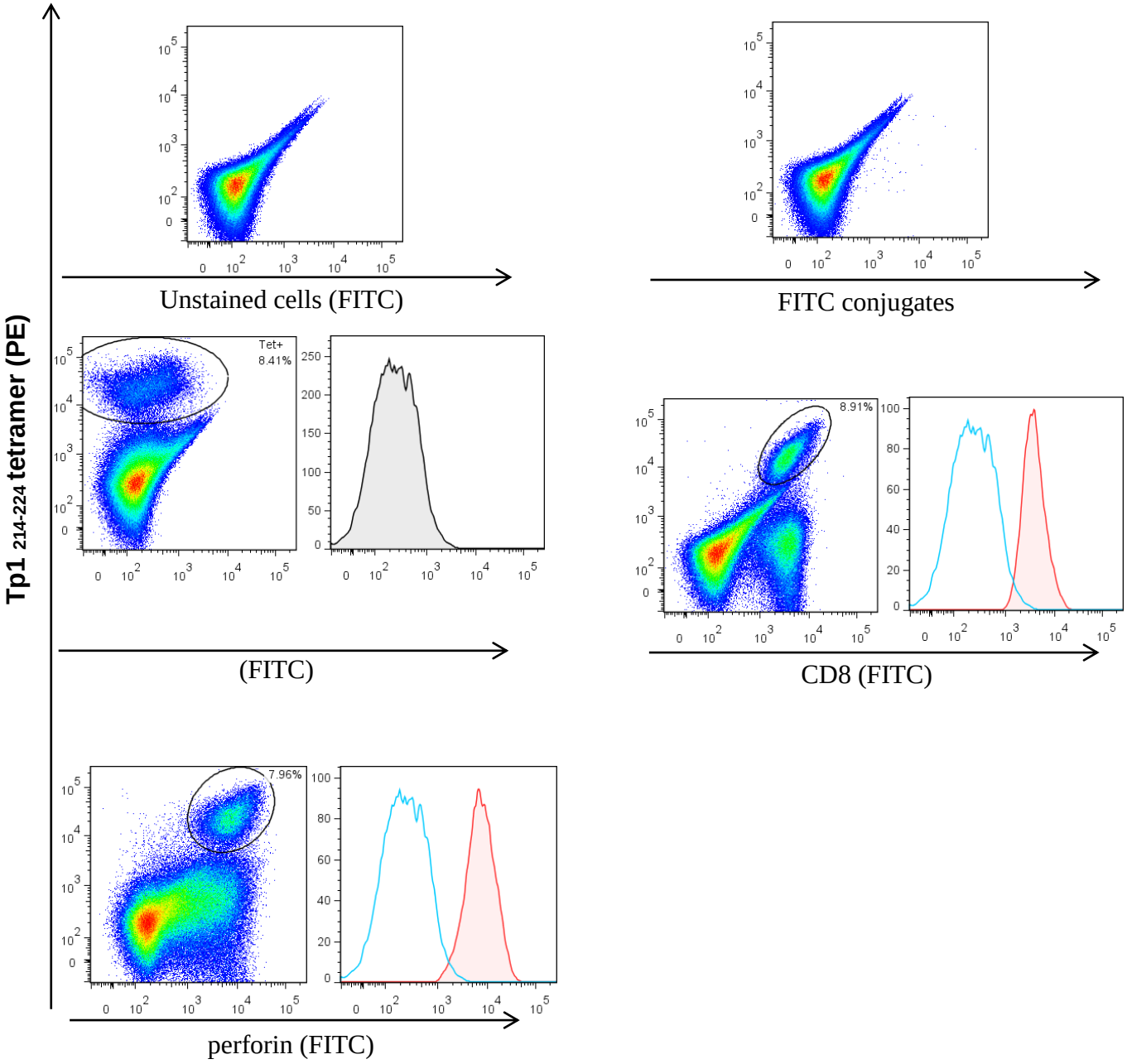
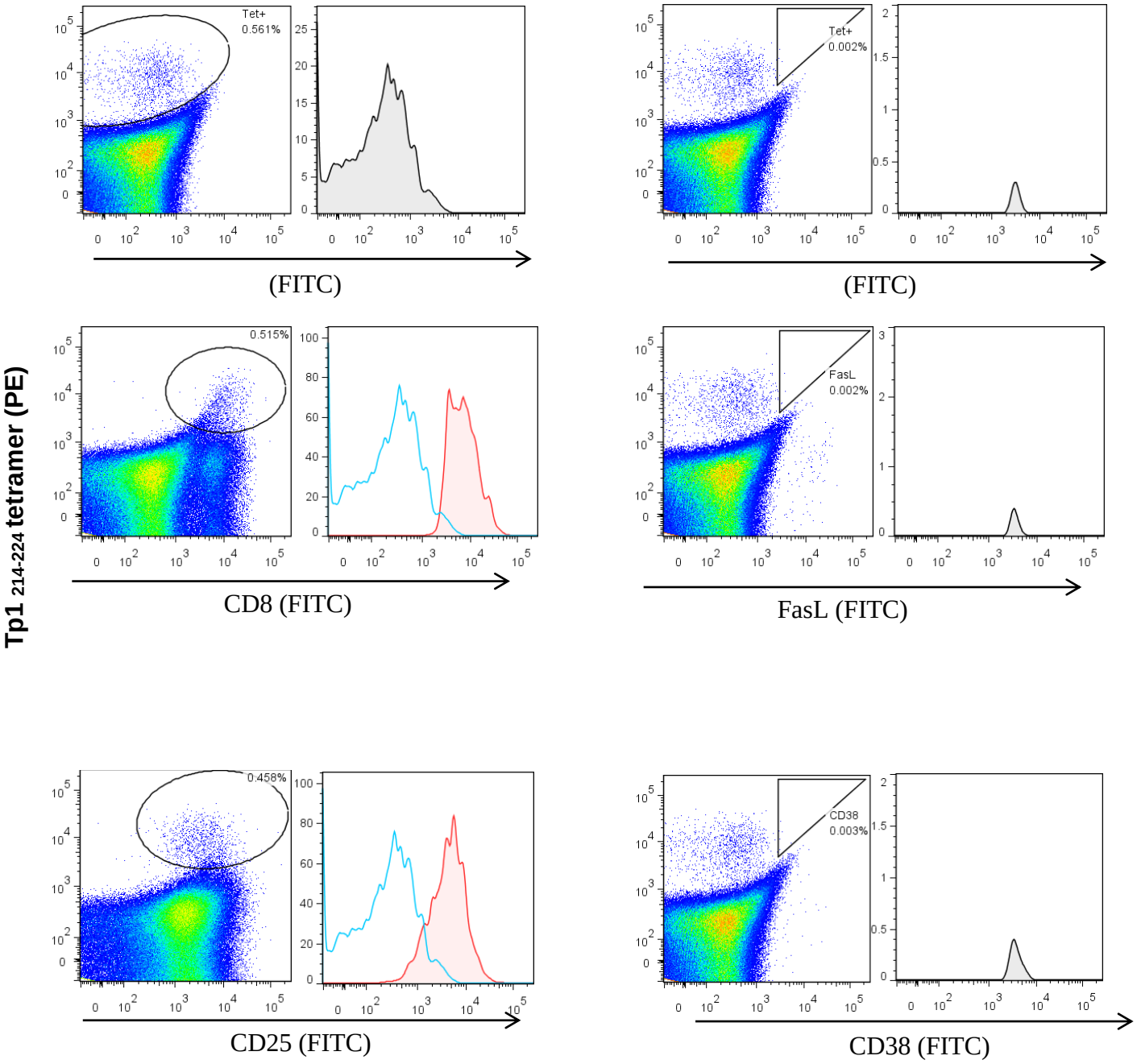


Figure 6.3 (a-v): FACS Staining for surface and intracellular biomarkers for BB007 PBMC Cell lines.

