EVALUATION OF THE VACCINE POTENTIAL OF RECOMBINANT LIVE ATTENUATED *LISTERIA MONOCYTOGENES* EXPRESSING $CD8^+$ T CELL EPITOPES OF *THEILERIA PARVA* IN CATTLE

Kerage Daniel Atumba BSc (Hons)

I56/13054/05

A thesis submitted in partial fulfillment for the requirement for the award of the degree of Master of Science (Biotechnology) in the School of Pure and Applied Sciences of Kenyatta University

SEPTEMBER 2008

DECLARATION

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

Signed.....Date.....07/09/2008.....

Kerage Daniel Atumba.

We confirm that the work reported in this thesis was carried out by the candidate under our supervision.

Signed.....Date.....27/10/2008.....

Dr. Eucharia U. Kenya, PhD.

Department of Biochemistry and Biotechnology, Kenyatta University

Signed.....Date.....08/09/2008.....

Dr. Duncan M. Mwangi, PhD.

International Livestock Research Institute, Nairobi

Signed.....Date.....15/9/08.....

Dr. Simon P. Graham, PhD.

Veterinary Laboratories Agency, UK.

DEDICATION

This work is dedicated to my mum, Madam Agnes Nyasuguta for educating me through thin and thick.

ACKNOWLEDGEMENTS

I am highly indebted to my supervisors Dr. Duncan Mwangi for his friendly manner, being freely available for me and his invaluable intellectual in-put into this work, Dr. Simon Graham who gave me the first needed opportunity for this work, his guidance, quick e-mail responses that eased communication and his contribution that gave this thesis the initial direction when we had face-to-face discussion, Dr. Eucharia Kenya for her lots of support and efficient communication that saved me a lot of time, Prof. John Harty for accepting to recommend me for Borlaug fellowship, contributing his expertise about *L. Monocytogenes* and for collaborating with Cerus corporation to give us the vaccine construct. I also want to thank Dr. Keith Bahjat through whom John Harty collaborated to generously donate to us the *Listeria* vaccine tried in this work, and for sharing profusely his experience with *L. Monocytogenes*. I am also grateful to the support I received from Lab 5 and 6 technical staff, at the International Livestock Research Institute (ILRI) specifically Rosemary Saya from whom I gained tremendous experience. Others on the list include James Gachanja, Gideon Ndambuki, Ferdinand Mbwika, Thomas Njoroge and John Wasilwa and also my fellow students in lab 5 and 6 for their contributions during intellectual discussions; Mulongo, Tinega, Tumusiime, Tindi, Musembi, Tinah, Kapere, Rono, Riitho, Awuoche and Jimbo. Thanks also to my friends Geoffrey Bosire, Migiro, Eric and Kiyondi for the passionate discussions we used to have while at the University and Kahawa apartments that have been my driving force academically and hope for life. Finally, thanks to my family particularly my brother Luke and uncle my Mr. Mauti, and Mr. Alloice mogunde for all your much needed support through out my education. Last, is to my almighty father for keeping me alive.

TABLE OF CONTENTS

CHAPTER THREE: MATERIAL AND METHODS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

LLO	Listeriolysin-O
Lm ΔactAΔinlB	Live attenuated *Listeria monocytogenes*
ECF	East Coast fever
PBMC	Peripheral blood mononuclear cells
TpM	*Theileria parva* (Muguga) infected cell line
APC	Antigen presenting cells
BoLA	Bovine leukocyte antigen (bovine MHC)
ELISpot	Enzyme linked immunospot
IFN-γ	Interferon-gamma (γ)
IL-2	Interleukin-2
ITM	Infection and treatment method
MHC	Major histocompatibility complex

ABSTRACT

East Coast fever (ECF) is a highly fatal lympho-proliferative disease of cattle caused by tick-borne protozoan *Theileria parva*. The intra-lymphocytic schizont stage induces a cancer-like transformation of the infected cell which is responsible for the pathology and ultimately death of infected animals. Immunity from the cattle that recover from ECF or treated using live vaccines is mediated by parasite-specific $CD8^+$ cytotoxic T lymphocytes (CTL), which target and clear schizont-infected lymphocytes. Although the identified CTL target antigens have been evaluated using DNA and attenuated pox virus vectors and have been shown to induce $CD8^+$ CTL responses in a proportion of cattle that have correlated with reduced disease severity upon parasite challenge, the inability to consistently trigger the protective CTL responses is a major impediment that needs to be overcome. This study aimed at evaluating the potential of recombinant live attenuated *Listeria monocytogenes* (*Lm ΔactAΔinlB)* expressing three defined *T. parva* CTL epitopes to stimulate protective CTL responses against *T. parva* in cattle upon lethal parasite challenge. *In vitro* studies using recombinant *Lm ΔactAΔinlB* indicated that one of the *T. parva* CTL epitopes could be weakly expressed and recognized by antigen-specific $CD8^+$ T cell lines in one of the experiments. Cattle were screened against *L. monocytogenes* T cell target antigenic peptide pool, listeriolysin-O (LLO), and the vaccine select animals based on those with no or low background responses to LLO. Induction of epitope-specific $CD8^+$ T cell IFN-γ immune responses were detected in three out of nine vaccinated animals while T cell responses in PBMC to LLO were observed in eight animals immunized with either wild type or recombinant *Lm ΔactAΔinlB*. Cytotoxic T cell responses were detected after boosting in only one of nine immunized animals. However these responses did not translate into a significant protective effect after challenge. This may be in part due to poor expression of recombinant antigens leading to sub-optimal induction of protective antigen-specific $CD8^+$ T cells. Although this immunization regime failed to induce protective $CD8^+$ T cells against *T. parva* in all the animals. By modifying the antigen formulation, dose, mode and route of inoculation a more efficient protocol can be developed. This kind of formulation will find application in vaccine preparation in other diseases including cancer where the induction of $CD8^+$ T-cell immunity is critical.

CHAPTER ONE

INTRODUCTION

1.1 Background

The intracellular tick-borne apicomplexan parasite *Theileria parva* is the causative agent of East Coast fever (ECF), an acute and often fatal lympho-proliferative disease of cattle, which is endemic in 12 countries in Eastern, Central and Southern Africa (Norval *et al.*, 1992). In susceptible cattle populations in endemic countries, one cow succumbs to the disease every thirty seconds resulting in economic losses of more than $300 million per annum (CGIAR Science Council, 2005). At least 28 million cattle are at risk, majority of which are kept under smallholder dairy systems (Moll, *et al.*, 1986).

Cattle that recover naturally from ECF or immunized using live vaccines, exhibit strong $CD8^+$ T cell responses against the schizont stage (Eugui and Emery, 1981), and a body of data exists that suggest that $CD8^+$ CTL are the dominant protective mechanisms against *T. parva*. It has been shown that in immune cattle, *T. parva* specific $CD8^+$ CTL are detected transiently in the peripheral blood and efferent lymph and increase in number during challenge around the time parasites are cleared (Eugui and Emery, 1981, Morrison *et al.*, 1987, McKeever *et al.*, 1994). Additionally, it has been demonstrated that the capacity of an immune animal to mount resistance to heterologous challenge is associated with the induction of the parasite strain-specific $CD8^+$ CTL (Taracha *et al.*, 1995). Direct evidence for the involvement of $CD8^+$ T cells in protection is provided through adoptive cell transfer studies, lethally infected non-immune calves received inoculations of responding $CD8^+$ T cells from their immune monozygous twins and the challenge

infection was subsequently confined to the regional lymph node and then cleared (McKeever *et al.*, 1994). Currently, the only method of vaccination available for the control of ECF is the use of live vaccines. However, this method has a number of practical constraints including the material expenses involved, the constant need for supply of a cold chain and the induction of carrier states in immunized animals (Radley *et al.*, 1975a).

In an attempt to improve ECF control, recent research has focused on the development of a sub-unit vaccine that would target both the sporozoite and schizont-stages of *T. parva* life-cycle. The first step in designing a sub-unit vaccine utilized the major sporozoite surface coat protein, p67, which has been shown to have significant vaccine potential in the reduction of the incidence of severe ECF (Musoke *et al.,* 1992, Musoke *et al.*, 2005). A number of *T. parva* schizont antigens targeted by $CD8^+$ CTL have been identified and immunization of cattle with these antigens using live vectored delivery systems has shown the potential to induce $CD8^+$ CTL responses (Graham, *et al.*, 2006, Graham *et al.,* 2007). However, the exploitation of these antigens to develop a subunit vaccine is limited by the inability to consistently trigger the protective $CD8^+$ CTL responses. This study explored an alternative vaccine antigen delivery system by evaluating the potential of a recombinant live attenuated *Listeria monocytogenes* (*Lm ΔactAΔinlB)* expressing three defined $CD8^+$ T cell epitopes from *T. parva* vaccine candidate antigens to induce CTL responses. Furthermore, the research investigated whether these responses could confer protection against a lethal parasite challenge. Like *T. parva*, *L. monocytogenes* replicates within the intra-cytoplasmic environment of leukocytes facilitating delivery of expressed

antigen into the endogenous antigen processing and presentation pathway which subsequently provides stimulation of peptide specific MHC class I-restricted $CD8^+$ CTL (Starks, *et al.*, 2004, Hamilton, *et al.*, 2006). The vaccine formulation used in this study has been evaluated in mice and is being currently tried in humans against cancer (S. K. Bahjat personal communication)

1.2 Justification

The infection and treatment method (ITM) of immunization against ECF is the most successful and the only one available that can provide protection in the field (Uilenberg, 1999, Radley *et al.*, 1975b, Radley, 1981). This method confers sufficient immunity to homologous and in many instances to heterologous strains (Brown *et al*, 1995, Radley *et al*, 1975a) following parasite challenge. However this approach is limited by the requirement for a cold chain, introduction of new pathogen strains, and the induction of carrier states in animals (Uilenberg, 1999). The most promising solution to these constraints will be the development of the ECF sub-unit vaccine. Current research is focusing on the sporozoites and schizont stages of the parasite (Mckeever *et al.*, 1999). Although the p67 subunit vaccine has shown potential to induce protection under laboratory conditions, it has failed to show sufficient efficacy under field-conditions (Kaba *et al.*, 2003, Musoke *et al.*, 1992). $CD8^+$ CTL specific for the intracellular schizont stage of the parasite have been shown to be the main protective mechanisms against ECF following ITM or recovery from infection (Eugui and Emery, 1981, Morrison *et al.*, 1987, McKeever and Morrison, 1994, Taracha *et al.*, 1995), and provide a complimentary approach to subunit vaccine development.

The identification of $CD8^+$ T cell target antigens from the schizont stage of *T. parva* (Graham *et al.*, 2006, Graham *et al.*, 2007) has raised hopes for the development of an ECF sub-unit vaccine based on the stimulation of CTL responses. Evaluation of these antigens using plasmid DNA and attenuated pox viral vectors has shown the induction of $CD8^+$ CTL responses in a proportion of cattle that associated with a reduction in the severity of the disease (Graham *et al.*, 2006, Graham *et al.*, 2007). Following sub-optimal performance of these antigen delivery systems, there is need to evaluate other systems that might consistently induce $CD8^+$ CTL responses. The bacterium *L. monocytogenes* has a tremendous potential to replicate intra-cellularly and has been shown to be a robust inducer of protective $CD8^+$ CTL responses in murine (Harty and Bevan, 1992, Jensen *et al.*, 1997, Starks *et al.*, 2004, Brockstedt *et al.*, 2004) and non-human primate models (Boyer *et al.*, 2006). Immunity to *L. monocytogenes* in mice is $CD8^+$ T cell-mediated (Pamer, 2004), which contrasts to pox viruses, where protective immunity is often antibody-mediated (Farina *et al.*, 2001, Pinto *et al.*, 2003). These reasons plus the ease of production in cheap media, make *L. monocytogenes* an attractive system to be evaluated as a $CD8^+$ CTL targeted subunit vaccine carrier against *T. parva.*

1.3 Research questions

i) Do *T. parva* specific bovine $CD8^+$ T cells respond to autologous cells infected with a recombinant *Lm ΔactAΔinlB* expressing *T. parva* $CD8^+$ T cell epitopes, *in vitro*?

ii) Does immunization of cattle with recombinant *Lm ΔactAΔinlB* expressing *T. parva* $CD8^+$ T cell epitopes induce $CD8^+$ CTL responses?

iii) Do the immune responses induced by recombinant *Lm ΔactAΔinlB* expressing *T. parva* $CD8^+$ T cell epitopes protect cattle against a lethal *T. parva* challenge infection?

1.4 Study objectives

1.4.1 General objectives

The main objective of this study was to evaluate whether a recombinant live *L. monocytogenes* attenuated through a deficiency in the Act-A and internalin-B proteins (Lm *ΔactAΔinlB)* expressing three defined CTL epitopes from *T. parva* vaccine candidate antigens could induce CTL responses in cattle and to determine whether these immune responses could protect against a lethal parasite challenge.

1.4.2 Specific objectives

i) To assess the ability of autologous bovine antigen presenting cells infected with recombinant *Lm ΔactAΔinlB* to be recognized by specific $CD8^+$ T cell lines.

ii) To evaluate the immunogenicity of the recombinant *Lm ΔactAΔinlB* bearing *T. parva* CTL epitopes in cattle.

iii) Evaluate the vaccine protective efficacy of recombinant *Lm ΔactAΔinlB* expressing *T. parva* CTL epitopes in cattle.

CHAPTER TWO

LITERATURE REVIEW

2.1 East Coast Fever (ECF)

The apicomplexan parasite *T. parva* is the causative agent of ECF. It is an often-fatal disease of cattle that results from the proliferation of parasite infected host lymphocytes that invade lymphoid and non-lymphoid tissues (Dobbelaere *at al.,* 2000). ECF is transmitted by the brown ear tick, *Rhipicephalus appendiculatus,* and leads to enormous economic losses in Eastern, Central and Southern Africa (Mukhebi, 1992). It is one of the most lethal of cattle diseases in the region with death rates approaching 100% in susceptible cattle in endemic areas (Moll *et al.*, 1986). In addition to mortalities, the losses accounted for include the overheads of procuring agro-chemicals for controlling the tick vectors and production costs. Furthermore, the disease constrains the livestock industries in areas where ECF is prevalent since non-indigenous productive breeds are more susceptible (Mukhebi, 1992). The escalating resistance to acaricides in tick populations has intensified the need for searching alternative control measures (Uilenberg, 1999, Redondo *et al.*, 1999). Current research efforts are aimed at developing vaccines based on sub-unit components of *T. parva* that could protect against ECF (McKeever *et al.*, 1999).

2.1.1 Life-cycle of *Theileria parva*

As illustrated in Fig 1, the life-cycle of *T. parva* involves both mammalian and tick hosts (Mehlhorn and Schein, 1984). The feeding tick inoculates sporozoites that infect host lymphocytes through a process of receptor-mediated endocytosis (Fawcett *et al.*, 1982b). The sporozoites differentiate in the host cytosol into a schizont stage that is responsible for much of the pathology of ECF since it transforms the bovine host cell into a leukemia-like state (Hulliger *et al.*, 1964). Upon lymphocytolysis, uninucleate merozoites are released and they invade the erythrocytes where they develop into piroplasms (Mehlhorn and Schein, 1984, Shaw and Tilney, 1995). The majority of cattle that recover from *T. parva* infection become carriers (Young *et al* 1981, Kariuki *et al.,* 1995), and ingestion of piroplasm infected erythrocytes by blood sucking ticks completes the mammalian phase of the life cycle.

Erythrocytolysis results in the release of piroplasms into the tick gut which differentiates to male and female gametes (Mehlhorn and Schein, 1984). These undergo syngamic fusion to form epithelial gut invading diploid zygotes that develop into motile kinetes (Young and Leitch, 1980). The kinetes are released into the tick haemocoel and then to the salivary gland where they invade type III acini cells (Walker, 1990, Mehlhorn and Schein, 1984, Fawcett *et al.*, 1982a). Upon the tick feeding on another host the kinete develops within the infected cell to a multinucleate sporoblast and finally to non-dividing infective sporozoites (Fawcett *et al.*, 1982b, Fawcett *et al.*, 1985).

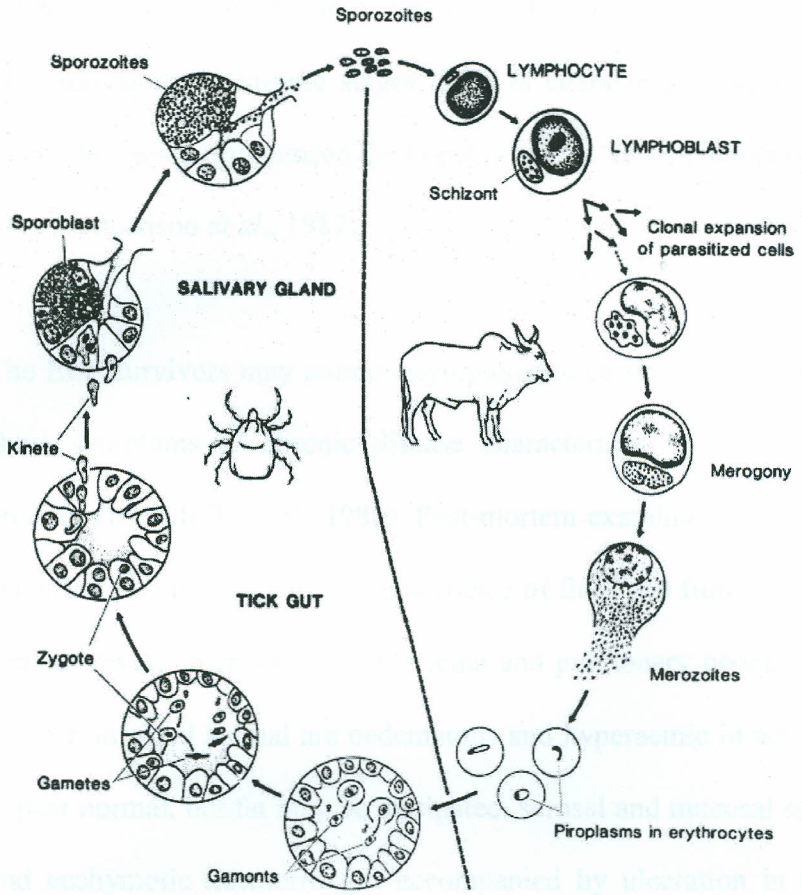


Figure 1: Life-cycle of *Theileria parva* (Norval *et al*, 1992).

2.1.2 Pathogenesis and clinical manifestation of ECF

The main cause of ECF pathology follows the invasion of the lymphoid and non-lymphoid tissues by *T. parva* (Dobbelaere *at al.,* 2000). *T. parva* infects all lymphocyte lineages and the schizont stage division is accompanied by lympho-proliferation with resultant phenomenal lymphocytolysis (Mckeever and Morrison, 1990). The lymph node swelling 7-15 days after tick infection is often the first clinical sign of ECF. The parasite-infected cells then spread to other lymphoid tissues and peripheral lymph nodes such as the parotid precapsular and prefermoral lymph nodes (Mckeever and Morrison, 1990).

Fever ensues (sometimes in excess of 42°C) and remains high throughout the course of ECF infection. In chronic stages, ECF is characterized with emaciation, diarrhea and even blindness, pronounced leukopenia and a fall in temperature occur in advanced stages (Morrison *et al.*, 1987).

The ECF survivors may remain asymptomatic carriers, in which only a small proportion show symptoms of chronic disease characterized by stunted growth and decreased productivity (Moll, *et al.,* 1986). Post-mortem examination reveals striking changes that occur in the lungs such as the appearance of fluid and froth in the trachea and bronchi as well as severe interlobular emphysema and pulmonary oedema. The lymph nodes from an ECF infected animal are oedematous and hyperaemic in acute cases. Muscles and fat appear normal, but fat may be dissipated, serosal and mucosal surfaces develop petechial and ecchymotic haemorrhages accompanied by ulceration in the gastrointestinal tract (especially abomasum) in more acute conditions (Irvin *et al.*, 1983).

2.1.3 Diagnosis of ECF

The diagnosis of ECF for the naturally infected animals involves the observation of clinical signs, laboratory confirmation of the presence of schizonts in the lymphoid cells through lymph node biopsy smears and the intra–erythrocytic piroplasms in blood smears from clinically sick animals. In extreme cases post-mortem lesions are observed and schizonts can be detected from spleen smears of dead animals. Where applicable, this diagnosis is complemented by the indirect fluorescent antibody test (IFAT) using

schizont antigen (Chinombo *et al.*, 1989). Although not all lymph node biopsy smears having schizonts are ECF cases (Malawi Government, 1975).

2.1.4 Treatment of ECF

Animals that suffer from ECF can recover if treated early enough using anti-theilerial drugs (Dolan *et al.*, 1984, Dolan, 1986). Infected animals treated with buparvaquone and parvaquone are immune to subsequent challenge with the same strain of the parasite (Radley *et al.*, 1975a). However, chemotherapy like live vaccines has been implicated with the development of the carrier state in the animals that recover from ECF (Dolan *et al.*, 1984, Dolan, 1986). In addition, occurrences of *Theileria annulata* strains resistant to buparvaquone treatment have been reported in Tunisia (Naouach, 2001, Mhadhbi, 2004) where this drug is mainly used. The report shows that calves were treated earlier enough (before disease had progressed) after thorough clinical and parasitological examination, but surprisingly the mortality reported was high by 12.5% (Naouach, 2001, Mhadhbi, 2004) compared with figures reported previously in the same farms in which there was no mortality case in 1995 (Soudani, 1995), while in 2000, 4.54% was reported (Karoui, 2002).

2.1.5 Prevention of ECF

The two main control measures employed in the prevention of ECF include the use of agro-chemicals such as acaricides to control the tick vector and live vaccination using *T. parva* sporozoites (ITM).

2.1.5.1 Vector Control

Tick vector control constitutes effective fencing, pasture management and application of acaricides (Young *et al.*, 1988). For control of the *R. appendiculatus* tick, acaricides should be applied at least weekly (Kariuki, 1990), however these chemicals are a burden to farmers in terms of cost and environmental pollution due to emission of toxic compounds (de Castro *et al.*, 1997, Mbogo *et al.*, 1996) while their inappropriate use leads to development of ticks resistant to acaricide (Uilenberg, 1999). The methods used for the application of acaricides include plunge dipping, hand spraying and hand dressing (Redondo *et al.*, 1999). The principal objective of tick control is to kill the infesting ticks in order to break the life cycle and to ensure total coverage of all predilection sites of the various tick species (Uilenberg, 1999, Redondo *et al.*, 1999).