In this experiment, PBMC bulk cell lines that had been restimulated twice with *T. parva* infected lymphocytes in culture were harvested and used to determine the surface and intracellular marker expression profiles of *T. parva* antigen specific CD+ cells.

PBMC Bulk Cell Lines' FACS Data



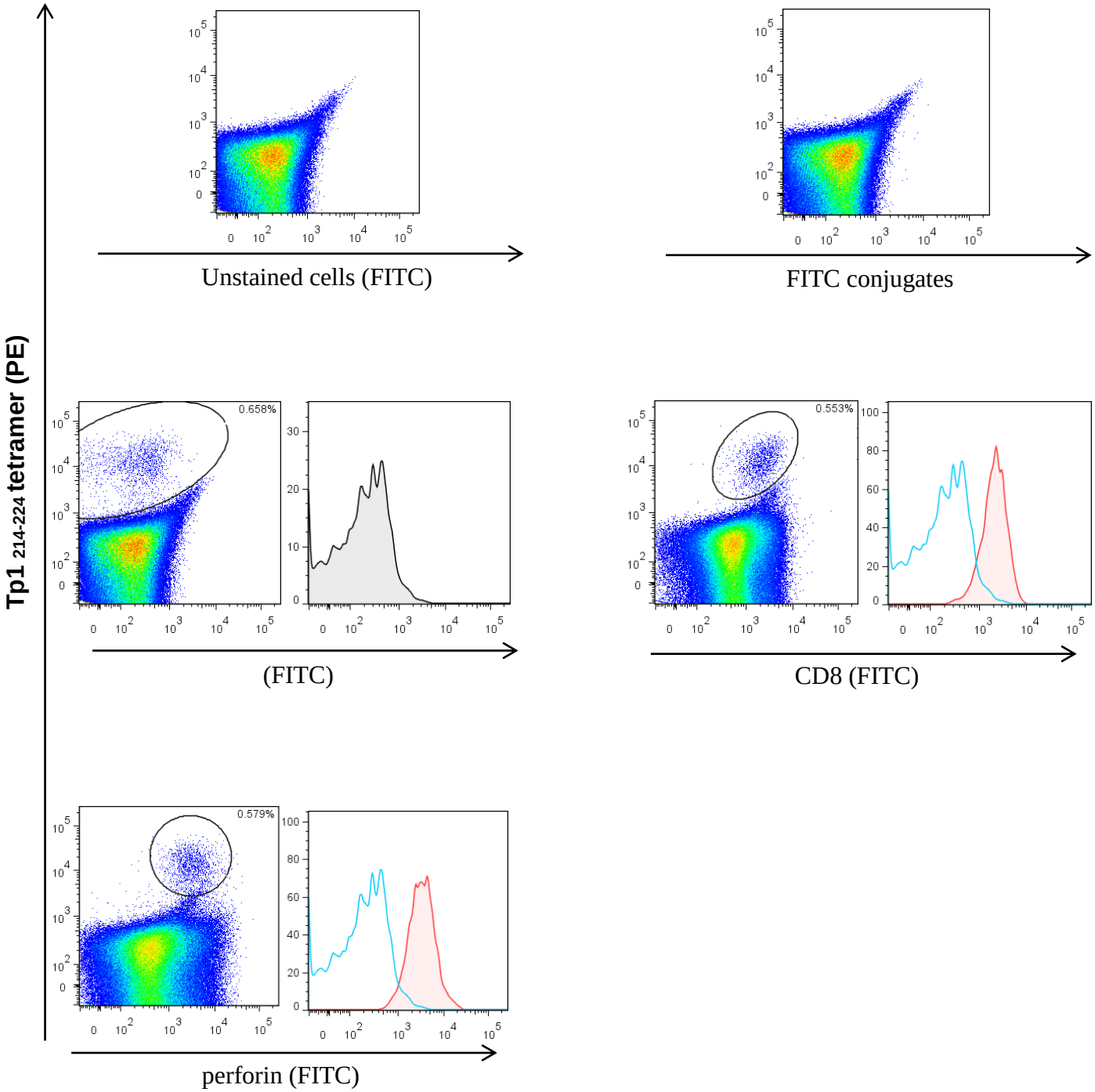
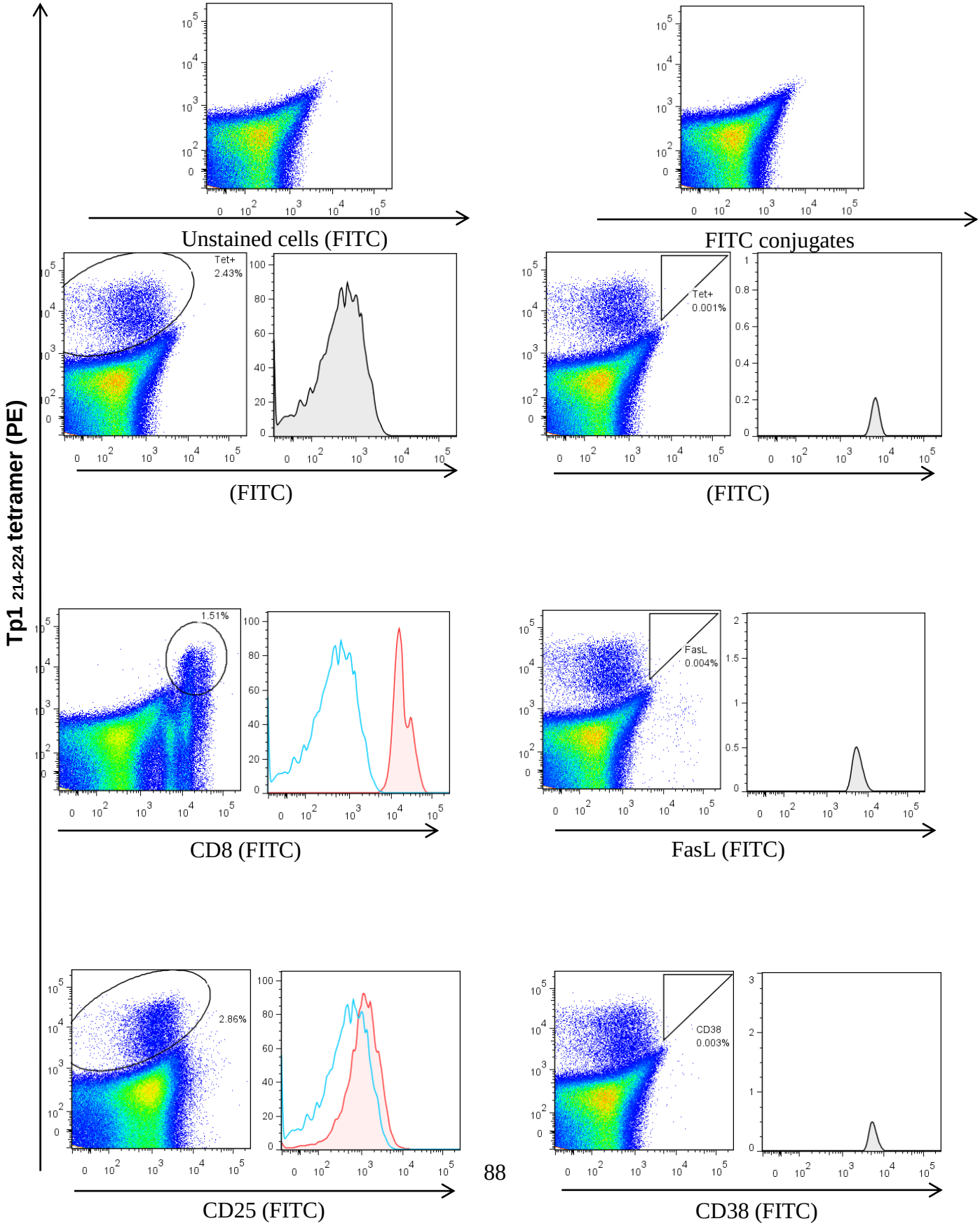


Figure 6.4: FACS Staining for surface and intracellular biomarkers for BA219 PBMC Cell lines.

In this experiment, PBMC bulk cell lines that had been restimulated twice with *T. parva* infected lymphocytes in culture were harvested and used to determine the surface and intracellular marker expression profiles of *T. parva* antigen specific CD+ cells.

Figure 6. 4: Animal BF091 PBMC Bulk Cell Lines' FACS Data



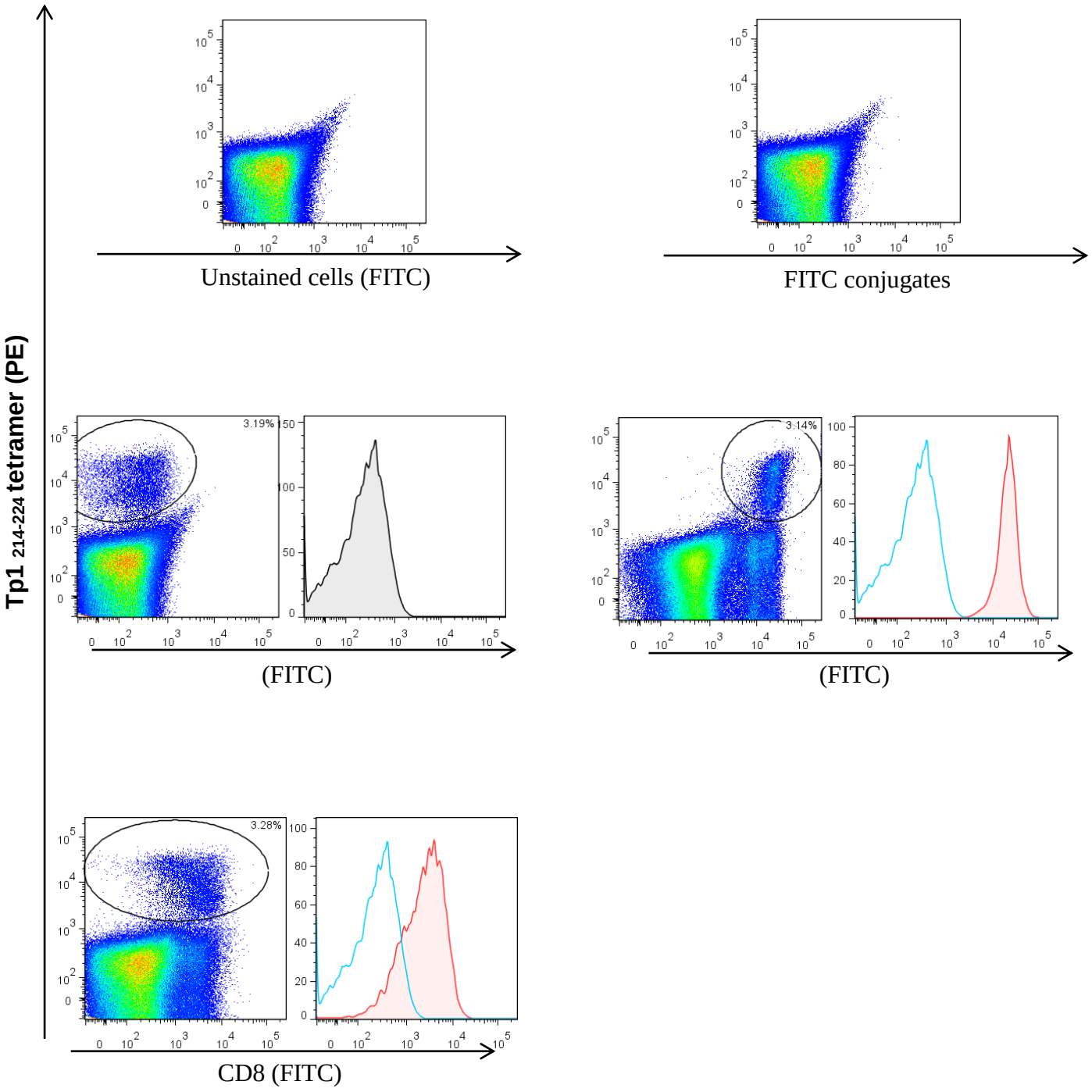
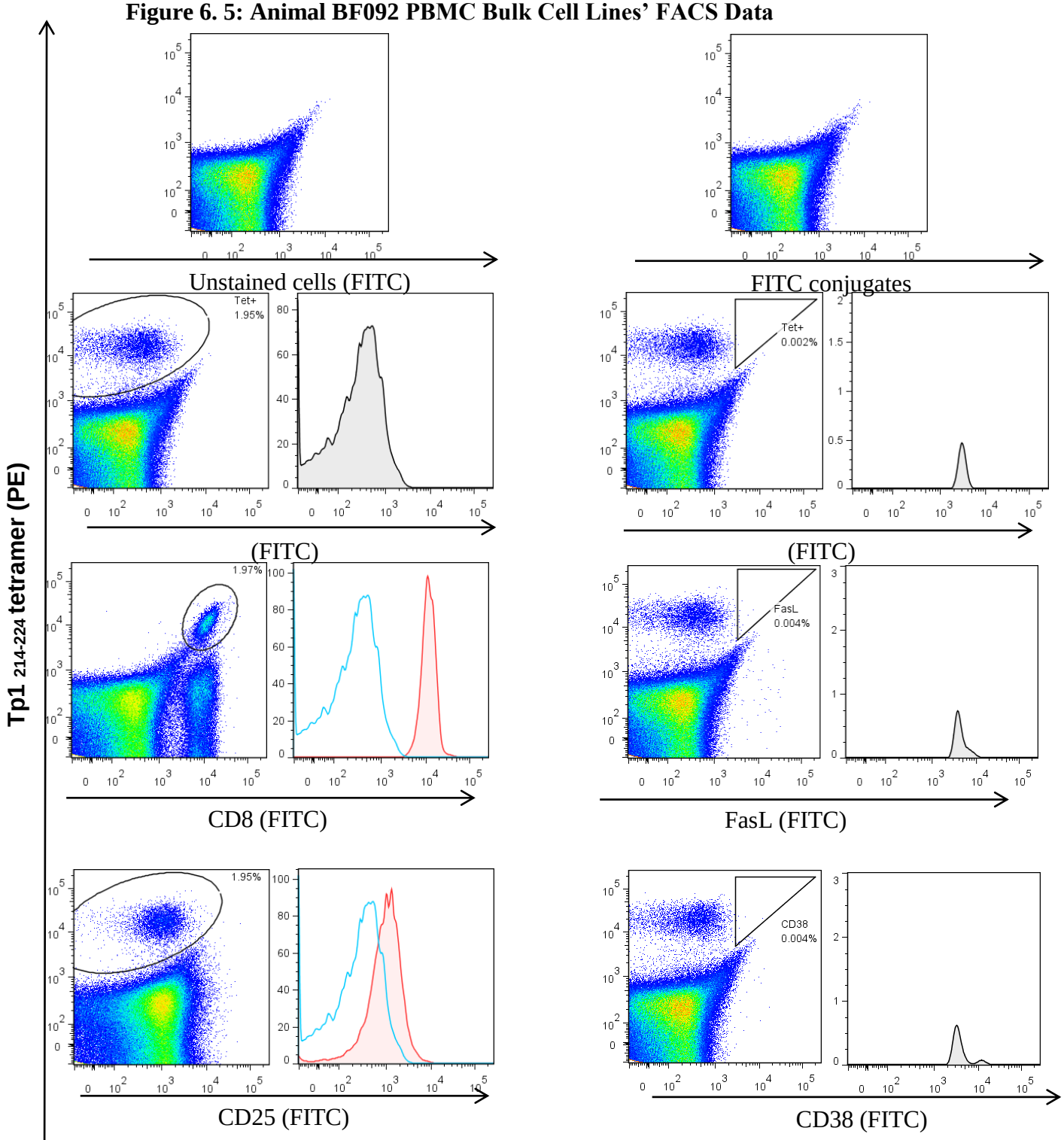