2.1.5.2 Infection and Treatment Method (ITM)

In ITM, animals are inoculated with a potentially lethal dose of infective sporozoites of *T. parva* and simultaneously treated using long-acting antibiotic oxytetracycline (Radley *et al.*, 1975b, Radley, 1981). This strategy confers effective and long term immunity to homologous and sometimes heterologous parasite challenge (Radley *et al.*, 1975a), and it has been adopted as a live vaccine for the control of ECF. It has also been shown that it is possible for an animal to recover from *T. parva* infection by clearing parasitosis while remaining immune (sterile immunity). An example of this is immunization using *T. parva* Muguga (Bishop *et al.*, 1992).

Immunization with other *T. parva* stocks creates a state of low parasite levels (pre-immunity) and is thought to remain carriers (Maritim *et al.*, 1989, Bishop *et al.*, 1992,

Kariuki *et al.*, 1995). However, this idea seems to contradict with recent findings that after immunization of cattle with Muguga cocktail most of the parasites carrier states were from Kiambu 5 component (Oura *et al.*, 2007), and there was no evidence for transmission of the Muguga or Serengeti stocks.

2.2 Immune responses to *T. parva* and protective immunity

Cattle that either recover from ECF or immunized using ITM are solidly protected against homologous and sometimes heterologous parasite challenge (Radley *et al.*, 1975a). Broad protection can be achieved by immunization with relatively few strains (Radley *et al.*, 1975b), suggesting that the immune system is directed against relatively few antigenic determinants or that the antigenic differences are few. However, direct evidence indicates that protection is provided by cellular immunity directed against cells infected with the schizont stage (Morrison *et al.*, 1987, McKeever *et al.*, 1994). The antibody responses against *T. parva* sporozoites inoculated by the tick on primary infection is very low, but cattle challenged repeatedly with the same parasite, show increased levels of serum antibodies with the potential to neutralize sporozoite infectivity *in vitro* (Musoke *et al.*, 1982).

2.2.1 Humoral responses against *T. parva*

Sera from hyperimmunised animals with *T. parva* sporozoite lysates or those from endemic areas have indicated an ability to neutralize sporozoite infectivity *in vitro* (Musoke *et al.*, 1984). The majority of these antibodies are directed against the major surface coat sporozoite protein termed p67 with B cell epitopes on the antigen that is

conserved among different parasite stocks (Dobbelaere *et al.*, 1984, Musoke *et al.*, 1984,). Monoclonal antibodies that recognize p67 also neutralize sporozoite infectivity (Dobbelaere *et al.*, 1984, Webster *et al.*, 1985). In contrast, Eugui and Emery (1981) have shown that sera obtained from schizont parasitized animals does not confer protective immunity since immune animals develop similar symptoms upon challenge, it is therefore believed that antibodies against *T. parva* schizont stage can not provide protective immunity.

2.2.2 Cellular immune responses against *T. parva*

Lymphocytes derived from immune cattle or those generated *in vitro* using *T. parva* infected cell-lines, have been shown to recognize and destroy autologous but not allogeneic infected cells (Eugui and Emery, 1981). The effector activity of these cells was found by Emery, (1981) to be associated with $CD8^+$ ligand molecules expressed by T-cell population present in the peripheral blood. It has been reported that in immunized animals, *T. parva* specific $CD8^+$ CTL are present in low frequencies in the peripheral blood and efferent lymph and expands during challenge around the time the parasites are controlled (Eugui and Emery, 1981, Morrison *et al.*, 1987, McKeever *et al.*, 1994).

Protection in immune cattle based on the elimination of *T. parva*-infected cells has been demonstrated to be provided by MHC class I restricted $CD8^+$ T cells (McKeever *et al.*, 1994). There is direct evidence of the involvement of cytotoxic $CD8^+$ T-cells in protection through kinetic and adoptive cell transfer studies (Mckeever *et al.*, 1994). The efferent lymphoid $CD8^+$ T-cells from an immune calf was transferred to its naïve twin 1-3 days following *T. parva* sporozoite infection, resulting in attenuation and recovery from

infection (Mckeever *et al.*, 1994). Further support for the role of $CD8^+$ T cells as the predominant mechanism of immunity was provided by studies using two strains of *T. parva* that demonstrated a correlation between the strain-specificity of the CTL responses and protection against challenge (Taracha *et al.*, 1995). Additionally, it has been demonstrated that the capacity of an immune animal to mount resistance to heterologous challenge, the induction of specific $CD8^+$ CTL is associated with the parasite strain (Taracha *et al.*, 1995). Besides, MHC class II restricted $CD4^+$ T cells with helper and cytolytic activity is reported to be detectable following ITM immunization (Baldwin *et al.*, 1987, Taracha *et al.*, 1997). However, this T cell sub-population has no proven direct effector mechanism against *T. parva*. Antigen specific $CD4^+$ T cells are nevertheless required for the necessary factors needed for the activation of naïve and immune parasite specific $CD8^+$ CTL against *T. parva* (Taracha *et al.*, 1997). While $CD4^+$ T cells provide the lymphokines (IL-2) and other signals for the expansion of primed $CD8^+$ T cells, the conditions for activation of naïve CTL precursors are more stringent than immune $CD8^+$ CTL precursors, these cells requires that both the $CD4^+$ and $CD8^+$ T cell epitopes be presented on the same cell (Taracha *et al.*, 1997).

Bovine γδ T cells from both naïve and immune animals have also been shown to recognize and respond to *T. parva*–infected cells during the course of primary infection (Daubenberger *et al.*, 1999). The association of γδ T cells with infections caused by intracellular parasites, has led to the suggestion that these cells constitute the innate surveillance system that acts as the first line of defense against infectious diseases (Kaufmann, 1996), and *T. parva* in young cattle (Daubenberger *et al.*, 1999). The

existence of cytotoxic γδ T cells has been thought to be involved in protection in young cattle against *T. parva*. The γδ T cells derived from cultures of PBMC from immune cattle and then restimulated with *T. parva*-infected lymphoblasts have been shown to lyse parasitized cells but not con A blasts. Such cytolysis are not blocked by MAbs directed against MHC class I or class II molecules nor parasite strain specific meaning that the effector activity of these cells are unrestricted (Daubenberger *et al.*, 1999).

On the other hand, T cell cytokines TNF-α and IFN-γ have been demonstrated not to have profound inhibitory effects in the establishment of *T. parva* infection *in vivo* (DeMartin and Baldwin, 1991). However, the synergistic anti-proliferative effect of TNF and IFN-γ on *T. parva*-infected cells indicates that these cytokines may inhibit *T. parva* infections if they are present at the time of sporozoite deposition (DeMartin and Baldwin, 1991).

3.0 ECF sub-unit vaccine development

The *T. parva* sporozoite surface coat protein, p67, has been evaluated as a candidate vaccine antigen against ECF (Musoke *et al.*, 1992, Nene *et al* 1995, Nene *et al*, 1996, Kaba *et al.*, 2003) and has demonstrated protective efficacy of up to 70-100% under laboratory conditions when challenged with a lethal dose of 70 (LD_{70}, which should kill 70% of animals). However, when this vaccine was field-tested its efficacy reduced the incidence of severe disease in 50% of animals (Musoke *et al.*, 2005). Therefore, there is need to identify complementary antigens that could be combined with p67 to provide a sub-unit vaccine that induces full protection. A major set-back to the p67-based vaccination approach, has been the inability to evoke cell-mediated immunity essential

for generating effective immunity against *T. parva* (McKeever *et al,* 1999). Schizont-specific bovine CTLs recognize parasite antigens expressed as peptides complexed with bovine MHC class I molecules on the surface of schizont-infected cells (McKeever *et al.*, 1994). Recent ECF vaccine research has focused on identifying these CTL target antigens and formulation of these antigens to evaluate their vaccine potential in cattle. This has resulted in the identification of antigens recognized by $CD8^+$ T cells from immune cattle (Graham *et al.*, 2006, Graham *et al.*, 2007).

Using preliminary data from the *T. parva* genome sequence (Gardner *et al.*, 2005) it was possible to generate a set of candidate antigen genes for cloning and immunoscreening. The genes were selected on the basis of encoding secreted or membrane bound proteins, because these parasite proteins should access the cytosol of the infected cell and be available for MHC class I processing and presentation to $CD8^+$ T cells. Of the selected genes, fifty-five open reading frames (ORFs) encoding candidate antigens were selected for screening, out of which 36 were successfully cloned into a eukaryotic expression vector and then screened with fully characterized polyclonal or clonal CTL lines from 13 cattle immunized by ITM (Graham *et al.*, 2006, Graham *et al.*, 2007). To compliment this approach, a cDNA library was generated from schizont-stage parasites and pools of cDNA were similarly screened for recognition by CTL lines. Through these approaches six parasite antigens recognized by CTL were identified and termed Tp1, Tp2, Tp4, Tp5, Tp7 and Tp8 (Graham *et al.*, 2006, Graham *et al.*, 2007). By comparing the target cDNA sequences with the predicted *T. parva* gene models (Gardner *et al.*, 2005) the antigens Tp4, Tp5, Tp7 and Tp8 were annotated as ε-subunit of T complex protein 1, elongation translation initiation factor 1A, heat-shock protein 90, and cysteine proteinase

respectively, whereas Tp1 and Tp2 were predicted as hypothetical proteins (Gardner *et al.*, 2005).

Vaccine formulation of five of these antigens was done using attenuated pox viral vectors (canary pox (CP), modified vaccinia virus Ankara strain (MVA) and a plasmid DNA. Evaluation of these vaccine constructs in cattle using heterologous prime/boost (DNA/MVA or CP/MVA) system, demonstrated the induction of protective $CD8^+$ CTL responses in a proportion of vaccinated cattle (Graham *et al.*, 2006). BoLA defined cattle that should be capable of recognizing at least one of the antigens were immunized. Majority (79%) of the vaccinated animals elicited $CD8^+$ T cell IFN-γ ELISpot responses out of which only a minority (30%) showed CTL responses that were associated with survival and reduced disease severity (Graham *et al.*, 2006). The $CD8^+$ T cell responses to these antigens following challenge showed kinetics and frequencies similar to those described for schizont specific CTL in efferent and peripheral blood and peaked at the predicted time of parasite clearance (Morrison *et al.*, 1987, McKeever *et al.*, 1994), demonstrating that these antigens are recognized by $CD8^+$ T cells during resolution of a parasite challenge (Graham *et al.*, 2006). Efforts are currently being made to identify additional antigens and to develop an effective antigen delivery system to deliver these antigens for elicitation of protective CTL responses (S. P. Graham and D. M. Mwangi, personal communication).

3.1 Alternative delivery systems for the induction of $CD8^+$ T cell responses

A number of antigen delivery systems have been evaluated for induction of $CD8^+$ T cell responses both in mice and large animal models. Adenoviruses have been demonstrated to be highly efficacious vaccine carriers both in murine and primate models (Gallichan *et al.*, 1993, Babiuk and Tikoo, 2000, Sullivan *et al.*, 2000, Shi *et al.*, 2001). Additionally, the ability of adenovirus vectors to induce $CD4^+$ and $CD8^+$ T cells is well described (Tripathy *et al.*, 1996, Yang and Wilson, 1996, Jooss *et al.*, 1998). Recently, Yang *et al.*, (2003) described the kinetics and nature of immune responses induced by adenovirus vectors where they were shown to induce robust long-lived $CD8^+$ T cell IFN-γ responses. On the other hand, other vaccine vectors such as MVA used alone or in combination with DNA priming have elicited potent $CD8^+$ T cell IFN-γ responses and provided the highest degree of protection in non-human primates against HIV-1 (Amara *et al.*, 2001, Barouch *et al.*, 2001a, Barouch *et al.*, 2001b, Shiver *et al.*, 2002, Earl *et al.*, 2002, Casimiro *et al.*, 2003). Furthermore, studies using murine and non-human primate animal models of malaria, tuberculosis and HIV have shown that the immunogenicity due to plasmid DNA, influenza virus or adenovirus priming using defined antigens can be greatly enhanced with MVA or fowlpox virus boosting expressing the same antigen (Kent *et al.*, 1998, Gilbert *et al.*, 1999, McShane *et al.*, 2001). However a major limitation of the adenoviral and poxviral vectors is that neutralizing antibodies to the vaccine carrier elicited by previous natural infections or vaccination impact greatly on their efficacy (Farina *et al.*, 2001, Pinto *et al.*, 2003).

In addition to viral vectors, live bacteria are also being tested as carrier systems for DNA vaccines. In this approach, attenuated or mutant strains of intracellular bacteria such as

BCG, *L. monocytogenes*, *Salmonella typhi, S. typhimurium*, or *Shigella flexnerii* are used to deliver DNA vaccines (Dietrich *et al.*, 2001) both in small and other large animal models. Moreover, vaccines based on plasmid DNA elicit strong antibody and $CD8^+$ T-cell responses in animal models, including mice and non-human primates. In contrast, when used in humans to immunize against HIV 1 or *P. falciparum* antigens, these vaccines failed to elicit antibodies, even when cellular immune responses (CTLs) are detected (Calarota *et al.*, 1998, Wang *et al.*, 1998).

Protein-based cancer vaccines have also been shown to have the potential of inducing both $CD8^+$ and $CD4^+$ T cells by presenting multiple epitopes, but they are inefficient in sensitizing $CD8^+$ T cells because these proteins are hardly processed by MHC class I pathway (Ikuta *et al.*, 2002).

3.2 *Listeria monocytogenes*

L. monocytogenes is a Gram-positive bacterium whose natural route of infection is the gastro-intestinal tract. The bacterium infection requires the interaction of internalin-A which is expressed on the surface of the bacteria with the epithelial cadherin (E-cadherin) expressed on the surface of epithelial cells (Gaillard *et al*,. 1991). The bacteria then traverses the epithelial layer into the blood stream from which it invades the liver and spleen. In the liver, the internalization by liver macrophages require *L. monocytogenes* to express another surface protein internalin-B, which binds to the hepatocyte growth factor on the cell surface of hepatocytes (Shen *et al.*, 2000). Upon cell invasion, *L. monocytogenes* secretes listeriolysin-O which enables the bacterium to escape from the phagosome (Bielecki *et al.*, 1990). The bacterium has a robust potential of intracellular

replication (Pamer, 2004), and mobility in the cytosol and cell to cell locomotion is enabled by the expression of actin–assembly inducing protein (Domann, 1992, Kocks, 1992).

L. monocytogenes has been extensively used to characterize T cell-mediated immune responses. Mice infected with *L. monocytogenes* have been used to demonstrate the cellular basis of protective immunity against *L. monocytogenes* (Mackaness, 1962). In these animals lytic $CD8^+$ T cells have been demonstrated to provide more long term immunity than $CD4^+$ T cells (Ladel *et al.*, 1994). In addition, $CD8^+$ T cells have been shown to mediate immunity against *L. monocytogenes* upon infection or following re-challenge in mice and non-human primate models (Jensen *et al.*, 1997b, Brockstedt *et al.*, 2004, Boyer *et al.*, 2006). These features have made *L. monocytogenes* an attractive organism to use as a vaccine vector capable of inducing $CD8^+$ T cell responses. However, since *L. monocytogenes* is known to be a food-borne pathogen, it is essential to retain immunogenicity as a vector while attenuating its pathogenicity for eventual efficacious clinical trials *in vivo* (Brockstedt *et al.*, 2004).

3.2.1 Attenuated *Listeria monocytogenes* as a vaccine vector

While there are safety concerns regarding the use of wild-type and recombinant *L. monocytogenes* as a vaccine vector, the natural biology of this bacterium offers a number of features that make it an attractive platform as a vaccine carrier. The intra-cellular life-cycle of *L. monocytogenes* enables effective expression, endogenous processing and presentation of foreign antigens to the target effector cells. This induces both $CD4^+$ and $CD8^+$ T cell immunity (Brockstedt *et al.*, 2004). These coupled to its ease

of production in defined media, simple vaccine formulation using well developed techniques for bacterial engineering, validates *L. monocytogenes* as an attractive system for vaccine construction.

Several lines of evidence support the use of *L. monocytogenes* as a vaccine vector; murine models immunized with recombinant *L. monocytyogenes* expressing choriomeningitis virus (CMV) nucleoprotein (NP) CTL epitope provokes specific cytolytic $CD8^+$ T cell responses that provides protection against lethal viral challenge (Shen *et al.*, 1995). Rabbits inoculated with *L. monocytogenes* expressing an antigen from papilloma virus are protected against papilloma infection (Jensen *et al.*, 1997a), and tumor bearing mice immunized with *L. monocytogenes* strain expressing a tumor antigen stimulates protective $CD8^+$ CTL against colonic or renal tumors (Pan *et al.*, 1995). Significant reduction of B16F10 solid carcinomas has been demonstrated upon immunization with *L. monocytogenes* expressing B16F10 specific nucleoprotein (Pan *et al.*, 1995). Similarly immunization with recombinant *L. monocytogenes* expressing human papilloma virus E7 gene elicits cellular immunity that regresses an E7-gene transformed cell-line (Gunn *et al.*, 2001).

A major limitation to viral vectors is thought to be as a result of neutralizing antibodies to the vaccine carrier due to previous exposure or repeat immunizations that implicate their efficacy (Farina *et al.*, 2001). In *L. monocytogenes* immunity due to re-infection is believed to be $CD8^+$ T cell mediated (Bouwer *et al.*, 1992). These data collectively provide a solid basis for the continued evaluation of *L. monocytogenes* as a vaccine platform for the induction of lytic $CD8^+$ T cell mediated immunity.

CHAPTER THREE

MATERIALS AND METHODS

3.1. Experimental animals and MHC class I BoLA typing

Cattle that express one of the BoLA class I alleles N*01301, N*01201 and N*00101 were used in this study. The MHC class I BoLA typing was conducted with PBMC derived from the selected animals using a panel of specific monoclonal antibodies. The PBMC were resuspended in FACS medium (containing RPMI 1640 supplemented with 2% horse serum and 0.02% Sodium Azide) and adjusted to $1x10^7$/ml. The cells and labeling antibodies were each dispensed at 50µl/well and then incubated for 30 min at 4°C. A secondary antibody that is, FITC-conjugated goat anti-mouse Ig was added and then the culture incubated for 30 min at 4°C. The results were analyzed using a FACScan machine (BD Biosciences, San Jose, USA) following three wash steps.

MHC class I BoLA typed cattle were watered and fed on a daily basis on a recommended diet. These animals were obtained from areas free from ECF and had been screened for presence of major tick-borne diseases (ECF, Babesiosis, and Anaplasmosis) as shown in Table 1. All animal work was approved and endorsed by ILRI's Institute of Animal Care and Use Committee (IACUC). Table 1 shows details of the 12 cattle that were recruited for the vaccine trial. The animals were maintained in a BL2 confinement facility throughout the entire period of the experiment with daily temperature recordings.

3.1.1 Screening of cattle for T cell reactivity against *L. monocytogenes* by IFN –γ ELISpot assay

Thirty milliliters of venous blood was collected from animals using a 16-gauge needle into a 60ml syringe containing 30ml of anticoagulant Alsevers' solution. PBMCs were isolated and purified by layering blood on Ficoll/paque (Pharmacia Fine Chemicals, Uppsala, Sweden) according to the methods described by Goddeeris and Morrison (1988). Viable cells were enumerated by trypan blue exclusion on an improved Neubaeur Chamber (Profondeur, Germany). PBMC were screened against a pool of overlapping synthetic peptides representing the full length of listeriolysin-O (LLO) by quantitative IFN-γ release on an ELISpot assay. A high protein binding membrane ELISpot plate (Multiscreen, Millipore, Bedford USA) was coated with 50µl/well mouse anti-bovine IFN-γ monoclonal antibody (Serotec, Germany) and incubated overnight at 4°C or for 1hr at 37°C. Wells were washed twice in RPMI-1640 medium (200µl/well) and then blocked with RPMI-1640 supplemented with 10% Fetal Calf Serum (200µl/well) and incubated at 37°C for 1 hr. The blocking medium was removed and PBMC ($2.5x10^5$/well in 100µl) and LLO peptide pool (2µg/ml) were added (25µl/well).

The ELISpot plate was incubated at 37°C humidified 5% CO_2 for 20 hours. The plates were washed three times in 0.1% Tween 20 in water and three times in 0.1% Tween20 in PBS (PBS-T) while shaking for 30 seconds between each wash. Rabbit anti-bovine IFN-γ polyclonal antisera (ILRI) diluted 1/1500 in 1% BSA in PBS-T (BSA/PBS-T) was added (50µl/well) and the plates incubated for 1hr at room temperature (RT). Using PBS-T

(200µl/well), the wells were washed 6 times, and then anti-rabbit IgG conjugated to alkaline phosphatase enzyme (Sigma-Aldrich, Steinheim Germany) diluted 1/2000 in BSA-PBS-T (50µl/well) was added and incubated for 1hr at RT. After washing the plate six times with PBS-T (200µl/well), BCIP/NBT substrate (Sigma-Aldrich, Steinheim Germany) was added and incubated for 10 minutes in the dark, at room temperature. Excess substrate was washed off using water and the plate dried in the dark. The spot forming cells (SFC) were enumerated using an ELISpot reader (AID, Strassberg, Germany).

Table 1: A list of BoLA defined LLO screened animals used for the vaccine trial experiment

Animal no.	Sex	Breed:	BOLA type	Source
BB003	F	Friesian Boran Cross-breed	N*01301	ILRI
BB061	M	Boran	N*00101	Kapiti Ranch
BB068	F	Boran	N*00101	Kapiti Ranch
BB166	M	Boran	N*00101	Kapiti Ranch
BB167	F	Boran	N*00101	Kapiti Ranch
BA207	M	Friesian	N*01201	Shama, Nyeri
BA228	M	Friesian	N*01201	Shama, Nyeri
BA229	M	Ayshire	N*01201	Shama, Nyeri
BA209	M	Ayshire	N*01201	Shama, Nyeri
BA225	M	Friesian	N*01301	Shama, Nyeri
BA200	M	Friesian	N*01301	Shama, Nyeri
BA127	M	Friesian	N*01301	Shama, Nyeri

3.2 Maintenance of *T. parva* (Muguga) infected cell lines (TpM)

Autologous *T. parva* Muguga infected lymphoblasts (TpM) had been established prior to this study as previously described (Goddeeris and Morrison, (1988), by infecting $2x10^7$ bovine PBMC with 200µl of *T. parva* sporozoites suspension from triturated salivary glands dissected from infected adult *R. appendiculatus* ticks. This was incubated

at 38°C in a humidified incubator for $1^{1}/_{2}$ h, 5%CO_2 while mixing every 30min. The cells were centrifuged and resuspended in a culture medium (with HEPES) Goddeeris and Morrison, (1988) (J. Gachanja, personal communication). TpM were then maintained by removal and discarding ¾ of the culture medium and replacing with fresh RPMI-1640 medium (with HEPES) at a pH of 7.0 supplemented with; 50mg/ml Gentamicin, 200mM L-Glutamine, 150µg/ml penicillin, 200µg/ml streptomycin and 10% gamma-irradiated filtered fetal calf serum (FCS; Harlan, Loughborough, UK) every two days (Goddeeris and Morrison, 1988).

3.3 Maintenance of $CD8^+$ CTL lines specific for *T. parva* antigens Tp1, Tp2 and Tp8

T. parva antigen specific $CD8^+$ T cell lines had been established preceding this study by subjecting PBMC from ITM-immunized animals of appropriate MHC class I genotypes to three rounds of restimulation by autologous TpM every seven days (Graham *et al.*, 2006). Briefly, *T. parva* specific-$CD8^+$ T cells were purified from re-stimulated cultures using MACS magnetic column chromatography (Miltenyi Biotech, Germany). Enriched $CD8^+$ T cells were resuspended in CTL medium RPMI 1640 (without HEPES) containing 50mg/ml Gentamicin, 200mM L-Glutamine, 150µg/ml penicillin, 200µg/ml streptomycin, 2% recombinant bovine IL-2 and 10% fetal calf serum. For this study, CTLs were maintained by seeding 100µl (containing $5x10^3$ cells) into U-bottomed 96-well culture plates. Irradiated autologous TpM were added at a density of $2.5x10^4$cells (in 100µl) to each well containing CTL. Restimulation was repeated every two to three weeks post-stimulation.

3.4 Recombinant live attenuated *L. monocytogenes* expressing $CD8^+$ T cell epitopes from *T. parva*

The *L. monocytogenes* strains used in this study included the live attenuated wild type *L. monocytogenes Lm ΔactAΔinlB* CRS-100 and a recombinant *Lm ΔactAΔinlB* BH711 (Cerus corp., Concord, USA) expressing three CTL epitopes from *T. parva*; $Tp1_{214-224}$, $Tp2_{98-106}$ and $Tp8_{379-387}$ restricted by MHC Bovine Leukocyte Antigen (BoLA) alleles, N*01301, N*01201 and N*00101 respectively (Graham *et al.*, 2006). The constructs were carrying an additional tag of ovalbumin (OVA) expressing gene (SIINFEKL). The *L. monocytogenes* strains used in this experiment *(Lm ΔactAΔinlB* CRS-100 and *Lm ΔactAΔinlB* BH711) were quality controlled by evaluating their potential to induce antigen-specific $CD8^+$ T cell responses *in vivo.* Mice C57BI/6 were immunized with both *Lm ΔactAΔinlB* CRS-100 and *Lm ΔactAΔinlB* BH711 and seven days post immunization the spleens were harvested to quantify SIINFEKL-specific $CD8^+$ T cells through intracellular cytokine staining (ICS). Large numbers of $CD8^+$ T cells specific to ovalbumin SIINFEKL were elicited as a confirmatory test for expression of epitopes from recombinant *Lm ΔactAΔinlB* BH711 unlike the wild type attenuated *Lm ΔactAΔinlB* CRS-100 (K.S. Bahjat, personal communication).

3.5 Assessment of $CD8^+$ T cell recognition of cells infected with *L. Monocytogenes* by IFN-γ ELISpot

The ability of *Lm ΔactAΔinlB* to infect cells *in vitro* and express recombinant epitopes was assessed using an IFN-γ ELISpot assay as a read-out, as described above. Monocytes for antigen presentation were freshly prepared from peripheral blood of animal BV115 and BX064. Briefly, PBMC were incubated for 30 minutes at 4°C in the presence of anti-CD14 antibody coupled to magnetic micro-beads (Miltenyi Biotec, Germany) added at 100µl/10^7 PBMC. Following two wash steps, the cultured cells were resuspended in MACS buffer and monocytes sorted using MACS magnetic columns (Miltenyi Biotech, Germany). Enumerated magnetically sorted $CD14^+$ monocytes were resuspended at $1x10^7$/ml in antibiotic free RPMI-1640 (without HEPES) medium containing 10% FCS. Monocytes at a density of $2.5x10^4$/well (50µl/well) were infected with recombinant and wild type *Lm ΔactAΔinlB* at a range of multiplicities of infection (MOI) of between 10 and 400, and then rotated at 180g, 37°C for 15 minutes. The cells were washed three times in medium without antibiotics by centrifuging at 180g for 8 min and the cell pellet resuspended in a titration of gentamicin diluted from 0.1-50mg/ml in cytotoxic T lymphocyte medium (CTL medium). The $CD8^+$ T cell lines specific for Tp1 from animal BV115 and Tp8 from animal BX64 were seeded into ELISpot plates at a $1.0x10^4$/well (50µl/well), autologous infected monocytes at $2.5x10^4$/well (25µl/well), and the synthetic Tp1 and Tp8 epitopes from *T. parva* as positive controls at 25µl/well. The ELISpot plate was then incubated at 37°C, 5% CO_2 for 20 hours and developed as described above.

Already established immortalized skin fibroblasts (iSF) were harvested by removing DMEM culture medium (supplemented with 50mg/ml Gentamicin, 200mM L-Glutamine, 150µg/ml penicillin, 200µg/ml streptomycin, and 10% FCS). The iSF were washed two

times with phosphate buffered saline (PBS)-EDTA, and then detached using Trypsin-EDTA (Sigma, Steiheim, Germany). The harvested iSF were resuspended in CTL medium and then enumerated. The cells were seeded into ELISpot plates and the procedure repeated typically as explained for monocytes. Infectivity of monocytes and iSF by recombinant *Lm ΔactAΔinlB* was determined by microscopy following Giemsa staining of cytospins. Slides containing the smears were fixed in absolute methanol for five minutes and then stained with 5% Giemsa stain that had been diluted in water/ buffer for 30 min at room temperature, RT. The slides were stained for 30 min, and then washed with water for 2 min before allowing them to air-dry. They were examined microscopically (oil emersion) for detection of *Listeria.*

3.6 Immunization of cattle with recombinant live attenuated *L. monocytogenes* and challenge with *T. parva*

The experiment was conducted in a double-blind fashion in which animals were re-tagged between immunization and challenge and the code managed by the ILRI/ICRAF Research Methods Group. The animals were randomized into 2 groups; one group received recombinant *Lm ΔactAΔinlB* while the other group received the wild type *Lm ΔactAΔinlB.* Cattle were primed on day 0 by intravenous inoculation of either recombinant or wild type *Lm ΔactAΔinlB* $1x10^{10}$ cfu/ ml. They were boosted 28 days later by intravenous inoculation of $1x10^{11}$ cfu/ ml of either the recombinant or wild type *Listeria.* The bacterial inoculum was diluted at a ratio of 1: 20 of *Lm ΔactAΔinlB* to normal saline, each animal receiving 10ml of the inoculum that included 1ml of *Lm ΔactAΔinlB.* On day 49, all the cattle were challenged with a lethal dose of *T. parva* (1:20

dilution, LD_{100}) Muguga stock sporozoite (stabilate # 4133, ILRI, Nairobi, Kenya). Reactions to challenge were calculated as an ECF reaction index by combination of thirteen parameters, including temperature, hematological and parasitological measurements, using first principle component analysis (Rowlands *et al.*, 2000).

3.6.1 Hematological responses of cattle following immunization with recombinant live attenuated *L. monocytogenes*

Venous blood in EDTA was collected daily for 3 days a week after immunization for assessing the leukocyte levels following priming and booster immunization. The cells were evaluated on the normal scale of 4.0-15.0 million cells per cubic millimeter (4.0-15.0 m/mm^3) of blood in which below 4.0 m/mm^3, the animal was marked as leucopoenia while above 15.0 m/mm^3 the condition was recognized as leukocytosis. However, following challenge WBC levels were determined twice a week. The changes in platelet levels were also monitored for the first three days after both primary and booster immunization. The enumeration and referencing of the thrombocytic levels was based on a normal range scale of 100-800 million cells per cubic millimeter (100-800 m/mm^3) in cattle. In addition venous blood was collected two times weekly following challenge to determine the red blood cell (RBC) levels and the packed cell volume (PCV). The cells were counted using full automated hematology cell analyzer (Mellet Schloesing laboratories, France).

3.7 Immune responses to vaccination of cattle with recombinant live attenuated *L. monocytogenes* and challenge with *T. parva*

Over the course of vaccination and challenge, panels of immunological assays were conducted to determine the induction of peptide-specific T cell responses. This included $CD8^+$ T cell and PBMC IFN-γ responses using an ELISpot assay responses, MHC class I tetramer staining for detection of the frequency of *T. parva* epitope-specific $CD8^+$ T cells and the ^{111}Indium release assays for the detection of cytotoxic activity.

3. 7.1 Assessment of Tp and LLO specific T cell IFN-γ responses by ELISpot assay

Sampling from experimental animals was conducted every two weeks over the course of the study period. Peripheral mononuclear cells (PBMC) and monocytes were prepared as described above. $CD8^+$ T cells were purified from PBMC by incubating with an anti-bovine $CD8^+$ monoclonal antibody (IL-A105 and IL-51, ILRI, Nairobi Kenya) for 30 min at 4°C followed by two wash steps. The cells were incubated with anti-mouse IgG microbeads (Miltenyi Biotech) and $CD8^+$ T cells were enriched using MACS columns (Miltenyi Biotech) as described by the manufacturer. $CD8^+$ T cells were seeded into the ELISpot plate wells at 50µl/well ($2.5x10^5$/well) together with purified monocytes at 50µl/well ($2.5x10^4$/well). Alternatively, PBMC were added at a density of $2.5x10^5$/well (50µl/well). Synthetic antigenic peptides ($Tp1_{214-224}$, $Tp2_{98-106}$ and $Tp8_{379-387}$ and LLO pool) were added to wells at a final concentration of 1ug/ml and the plates incubated for 20hr at 37°C after which the ELISpot assay procedure was conducted as detailed above.

3.7. 2 Assessment of antigen specific cytotoxic T cell responses

PBMCs from immunized animals were seeded $4x10^6$/well with $2x10^5$/well γ-irradiated autologous TpM in 24 well-plates and cultured for 7 days at 37°C in a humidified 5%

CO_2 atmosphere. Viable cells were harvested from the culture on day 7 and re-stimulated with TpM as before. $CD8^+$ T cells were purified 7 days after secondary restimulation using MACs columns (Miltenyi Biotech) and maintained by restimulation using TpM for another six days before being used in a CTL assay. CTL activity against autologous *T. parva*-infected (TpM) and peptide-pulsed concanavalin-A (Con-A) lymphoblasts was assessed using ^{111}In release-assay. Con-A blasts were plated ($5x10^5$cells/well) into a 96-well flat-bottom plate which was then pulsed with *T. parva* and LLO peptides (1μg/ml) by overnight incubation at 37°C, in a humidified 5% CO_2 atmosphere. The target antigen loaded Con-A blasts were harvested, centrifuged and resuspended in RPMI 1640 with HEPES supplemented with 5% FCS (cytotoxicity medium) at $2x10^7$ cells/ml.

Radioisotopes (100μl of ^{111}In was added to 10ml tube containing 100ul ($5x10^4$ cells) of either con-A blast antigen targets, autologous and allogeneic TpM followed by incubation for 15 min at 37°C. Cytotoxicity medium was added followed by centrifugation of the labeled cells at 150g for 5 min at room temperature. The supernatant was removed and the cells resuspended in 7ml cytotoxicity medium and spun at 150g for 5 min at room temperature. This wash step was repeated three times before the labeled targets were resuspended in cytotoxicity medium at a concentration of $1x10^6$/ml. The labeled targets (50μl) were then added at $5x10^4$/well to the effector $CD8^+$ T cells that had been diluted to give different effector: target ratios. This was followed by incubation for 4 hrs. After incubation, the contents of the wells were mixed by pippetting and then spun at 150g for 2 min. Seventy five microliters (75μl) of supernatant was removed and gamma-emissions counted on a gamma-counter (Burnham-on-Crouch, Essex, England). Maximum and

spontaneous release was determined by incubating 50µl target cells in 100µl 0.1% Tween20 solution or cytotoxicity medium respectively. The % lysis was calculated using the formula:

$$\% \text{ Lysis} = \frac{\text{Specific Release} - \text{Spontaneous Release}}{\text{Maximum Release} - \text{Spontaneous Release}} \times 100$$

3.7.3 Analysis of epitope-specific $CD8^+$ T cell responses with tetramer staining

Enumerated PBMC from cattle were resuspended in PBS/0.5%BSA and plated at $2.5x10^5$cells/well in a 96-well round-bottomed plate. This was spun at 150g for 2 min and the supernatant discarded. Bovine MHC class I/*T. parva* tetramers; BoLA-N*01301/Tp1$_{214-224}$, BoLA-N*01201/Tp2 $_{98-106}$ and BoLA- N*00101/Tp8$_{379-387}$ (BD Biosciences, USA), at a concentration of 40nM were added to the wells (25ul/well) and incubated for 10min at room temperature in the dark. The cells were washed two times by spinning at 150g for 2 min, and the supernatant discarded followed by addition of 200µl/well of PBS/0.5%BSA. Rat anti-mouse IgG conjugated to PerCP (BD Biosciences, San Jose, USA) at 6.25µg/ml was added 1.25µl/well and then incubated and washed as before. The cells were finally resuspended in 200µl of PBS/0.5%BSA and analysed using a FACSCantoII flow cytometer (BD Biosciences, San Jose, USA).

3.8 Assessment of responses of cattle to challenge infection with *Theileria parva* sporozoites.

The animals were monitored clinically and parasitologically daily following parasite challenge. The cattle were clinically assessed daily by two independent veterinarians following challenge and animals displaying clinical signs of severe ECF including persistent coughing/dyspnoea, pyrexia, parasitosis, hematological indicators, nasal discharge, diarrhea and recumbence were euthanized by captive bolt based on the veterinarian's clinical decision. The establishment and manifestation of parasitosis and hematological indicators such as WBC levels, RBC and PCV were monitored periodically over the course of the experiment following challenge. The animals were monitored closely for development of parasites from day five of challenge. Detection of schizonts was confirmed by lymph node biopsy smears, while parasitaemia due to intra-erythrocytic piroplasm was revealed through blood smears.

3.9 Statistical Analysis.

A combination of parameters, including temperature, hematological and parasitological measurements was used to calculate the ECF reaction index using first principle component analysis (Rowlands *et al.*, 2000). Statistical analysis was also performed using Genstat program version 9 (Rothamsted Agricultural Station, Harpenden, UK) using generalized linear models of regressional analysis of variance. The probability (p-value) at each time point between the two vaccine groups was calculated using student t-test.

CHAPTER FOUR

RESULTS

4.1 Determination of the recognition of CTL *T. parva* epitopes expressed by recombinant *L.monocytogenes* using antigen–specific $CD8^+$ T cell lines

The assessment of whether the recombinant *Lm ΔactAΔinlB* expressing the *T. parva* CTL target epitopes could stimulate antigen-specific $CD8^+$ T cell responses *in vitro* was assayed using autologous monocytes and immortalized skin fibroblasts (iSF) as antigen presenting cells. From the results obtained it was evident that in the absence of gentamicin, there were high background IFN-γ responses induced by both recombinant and wild type *Lm ΔactAΔinlB* infected monocytes (Fig 2). However, although not as obvious, it was noted that the responses provoked by recombinant *Lm ΔactAΔinlB* infected monocytes were higher than that of the wild type in the absence of gentamicin (Fig 2A). The number of IFN-γ secreting cells quantified in cultures containing monocytes infected with wild type *Lm ΔactAΔinlB* decreased to zero with increasing antibiotic concentration up to 12mg/ml (Fig 2B). On the other hand, although recombinant *Lm ΔactAΔinlB* induced $CD8^+$ T cell IFN-γ responses that decreased with the increasing gentamicin concentration, weak but significant responses were observed at 12mg/ml (Fig 2A).

Atypical decline in the ELISpot CTL IFN-γ release was observed in the antibiotic middle range of 5-12 mg/ml (in both cultures), although the positive control (Tp1 epitope) behaves almost in the same manner at this concentration (5-12mg/ml) (Fig 2). Increasing the gentamicin concentration to 25 mg/ml elicited greater numbers of IFN-γ secreting

cells to recombinant but not wild-type *Lm* Δ*actA*Δ*inlB* infected monocytes. These responses were more marked at the three highest multiplicities of infections (MOI) (Fig 2A). In all cases, the positive control (Tp1 epitope) induced comparatively greater responses than that of either the wild type or recombinant *L. monocytogenes* infected cells (Fig 2) and were highest at 25 mg/ml gentamicin. However, it is essential to note that the result illustrated in Figure 2 was not reproducible even after repeating a number of times.

When the experiment reported in Figure 2A and B was repeated using autologous monocytes as antigen presenting cells co-cultured with Tp8-specific polyclonal CTL line, very low responses were induced (data not shown). It is also worthwhile noting, that it was difficult raising a Tp2- specific CTL line *in vitro*. Thus it was not possible to assay the expression and recognition of Tp2 CTL *T. parva* epitope by specific CTL lines *in vitro*. In addition, although a Tp8-specific polyclonal CTL could be raised *in vitro* it was not possible to obtain a convincing result to show that Tp8 was being expressed and recognized by specific CTL line.

The results of a repeat experiment conducted using immortalized autologous skin fibroblasts pulsed with both wild type and recombinant *Lm* Δ*actA*Δ*inlB* failed to induce detectable responses when co-cultured with Tp1-specific CTL lines (Fig 2C). However, the Tp1 epitope pulsed iSF (positive control) from this experiment (Fig 2C) induced responses whose magnitudes were indistinguishable with that conducted using peptide pulsed monocytes as antigen presenting cells (Fig 2 A and B). It was also noted that

cytospins of monocyte-infected cells from; 15min, 30 min and overnight cultures did not illustrate clear evidence of intracellular bacteria due to infectivity.

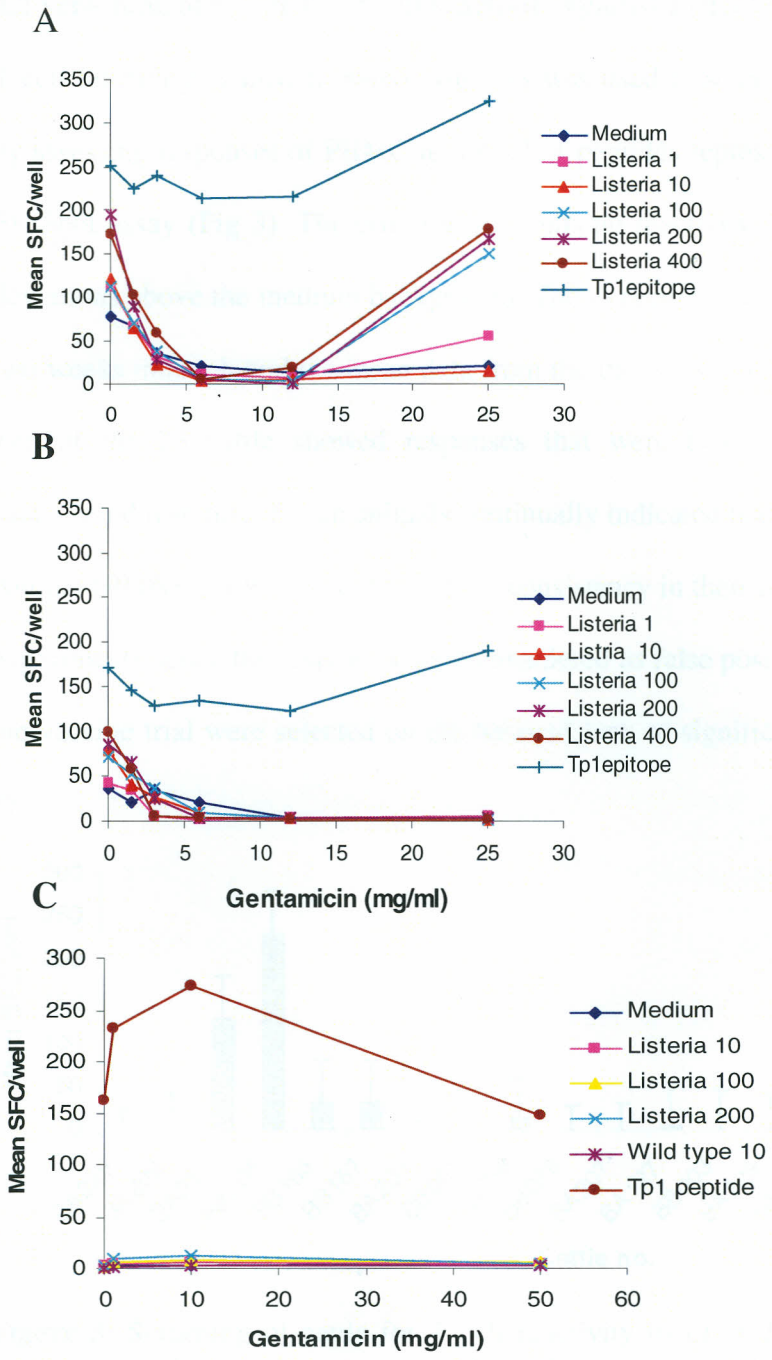


Figure 2: Responses of Tp1 specific cytotoxic T-lymphocytes (CTL) to autologous monocytes infected *in vitro* with wild type and recombinant *L. monocytogenes* expressing $CD8^+$ T cell epitopes from *T. parva*. Autologous monocytes were infected *in vitro* for 30 minutes with recombinant *Lm ΔactAΔinlB* (**A**) and wild type *L ΔactAΔinlB* (**B**) at a range of multiplicity of infections (MOI) and cultured in varying gentamicin concentrations. The experiment was repeated with immortalized autologous skin fibroblasts (**C**). As a positive control, CTL were co-cultured with monocytes pulsed with a synthetic Tp1 antigenic peptide (Tp1 epitope) in all the three experiments (**A, B and C**).