Figure 6.5: FACS Staining for surface and intracellular biomarkers for BF091 PBMC Cell lines.

In this experiment, PBMC bulk cell lines that had been restimulated twice with *T. parva* infected lymphocytes in culture were harvested and used to determine the surface and intracellular marker expression profiles of *T. parva* antigen specific CD+ cells.

Figure 6. 5: Animal BF092 PBMC Bulk Cell Lines' FACS Data



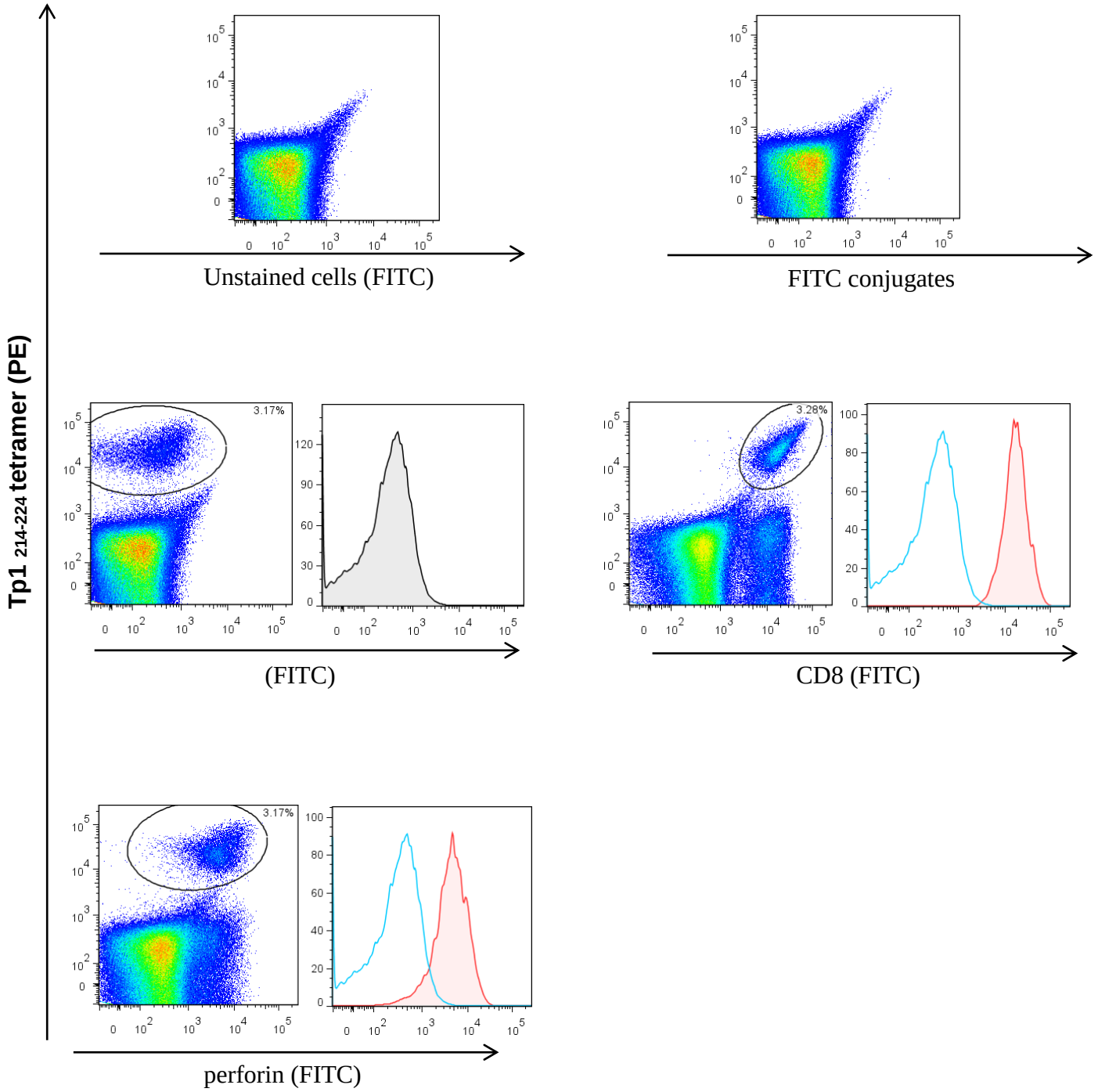


Figure 6.6: Additional FACS results from 3 animals; BA219, BF091 and BF092

In these experiments, bulk PBMC cell lines in culture were harvested and stained to determine the surface and intracellular marker expression profiles of *T. parva* antigen specific CD+ cells.

Appendix 2: Protocol for Preparation of Peripheral Blood Mononuclear Cell (PBMC) by Gradient Density Centrifugation.