4.2 Screening of Cattle for T cell reactivity against Listeriolysin-O (LLO).

T cell reactivity against *L. monocytogenes* was used to screen the experimental animals by assessing responses of PBMC to a pool of peptides representing LLO using an IFN-γ ELISpot assay (Fig 3). The criterion for immune reactivity was based on two standard deviations above the medium background. The experiment was repeated three times with two weeks interval, and the mean data from the three experiments are shown in Figure 3. Ten of the 25 cattle showed responses that were below or equal to the medium background response. Seven animals continually indicated high reactivity to LLO (Fig 3). Animal BB166 and BB167 did not show consistency in their responses in the three repeat experiments hence the responses were considered as false positives. The animals used for the vaccine trial were selected on the basis of lack of significant responses to LLO (Fig 3).

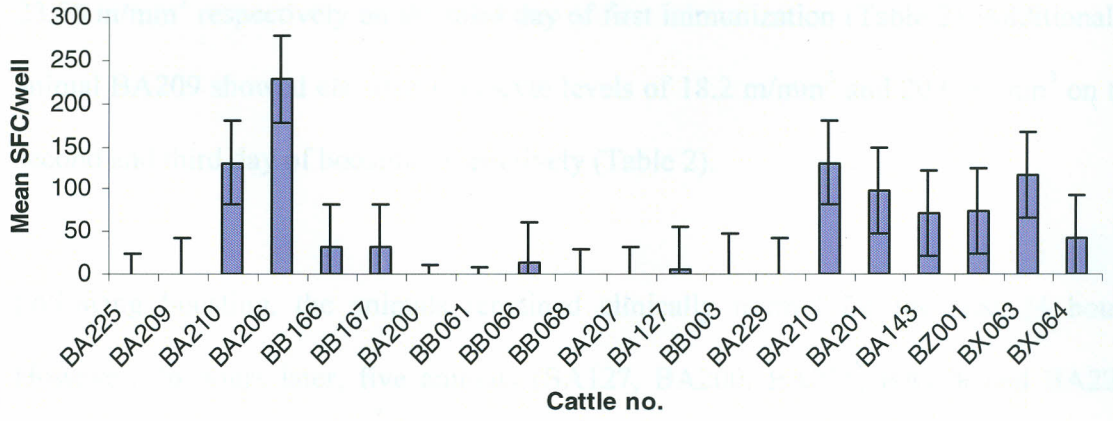


Figure 3: Screening of cattle for T cell reactivity to LLO. IFN-g responses of PBMC were measured in response to a peptide pool representing LLO using an IFN-g ELISpot assay. Mean responses from three repeat experiments are presented and expressed as mean medium corrected spot forming cells (SFC)/1x10^6 PBMC.

4.3 Clinical reactions of cattle to immunization with recombinant live attenuated *L. monocytogenes.*

All the animals were vaccinated at day zero with a primary dose of $1x10^{10}$cfu of the wild type and recombinant *Lm ΔactAΔinlB* for the controls and vaccinates respectively. There were no adverse clinical reactions following primary immunization. However, while most animals had leukocyte levels within the normal range, animal BA207 showed an increase in leukocyte numbers to 16.12 million cells per cubic millimeter of blood (16.12 m/mm^3) one hour post priming which was above the normal range (4.0-15.0 m/mm^3) and the value pre-immunization (14.55m/mm^3) (Table 2). A day later three other animals were shown to have leukocyte levels above the normal limits (BB003 -16.9 m/mm^3, BA127-17.57 m/mm^3 and BA209 19.94 - m/mm^3). Animal BB003 reverted to normal levels a day later while BA127 and BA209 maintained high leukocyte responses to 20.5 m/mm^3 and 23.58 m/mm^3 respectively on the third day of first immunization (Table 2). Additionally, animal BA209 showed elevated leukocyte levels of 18.2 m/mm^3 and 20.0 m/mm^3 on the second and third day of boosting respectively (Table 2).

Following boosting, the animals remained clinically normal for the first 24 hours. However, 36 hours later, five animals (BA127, BA200, BA225, BA228 and BA229) became anorexic and had elevated respiratory rate. One animal, BA225, developed severe complications associated with labored breathing/dyspnoea, nasal discharge and froth from the mouth. The rectal temperature of these animals remained between 37.5 to 39.2°C, well within normal limits. All these animals were treated with Dexamethasone and anti-histamines. Animal BA225 was additionally treated with Penicillin-

Streptomycin (Kela, Germany). Four of these animals recovered within 2 hours while BA225 died an hour after treatment. Post-mortem evaluation indicated that the animal had suffered from interstitial pneumonia and emphysema characteristic of reactions to intravenous inoculation of bacterial preparations.

Thrombocyte levels remained within the normal limits (100-800 m/mm^3) in these animals post priming and boosting. The rest of the animals had also normal thrombocyte levels except animal BB166 which recorded a drastic increase to 2359 million cells per cubic millimeter of blood (2359 m/mm^3) within one hour of immunization. Another animal, BA209, developed thrombocytopenia (92 m/mm$^{3)}$ after day 3 of priming (Table 3). This animal again showed low platelets counts of 79 m/mm^3 and 78 m/mm^3 after the second and third day of boosting respectively. In addition animal BB068 had high thrombocyte levels of 1235 m/mm^3 after day 3 of boosting (Table 3).

Table 2: Changes in leukocyte levels representing a million cells per cubic millimeter (m/mm^3) of blood between vaccinated and control animals following immunization with wild type and recombinant live attenuated *L. monocytogenes*.

		Days post priming				Days post boosting			
Group	**Day**	**0**	**1**	**2**	**3**	**0**	**1**	**2**	**3**
Vaccinated animals	BB003	12.03	14.32	16.9	9.57	8.88	7.09	9.46	9.76
	BB68	5.71	9.62	7.04	8.42	5.1	5.35	10.27	7.59
	BB166	10.18	14.09	11.3	12.16	5.63	6.12	7.92	8.78
	BB 167	9.99	11.44	13.69	13.4	11.81	5.42	9.43	10.86
	BA127	12.18	10.02	17.57	20.5	9.27	6.79	14.03	8.95
	BA200	12.06	11.28	12.73	12.71	7.31	5.46	10.61	7.02
	BA207	14.55	16.12	14.91	15.71	11.26	7.5	11.09	10.4
	BA228	14.28	12.06	13.36	14.56	7.69	6.54	8.15	11.02
	BA229	11.39	13.11	11.78	12.6	9.95	5.96	6.02	6.93
Controls	BB61	7.13	9.59	9	7.66	6.24	3.15	7.87	8.21
	BA209	15.32	13.87	19.94	23.58	22.13	10.58	18.2	20
	BA225	7.22	12.93	12.23	13.66	8.64	8.83	13.32	15.97

Table 3: Changes in thrombocyte levels representing million cells per cubic millimeter (m/mm^3) of blood between vaccinated and control animals following priming and boosting with wild type and recombinant live attenuated *L. monocytogenes*.

		Days post priming				Days post boosting			
Group	**Day**	**0**	**1**	**2**	**3**	**0**	**1**	**2**	**3**
Vaccinated animals	**BB003**	**270**	**485**	**788**	**324**	**310**	**252**	**160**	**248**
	BB68	**481**	**476**	**539**	**396**	**656**	**474**	**445**	**1235**
	BB166	**337**	**2359**	**239**	**293**	**151**	**160**	**152**	**232**
	BB 167	**413**	**621**	**874**	**356**	**966**	**400**	**174**	**197**
	BA127	**277**	**286**	**410**	**279**	**383**	**473**	**238**	**436**
	BA200	**350**	**531**	**582**	**424**	**434**	**132**	**141**	**241**
	BA207	**457**	**539**	**606**	**333**	**271**	**196**	**137**	**295**
	BA228	**187**	**394**	**332**	**308**	**250**	**426**	**228**	**272**
	BA229	**1400**	**547**	**352**	**190**	**162**	**256**	**210**	**179**
Controls	**BB61**	**321**	**424**	**384**	**508**	**383**	**220**	**229**	**331**
	BA209	**456**	**293**	**226**	**92**	**62**	**163**	**79**	**78**
	BA225	**174**	**337**	**223**	**278**	**183**	**208**	**168**	**303**

4.4 Immunological responses following immunization with recombinant *L. monocytogenes* and challenge with *T. parva*

4.4.1 Antigen specific T cell IFN-γ ELISpot responses

An IFN-γ ELISpot assay was employed to assess responses of PBMC and purified $CD8^+$ T cells to the *T. parva* epitopes and LLO after immunization and parasite challenge. Responses were considered positive when the numbers of IFN-γ ELISpot spot forming cells (SFC) responding to the antigen was three times greater than that of medium alone (Graham *et al.*, 2006). In general, there was poor $CD8^+$ T cell IFN-γ responses observed in seven out of nine vaccinated cattle after immunization and challenge (Fig 4). Following primary vaccination, two of the nine immunized animals showed $CD8^+$ T cell ELISpot IFN-γ responses to *T. parva* antigens that correlated with the MHC haplotypes that restricts these CTL epitopes. BA127 (BoLA-N*01301$^+$), and BB068 (BoLA-N*00101$^+$) responded to Tp1 and Tp8 respectively (Fig 4). In these two animals, the responses were elevated following boosting (Fig 4). In BB068, the responses increased six fold while that of BA127 increased almost twice following booster immunization (Fig 4). It is worth noting that there were no responses to Tp2 antigen in any of the experimental animals from enriched $CD8^+$ T cells population after priming and boosting (Fig 4).

Weak $CD8^+$ T cell responses to LLO were observed in the vaccinated animal BB068 after booster immunization (Fig 4). Weak but significant responses to LLO were also detected in two animals after priming (BA207) and boosting (BA127) respectively (Fig 4). Lack of responsiveness to antigenic peptides including LLO was generally seen across all

controls in the enriched $CD8^+$ T cell pool (Fig 4). In general, the LLO peptide pool was weakly recognized in purified $CD8^+$ T cell sub-population (Fig 4). However, strong recall responses to LLO unlike *T. parva* antigens were more evident in the PBMC population (Fig 5). Seven cattle including; two control animals (BB061, BA209) and five vaccinated animals (BB003, BB068, BA207, BA228 and BA229) elicited strong IFN-γ responses against the LLO peptide pool in the PBMC population following booster immunization (Fig 5).

Following challenge, recall or boosting of antigen specific IFN-γ responses from $CD8^+$ T cells were weakly detected in only two vaccinated animals BB003 and BB068 against Tp1 and Tp8 respectively (Fig 4). However strong responses were provoked from four vaccinated animals (BB003, BB068, BB167 and BA228) and one control animal (BA209) against synthetic peptide pool representing LLO following challenge in the PBMC population (Fig 5). In addition weak responses were also noted in one control animal (BB61) and three vaccinated animals (BB166, BA207 and BA229) from intact PBMC population after challenge (Fig 5). As a general observation, the $CD8^+$ T cell IFN-γ responses were inadequately induced and sub-optimal.

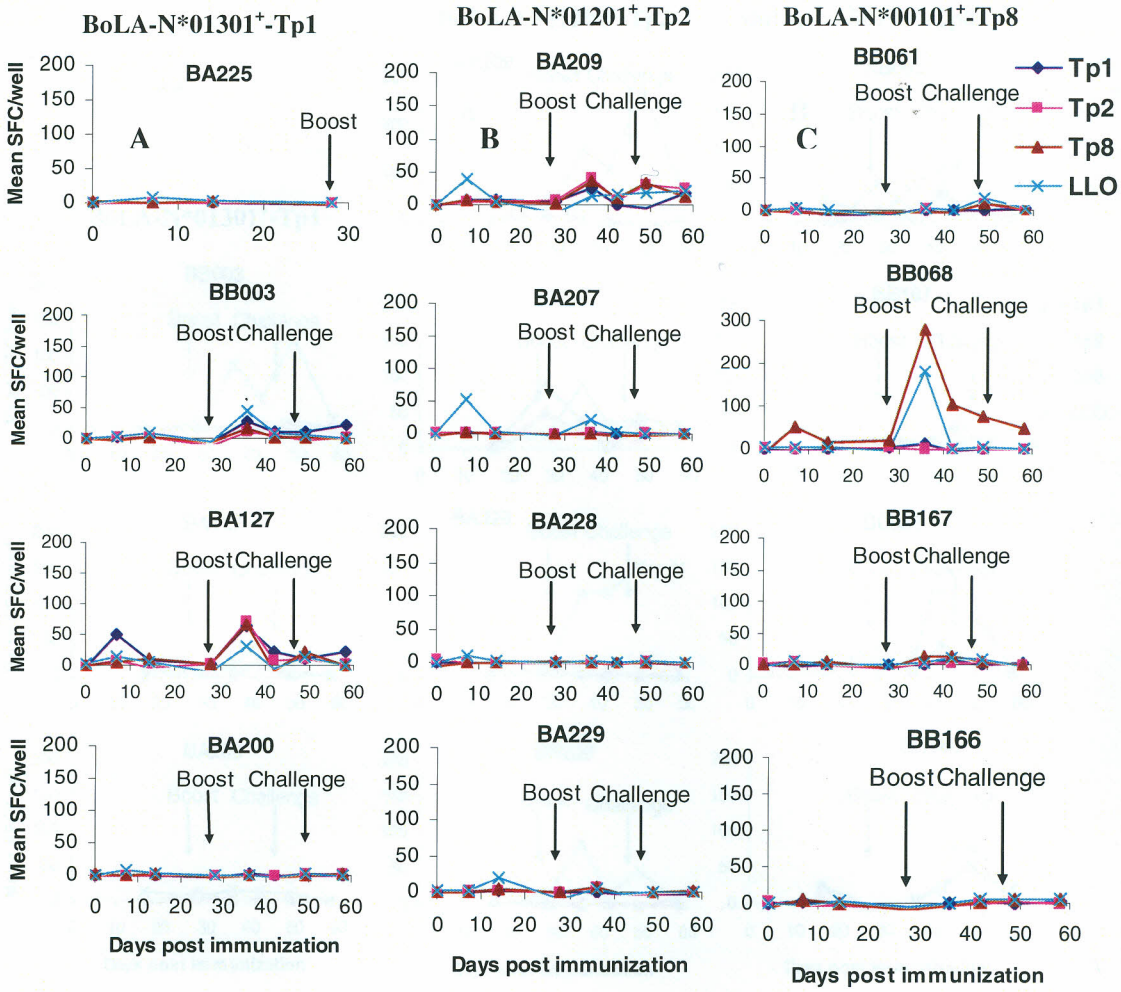
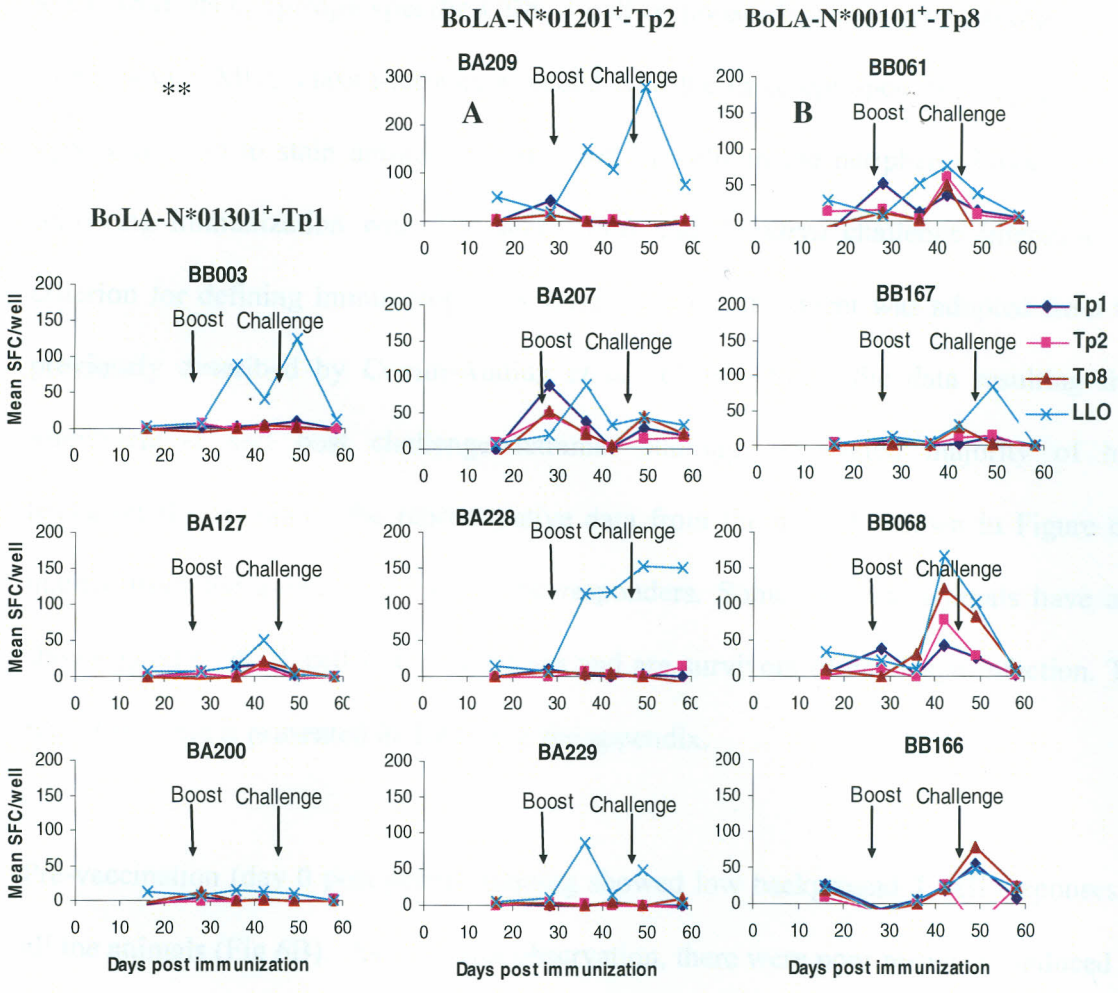


Figure 4: CD8$^+$ T cell IFN-γ ELISpot responses against CTL epitopes from *T. parva* and LLO peptide pool from *L. monocytogenes*. Antigens were co-cultured with purified CD8$^+$ T cells in the presence of autologous monocytes from animals vaccinated with recombinant *Lm ΔactAΔinlB*) and three control animals (**A, B** and **C**) representing different BoLA types receiving wild type *Lm ΔactAΔinlB*.



** The control animal (BA225) missing, due to death after booster immunization.

Figure 5: PBMC T IFN-γ ELISpot responses against $CD8^+$ T cell epitopes from *T. parva* and LLO peptide pool from *L. monocytogenes* following immunization and challenge. Antigens were co-cultured with intact PBMC from animals immunized with recombinant *Lm* Δ*actA*Δ*inlB*) and two control animals (**A** and **B**) receiving wild type *Lm* Δ*actA*Δ*inlB*.

4.4.2 Analysis of epitope-specific $CD8^+$ T cell responses by tetramer staining

Three bovine MHC class I-tetramers loaded with the three epitopes from Tp1, Tp2 and Tp8 were used to stain antigen-specific $CD8^+$ T cells in the peripheral blood of cattle following immunization with *Lm* Δ*actA*Δ*inlB* and *T. parva* challenge infection. The criterion for defining immunologic responses in this experiment was adopted from that previously described by Comin-Andiux *et al.*, (2006). Since the data resulting from immunization and post challenge tetramer staining constituted majority of high background responders, the representative data from the animals shown in Figure 6 is mainly from the animals thought to be responders. Some of these animals have also shown good $CD8^+$ T cell INF-γ responses and are survivors of challenge infection. The rest of the data is presented in Table 4 at the appendix.

Pre-vaccination (day 0 post prime) staining showed low background T cell responses in all the animals (Fig 6B). As a general observation, there were poor responses induced by Tp2 as compared to Tp1 and Tp8 tetramers. In addition, animals that showed respectable tetramer staining (BA127-Tp1 and BB068-Tp8) corresponded to those animals which had previously shown IFN-γ ELISpot responses (Fig 7). Seven days following vaccination, a few of the animals stained positively to *T. parva* tetramers (Fig 7). These responses were detected mainly in vaccinated animals BB068 (Tp8) and BA127 (Tp1) indicating tetramer populations of 3.5% and 0.6% respectively (Fig 7). While strong responses were noted in animal BB068 (1.2%), weak but significant responses were observed in two animals BA200 (0.6%) and BA127 (0.5%) following boosting (Fig 7). After 9 days of challenge infection, two animals BA127 and BB068 indicated PBMC staining

frequencies of 2.1% and 1.6% respectively, while the vaccinated animal BA200 and the control BA209 staining very poorly (Fig 7).

There was generally poor staining from the control animals throughout the course of the experiment, however animal BB061 had good responses (1.1%) at day 70 post infection (Fig 7). Equally notable was the tetramer results of the post-infection survivors of a vaccinated animal BB068 that stained with magnitudes of 1.6% (Fig 7), although this is a bit lower than what was observed in day seven post priming (3.5%). The other vaccinated survivor animal BA200 did not show any detectable tetramer staining positive events at day 70 of challenge (Fig 7).

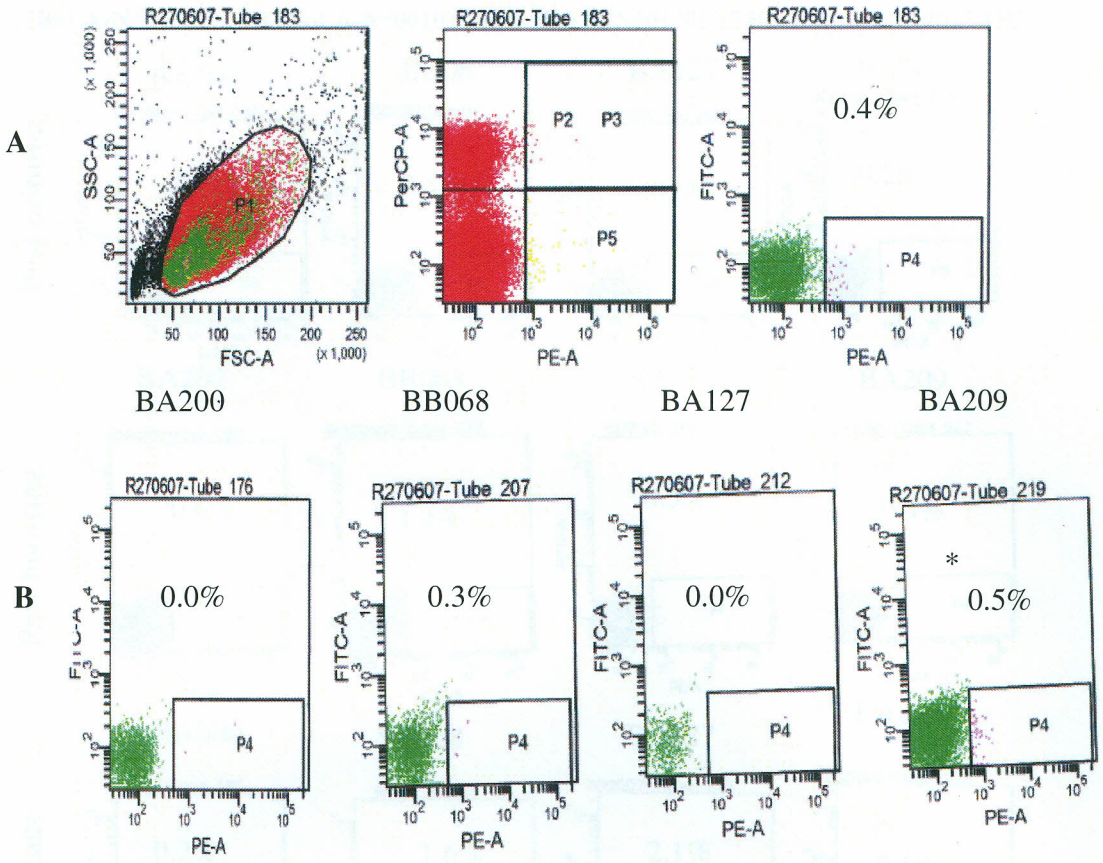
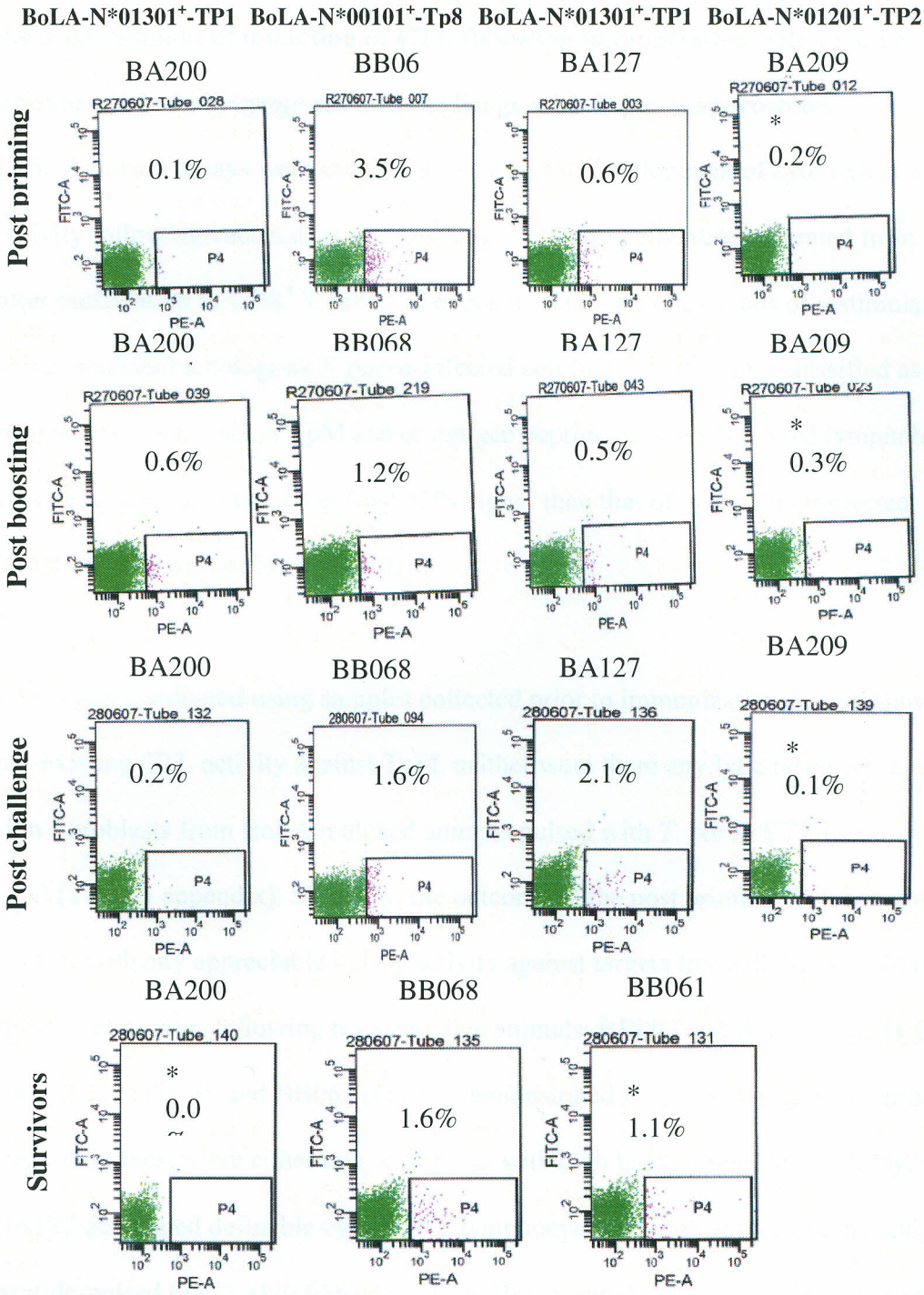


Figure 6: FACS analysis profiles of pre-vaccination tetramer results from animals of different BoLA types; BoLA-N*01301^{+} (BA200 and BA127), BoLA-N*00101^{+} (BB068 and BB061). The PBMC were tested against specific tetramers (Tp1, Tp2 and Tp8) in the presence of defined CD8^{+} and tetramer specific monoclonal antibodies. The gating strategy (**A**) of PBMC involved 100, 000 events, (P1) representing the live T cell population, from which those that stained CD8^{+} (P2) and tetramer positive (P4) were gated, and (P5) representing background noise. Representative pre-immunization animal results (**B**) are shown.



* Represents control animals in the experiment.

Figure 7: Dot plots of the frequency of tetramer specific $CD8^+$ T cells from vaccine trial animals. The PBMC were co-cultured with MHC: *T. parva* tetramers (Tp1, Tp2 and Tp8) using $CD8^+$ T cell and tetramer specific monoclonal antibodies. The tetramer-specific $CD8^+$ T cell population profiles (p4) were quantified using automated FACScanto II machine, and the results from representative animals are shown.

4.4.3 Assessment of induction of CTL following immunization with recombinant live attenuated *L. monocytogenes* and challenge with *T. parva* sporozoites

Indium-release assays were employed to assess the development of cytotoxic T cell activity following vaccination and challenge. Effector cells were generated from PBMC after purification of $CD8^+$ T cells and exposing them to three rounds of restimulation using irradiated autologous *T. parva*-infected cell lines. Cattle were classified as CTL responders when lysis of TpM and or antigen peptide-pulsed uninfected lymphoblasts was three times greater and at least 15% higher than that of unpulsed uninfected lymphoblast (Graham *et al.*, 2006).

CTL assay conducted using samples collected prior to immunization did not show any pre-existing CTL activity against TpM, neither were there any lytic responses against skin fibroblasts from BoLA matched animals pulsed with *T. parva* CTL target epitopes or LLO (Table 5 appendix). Similarly, the outcome of the post priming CTL assay was not evident with any appreciable killing activity against targets from all the animals (data not shown). However, following boosting, five animals; BB003 and BA200 (Fig 8), BA 209 and BA229 (Fig 9), and BB061 (Fig 10) demonstrated CTL activity against various targets but these were either non-specific or with high background. In this assay, animal BA127 developed definable cytotoxic T lymphocytes capable of recognizing and lysing peptide-pulsed (Tp1) skin fibroblasts from BoLA matched animal BV115 up to 64% (at E: T of 16:1) while lysing the autologous TpM up to 34% at the same E: T ratio (Fig 8). The CTL from this animal did not show any background activity or non-specific responses by lysis of TpM from a mismatched animal (Fig 8).

Unlike BA127, animal BA200 with MHC phenotype that restrict $CD8^+$ T cell against Tp1 lysed autologous TpM up to 41% at E: T ratio of 12:1 but did not lyse allogeneic TpM. This animal did not recognize immortalized skin fibroblast pulsed with Tp1 from a BoLA matched animal BV115 (Fig 8). It was also noted that animal BA207 (Fig 9), like animal BB068 (Fig 10) did not demonstrate any CTL activity either to peptide-pulsed lymphoblasts or autologous TpM from post boost results. The T cell bulks obtained from two animals BB003 (Fig 8A) and BB068 (Fig 8B) based on their responses from PBMC and purified $CD8^+$ T cell populations to LLO and then restimulated three times using the *L. monocytogenes* antigen peptide pool (LLO) lacked killing activity against LLO antigen-pulsed targets (Fig 8A, B).

Post challenge CTL assay demonstrated a number of non-specific responses and none of the animals acquired definable CTL activity (Fig 8, 9, 10). The behavior of CTL activity from this assay for animal BB068 and BA200 was similar to that observed after booster immunization (Fig 8, 10). It was not possible to assay the CTL activity of animal BA127 following challenge due to microbial contamination of the T cell bulk cultures. There was also no parallel relationship drawn between the induction of CTL activity and the eventual outcome of the post survival cytotoxicity assay (Table 6 appendix). In general, the induction of CTL activity was disappointing across the immunization and post challenge experiment. The animals whose results is presented below is due to the death of a control post-immunization and microbial contamination of the T cell bulk cultures, hence CTL activity not assayed (Fig 8, 9, 10).

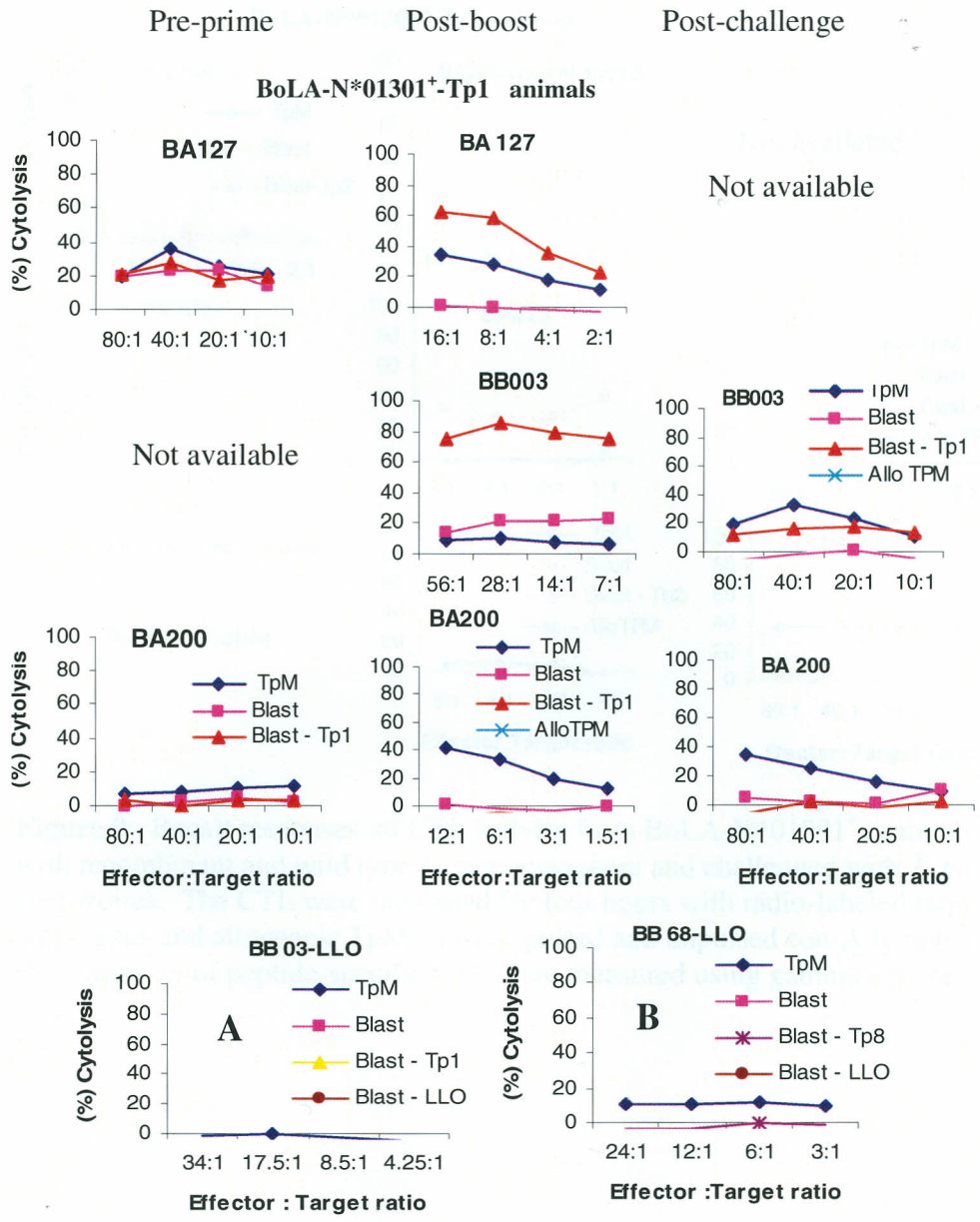


Figure 8: CTL activity against various targets of BoLA-N*01301^{+}-animals post immunization and challenge. CTL were cultured for 4 hours with autologous and allogeneic TpM and peptide pulsed CD8^{+} T cell target epitopes from *T. parva*. The activity of LLO restimulated CTL from animal BB003 (**A**) and BB068 (**B**) were tested against similar antigens including LLO pulsed to autologous skin fibroblasts.

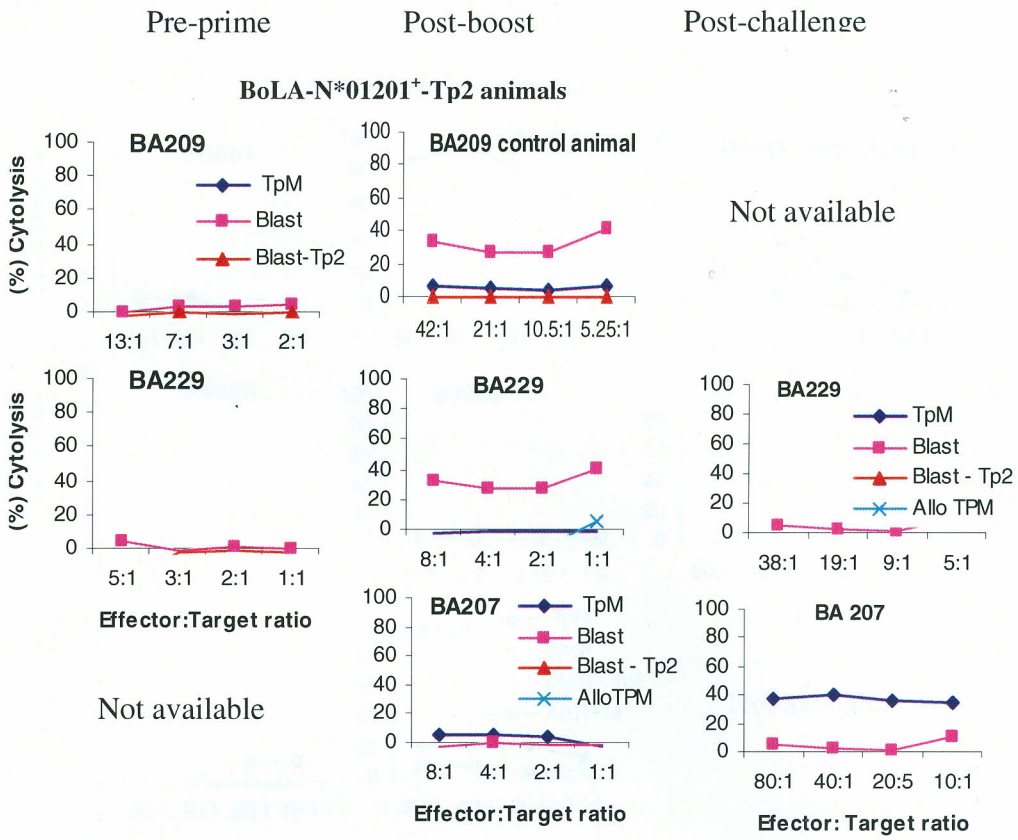


Figure 9: Recall responses of CTL activity from BoLA-N*01201^{+}- animals immunized with recombinant and wild type *L. monocytogenes* and challenged with *T. parva* sporozoites. The CTL were incubated for four hours with radio-labeled targets including autologous and allogeneic TpM, antigen pulsed and unpulsed con-A lymphoblasts and the frequency of peptide-specific CTL were measured using gamma-counter.

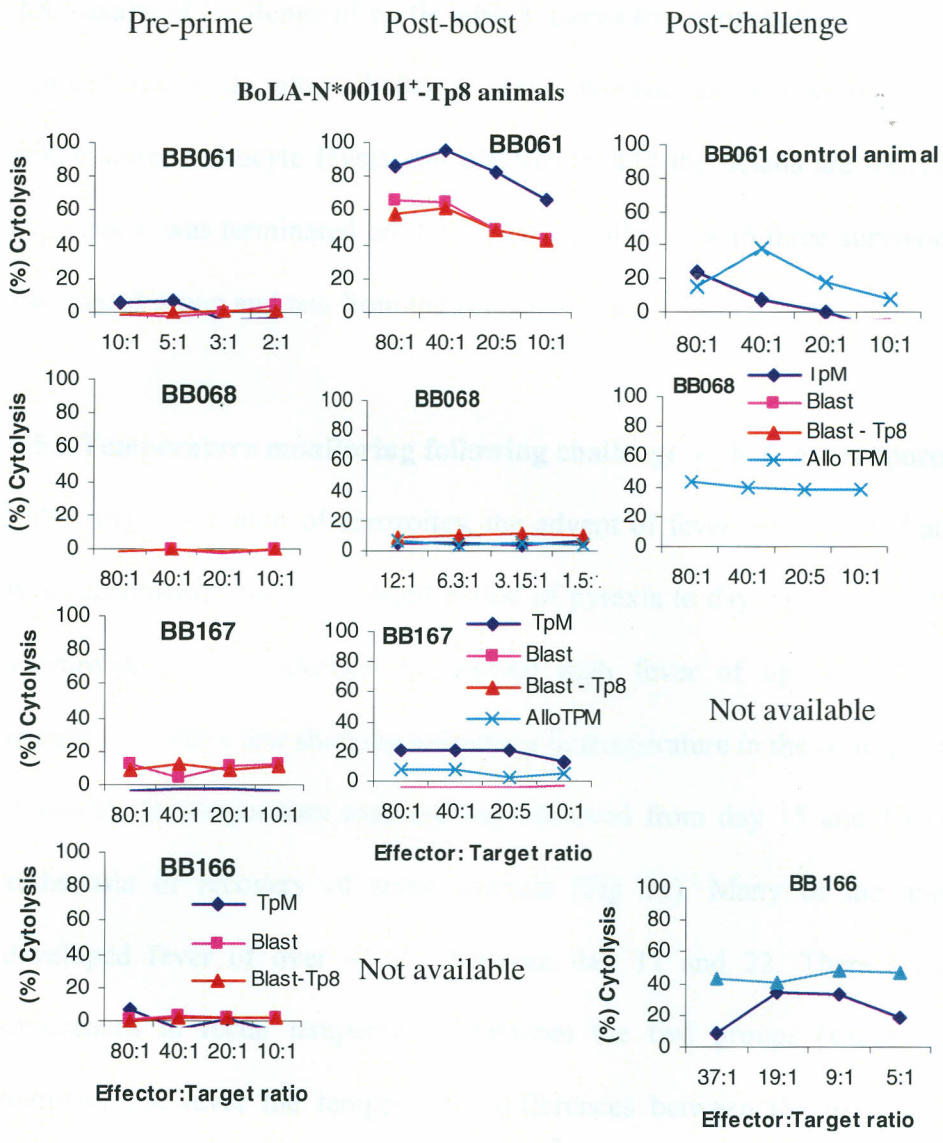


Figure 10: CTL activity from BoLA-N*00101^{+}- animals tested over a range of target antigens post immunization and challenge. The CTL were incubated for four hours with Indium radio-labeled CTL targets and the activity measured using gamma-counter.

4.5 Results of challenge of cattle with *T. parva* live sporozoites

Clinical responses after challenge were assessed by monitoring changes in rectal temperature, leukocyte levels and parasitosis, and the details are described below. The experiment was terminated on day 23 post-challenge with three survivors, two from the vaccinated group and one from the control group.