ILRI INTERNATIONAL LIVESTOCK RESEARCH INSTITUTE	STANDARD OPERATING PROCEDURE (S.O.P)	THEME: Biotechnology
		ACTIVITY: Immunology and Tissue Culture
		NUMBER: BT-ITC-004-01
		REVIEW DATE: APRIL 2011

Title: Preparation of peripheral blood mononuclear cell (PBMC) by density gradient centrifugation

Effective Date: April 2008	Supersedes:	TH4-LAB6-067-00
Written By: Elias Awino	Date: 05/03/08	
Number Allocated By: EOHSO	Date: 05/03/08	
Reviewed By: Dr. Duncan Mwangi (Scientist)	Date: 17/03/08	
Approved By: Dr. Phil Toye (OPL)	Date: 17/03/08	

1.0 PURPOSE

To describe Preparation of peripheral blood mononuclear cell (PBMC) by density gradient centrifugation

2.0 SCOPE

It covers the Preparation of peripheral blood mononuclear cell (PBMC) by density gradient centrifugation within the lab complex

3.0 RESPONSIBILITIES

It is the responsibility of the technicians and the scientists to ensure that the procedure is followed.

4.0 PROCEDURE

Note: all operations must be performed under sterile conditions

1. Collect blood from the jugular vein into a syringe containing an equal volume of Alsever's solution and mix gently.
2. Layer blood onto Ficoll Paque solution and centrifuge at 2000 g for 30 minutes at room temperature, switch off the brakes of the centrifuge.
3. Using a sterile pipette aspirate the PBMC from the interface, transfer to a sterile tube and top with warm Alsever's solution.
4. Centrifuge the PBMC at 1200 g for 10 minutes at room temperature.
5. Wash the PBMC 3 times with Alsever's solution by centrifugation at 800 g at room temperature, to remove platelets.
6. Re-suspend the pellet of PBMC in medium of choice, depending on further use.

5.0 DEFINITIONS

EOHSO- Environmental Occupational Health and Safety Officer

OPL- Operating Project Leader

Appendix 3: Protocol for Generation of *T. parva* Specific Bulk Cultures

ILRI INTERNATIONAL LIVESTOCK RESEARCH INSTITUTE	STANDARD OPERATING PROCEDURE (S.O.P)	THEME: Biotechnology
		ACTIVITY: Immunology and Tissue Culture
		NUMBER: BT-ITC-021-01
		REVIEW DATE: APRIL 2011

Title: Generation of *T. parva* specific bulk cultures			
Effective Date: April 2008		Supersedes:	TH4-LAB6-084-00
Written By: Elias Awino		Date: 05/03/08	
Number Allocated By: EOHSO		Date: 05/03/08	
Reviewed By: Dr. Duncan Mwangi (Scientist)		Date: 17/03/08	
Approved By: Dr. Phil Toye (OPL)		Date: 17/03/08	

1.0 PURPOSE

To describe the generation of *T. parva* specific bulk cultures

2.0 SCOPE

It covers the generation of *T. parva* specific bulk cultures within the lab complex.

3.0 RESPONSIBILITIES

It is the responsibility of the technicians and scientists to ensure that the procedure is followed.

4.0 PROCEDURE

Primary Stimulation:

1. Obtain blood from an immunised animal and process for PBMC as described herein.

2. Re-suspend PBMC at $4x10^6$ per ml in culture medium (without HEPES).

3. Distribute 1ml into 24-well tissue culture plate.

4. Prepare cell suspension of autologus *T. parva* infected cells at 2×10^5 per ml.

5. Irradiate at the Ceasium source for 15 minutes (expose to 5000 rads). Add 1 ml into the wells containing the PBMC.

6. Incubate the cells for 7 days in a CO2 incubator.

Second stimulation:

1. Mix the cells and aspirate with a 5 or 10 ml pipette. Layer on ficoll and centrifuge at 2000 g for 20 minutes at RT to remove the dead cells. HarvesT-cells from the interface, dilute with an equal volume of culture medium and pellet at 1200 g for 10 minutes. Re-suspend the pellet and wash once at 800 g for 10 minutes.

2. Re-suspend the cells at a concentration of 4 $x10^6$ and stimulate for a second time with irradiated autologus (or MHC matched) infected cells as in primary stimulation.

Generation of *T. parva specific bulk cultures*

3. Incubate at 37^oC for 7 days. Harvest the cells from the plates, separate viable cells from the culture by spinning on ficoll and stimulate for the third time as described above. However, for tertiary stimulation, the cell inputs are as follows: stimulated (responder) cells 2 $x10^6$ per well with 4 $x10^5$ autologus infected cells (stimulator) and 2 $x10^6$ irradiated autologous PBMC as filler cells.

4. If there is a need to continue propagating the cultures then after incubation for 7 days, separate viable cells from the cultures as before and stimulate weekly using 1 $x10^6$ responder cells per well, 4 $x10^5$ stimulator cells and 3 x 10^6 filler cells.

Appendix 4: Protocol for Staining of Cells for Analysis with Flow Cytometry (FACS)

ILRI INTERNATIONAL LIVESTOCK RESEARCH INSTITUTE	STANDARD OPERATING PROCEDURE (S.O.P)	THEME: Biotechnology ACTIVITY: Immunology and Tissue Culture NUMBER: BT-ITC-012-01 REVIEW DATE: APRIL 2011	
Title: Staining of cells for analysis with flow cytometry (FACS)			
Effective Date: April 2008		Supersedes:	TH4-LAB6-077-00
Written By: Elias Awino		Date: 05/03/08	
Number Allocated By: EOHSO		Date: 05/03/08	
Reviewed By: Dr. Duncan Mwangi (Scientist)		Date: 17/03/08	
Approved By: Dr. Phil Toye (OPL)		Date: 17/03/08	

1.0 PURPOSE

To describe the procedure of staining cells for analysis with flow cytometry (FACS).

2.0 SCOPE

It covers the staining cells for analysis with flow cytometry (FACS) within the lab complex.

3.0 RESPONSIBILITIES

It is the responsibility of the technicians and scientists to ensure that the procedure is followed.

4.0 PROCEDURE

This assay is also referred to as indirect fluorescent antibody test (IFAT). Basically, cells are stained with the antibody(s) of choice for the determination of molecules /biomarkers expressed on the cell surface and analysed with Flow Cytometry.

1. Prepare cells from blood (PBMC) or cultures, re-suspend in FACS medium (RPMI 1640 supplemented with 2% horse serum and 0.02% Sodium Azide Na3N) and adjusT-cell concentration at 1-2x10^7 per ml.

2. Dispense 50ul of the cell suspension into each well of a 96 plate with round bottom. Note; remember to set a well with cells and medium only to serve as a negative control.

3. Add 50 ul of the labelling or test antibodies into each well. Usually monoclonal antibodies are used at 1:500 dilutions (i.e. ascitic fluid).

4. Incubate the preparation for 30 minutes at 4^{o}C / on ice.

5. Pellet the cells by centrifugation at 1000 g for 3 minutes at 4 0 C, flick medium and break pellet on a Mixer. Re-suspend cells in 100-200ul of FACS medium and wash 2 times. Staining of cells for analysis with flow cytometry (FACS) BT-ITC-012-01

6. Re-suspend cells in 25ul or 50ul of wash medium then add equal volume of second

antibody i.e. FITC – conjugated goat anti mouse Ig (polyvalent) diluted at 1:200 in FACS medium.

7. Incubate for 30 minutes on ice (at 4^{o}C).

8. Wash 3 times as in step 5 above. Re-suspend the cells in 100 ul of FACS medium and analyse with the FACSCAN.

9. If cells cannot be analysed on the same day, wash 2 times with PBS containing 0.2% Sodium Azide and fix in 2% formalin in PBS (100 ul) and store at 4^{o}C in the dark. Analyse on the FACSCANTO later.

Appendix 5: Protocol for Tetramer Staining

1- Resuspend PBMC in PBS- 0.5% BSA and plate $4x10^5$ PBMC/ well in 96-well round-bottom plates

2- Spin at 1500 G, room temperature, 2 min.

3- Add 25 µl tetramer at 40 nM and incubate for 10 min, RT, dark

4- Add 25 µl mAb ILA51 (IgG1) diluted 1/ 250 PBS/ 0.5% BSA and incubate for 10 min, RT, dark

5- Wash 2 times by adding 200 µl PBS-0.5% BSA per well and - Spin @ 1,500 for 2 min@RT- flick off supernatant, and break pellet on mixer.

6- Add 1µl rat anti-mouse IgG1 PerCP (BD Cat. No. 340272) (6.25 µg/ml) and incubate for 10 min, RT, dark

7- Wash 2 times as above

8- Resuspend in 200 µl PBS/ 0.5% BSA for FACS analysis

Appendix 6: Protocol for Intracellular Staining

To measure intracellular cytokines production, cells secretion must be blocked (if cell constitutively produce the cytokine); otherwise cells need to be activated as well as blocked before staining.

Blocking is done by incubating cells in culture medium supplemented with monensin (blocking agent), whereas activation is carried out by incubating cells with phorbol myristate acetate (PMA) and ionomycin (activating agents) in presence of monensin. Incubation time is usually around 6 hrs.

Stimulation protocol

On whole blood or cell suspension (PBMCs, LN and spleen cells)

- **whole blood**: dilute blood collected in Heparin 1/3 in C- RPMI[1] (1 blood + 2 medium); add PMA[2], ionomycin[3] and monensin[4] to one tube (or well) and monensin only to the control tube. Incubate 6 hours at 37 *C, 5% humidity. Recover cells by Ficoll, wash once in D'PBS[5], once in L15[6] and proceed for the staining.

After PMA activation CD4 antigen disappear from the cell surface, to be internalized in the cytoplasm. CD4 + cells can be detected by means of a simultaneous double cytoplasmic staining (see staining protocol n. 3).

-Cell suspension: resuspend cells in C-RPMI at 1-4 x 10^6/ml and add PMA, inonomicyn and monensin to one well and monensin only to the control well. Incubate 6 hours at 37 *C, 5% humidity. Recover cells, wash once in D'PBS, once in L15 and proceed for the staining.

Staining protocol

1) Surface staining followed by intreacellular staining.

- ❖ Resuspend the pellet at 5 x 10^6 cells/ml in ice-cold L15;
- ❖ Dispense 50 µl of cells suspension in each well of a 96 wells plate;
- ❖ Add 50 µl of anti-surface antigen antibody diluted in L15 (ice-cold);
- ❖ Incubate for 30' at 4ºC.
- ❖ Wash twice with L15; for the first was add 150 µl of ice-cold L15 to each well, spin (2500/2') in refrigerated bench centrifuge, aspirate supernatant and vortex to resuspend the pellet; add 200 µl of ice-cold L15 and repeat the wash;

❖ Add 25 µl of second step isotype-specific fluorochrome-conjugated antibody (usually FITC conjugate) diluted in ice-cold L15;

❖ Incubate for 30’ at 4 ºC;

❖ Wash twice with 200µl of L15 as precious and once in 200µl of D’PBS;

❖ Fixation step: add 200µl of 0.25% PFA7 to each plate well and incubate at room temperature in the dark for exactly 10 minutes. Spin in bench centrifuge, remove supernatant and vortex to resuspend the pellet; wash once with 200 µl of ice-cold D’PBS;

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[1] C-RPMI: complete RPMI.RPMI 1640 without phenol red, pen/strep, L-glutamine, ß- mercaptoethanol, 10% FCS.

[2] PMA: final concentration 50 ng/ml. Prepare stock solution 2000x in DMSO. Further dilute in C-RPMI to 100x. filter sterilise and store in small aliquots at -20 *C. Light sensitive.

[3] Ionomycin: final concentration 1 µg/ml. Prepare stock solution 1000x in methanol or DMSO.

Further dilute in C-RPMI to 100x. Filter sterilise and store in small aliquots at -20 *C.

[4] Monensin: final concentration 2µM. Prepare stock solution 1000x in ethanol or DMSO. Further dilute in C-RPMI to 100x. Filter sterilise and store in small alilquots at -20 *C.

[5] D’PBS: Dulbecco PBS.[6] L15: Lebovitz’s 15 medium, 5% heat-inactivated FCS, 0.2% (w/v) sodium azide

❖ Permeabilization step: add to each well 200 µl of PBS-Sap[8] supplemented with 20% heat inactivated normal goat serum (Fc receptor blocking) and incubate at room temperature for 30’ or overnight at 4ºC;

❖ Spin cells down, remove supernatant and add 50 µl of anti-cytokine antibody diluted in PBS-Sap;

❖ Incubate for 30’ at 4 ºC;

❖ Wash twice with PBS-Sap; for the first wash add 150 µl of ice-cold PBS-Sap to each well, spin (2500/2') in refrigerated bench centrifuge, aspirate supernatant and vortex to resuspend the pellet; add 200 µl of ice-cold PBS-Sap and repeat the wash;

❖ Add 25 µl of second step isotype-specific fluorochrome-conjugated antibody (usually PE conjugate) diluted in ice-cold PBS-Sap;

❖ Incubate for 30' at 4 ºC;

❖ Wash twice with 200 µl of PBS-Sap as previous, and once more with 200 µl of D'PBS. Resuspend in 400 µl of PBS-Sap and read immediately at the FACS machine. If impossible to read immediately fix once again with 200 µl of 0.25% PFA and store at 4 ºC in the dark for 1 day maximum.

2) Intracellular staining only

If only cytoplasmic straining is required, wash cell twice in PBS-NaN_3 and begin the protocol from the fixation step

3) Simultaneous surface and intracellular staining

❖ Recover the cells, pellet and remove culture medium;

❖ Resuspend in 200 µl of D'PBS for each sample to be stained and dispense in 96 well plates; pellet and remove D'PBS;

❖ Begin the protocol from the fixation step (fix in 0.25% PFA);

❖ After permeabilization spin cells down, remove supernatant and add 25 µl of each monoclonal antibody (anti-cell surface and anti-cytokine) diluted in PBS-Sap to the pellet; usually the antibodies dilution is half of that one used in single staining (if amntibody is diluted 1/1000 for single, dilute 1/500 for double), But each combination must be titrated.