4.5.1 Temperature monitoring following challenge with *T. parva* sporozoites

Following inoculation of sprozoites, the advent of fever was reported at day 10, which was accompanied by a prolonged period of pyrexia to day 23 (Fig 11). The animals that succumbed to the infection maintained high fever of up to 42°C throughout the experiment with a few showing a decrease in temperature in the course of the experiment. A decline in temperature reaction was observed from day 15 and 19 in the advent of euthanasia or recovery of some animals (Fig 11). Many of the animals generally developed fever of over 41.0°C between day 11 and 22. There were no statistical differences in rectal temperatures between the two groups (vaccinated and control animals). However the temperatures differences between the days were significantly higher from day 8 ($p<0.05$), and the mean temperatures for both vaccinated and control groups hit at 39.5°C around day 9 post challenge.

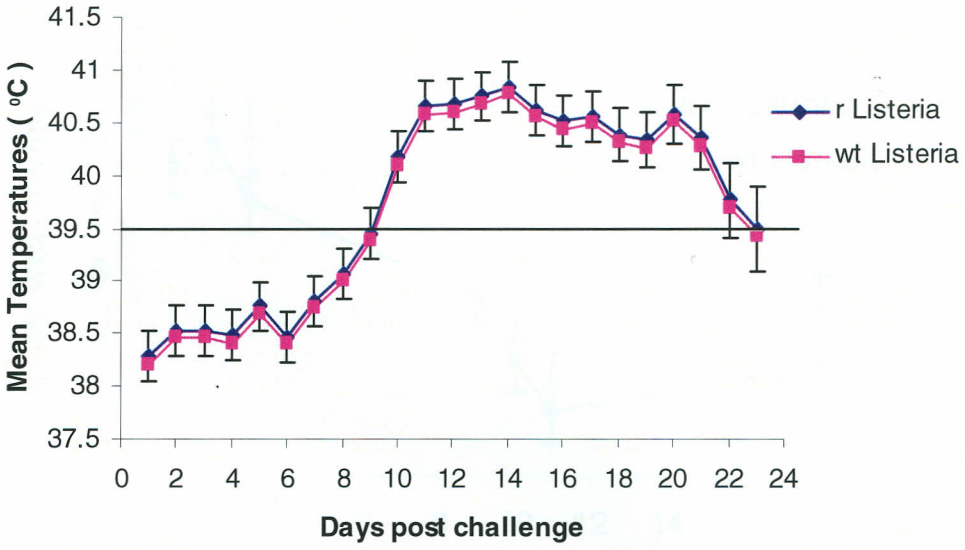


Figure 11: Mean daily temperature changes between two groups of animals that had been vaccinated with recombinant *Listeria* (r *Listeria*) containing *T. parva* antigens and those receiving attenuated wild type *Listeria* (wt *Listeria*) following challenge with *T. parva* sporozoites. Rectal temperatures were measured daily for all experimental animals, and the temperatures exceeding 39.5°C were considered as pyrexia.

4.5.2 Monitoring of leukocyte levels following challenge with *T. parva* sporozoites

An increase of the leukocyte levels was reported a day after challenge (day 2). This was followed by leucopenic phase between day 3 and 11 after which the levels returned to normality (Fig 12). In many of the animals that succumbed, they were reported severely leucopenic between day 10 and 20 post parasite inoculation. The level of white blood cell (leukocyte) counts in these animals went as low as $0.4m/mm^3$ and they did not show indications of bouncing back. On the other hand, the survivors had leucopenia between day 10 and 15 but returned to normal levels by day 20. The WBC counts post challenge for all the time points between the groups was not significantly different, but there was significant differences between the days ($p<0.001$) (Fig 12).

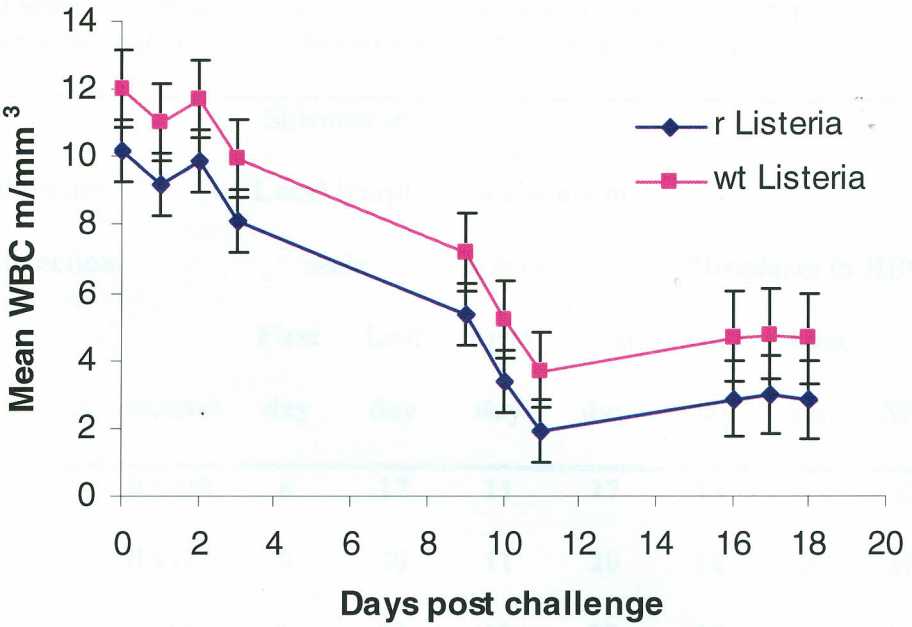


Figure 12: Changes in white blood cell (WBC) counts of animals following parasite challenge. The blood for assessing the WBC levels was collected in EDTA and the monitoring was done three times a week.

4.5.3 Parasitosis

The presence of the *T. parva* parasite infection was confirmed by needle puncture biopsy smears from day 6 post-challenge inoculations. Schizonts were first detected in the draining lymph node in two animals (BB061 and BB167) on day 6, four in day 7 and 8, and one animal on day 9 following challenge (Table 7). The presence of schizonts in the contra-lateral lymph node was detected in three animals on day 10 and seven animals on day 11 while one animal on day 12 (Table 7). The piroplasm parasitaemia was recorded first between day 13 and 16 after challenge (Table 7). From the result it was observed that the survivors which included one control animal had comparatively lower parasite scores in the lymph nodes and erythrocytes. These animals cleared the parasites much earlier than the control and vaccinated animals that succumbed.

Table 7: Summary of parasitological measurements in the lymph node and blood of animals challenged by *Theileria parva* sporozoites following *Listeria*-based vaccination.

Parasite detection		Shizonts in Local lymph node		Schizonts in Contra-lateral		Piroplasm in RBC		
Group	Animal	First day	Last day	First day	Last day	First day	Last day	Max
Vaccinated animals	BA228	8	17	11	17	13	17	68
	BA127	8	20	11	20	14	20	166
	BB167	6	23	10	23	14	23	196
	BA229	7	19	10	19	14	19	40
	BA200	7	19	11	15	14	21	2
	BB03	7	22	11	22	14	22	296
	BB166	8	21	10	21	14	21	180
	BB068	7	13	11	13	16	17	1
	BA207	8	18	11	18	14	18	32
Control	BA209	9	21	12	21	14	21	114
	BB061	6	14	11	14	14	21	1

4.5.4 ECF reaction Index

An ECF reaction index was calculated from several clinical and parasitological parameters of 11 experimental animals that were infected with *T. parva*. Among the parameters taken into account include pyrexia by measuring rectal temperature changes, parasitological responses and hematological measurements as described above. From the data generated two animals of the vaccine group and one of the control group were classified as mild/moderate reactors and these animals survived the challenge infection (Table 8). Eight animals were severe reactors, seven of which were immunized and one a control animal, all of these animals had to be euthanized between day 17 and 23 post-parasite challenge due to their severe reactions (Table 8).

Table 8: Summary of disease severity (ECF reaction index and Euthanasia) in cattle following challenge with *T. parva* sporozoites.

Animal no.	Breed	MHC class I BoLA type	ECF reaction index*	ECF disease Severity ~	Day of Euthanasia post-challenge
BB03	Friesian	N*01301	9.33	SR	23
BA127	Friesian	N*01301	9.52	SR	20
BA200	Friesian	N*01301	5.65	MR / MODR	
BA207	Friesian	N*01201	9.53	SR	18
BA209	Ayshire	N*01201	9.42	SR	21
BA228	Friesian	N*01201	9.21	SR	17
BA229	Ayshire	N*01201	9.83	SR	19
BB61	Boran	N*00101	4.63	MR / MODR	
BB68	Boran	N*00101	4.77	MR / MODR	
BB166	Boran	N*00101	10	SR	21
BB167	Boran	N*00101	10	SR	23

**Classification of experimental animals into different clinically defined ECF reaction indices using first principal component statistical analysis was based on thirteen parasitological, clinical and hematological findings.

~Classification of animals into mild/moderate (MR/MODR) and severe reactors (SR) following parasite challenge was based on the ECF reaction indices.

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

The aim of the present study was to evaluate the potential of recombinant live attenuated *Listeria monocytogenes* (*Lm ΔactAΔinlB)* as a vaccine carrier expressing $CD8^+$ T cell epitopes ($Tp1_{214-224}$, $Tp2_{98-106}$ and $Tp8_{379-387}$) of the bovine parasite *Theileria parva*. There is lack of clear evidence of effective antigen expression by recombinant *Lm ΔactAΔinlB* from this experiment despite rigorous validation efforts. Nevertheless, this vaccine formulation was shown to weakly express and stimulate $Tp1_{214-224}$-specific $CD8^+$ T cell responses *in vitro* in one of the experiments. This was only detected in pulsed monocytes but not skin fibroblasts. Recombinant *Lm ΔactAΔinlB* infected monocytes were co-cultured with Tp1-specific $CD8^+$ T cells in the presence of sodium bicarbonate and recombinant bovine IL-2. This followed failure to induce significant responses in earlier experiments in the absence of these additives. This may reflect the stringent conditions required to induce gene expression by recombinant *L. monocytogenes in vitro*. This hypothesis is partly supported by the fact that the result obtained from this work was not reproducible. Furthermore, recent studies have indicated that macrophages effectively infected with a live attenuated recombinant *L. monocytogenes* expressing feline immunodeficiency virus (FIV) protein (LM-17), failed to express and secrete detectable levels of recombinant FIV antigen *in vitro,* and in addition, the presence of mRNA could not be demonstrated. The reasons for these observations are unknown (Stevens *et al.*, 2005).

The inability of autologous immortalized skin fibroblasts (iSF) to induce significant responses when pulsed with both wild type and recombinant *L. monocytogenes* could be associated with low efficiencies for infection of iSF by *Lm ΔactAΔinlB* or the inability of iSF to support bacterial gene expression and processing capacity. This idea explanation supported by infection and cell to cell spread inefficiencies of avirulent strains of *Mycobacterium tuberculosis* in human skin fibroblasts (Byrd *et al.*, 1998). Furthermore, the fact that the antigen presenting cells (iSF) pulsed with positive control of a Tp1 epitope were readily recognized by specific $CD8^+$ T cells whose magnitude was comparable with those observed using monocytes, could be a good indication of poor uptake and processing-presentation efficiencies by iSF *in vitro*. Recent work has shown that expression of recombinant *L. monocytogenes* genes may occur more rapidly in phagocytic cells than in non-professional phagocytic cells (Jacobs and Freitag, unpublished observations). Further work has demonstrated that, absence of gene expression inducing compounds such as IPTG has implications leading to poor expression of *L. monocytogenes* genes (LLO), intracellular growth and cell to cell spread in murine L2 skin fibroblasts cultures (Dancz *et al.*, 2002).

The Tp1-specific $CD8^+$ T cell IFN-γ responses provoked *in vitro* by recombinant *Lm ΔactAΔinlB* using monocytes was only evident at 25mg/ml concentrations of gentamicin. This is seemingly a specific response induced at a gentamicin concentration, when all the extra-cellular bacteria had been cleared. In the absence of gentamicin, both the recombinant and wild-type *Lm ΔactAΔinlB* stimulated a low-level IFN-γ response, presumably a non-specific response even stimulated by unrestricted bacterial growth. The

absence of responses to recombinant *Lm ΔactAΔinlB* at lower gentamicin concentrations could be difficult to explain, while non-specific responses were induced by wild type *Lm ΔactAΔinlB* at this concentration.

The inability to detect responses by Tp8 specific CTL against monocytes infected with both recombinant and wild type *Lm ΔactAΔinlB* may reflect that the *in vitro* assay was suboptimal, and differences in the polyclonality of the CTL used may mean that responses were below the detection level. The data from Cerus Biotech Corporation who constructed the *L. monocytogenes* strains used in this experiment, demonstrated induction of antigen-specific $CD8^+$ T cell responses to the tag sequence *in vivo.* Mice C57BI/6 that were immunized with either recombinant or wild type *Lm ΔactAΔinlB* demonstrated large numbers of $CD8^+$ T cells specific for the tag antigen ovalbumin (SIINFEKL) as confirmatory inference test for expression and secretion of *T. parva* epitopes from recombinant *Lm ΔactAΔinlB* as opposed to wild type (data not shown) seven days after immunization. Such responses are seldom induced *in vitro* but significant immunogenic vaccine potential is observed *in vivo* (K.S. Bahjat, personal communication). However, the poor $CD8^+$ T cell IFN-γ responses demonstrated by recombinant *Lm ΔactAΔinlB in vitro*, is in total contrast with earlier observations using recombinant plasmid DNA/MVA/CP expression systems of *T. parva* epitopes. These regimens have provoked strong and clear $CD8^+$ T cell IFN-γ responses *in vitro* even when iSF have been used as antigen presenting cells (Graham *et al.*, 2006). This leaves minimal doubt in implicating the *Lm ΔactAΔinlB* vaccine construct for poor recombinant antigen expression. This may indicate that the problem is not with the antigen presenting cells but the vaccine

formulation itself. Furthermore, it was also difficult to demonstrate infectivity of APC (monocytes and skin fibroblast) by detecting intracellular *Listeria* using cytospins.

The experimental animals that were selected after screening against pre-existing anti-*Listeria* T cell responses against the major *L. monocytogenes* T cell target antigen LLO had varying responses. From the results it came out that, it is possible to get significant responses even when an animal has low-level responses to *L. monocytogenes* prior to immunization. In addition, *L. monocytogenes* as a vaccine vector can be used repeatedly and pre-existing anti-*Listeria* immunity does not abrogate the boosting potential of recombinant *L. monocytogenes* (Starks *et al.*, 2004, Stevens *et al.*, 2005).

Following immunization, surprisingly only a few animals exhibited changes in WBC and platelets as opposed to observations in murine and primate models (Farber and Peterkin, 1991, Angelakopoulos *et al.*, 2002, Bahjat K, personal communication). However, booster immunization was accompanied with complications characterized with anorexia and respiratory problems in five animals. This followed 36 hours later despite being clinically stable for the first 24 hours. One animal, developed severe labored breathing/dyspnoea, nasal discharge and froth from the mouth. Notwithstanding all these complications, the rectal temperatures of these animals were within the normal limits (37.5 to 39.2°C). In addition, it was also surprising that the leukocyte and platelet levels in all these animals remained within the normal range. Although four of these animals recovered within 2 hours after chemotherapeutic intervention with appropriate drugs, one animal BA225 succumbed an hour after treatment. Post-mortem evaluation indicated that

the animal death was associated with interstitial pneumonia and emphysema. These complications were attributed to the reactions induced following intravenous inoculation of bacterial preparations.

Adverse pulmonary reactions have been observed in calves following intentional or inadvertent intravenous injection of bacteria (killed) and toxoid vaccines (Lay and Slauson, 1982, Ramsay *et al.*, 2005). Earlier experiments have demonstrated that intravenous inoculation of calves with Freund's complete adjuvant induced acute multi-focal vasculitis and edematous-exudative pneumonitis, which escalated to granulomatous interstitial pneumonitis. These reactions resulted to death of four calves post adjuvant immunization (Lay and Slauson, 1982). Further evidence is provided by studies involving injection of lipopolysaccharides (LPS) isolated from *P. haemolytica* which caused severe pulmonary edema with intra-alveolar macrophages, hyper-cellular interalveolar septa and multi-focal alveolar collapse in calves (Slocombe *et al.*, 1990). In addition, studies involving a group of 75 Red Angus calves immunized with two different bacterial vaccines (Somnustar Ph and Tasvax 8), have been shown to provoke severe responses in 19 calves, ranging from mild depression to severe dyspnoea with red foamy nasal and oral discharge two hours post immunization. Three of the affected calves died before medical attention, while the rest survived after they were treated with dexamethazone and epinephrine. Post mortem examinations revealed that the three calves had suffered from severe diffuse pulmonary edema, congestion, multi-focal-to-coalescing hemorrhages in the dorsal pulmonary parenchyma which failed to collapse when the thoracic cavity was opened. Histo-pathological evaluation showed that the lungs were affected with acute

interstitial pneumonia with multi-focal-to-coalescing severe intra-alveolar and interstitial hemorrhages (Lay and Slauson, 1982, Ramsay *et al.*, 2005). These findings were consistent with our earlier speculations that the reactions leading to animal death in this experiment could have been due to anaphylaxic responses, endotoxaemia/septicemia, or other factors that could trigger a fatal cytokine cascade (Lay and Slauson, 1982, Ramsay *et al.*, 2005).

We have previously observed severe reactions following intravenous injection of sheep red blood cells or tetracyclines. These reactions are either immediate or delayed (D. M. Mwangi personal communication). We have also employed this technique before to inject drugs (adverse reactions with Oxytetracyclines observed) and to immunize cattle against *Ehrlichia ruminantium* with 5mls of culture medium containing live Erlichial elementary bodies (No adverse reactions observed). For all our other immunizations using live bacterial, viral, toxoid, or DNA immunizations we have only used either the sub-cutaneous or intradermal routes with no adverse reactions (D. M. Mwangi personal communication). These data taken together confirms that the inoculations using *L. monocytogenes* preparations were accompanied with adverse reactions in a proportion of the animals that led to death of one animal. These facts are useful to pharmaceutical companies in providing specific guidelines for administration of vaccine products in the light of improving safety margin and decrease undesired reactions post vaccination.

The poor $CD8^+$ T cell immune responses induced after vaccine delivery could be associated with a number of reasons. Although the animals had defined MHC BoLA

types, other genetic variations due to the animal's inherent potential may have contributed to the differences in the magnitudes of immune responses induced in the minority of the animals. Key on the reasons leading to these poor immune responses is lack of sufficient antigen expression by the vaccine regime. This is supported by the failure to replicate the result obtained *in vitro* after stimulating Tp1-specific $CD8^+$ T cell lines using recombinant *Lm ΔactAΔinlB* infected monocytes. In addition, low-level responses were detected when Tp8-specific polyclonal $CD8^+$ T cell lines were employed together with monocytes as antigen presenting cells. Although it was not possible to raise Tp2-specific $CD8^+$ T cell lines *in vitro*, the *in vivo* experiment was good evidence to show that this antigen was most poorly recognized following immunization. The reasons for poor expression may be the way the construct was made or the intrinsic factors of *L. monocytogenes* to support effective heterologous protein expression. It is also possible that the poor recombinant antigen expression *in vitro* may have been replicated *in vivo*, in the cattle experiment. Other theories suggest that *L. monocytogenes* encounters several different host cell compartment environments during the course of infection, and these environments are thought to influence bacterial gene expression (Freitag and Jacobs, 1999). These data collectively argue strongly for lack of efficient and effective recombinant *Lm ΔactAΔinlB* antigen expression *in vivo* as the basis leading to weak responses post vaccination and challenge.

Assuming that the construct was expressing then the dose used was too low or insufficient to induce robust $CD8^+$ T cell immune responses. In animal models, inoculations involving *L. monocytogenes* is normally based on body weight, for instance

studies describing intra-peritoneal and intragastric infection of mice (16-21g) or guinea pigs have been shown to require relatively high numbers of *L. monocytogenes* (10^9 CFU), that have been tolerated (Pine *et al.*, 1990, Barbour *et al.*, 1996, Linman *et al.*, 1998, Lecuit *et al.*, 2001, Barbour *et al.*, 2001, Sleator *et al.*, 2001). In addition, other dose-survival studies have shown that mice can survive with i.v dose of 10^5 CFU and guinea pigs 10^7 CFU *L. monocytogenes* (Lecuit *et al.*, 1999). Both animals survive with 10^{10} CFU dose in oral inoculation of virulent *L. monocytogenes* (Lecuit *et al.*, 1999, Lecuit and Cossart, unpublished observations). On the basis of these numbers of bacteria used in mice and other animals; it could be coherent to argue that the dose used for immunization in this experiment was 10,000 fold lower. Besides, the fact that the vaccine construct did not demonstrate respectable antigen expression *in vitro* could mean that even much higher doses were needed to realize significant responses *in vivo*.

It is also thought that the intravenous route of vaccine inoculation may have bypassed routes rich in professional antigen presenting cells. Routes such as subcutaneous or intra-dermal have been associated with abundance of antigen presenting cells such as the dendritic and Langerhans cells (Bos and Kapsenberg, 1993). Previous immunogenicity studies using DNA/ modified vaccinia virus Ankara strain (DNA/MVA) or canarypox/MVA (CP/MVA) prime-boost systems showed induction of strong cellular $CD8^+$ IFN-y responses (Graham *et al.*, 2006) when the recombinant pox viruses were delivered subcutaneously and plasmid DNA through intra-dermal route. A similar regime to viral pox/DNA in cattle induced undetectable immune responses when the target site of delivery of *Mycobacterium tuberculosis* antigen 85A was the muscle, but

the animals mounted enhanced T cell responses several fold, when the site of inoculation targeted the skin (Taracha *et al.*, 2003). These may highlight the role played by the route of administration in any vaccine delivery system. However, the dose-route response data from CP/MVA or CP/MVA prime-boost regimes targeting the subcutaneous and intra-dermal routes have been demonstrated not to have significant differences in terms of the responses they induce, but the intra-venous route has not been evaluated using these constructs in cattle (D.M. Mwangi personal communication). The intra-venous route may have also led to rapid clearance and degradation of the vaccine construct to the extent that it could affect induction of immune responses. It is believed that antigen persistence and exposure is a key driver in shaping the immunological memory during chronic infection (Hayflck, 1965, Brake, 2003). This may have affected the quality and quantity of immune responses induced.