❖ Incubate for 30' at 4 ºC;

❖ Wash twice with PBS-Sap; for the first wash add 150 µl of ice-cold PBS-Sap to each well, spin (2500/2') in refrigerated bench centrifuge, aspirate supernatant andchg vortex to resuspend the pellet; add 200 µl of ice-cold PBS-Sap and repeat the wash;

❖ For second step combine both isotype-specific fluorochrome-conjugated antibodies in the same volume of PBS-Sap; add 25 ul/well and incubate for 30 at 4 ºC

❖ Wash twice with 200 µl of PBS-Sap as previous, and once more with 200 µl of D'PBS. Resuspend in 400 µl of PBS-Sap and read immediately at the FACS machine. If impossible to read immediately fix once again with 200 µl of 0.25% PFA and store at 4 ºC in the dark for 1 day maximum.

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[7] PFA: make fresh 0.25% paraformaldehyde solution in D'PBS. Use a 20% stock solution prepared dissolving PFA (powder, electron microscopy grade) in water; leave the powder to dissolve for two hours at 56 *C, until the solution is clear. To help PFA to dissolve add few pellets of sodium hydroxide, when heating. Adjust the pH at 7.00 (use pH paper); aliquot in 1ml tubes and store at -20 *C; warm up 20% solution in waterbath (37 *C), and prepare 0.25% solution adding 1 ml of 20% PFA to 79 ml of D'PBS. Do not re-use dilute PFA or stock solutions.

[8] PBS-Sap: Dulbecco's PBS, 0.1% (w/v) saponin, 0.2% NaN_3, 10mM HEPES, 10% FBS

4) Surface straining before cryopreservation followed by intracellular straining

❖ Strain cells for surface antigens as in protocol 1) up to the fixation step;

❖ Fixation step: add 200 µl of 1% PFA to each plate well and incubate at room temperature in the dark for exactly 10 minutes. Spin in bench centrifuge, remove supernatant and vortex to resuspend the pellet; wash once with 200 µl of ice-cold D'PBS;

❖ Wash once in 200 µl of PBS-BSA9 and resuspend in 200 ml of PBS-BSA-DMSO10; cover the plate with parafilm, cool it for 20' on ice and freeze at -20 ºC;

❖ DefrosT-cells at room temperature (dark), pellet, aspirate supernatant, wash once with D'PBS and restart protocol 1 from the permeabilization step.

5) Cryopreservation before surface and intracellular straining

This staining could be done only after checking that the epitope recognised by the surface-specific antibody resist to fixation, permeabilization and cryopreservation procedures.

- Recover the cells, pellet and remove culture medium (use 10 ml tubes);
- Wash once in 10 ml of PBS-NaN_3;
- Fix in 2 ml of 1% PFA for 10' at room temperature; pellet and remove PFA;
- Wash once in 10 ml of D'PBS and once in 5 ml of PBS-BSA;
- Resuspend in a small volume of PBS-BSA-DMSO (usually 1-2 ml) in Eppendorf tubes;
- Cool tubes for 20'on ice and freeze at -20 ºC;
- DefrosT-cells at room temperature. Pellet, aspirate supernatant, wash once with D'PBS and restart protocol 1 from the beginning.

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[9] PBS-BSA: Dulbecco's PBS, 2% (w/v) Bovine serum albumine, 0.2% NaN_3,

[10] PBS-BSA-DMSO: PBS-BSA supplemented with 10% DMSO.

Appendix 7: Protocol for MiniMACS Sorting

1) Separate cells into a clean, viable single cell suspension

2) Wash in PBS (without magnesium and calcium) containing 2% FCS (PBSa/FCS)

3) PelleT-cells

4) Add 12.5 µl mAb diluted in PBSsFCS (or complete RPMI) per 10^6 cells (i.e. 125 µl 1/500 per 10^7 cells use **IL-A30**, ILA- A58 or IL-A59

5) Incubate 10 minutes at RT

6) Wash twice (6-8 minutes 300g) – fill falcon with buffer

7) Pellet the cells

8) Add 10 µl of anti-mouse MiniMACS beads pe r 10^7 cells, mix and incubate at RT for 10 minutes (or for 30 mins on ices).

9) Prepare the MiniMACS column – mount on the magnet and flush through with 0.5m. PBSa/FCS + 2mM EDTA (M ACS buffer – ice cold).

10) Wash cells 2 twice (6-8 minutes 300 g) PBSa/FCS

11) Resuspend cells in 0.5 -1 ml FACS buffer and apply this to the column

12) Wash the column with 3 times with 0.5ml FACS buffer

13) Remove the column from the magnet and apply 2ml RPMI /10% FCS

14) Elute the cells reapidly with the plunger into a tube containing complete RPMI

15) Stain an aliquot of cells with Ga M Ig FITC to assess purity

16) Spin cells down, resuspend in complete RPMI then count.

N.B The volumes and columns change depending on the number of cells you wish to isolate (i.e. target T-cells). Mini macs column should be used if you have upto 10^7 target T-cells or approx 10^8 cells in total population). If you wish to obtain more than 10^7 target T-cells you should use MIDI macs columns – and the volume for buffer and beads etc change with the increasing size of the column.

Appendix 8: Protocol for Direct Elispot Assay for Detection of Bovine IFN-γ

ILRI INTERNATIONAL LIVESTOCK RESEARCH INSTITUTE	STANDARD OPERATING PROCEDURE (S.O.P)	THEME: Biotechnology
		ACTIVITY: Immunology and Tissue Culture
		NUMBER: BT-ITC-025-01
		REVIEW DATE: APRIL 2011

TITLE: Direct elispot assay for detection of bovine IFN-γ			
Effective Date: April 2008		Supersedes:	TH4-LAB6-020-00
Written By: Elias Awino		Date: 05/03/08	
Number Allocated By: EOHSO		Date: 05/03/08	
Reviewed By: Dr. Duncan Mwangi (Scientist)		Date: 17/03/08	
Approved By: Dr. Phil Toye (OPL)		Date: 17/03/08	

1.0 PURPOSE

To describe the Direct Elispot Assay for Detection of Bovine IFN-γ

2.0 SCOPE

It covers the Direct Elispot Assay for Detection of Bovine IFN-γ

3.0 Responsibilities

It is the responsibility of the technicians and scientists to ensure that the procedure is followed.

4.0 PROCEDURE

1. Coat Millipore MAIP S45 plates over night at 4^{o}C with 50µl / well with anti-bovine IFN-γ capture monoclonal antibody CC302 diluted at 1 µg/ml in sterile

coating buffer. Shake plate on plate mixer to evenly spread the antibody over well surface. Wrap plates in aluminium foil and place in fridge/ cold room.

2. Wash plates twice with un-supplemented RPMI 1640 with HEPES. Use multi-pipette to add 200µl wash fluid. Block plate with 10% FBS in RPMI for 2 hours at 37^{o}C, (add 200ul per well sterile). Then replace with 50µl /well antigens at appropriate concentrations. Do not forget to include medium control and mitogen (Concanavalin A) control wells.

3. Prepare cells at appropriate dilutions in 4% FBS in RPMI 1640.Make cell dilution starting from 5-10x10^{6} in 96 well plate and transfer 50 ul per onto ELISPOT plate. Include control wells with no cells.