The immunization results were generally poor. Two animals (BA127, BB68) with significant *L. monocytogenes ΔactAΔinlB* $Tp1_{214-224}$ and $Tp8_{379-387}$-specific $CD8^+$ T cell responses respectively were detected following seven days of primary immunization. The ELISpot and tetramer results of these two animals plus an additional one (BB03) with MHC phenotype capable of recognizing Tp1 were enhanced with a boosting dose. The priming and booster dose results indicate that the antigens are being recognized by the bovine system and can provoke an immune response albeit in a few animals (Graham *et al.*, 2006). However, it is not clear why some animals did not elicit an immune response at least for the antigenic peptides that induced immune responses in others of the same BoLA type. One implication of such observation, could be that low frequencies of

antigen specific T cells were primed that could not be detected by tetramer staining and IFN-γ ELISpot assays. The reasons for undetectable priming and boosting in many of BoLA-N*01301$^+$ and BoLA-N*00101$^+$ animals that restricts responses to Tp1 and Tp8 respectively could be associated with poor expression and secretion of recombinant antigens. This is supported by the fact that in other delivery systems over 80% of the immunized animals have demonstrated consistently, CD8$^+$ T cell IFN-γ ELISpot responses (Graham *et al.*, 2006, Graham *et al.*, 2007). Additionally, it could be difficult to tell from the result obtained if priming ever occurred in the animals with BoLA-N*01201$^+$ since the Tp2 antigen has not induced significant immunogenicty previously (Graham *et al.*, 2006).

Other than the *T. parva* CTL derived antigens, the induction of responses by the *Listeria*-based antigen LLO was equally poor with the purified CD8$^+$ population but strong responses were detected in nine of eleven animals from the intact PBMC population. When PBMC obtained from select animals and then restimulated three times using LLO peptide pool, the cells proliferated well *in vitro* but failed to demonstrate any CTL lytic activity. These findings indicate that T cell responses to LLO in cattle are most likely confined to CD4$^+$ T cell compartment. Although previous studies in mice have implicated LLO as the major CD8$^+$ T cell target antigen of *L. monocytogenes* (Bouwer *et al.*, 1992, Badovinac and Harty, 2000), it is possible that cattle CD8$^+$ T cells may not recognize these epitopes.

The cytokine response data obtained did not translate into CTL activity as evidenced from the ^{111}In-release assays even for the animals that had demonstrated consistent specific $CD8^+$ IFN-γ responses. This has also been observed previously in which about 80% of immunized animal showed induction of IFN-γ responses but only approximately 30% of these animals had detectable CTL with cytotoxic activity (Graham *et al.*, 2006). Animal BB68, whose cytokine response was boosted except post-challenge, and who survived the challenge infection, failed to show any cytotoxic activity. This may mean that the frequency of the cytotoxic $CD8^+$ T cells were too low to be detected but were sufficient to provide protection. Animal BA207 showed poor immunogenicity from the ELISpot data but stained well for the Tp2 $_{98\text{-}106}$ tetramer specific populations. CTL activity was detected against autologous TpM but not peptide-pulsed targets in this animal. This could mean that the TPM lysis in such responses might have been non-specific. In the contrast, animal BA200, a survivor of the lethal dose challenge demonstrated no immunogenicity at all, both from the cytokine data or tetramer staining. However this animal had CTL against TpM but not for peptide-pulsed targets after boosting and challenge. This could be associated with induction of primary CTL responses against other antigens following the *in vitro* stimulation with TpM.

Only one animal BA127, displayed low level definable CTL activity against both TpM and peptide (Tp1) pulsed targets after boosting albeit at a low level. Due to microbial contamination of T cell bulk cultures, it was not possible to assay the post challenge CTL activity. Nevertheless, this animal succumbed to the challenge infection. It is possible that following challenge, the animal failed to mount a secondary CTL response which

could translate into protection. This is supported by the fact that earlier vaccinations using DNA/pox viruses, detection of a CTL response correlated with survival following a potentially lethal challenge infection (Graham *et al.*, 2006).

5.2 Conclusions

In summary this study failed to convincingly demonstrate that recombinant live attenuated *L. monocytogenes* was expressing recombinant antigenic peptides, which were effectively processed and presented to specific $CD8^+$ T cell lines *in vitro*. The result of *in vitro* work generated using Tp1 specific $CD8^+$ T cell lines and monocytes as APC could not be reproduced. It was also not possible to obtain reliable data using Tp8 specific $CD8^+$ T cell lines. Although Tp2 gene expression was not assayed, the failure of the *in vitro* experiment was generally associated with poor antigen expression and to a lesser extent infectivity. Moreover, the low infection potential of *L. monocytogenes in vitro* may be demonstrated by cytospins from monocytes infected with either wild type or recombinant *Lm ΔactAΔinlB* at different time points (15 min, 30 min and overnight cultures), the data obtained had no convincing evidence of intracellular (monocyte) bacteria due to infectivity.

Immunization of cattle with recombinant live attenuated *L. monocytogenes* demonstrated the induction of $CD8^+$ T cells responses to the Tp1 and Tp8 peptides, although this was only detected in a proportion of animals that were also boosted. There were three survivors after parasite challenge, two of which were vaccinated and one control animal. One of the vaccinated animals (BB068) demonstrated strong immunogenicity against Tp8

but lacked CTL activity, while the other one (BA200) had CTL only recognizing the autologous TpM but had no antigen-specific IFN-γ responses. Nevertheless, the two animals survived. There is a strong thinking that the frequencies of the $CD8^+$ T cells were too low to be detected given that the animals were protected or lyses of TPM may have been due to primary responses that recognize other antigens following *in vitro* restimulation, and in addition the sensitivity of the CTL assay may not have been optimal.

Definable CTL activity was detected in only one animal (BA127) after boosting, which did not correlate with survival or reduced disease severity as previously observed in other systems. This indicates differences in functionalities of the $CD8^+$ T cells induced in relation to those induced by live vaccines. The survival of the control animal makes it difficult to draw a direct conclusion of the involvement of the vaccine construct in protection of the vaccinated animals that survived.

5.3 Recommendations

Following failure of the recombinant live attenuated *L. monocytogenes* to sufficiently express $CD8^+$ T cell epitopes *in vitro* and inability to induce $CD8^+$ T cell immune responses in majority of the animals, the following are recommended:

1. Extra work is needed to make *L. monocytogenes* a more robust system that can express and deliver antigens more effectively.
2. Increase the injectable inoculation dose in a proportion that correlates with body weight.
3. Formulate a good vaccine construct that induces strong responses at lower doses.
4. Change the route of inoculation or mode of administration. It would be recommendable to target the route(s) that is APC rich such as subcutaneous route.
5. Consider the option of using a perforin ELISpot to the current CTL assay, which requires repeated stimulation of PBMC with TpM with the possible risk of stimulating primary CTL responses. This could help avoid problems associated with the peptide-loaded target cells.

REFERENCES

Amara, R.R., Villinger, F., Altman, J.D., Lydy, S.L., O'Neil, S.P., Staprans, S.I Montefiori, D.C., Xu, Y., Herndon, J.G., Wyatt, L.S., (2001). Control of a mucosal challenge and prevention of AIDS by a multiprotein DNA/MVA vaccine. *Science* **292**:69.

Angelakopoulos, H., Loock, K., Sisul, D.M., Jensen, E.R., Miller, J.F., Hohmann, E.L., (2002). Safety and shedding of an Attenuated Strain of *Listeria monocytogenes* with a Deletion of actA/plcB in Adult Volunteers: a Dose Escalation Study of Oral Inoculation. *Infection and Immunity* **70**: 3592-3601.

Babiuk, L.A., and Tikoo, S.K., (2000). Adenoviruses as vectors for delivering vaccines to mucosal surfaces. *Journal of Biotechnology* **83**:105-113.

Badovinac, V.P., Harty, J.T., (2000). Adaptive Immunity and Enhanced CD8+ T Cell Response to *Listeria monocytogenes* in the Absence of Perforin and IFN-{gamma}. *Journal of Immunology* **164**: 6444-6452.

Baldwin, C.L., Black, S.J., Brown, W.C., Conrad P.A., Godderis, B.M., Kinuthia, S.W., Lalor, P.A, MacHugh, N.D., Morrison, W.I., Morzaria, S.P., Naessens, J., and Newson, J., (1987). Bovine T cells, B cells, and Null cells are transformed by protozoan parasite *Theileria parva*. *Infection and Immunity* **56:** 462-467.

Barbour, A.H., Rampling, A., and Hormaeche, C.E., (1996). Comparison of the infectivity of isolates of *Listeria monocytogenes* following intragastric and intravenous inoculation in mice. *Microbial Pathology* **20:**247-253.

Barbour, A.H., Rampling, A., and Hormaeche, C.E., (2001a). Variation in the infectivity of *Listeria monocytogenes* isolates following intragastric inoculation of mice. *Infection and Immunity* **69:**4657-4660.

Barouch, D.H., Craiu, A., Santra, S., Egan, M.A., Schmitz, J.E., Kuroda, M.J., Fu, T.M., Nam, J.H., Wyatt, L.S., (2001b). Elicitation of high-frequency cytotoxic T-lymphocyte responses against both dominant and subdominant simian-human immunodeficiency virus epitopes by DNA vaccination of rhesus monkeys. *Journal of Virology* **75**:2462.

Barouch, D.H., Santra, S., Kuroda, M.J., Schmitz, J.E., Plishka, R., Buckler-White, A., Gaitan, A.E., Zin, R., Nam, J.H., (2001). Reduction of simian-human immunodeficiency virus 89.6P viremia in rhesus monkeys by recombinant modified vaccinia virus Ankara vaccination. *Journal of Virology* **75**:5151.

Bielecki, J., Youngman, P., Connelly, P., and Portnoy, D.A., (1990). *Bacillus subtilis* expressing a haemolysin gene from *Listeria monocytogenes* can grow in mammalian cells. *Nature* **345**: 175–176.

Bishop, R.P., Sohanpal, B.K., Kariuki, D.P., Young, A.S., Nene, V., Baylis, H., Allsopp, B.A., Spooner, P. R., Dolan, T.T., and Morzaria, S.P., (1992). Detection of a carrier state in *Theileria parva* infected cattle using the polymerase chain reaction. *Parasitology* **104**: 215–232.

Bos, J.D., and Kapsenberg, M.L., (1993). The skin immune system: Progress in cutaneous biology. *Immunology Today* 14:75-78.

Boyer, J.D., Maciag, P.C., Parkinson, R., Wu, L., Lewis, M.G., Weiner, D.B., Paterson, Y., (2006). Rhesus macaque with high levels of vaccine induced IFN-gamma producing cells better viral set-point following challenge with SIV239. Vaccine **24**: 4498-4502.

Brake, D.A., (2003). Parasite and Immune Responses. Memory Illusion? *DNA and Cell Biology* **22**: 405-419.

Bouwer, H.G., Nelson, C.S., Gibbins, B.L., Portnoy, D.A., and Hinrichs D.J., (1992). Listeriolysin O is a target of the immune response to *Listeria monocytogenes*. *Journal of Experimental Medicine* **175**: 1467-1471.

Brockstedt, D.G., Martin, A.G., Meredith, L.L., Keith, S.B., Yi, G., William, L., Weiqun, L., David, N.C., Daniel, A.P., and Thomas, W.D., (2004). *Listeria*-based cancer vaccines that segregate immunogenicity from toxicity. *Proceedings of the National Academy of Sciences* **101**: 13832– 13837.

Brown, D.J., Campbell, J.D., Russell, G.C., Hopkins, J., and Glass, E. J., (1995). T cell activation by *Theileria annulata*-infected macrophages correlates with cytokine production. *Clinical and Experimental Immunology* **102**:507–514.

Byrd, T.F., Green, G.M., Fowlston, S.E., and Lyons, R., (1998). Differential Growth Characteristics and Streptomycin Susceptibility of Virulent and Avilunt *Mycobacterium tuberculosis* in a Novel Fibroblast –*Mycobacterium* Microcolony assay. *Infection and Immunity* **66:**5132-5139.

Calarota, S., Bratt, G., Nordlund, S., Hinkula, J., Leandersson, A.C., and Wahren, B., (1998). Cellular cytotoxic responses induced by DNA vaccination in HIV1 infected patients. *Lancet* **351**: 1320–5.

Casimiro, D.R., Chen, L., Fu, T.M., Evans, R.K., Caulfield, M.J., Davies, M.E., Tang, A., Chen, M., Huang, L., Harris, V., (2003). Comparative immunogenicity in rhesus monkeys of DNA plasmid, recombinant vaccinia virus, and replication-defective adenovirus vectors expressing a human immunodeficiency virus type 1 gag gene. *Journal of Virology* **77**:6305.

CGIAR Science Council (2005) Science for agricultural development: Changing contexts, new opportunities. Rome, Italy: Science Council Secretariat.

Comin-Andex, B., Gualberto, A., Glaspy, J.A., Seja, E., Ontiveros, M., Reardon, D.L., Renteria, R., Englahner, B., Econmou, J.S., Gomez-Navarro, J., and Ribas, A., (2006). Definition of an Immunologic Response Using Major Histocompatibility Complex tetramer and enzyme linked Immunospot Assay. *Clinical Cancer Research* **12**: 107-116

Chinombo, D.O., Mzoma, F., and Musisi, F.L., (1989). Proceedings of a workshop on East Coast fever immunization held at Lilongwe, Malawi, 20–22 September Organized by ILRAD (International Laboratory for Research on Animal Diseases), FAO (Food and Agriculture Organization of the United Nations) and OAU (Organization for African Unity), with the support of the Government of Malawi. ILRAD, Nairobi, Kenya pp.12–16.

Dancz, C.E., Haraga, A., Portnoy, D.A., Higgins, D.E., (2002). Inducible Control of Gene Expression in *Listeria monocytogenes*: Temporal Requirements of Listeriolysin O during Intracellular Infection. *Journal of Bacteriology* 184: 5935-5945.

Daubenberger, C.A, Taracha, E.L.N., Gaiduls, L., Davis, W.C., McKeever, D.J., (1999). Bovine γδ T cell responses to the intracellular protozoan parasite *Theileria parva*. *Infection and Immunity* **67**: 2241-2249.

De Castro, J.J., James, A.D., Minjauw, B., Di Giulio, G.U., Permin, A., Pegram, R.G., Chizyuka, H.G., and Sinyangwe, P., (1997). Long-term studies on the economic impact of ticks on Sanga cattle in Zambia. *Experimental Applications in Acarology* **21**: 3-19.

DeMartini, J.C., and Baldwin, C.L., (1991). Effects of gamma interferon, tumor necrosis factor alpha, and interleukin-2 on infection and proliferation of *Theileria parva*-infected bovine lymphoblasts and production of interferon by parasitized cells. *Infection and Immunity* **59:** 4540–4546.

Dietrich, G., Kolb-Maurer, A., Spreng, S., Schartl, M., Goebal, W., and Gentschev, I., (2001). Gram-positive and Gram-negative bacteria as carrier systems for DNA vaccines. *Vaccine* **19**: 2506–12.

Dobbelaere, D.A., Fernandez, P.C., and Heussler, V.T., (2000). *Theileria parva*: taking control of host cell proliferation and survival mechanisms. *Cell Microbiology* *2*:91-99.

Dolan, T.T., Young, A.S., Leitch, B.L., and Stagg, D.A., (1984). Chemotherapy of East Coast fever: parvaquone treatment of clinical disease induced by isolates of *Theileria parva*. *Veterinary Parasitology* **15**: 103–116.

Dolan, T.T., (1986). Chemotherapy of East Coast fever: the long term weight changes, carrier state and disease manifestations of parvaquone treated cattle. *Journal of Comparative Pathology* **96**: 137–146.

Domann, E., (1992). A novel bacterial virulence gene in *Listeria monocytogenes* required for host cell microfilament interaction with homology to the proline-rich region of vinculin. *European Molecular Biology Organization Journal* **11**:1981–1990.

Earl, P.L., Wyatt, L.S., Montefiori, D.C., Bilska, M., Woodward, R., Markham, P.D., Malley, J.D., Vogel, T.U., Allen, T.M., Watkins, D.I., (2002). Comparison of vaccine strategies using recombinant env-gag-pol MVA with or without an oligomeric Env protein boost in the SHIV rhesus macaque model. *Virology* **294**:270.

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

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

Farber, J. M., and Peterkin, P. I., (1991). *Listeria monocytogenes*, a food-borne pathogen. *Microbiology Review* **55**: 476-511.

Farina, S.F., Gao, G.P., Xiang, Z.Q., Rux, J.J., Burnett, R.M., Alvira, M.R., Marsh, J., Ertl, H.C., Wilson, J.M., (2001). Replication-defective vector based on a chimpanzee adenovirus. *Journal of Virology* **75**:11603.

Fawcett, D.W., Buscher, G., and Doxsey, S., (1982a). Salivary gland of the tick vector of East Coast fever.IV. Cell type selectivity and host cell responses to *Theileria parva*. *Tissue Cell* **14**: 397–414.

Fawcett, D.W., Doxsey, S., Stagg, D.A., and Young, A.S., (1982b). The entry of sporozoites of *Theileria parva* into bovine lymphocytes *in vitro*. Electron microscopic observations. *European Journal of Cell Biology* **27**: 10–21.

Fawcett, D.W., Young, A.S., and Leitch, B.L., (1985). Sporogony in *Theileria* (Apicomplexa: Piroplasmida). A comparative Ultra-structural Study. *Journal of Sub-microbiological Cytology* **17**: 299–314.

Freitag, N.E., and Jacobs, K.E., (1999). Examination of Intracellular Gene Expression of *Listeria monocytogenes* using Green Flouresecent Protein of *Aequorea victoria*. Infection and Immunity **67**: 1844-1852.

Gaillard, J.L., Berche, P., Frehel, C., Gouin, E. and Cossart, P., (1991). Entry of L. monocytogenes into cells is mediated by internalin, a repeat protein reminiscent of surface antigens from Gram-positive cocci. *Cell* **65**: 1127–1141.

Gallichan, W.S., Johnson, D.C., Graham, F.L., and Rosenthal K.L., (1993). Mucosal immunity and protection after intranasal immunization with recombinant adenovirus expressing herpes simplex virus glycoprotein. *Brazilian Journal of Infectious Diseases* **168:**622-629.

Gardner, M.J., Bishop, R., Shah, T., de Villiers, E.P., Carlton, J.M., Hall, N., Ren, Q., Paulsen, I.T., Pain, A., Berriman, M., Wilson, R.J.M., Sato, S., Ralph, S.A., Mann, D.J., Xiong, Z., Shallom, S.J., Weidman, J., Jiang, L., Lynn, J., Weaver, B., Shoaibi, A., Wasawo, D., Crabtree, J., Wortman, J.R., Haas, B., Angiuoli, S., Creasy, T., Lu, C., Suh, B., Silva, J., Utterbacl, T., Feldblyum, T., Pertea, M., Allen, J., Taracha, E., Salzberg, S.L., White, O., Fitzhugh, H.A., Morzaria, S., Venter, J.C., Fraser, C.M., Nene, V., (2005). Genome sequence of *Theileria parva*, a bovine pathogen that transforms lymphocytes. *Science* **309**:134–137.

Gilbert, S. C., Schneider, J., Plebanski, M., Hannan, C.M., Blanchard, T.J., Smith, G.L., and Hill, A.V.S., (1999). Ty virus-like particles, DNA vaccines and modified vaccinia virus Ankara; comparisons and combinations. *Journal of Biological Chemistry* **380:**299-303.

Goddeeris, B.M., and Morrison, W.I., (1988). Techniques for cloning, characterization of bovine cytotoxic T cells specific for protozoan *Theileria parva*. *Journal of Tissue Culture Methods* **11**: 2.

Graham, S.P., Pelle,´ R., Honda, Y., Mwangi, D.M., Nyerhovwo, J.T., Yamage,M., Glew, E.J., de Villiers, E.P., Shah, T., Bishop, R., Abuya, E., Awino, E., Gachanja, J., Luyai, A.E., Mbwika, F., Muthiani, A.M., Ndegwa, D.M., Njahira, M., Nyanjui, J.K., Onono, F.O., Osaso, J., Saya, R.M., Wildmann, C., Fraser, C.M., Maudlin, I., Gardner, M.J., Morzaria, S.P., Loosmore, S., Gilbert, S.C., Audonnet, J.C., van der Bruggen, P., Nene, V., and Taracha, E.L.N., (2006). *Theileria parva* candidate vaccine antigens recognized by immune bovine cytotoxic T lymphocytes. *Proceedings of the National Academy of Sciences* **103**: 3286–3291.

Graham, S.P., Honda, Y., Pellé, R., Mwangi, D.M., E Jane Glew, E. J., de Villiers, E.P., Shah, T., Bishop, R., van der Bruggen, P., Nene, V., and Evans LN Taracha, E. L. N., (2007). A novel strategy for the identification of antigens that are recognized by bovine MHC class I restricted cytotoxic T cells in a protozoan infection using reverse vaccinology. *Immunome research* **3**: 2.

Gunn, G.R., Zubair, A., Peters, C., Pan, Z.K., Wu, T.C., and Paterson, Y., (2001). Two *Listeria monocytogenes* vaccine vectors that express different molecular forms of a human papilloma virus-16 (HPV-16) E7 induce qualitatively different T cell immunity that correlates with their ability to induce regression of established tumors immortalized by HPV-16. *Journal of Immunology* **167**: 6471.

Hamilton, S.E., Vladimir, P.B., Khanolkar, A., and Harty, J.T., (2006). Listeriolysin O-Deficient *Listeria monocytogenes* as a Vaccine Delivery Vehicle: Antigen-Specific $CD8^+$ T Cell Priming and Protective Immunity *Journal of Immunology* **177**: 4012-4020.

Harty, J.T., and Bevan, M.J., (1992). CD81 T cells specific for a single nonamer epitope of *Listeria monocytogenes* are protective *in vivo*. *Journal of Experimental Methods* **175**:1531–1538.

Hayflick, L., (1965). The serial cultivation of human diploid strains. *Experimental Cell Research*. **37**: 585-621.

Hulliger, L., Wilde, J.K.H., Brown, C.G.D., and Turner, L., (1964). Mode of multiplication of *Theileria* in cultures of bovine lymphocytic cells. *Nature* **203**: 728–730.

Ikuta, Y., Katayama, N., and Wang, L., (2002). Presentation of a major histocompatibility complex class 1-binding peptide bymonocyte-derived dendritic cells incorporating hydrophobized polysaccharide-truncated HER2 protein complex: implications for a polyvalent immunocell therapy. *Blood* **99**:3717-3724.

Irvin, A.D., Dobbelaere, D.A.E., Mwamanchi, B.M., Minami, T., Spooner, P.R., and Ocama, J.G.R., (1983). Immunization against East Coast fever: correlation between monoclonal antibody profiles of *Theileria parva* stocks and cross-immunity *in vivo*. *Research in Veterinary Science* **35**: 341–346.

Jensen, E.R., Selvakumar, R., Shen, H., Ahmed, R., Wettstein, F.O, and Miller, J. F., (1997a). Recombinant *Listeria monocytogenes* vaccination eliminates papillomavirus-induced tumors and prevents papilloma formation from viral DNA. *Journal of Virology*, **71**: 8467–8474.

Jensen, E.R.H., Shen, F.O., Wettstein, R., Ahmed., and Miller, J.F., (1997b). Recombinant *Listeria monocytogenes* as a live vaccine vehicle and a probe for studying cell-mediated immunity. *Immunology Review* **158**:147.

Jooss, K., Ertl, H.C., and Wilson J.M., (1998). Cytotoxic T-lymphocyte target proteins and their major histocompatibility complex class I restriction in response to adenovirus vectors delivered to mouse liver. *Journal of Virology* **72:**2945-2954.

Kaba, S.A., Hemmes, J.C., Nene, V., Musoke, A.J., Vlak, J.M., and Van Oers, M.M., (2003). Baculovirus surface display of *Theileria parva* antigen preserves the conformation of sporozoite-neutralizing epitopes. *Protein Engineering* **16**: 73 - 78.

Kariuki, D.P., (1990). Current status of Theileriosis in Kenya. Progress towards the control of East Coast fever (Theileriosis) in Kenya. KARI, Nairobi 17–26.

Kariuki, D.P., Young, A.S., and Morzaria, S.P., (1995). *Theileria parva* carrier state in naturally infected and artificially immunized cattle. *Tropical Animal Health and Production* **27**: 15–25.

Karoui, M., (2002). Suivi de l'infection sur les veaux en première saison de theiléríose tropicale dans l'état d'endémie stable: application à l'évaluation de l'intérêt de la vaccination contre la theiléríose tropicale, Thèse Doc. Méd. Vét., Sidi Thabet, Tunisie, **68** :18.

Kaufmann, S.H.E., (1996). Gamma/delta and other unconventional T lymphocytes: what do they see and what do they do? *Proceedings of the National Academy of Sciences* **93**: 2272-2279.

Kent, S. J., Zhao, A., Best, S.J., Chandler, J.D., Boyle, D.B., and Ramshaw, I.A., (1998). Enhanced T-cell immunogenicity and protective efficacy of a human immunodeficiency virus type 1 vaccine regimen consisting of consecutive priming with DNA and boosting with recombinant fowlpox virus. *Journal of Virology* **72:**10180-10188.

Kocks, C., (1992). *L. monocytogenes*-induced actin assembly requires the actA gene product, a surface protein. *Cell* **68**: 521–531.

Ladel, C.H., Flesch, I.E., Arnoldi, J., and Kaufmann, S.H., (1994). Studies with MHC-deficient knock-out mice reveal impact of both MHC I- and MHC II-dependent T cell responses on *Listeria monocytogenes* infection. *Journal of Immunology* **153**: 3116–3122.

Lay, J.C., and Slauson D.O., (1982). The bovine pulmonary inflammatory response: adjuvant pneumonitis in calves. *Veterinary Pathology* **19**: 506-520.

Lecuit, M., Dramsi, S., Gottardi, C., Fedor-Chaiken, M., Gumbiner, B., and Cossart P., (1999). A single amino acid in E-cadherin responsible for host specificity towards the human pathogen *Listeria monocytogenes*. *Journal of European Molecular Biology Organization* **18**:3956-3963.

Lecuit, M., Vandormael-Pournin, S., Lefort, J., Huerre, M., Gounon, P., Dupuy, C., Babinet, C., and Cossart, P., (2001). A transgenic model for listeriosis: role of internalin in crossing the intestinal barrier. *Science* **292:**1722-1725.

Linman, M.J., Mascola, L., Lou, X.D., Goulet, V., May, S., Salminen, C., Hird, D.W., Yonekura, M.L., Hayes, P., Weaver, R., Audurier, A., Plikaytis, B.D., Fannin, S.L., Kleks, A., and Broome, C.V., (1988). Epidemic listeriosis associated with Mexican-style cheese. *New England Journal of Medicine* **319:**823-828.

Mackaness, G.B., (1962). Cellular resistance to infection. *Journal of Experimental Methods.* **116**: 381–406.

Macro, A. J., Altimira, J., Prats, N., Lopez, S., Dominguez, L., Domingo, M., and Briones, V., (1997). Penetration of *Listeria monocytogenes* in mice infected by the oral route. *Microbial Pathology* **23:**255-263.

Malawi Government. (1975). Veterinary Department Annual Report. Zomba, Malawi: *Government Printer.*

Maritim, A.C., Young, A.S., Lesan, A.C., Ndungu, S.G., Mutugi, J.J., and Stagg, D.A., (1989). *Theileria* parasites isolated from carrier cattle after immunization with *Theileria parva* by infection and treatment. *Parasitology* **99**: 139–147.

Mbogo, S.K., Kariuki, D.P., Ngumi, P.N., and McHardy, N., (1996). A mild *Theileria parva* parasite with potential for immunization against East Coast fever. *Veterinary Parasitology* **61**: 41–47.

Mckeever, D.J., and Morrison, W.J., (1990). *Theileria parva*: the nature of immune response and its significance for immunoprophylaxis. *Revue scientifique et Technique de l'office international des Epizooties* **9**: 405-421.

McKeever, D.J., and Morrison, W.I., (1994). Immunity to a parasite that transforms T lymphocytes. *Current Opinions in Immunology* **6**: 564–567.

McKeever, D.J., Taracha, E.L.N., and Innes, E.A., (1994). Adoptive transfer of immunity to *Theileria parva* in the $CD8^+$ fraction of responding efferent lymph. *Proceedings of the National Academy of Sciences* **91**: 1959–1963.

McKeever, D.J., Taracha, E.L.N., Morrison, W.I., Musoke, A.J., Morzaria, S.P., (1999). Protective immune mechanisms against *Theileria parva:* evolution of vaccine development strategies. *Parasitology Today* **15**: 263–267.

Mckeever, D.J., (2006). *Theileria parva* and the bovine CTL response: down but not out? *Parasite Immunology* 28339-345.

McShane, H., Brookes, R., Gilbert, S.C. and Hill A.V.S., 2001. Enhanced immunogenicity of $CD4^+$ T-cell responses and protective efficacy of a DNA-modified vaccinia virus Ankara prime-boost vaccination regimen for murine tuberculosis. *Infection and Immunity* 69:681-686.

Mehlhorn, H., and Schein, E., (1984). The piroplasms: life cycle and sexual stages. *Advances in Parasitology* **23**: 37–103.

Mhadhbi, M., (2004). Caractérisation génétique des isolats tunisiens de Theileria annulata potentiellement résistants à la buparvaquone par électrophorèse isoenzymatique. Diplôme de Mastère en génétique et biologie moléculaire. Faculté des sciences de Tunis, Tunisie, 51 pp.

Moll, G., Lodhing, A., Young, A.S., and Leitch, B.L., (1986). Epidemiology of Theileriosis in calves in an endemic area of Kenya. *Veterinary Parasitology* **19**: 255–273.

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

Morzaria, S., Nene, V., Bishop, R., and Musoke, A., (2000). Vaccines against *Theileria parva*. *Annals of the New York Academy of Sciences* **916**: 464-473.

Mukhebi, A.W., Karuiki, D.P., Mussukuya, E., Mullins, G., Ngumi, P.N., Thorpe, W., and Perry, B.D., (1992). Assessing the economic impact of immunization against east coast fever: a case study in coast, Kenya. *Veterinary Research* **137**: 17–22.

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.

Musoke, A.J., Nantulya, V., Mrurangirwa, F.R., and Buscher, G., (1984). Evidence for a common protective antigenic determinant on sporozoites of several *Theileria parva* strains. *Immunology* **52**: 231 - 238.

Musoke, A., Morzaria, J.S., Nkonge, C., Jones, E., and Nene, V., (1992). A recombinant sporozoite surface antigen of *Theileria parva* induces protection in cattle. *Proceedings of the National Academy of Sciences* **89**: 514 - 518.

Musoke, A.J., and V. Nene., (1990). Development of recombinant antigen vaccines for the control of theileriosis. *Parasitologia* **32:**73-85

Musoke, A.J., Nene, V., Morzaria, S.P., (1993). A sporozoid based vaccine for *Theileria parva*. *Parasitology Today* 9: 385-388.

Musoke, A., Rowlands, J., Nene, V., Nyanjui, J., Katende, J., Spooner, P., Mwaura, S., Odongo, D., Nkonge, C., Mbogo, S., Bishop, R., and Morzaria, S., (2005). Subunit vaccine based on the p67 major surface protein of *Theileria parva* sporozoites reduces severity of infection derived from field tick challenge. *Vaccine* **23**: 3084–3095.

Naouach, A., (2001). Evaluation de l'effet de la buparvaquone sur deux souches ST1/13 et ST2/19 de *Theileria annulata* potentiellement résistantes. Thèse Doc. Méd. Vét., Sidi Thabet, Tunisie, 69 pp.

Nene, V., Inumaru, S., Mckeever, D., Morzaria, S., Shaw, M., and Musoke, A., (1995). Characterization of an insect cell-derived *Theileria parva* sporozoite vaccine antigen and immunogenicity in cattle. *Infection and Immunity* **63**: 503 -508.

Nene, V., Musoke, A.J., Godbright, E., and Morzaria, S., (1996). Conservation of the sporozoite p67 vaccine antigen in cattle-derived *Theileria* stocks with different cross-immunity profiles. *Infection and Immunity* **64**: 2056 - 2061.

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

Oura, C.A., Asiimwe, B.B., Weir, W., Lubega, G.W., and Tait, A., (2005). Population genetic analysis and sub-structuring of *Theileria parva* in Uganda. *Molecular and Biochemical Parasitology* **140**: 229–239.

Oura, C.A., Bishop, R., Asiimwe, B.B., Spooner, P., Lubega, G.W., and Tait, A., (2007). *Theileria parva* live vaccination: parasite transmission, persistence and heterologous challenge in the field. *Parasitology* 1-9.

Pamer, E.G., (2004). Immune resoponses to *Listeria monocytogenes*. *Immunology* **4**: 812-823.

Pan, Z.K., Ikonomidis, G., Lazenby, A., Pardoll, D., and Paterson. Y., (1995). A recombinant *Listeria monocytogenes* vaccine expressing a model tumour antigen protects mice against lethal tumour cell challenge and causes regression of established tumors. *Nature Medicine* **1**:471.

Pine, L., Malcolm, G.B., and Plikaytis, B.D., (1990). *Listeria monocytogenes* intragastric and intraperitoneal approximate 50% lethal doses for mice are comparable, but death occurs earlier by intragastric feeding. *Infection and Immunity* **58**:2940-2945.

Pinto, A.R., Fitzgerald, J.C., Giles-Davis, W., Gao, G.P., Wilson, J.M., Ertl, H.C.J., (2003). Induction of $CD8^+$ T Cells to an HIV-1 Antigen through a Prime Boost Regimen with Heterologous E1-Deleted Adenoviral Vaccine Carriers. *Journal of immunology* **171**: 6774-6779.

Radley, D.E., Brown, C.G.D., and Cunningham, M.P., Kirimi, I.M., Purnell, R.E., and Young, S.A., (1975a). Chemoprophylactic immunization of cattle against *Theileria parva* (Muguga) and five *Theileria* strains. *Veterinary Parasitology* **1**: 35–41.

Radley, D.E., Brown, C.G.D., Burridge, M.J., Kimber, C.D., Musisi, F.L., Payne, R.C., Purnell, R.E., Stagg, D.A., and Young, S.A., (1975b). East Coast fever: 3. Chemoprophylactic immunization of cattle using oxytetracycline and a combination of Theilerial strains. *Veterinary Parasitology* **1**: 51–60.

Radley, D.E., (1981). Infection and treatment method of immunization against theileriosis. In: Irvin AD, Cunningham MP and Young AS, eds. *Advances in the Control of Theileriosis: Proceedings of an International Conference Held at the International Laboratory for Research on Animal Diseases, Nairobi, 9-13 February 1981.* The Hague: Martinus Nijhoff, pp. 227-237.

Ramsay, J.D., Williams C.L., and Simko E., (2005). Fatal Adverse Pulmonary Reaction in Calves after Inadvertent Intravenous Vaccination. *Veterinary Pathology* **42**: 492-495.

Redondo, M., Fragoso, H., Montero, C., Lona, J., Medellín, J.A., Fría, R., Hernández, V., Franco, R., Machado, H., Rodríguez, M., and De La Fuente, J., (1999). Integrated Control of Acaricide-Resistant Boophilus Microplus Populations on Grazing Cattle in Mexico Using Vaccination with Gavac™ and Amidine Treatments 3: 10.

Rowlands, G.J., Musoke, A.J., Morzaria, S.P., Nagda, S.M., Ballingal, K.T., and McKeever, D.J., (2000).A statistically derived index for classifying East Coast fever reactions in cattle challenged with Theileria parva under experimental conditions. *Parasitology* **120**: 371-381.

Shaw, M.K., and Tilney, L.G., (1995). The entry of *Theileria parva* merozoites into bovine erythrocytes occurs by a process similar to sporozoite invasion of lymphocytes. *Parasitology* **111**: 455–461.

Shen, H., Slifka, M.K., Matloubian, M., Jensen, E.R., Ahmed, R., and Miller, J.F., (1995). Recombinant *Listeria monocytogenes* as a live vaccine vehicle for the induction of protective anti-viral cell mediated immunity. *Proceedings of the National Academy of Sciences* **92**: 3987.

Shen, Y., Naujokas, M., Park, M., and Ireton, K., (2000). InlB-dependent internalization of *Listeria* is mediated by the Met receptor tyrosine kinase. *Cell* **103**: 501–510.

Shiver J.W., Fu, T.M., Chen, L., Casimiro, D.R., Davies, M.E., Evans, R.K., Zhang, Z.Q., Simon, A.J., Trigona, W.L., Dubey, S.A., (2002). Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity. *Nature* **415**:331.

Sleator, R.D., Gahan, C.G.M., and Hill C., (2001). Mutations in the listerial *proB* gene leading to proline overproduction: effects on salt tolerance and murine infection. *Applied Environmental Microbiology* **67:** 4560-4565.

Slocombe, R.F., Mulks, F., Killingsworth, C.R., Derksen, F.J., Robinson, N.E., (1990). Effect of *Pasteurella haemolytica*-derived endotoxin on pulmonary structure and function in calves. *American Journal of Veterinary Research* **51** : 433-438.

Soudani, M.C., (1995). Contribution à l'étude épidémiologique de la theiléríose bovine à *Theileria annulata*: analyse clinique, parasitologique et sérologique de l'infection naturelle des veaux en première saison estivale, Thèse Doc. Méd. Vét., Sidi Thabet, Tunisia, 68 pp.

Starks, H., Bruhn, K.W., Shen, H., Barry, R.A., Dubensky, T.W., Brockstedt, D., Hinrichs, D.J., Higgins, D.E., Miller, J.F., Giedlin, M., and Bouwer, H.G.A., (2004). *Listeria monocytogenes* as a Vaccine Vector: Virulence Attenuation or Existing Antivector Immunity Does Not Diminish Therapeutic Efficacy. *The Journal of Immunology* **173**: 420-427.

Stevens, R., LaVoy, A., Nordone, S., Burkhard, M., and Dean, G.A., (2005). Pre-existing immunity to pathogenic *Listeria monocytogenes* does not prevent induction of immune responses to feline immunodeficiency virus by a novel recombinant *Listeria monocytogenes* vaccine. *Vaccine* **23**:1479-1490.

Taracha, E.L.N., Godderis, B.M., Morzaria, S.P., and Morrison, W.I., (1995). Parasite strain specificity of precursor cytotoxic T cells in individual animals correlates with cross-protection in cattle challenged with *Theileria parva*. *Infection and Immunity* **63**: 1258-1262.

Taracha, E.L.N., Bishop, R., Musoke, A.J., Hill, A.V.S., Gilbert, S.C., (2003). Heterologous prime-boosting immunization of cattle with *Mycobacterium tuberculosis* 85A induces antigen-specific T-cell responses. *Infection and Immunity* **71**: 6906–6914.

Tripathy, S. K., Black, H.B., Goldwasser, E., and Leiden J.M., (1996). Immune responses to transgene-encoded proteins limit the stability of gene expression after injection of replication-defective adenovirus vectors. *Nature and Medicine* **2:**545-550.

Uilenberg, G., (1999). Immunization against diseases caused by Theileria parva: a Review. *Tropical Medicine and International Health* **4**: 12–20.

Walker, A.R., (1990). Parasite adaptations in the transmission of *Theileria* by ticks. *Tropical Animal Health Production* **22**: 23–33.

Wang, R., Doolan, D., and Le Thong, P., (1998). Induction of antigen-specific cytotoxic T lymphocytes in humans by a malaria DNA vaccine. *Science* **282**: 476–80.

Webster, P., Dobbelaere, D.A.E., and Fawcett, D.W., (1985). The entry of sporozoites of *Theileria parva* into bovine lymphocytes *in vitro*. Immunoelectron microscopic observations. *European Journal of Cell Biology* **36**: 157–162.

Yang, Y., Su, Q., and Wilson J.M., (1996). Role of viral antigens in destructive cellular immune responses to adenovirus vector-transduced cells in mouse lungs. *Journal of Virology* **70:**7209-7212.

Yang, Z.Y., Wyatt, L.S., Kong, W.P., Moodie, Z., Moss, B., Nabel, G.J., (2003). Overcoming immunity to a viral vaccine by DNA priming before vector boosting. *Journal of virology* **77**:799.

Young, A.S., and Leitch, B.L., (1980). A probable relationship between the development of *Theileria* species and the ecdysis of their tick hosts. *Journal of Parasitology* **66**: 356–359.

Young, A.S., Leitch, B.L., and Newson, R.M., (1981). The occurrence of a *Theileria parva* carrier state in cattle from an East Coast fever endemic area of Kenya. In Advances in the Control of Theileriosis, *The Hague*: *Martinus-Nijhoff Publishers* 60–62.

Young, A.S., Groocock, C.M., and Kariuki, D.P., (1988). Integrated control of ticks and tick-borne diseases of cattle in Africa. *Parasitology* **96**: 403-432.

APPENDICES

1. Analysis of epitope-specific $CD8^+$ T cell responses by tetramer staining.

Table 4: Summary of $CD8^+$ T cell staining positive events of three MHC class I: *T. parva* tetramers between day 0 and 70 following immunization of cattle with recombinant live attenuated *L. monocytogenes* and challenge with *T. parva* sporozoites.

		Days post	prime		boost			challenge		
Tetramers and cattle stained	Cattle	0	7	14	28	37	42	49	58	70
MHC class I : Tp1 tetramer	BB003	0.2	0.5	0.7	0	0.7	0.3	0.3	1.2	-
	BA127	0	0.6	0.3	0.4	0.5	0.1	0.6	2.1	-
	BA200	0	0.1	0.1	0.6	0.2	0.6	0.2	0.4	0
	BA225	0.5	0	0.1	0	*	*	*	*	*
MHC class I : Tp2 tetramer	BA207	0.2	1.1	0	0.8	0.6	0.6	0.7	1.5	-
	BA228	0	0.3	0.3	0.8	0.7	1.2	0.5	0.6	-
	BA229	0	0.2	0.2	0.2	0.5	0.3	0.4	1.1	-
	BA209	0.5	0.2	0	0.1	0.3	0.2	0.3	0.1	-
MHC class I : Tp8 tetramer	BB68	0.3	3.5	0.6	0.6	1.2	1.5	0.5	1.6	1.6
	BB166	0.1	0.9	0.4	0.1	2.2	4.1	0.2	1.5	-
	BB167	0.3	1.3	0.4	0.2	0.5	0.2	0.3	1.8	-
	BB61	0.4	0.9	0.6	0.9	0.4	0.1	0.2	0.4	1.1

* The animal was not stained for the tetramer in the days indicated because he succumbed following the boosting dose.

- The animal died before day 70 post challenge and not assayed for tetramer staining.

2. Assessment of induction of CTL following immunization with recombinant live attenuated *L. monocytogenes* and challenge with *T. parva* sporozoites.

Table 5: % CTL activity against various targets at the highest possible effector: target ratio pre- and post- primary immunization.

		% lysis pre-prime			% lysis post-prime		
BoLA/Tp Type	**Animal**	**TpM**	**Blast**	**Tp**	**TpM**	**Blast**	**Tp**
BoLA-N*01301$^+$-Tp1	**BB003**	-	-	-	2	1.9	0
	BA127	20	20	21	13	3	0
	BA200	7	0	3	3	0	0
	BA225*	0	25	0	8	0	1
BoLA-N*01201$^+$-Tp2	**BA207**	-	-	-	9	0	0
	BA228	0	6	1	0	0	0
	BA229	0	4	0	-	-	-
	BA209*	0	0	0	0	0	0
BoLA-N*00101$^+$-Tp8	**BB068**	0	0	0	13	0	4
	BB166	7	1	0	1	0	3
	BB167	0	12	8	0	0	0
	BB061*	5	0	0	-	-	-

* Represents control animal in the experiment.
- Animal not tested due to microbial contamination of T cell cultures

Table 6: % CTL activity against different targets at the highest possible effector: target ratio for post challenge survivors' assay.

% lysis survivors' assay

BoLA/Tp Type	Animal	TpM	Blast	Tp
BoLA-N*00101^{+}-Tp8	BB061*	11	0	0
BoLA-N*00101^{+}-Tp8	BB068	16	0	0
BoLA-N*01301^{+}-Tp1	BA200	3	6	2