4. Incubate ELISPOT plate for 20 hours at 37^{o}C in humidified incubator with 5% CO2. Ensure that plates are level so that, cells are evenly distributed.

Direct Elispot assay for detection of bovine IFN-γ

5. Wash the wells 4 times with distilled water +0.05%Tween 20 and then 4 times with PBS-T. Place on plate shaker each time for 20-60 seconds before flicking off wash fluid. Add 50 µl/well rabbit anti-bovine IFN-γ (diluted 1:1500 in PBS-T/BSA) and incubate for 1 hour at RT. Then wash 3-4 times with PBS-T (without shaking); remove wash fluid as well as possible by tapping plate on paper towels.

6. Add 50 µl/well of monoclonal anti-rabbit 1gG-alkaline phosphatase conjugate (Sigma, clone R696, Cat. No. A-2556 diluted 1:2000 in PBS-T/BSA) and incubate for 1 h at RT.

7. Wash plates 4 times with PBS-T (without shaking between washes). Then add 50µl per well of BCIP/NBT substrate. To prepare the substrate dissolve by vortexing for 2-3 minutes 1 tablet (Sigma Fast BCIP/NBT buffered substrate tablet, cat. No. B-

5655) in 10ml distilled water. Pass through 0.2 um filter to remove un-dissolved substrate particles. Keep plate in dark during spot development (5-10 minutes).

8. Once spots have developed flick off substrate and wash with copious amounts of tap water for 5 min/plate, also remove plastic manifold and wash back of wells. Air-dry plates at RT in the dark for at least two hrs. Count spots on AID Elispot Reader.

MATERIALS

1. ELISPOT plates: Millipore MAIP S45.
2. RPMI 1640 (from central core).
3. FBS (heat-inactivated).
4. Coating buffer: Carbonate/bicarbonate buffer pH9.6 (1.6g Na2CO3, 2.93g NaHCO3 in 1litre-distilled water). Store at –20^{o}C). Filter before storage (0.2um filter).
5. Anti-bovine IFN-γ reagents: Mab CC302 and rabbit anti-bovine IFN-y
6. PBS-T (PBS/0.055 Tween 20): add 0.5 ml Tween 20 to 1 litre of PBS.
7. PBS-T/BSA (PBS-T/0.2% BSA): add 100 mg BSA to 100 ml PBS-T (0.1% BSA final), filter sterilise (0.2 μm filter)
8. Conjugate: anti-rabbit 1gG-alkaline phosphatase conjugate (Sigma, clone R696, Cat. No. A-2556).
9. Substrate tablets: Sigma Fast BCIP/NBT buffered substrate tablet, cat. No. B-5655.

Appendix 9: Protocol for Cytotoxicity Assay

ILRI INTERNATIONAL LIVESTOCK RESEARCH INSTITUTE	STANDARD OPERATING PROCEDURE (S.O.P)	THEME: Biotechnology ACTIVITY: Cellular Immunology and Tissue Culture NUMBER: BT-ITC-015-01 REVIEW DATE: APRIL 2011	
Title: Killing assay			
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Written By: Elias Awino		Date: 05/03/08	
Number Allocated By: EOHSO		Date: 05/03/08	
Reviewed By: Dr. Duncan Mwangi (Scientist)		Date: 17/03/08	
Approved By: Dr. Phil Toye (OPL)		Date: 17/03/08	

1.0 PURPOSE

To describe the cytotoxicity assay

2.0 SCOPE

It covers the cytotoxicity assay within the lab complex.

3.0 RESPONSIBILITIES

It is the responsibility of the technicians and scientists to ensure that the procedure is followed.

4.0 PROCEDURE

CTL: (51) Sodium Chromate Release Assay

Background notes:

Cytotoxicity assays can be used to study the defence mechanism in specific patients or to study target T-cells, depending on the whether the effector (or killer) cells are the unknown.

Chromium 51 is the most important marker for the labelling of target T-cells in cyto-toxicity assay. The common name for such assays "Chromium release" also describes the fundamental method behind the assay procedure. It is non-toxic (as Na2 CrO4) it is easily taken up by the cells and it's spontaneously released only in small amounts. It does not change the morphology and characteristics of the target T-cells.

Normally a number of concentrations of effector cells are used to give different ratios of effector to target T-cells (E: T ratios).

The incubation is performed at 37^{o}C in CO2 incubator for 4 hours.

After incubation the microtitre plates are centrifuged in order to separate the cell mass from supernatant containing the released Chromium.

Control samples; to determine the total amount of Chromium taken up by the cells, a known sample is added to 2% Triton (detergent) to completely disrupt the cells. An aliquot is taken from the resulting supernatant and measured. The value obtained is referred to as MAXIMUM CHROMIUM RELEASED.

A second control, the activity of the labelled cells (incubated in cytotoxicity medium) is measured and referred to as spontaneous release or medium background.

Calculation of results; the formula for calculating the percentage lysis of the cells of specific cytotoxicity of E:T ratio is;

$$\frac{\text{Sample 51Cr release value} - \text{spontaneous release value}}{\text{Maximum 51Cr release value} - \text{spontaneous release value}} \times 100$$

The result is also called % specific release.

Labelling of the target cells

1. Harvest *T. parva* infected lymphoblasT-cells which are in log phase of growth, centrifuge and re-suspend at 2 x 10^7 in cytotoxicity medium (5% FBS or Fetal clone II in RPMI 1640 with HEPES).

2. Mix 100ul of the target T-cells with 100 microcurie of 51Cr in 10 ml sterilin tube, incubate for 1 hour at 37o C.

3. Add 10ml of cytotoxicity medium to the cells and spin at 1000 g for 5 -10 minutes at RT.

4. Break the cell pellet on a vibrator, add 10 ml of medium and wash 3 times.

5. Re-suspend the labelled target T-cells in cytotoxicity medium at a concentration of $1X10^6$ per ml.

Cytotoxic T-cells (Effectors)

Harvest T-cells from cultures (bulk or clones), ficoll in order to remove dead cells and debris. Re-suspend in cytotoxicity medium, adjust T-cell concentration to 2×10^7 per ml. Clones are normally used at $1 – 5 \times 10^6$.

Assay

1. Distribute in duplicates 200-ul/ well twofold dilution of the effectors in 96 well with flat bottom culture plated.

2. Add to each well containing effectors cells 50ul of labelled target T-cell ($5x 10^4$) resulting effectors to target T-cell ration starting from 80:1.

3. Add in triplicate to separate wells, 50ul of the target T-cell suspension to 200ul of cytotoxicity medium, to measure spontaneous release.

4. Incubate the plate(s) for 4 hours at 37o C in a CO2 incubator.

5. Add in triplicate in eppendorf tubes, 50ul of the target T-cells to 200 µl of tap water to measure maximum release of the label. Freeze thaw twice, spin and harvest the supernatant.

6. After 4 hours of incubation, mix the cells with a multi-channel pipette and centrifuge the plate(s) for 5 minutes at RT.

7. Harvest 125ul of the supernatant from each well into a sample tube and count with gamma counter.

5.0 DEFINITIONS

EOHSO- Environmental Occupational Health and Safety Officer

OPL- Operating Project Leader