Development of a gamma glutamylcysteine synthetase vaccine to protect against *Leishmania* infection

A thesis presented by

Basma Doro

For the degree of Doctor of Philosophy

Of

University of Strathclyde

Copyright

ABSTRACT

Leishmaniasis is a public health problem and development of a vaccine to prevent infection is required. The overall aim of this study was to develop a vaccine to protect against *Leishmania* infection using L. *donovani*, *L. mexicana* and *L. major* gamma glutamylcysteine synthetase (γGCS) recombinant proteins.

Studies to optimise the expression of *L. donovani*, *L. mexicana* and *L. major* γGCS recombinant proteins showed that induction with 0.1 mM isopropyl β-D-1-thiogalactopyranoside produced the highest amounts of recombinant protein, and incubation of bacteria at 18ºC after induction increased the amount of soluble protein produced. Recombinant *L. mexicana* γGCS had significantly higher specific enzyme activity ($p < 0.001$) compared to *L. donovani* and *L. major* γGCS. *L. mexicana* γGCS was the most resistant to L-buthionine sulphoximine (BSO) inhibition, the specific irreversible inhibitor of γGCS, at the maximum concentration tested (1.5 mM) and *L. mexicana* promastigotes were the most resistant to the cytotoxic effects of BSO compared to *L. donovani* and *L. major* promastigotes ($p < 0.001$).

Vaccination with the recombinant γGCS proteins from *L. donovani*, *L. major* and *L. mexicana* (triple vaccine) induced significant parasite-specific Th1 and Th2 immune responses based on antibody titres and cytokine production by *in vitro* stimulated splenocytes from immunised mice. Vaccination by inhalation or subcutaneous injection with the triple vaccine was similar mean percentage reduction in parasite burdens compared to controls ± SE, was 98% ± 0.02 in *L. mexicana* infected mice. In *L. major* infected mice was 70% ± 0.1 by subcutaneous

immunisation and 65%± 0.01 for inhalation vaccination. Treatment with the triple vaccine by inhalation failed to protect mice against *L. donovani* infection but was effective in hamsters, where a significant reduction in liver and bone marrow parasite burdens compared to control values ($p < 0.05$; mean percentage reduction compared to controls ± SE*:* spleen 89 ± 1; liver 83 ± 0.3; bone marrow 77 ± 1).

In conclusion, the results of this study indicate that vaccination against leishmaniasis is feasible by the pulmonary route and that the triple vaccine is a potential vaccine candidate.

Scientific meetings attended

Posters

- Optimisation of *Leishmania donovani* gamma-glutamylcysteine synthetase recombinant protein expression in *Escherichia coli.* Basma Doro; M Wiese; A. B. Mullen; K. C Carter. Research day, SIPBS, February 2012.
- *In vivo* imaging of cutaneous leishmaniasis in BALB/c mice. B. Doro, R. Williams, M. Wiese and K.C. Carter IMB symposium March 2012 Integrative Mammalian Biology Initiative, meeting, 29th March 2012 University of Strathclyde.
- The effect of Buthionine sulphoximine on the survival of *Leishmania* species, B. Doro, G. Westrop, M. Wiese, R.A.M. Williams and K.C. Carter. BSP Spring Meeting 2012, in Glasgow.
- The effect of vaccination with recombinant *L. donovani* and *L. mexicana* gamma glutamyl cysteine synthetase by different routes of administration on host immune responses. B. Doro, M. Wiese, A. B. Mullen and K.C. Carter, European Congress of Immunology, 2012, Glasgow.
- Comparing the effect of buthionine sulphoximine on *Leishmania* gamma-glutamylcysteine synthetase from different species, B. Doro, G. Westrop, M. Wiese, C. Shaw, R.A.M. Williams, R. Burchmore, A.B. Mullen and K.C. Carter. BSP spring Meeting 2013 in Bristol.
- The Effect Of Buthionine Sulphoximine on the Survival of different *Leishmania* species and on the expression of gamma-glutamylcysteine

synthetase from different *Leishmania* species in the Poster sessions, Basma Doro; G Westrop; M Wiese; R Willams; C Shaw; R Burchmore; A.B. Mullen; K.C Carter. Worldleish5, 13th to May 17th, 2013, at Porto de Galinhas, PE, Brazil.

Oral presentations

- Optimisation of *Leishmania donovani* gamma-glutamylcysteine synthetase recombinant protein expression in *Escherichia coli.* Basma Doro; M Wises; A. B. Mullen; K. C Carter. Research day, SIPBS, February 2012.
- The ability of Vaccination with a recombinant *Leishmania* gamma glutamyl cysteine synthetase vaccine to protect against *L. mexicana* infection. Basma Doro; M Wiese; A. B. Mullen; K. C Carter Worldleish5, 17th, 2013, at Porto d. Galinhas, PE, Brazil.
- The ability of vaccination with a recombinant *Leishmania* gamma glutamyl cysteine synthetase vaccine to protect against leishmaniasis. B. Doro; M Wiese; A. B. Mullen; K. C Carter IIM Post-Graduate Symposium, June 2013.

DEDICATION

I would like to dedicate this thesis to my father and mother, who supported and encouraged me to achieve my ultimate goal.

ACKNOWLEDGEMENTS

This thesis would not have been possible without the help and support of many individuals. I would especially like to thank the following people. **Dr Katharine Carter** for her expert guidance, invaluable advice and unfailing support. I particularly appreciate her meticulous attention to detail, her patience in explaining things in the simplest terms and for her promptness in providing feedback to drafts and responding to email queries. I would like to thank **Dr. M. Wiese**, **Dr. G. Westrop and Prof A. B. Mullen**, I extend my continual appreciation for them keenness and support in all my studies.

I appreciate all the teachers and colleagues, in particular **Mireia Puig**, **Craig Shaw**, **Fatima Al-Gawhari and John Carruthers** for their support and kindness, I have learnt much from them during the time I spent at laboratory. I would like to thank you all for being there.

LIST OF ABBREVIATIONS

APC	Antigen presenting cell
ATP	Adenosine triphosphate
bp	Base pair
BCG	Bacillus Calmette-Guerin
BLI	Bioluminescence
BSA	Bovine serum albumin
BSO	L-buthionine sulphoximine
CL	Cutaneous Leishmaniasis
DC	Dendritic cell
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FCS	Fetal calf serum
γGCS	Gamma Glutamyl Cysteine Synthetase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GSH	Glutathione
HN	Hemagglutinine neuraminidase
HPRT	Hypoxanthine-guanine phosphorribosyl transferase
HRP	Horseradish peroxidase

IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IVIS	*In vivo* imaging system
KBMA	Killed but metabolic active
kDa	kilo Daltons
MΦ	Macrophages
MALD1	Matrix-assisted laser desorption/ionization mass spectrometry
MHC	Major histocompatibility complex
mRNA	messenger RNA
MCL	Mucocutaneous Leishmaniasis
NK	Natural killer
NADPH	Nicotinamide Adenine Dinucleotide Phosphate Oxidase
NO	Nitric oxide
LACK	*Leishmania* activated C kinase
LdγGCS	*L. donovani* γGCS
LmγGCS	*L. mexicana* γGCS
LjγGCS	*L. major* γGCS
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
SDS PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SE	Standard error

Th	T-helper cells
TNF-α	Tumor necrosis factor-α
T reg	T regulatory
TSH	Trypanothione
VL	Visceral Leishmaniasis
X-gel	5-bromo-4-chloro-3-indoyl-beta-D-galactopyranoside

Table of Contents

Figures

Tables

Chapter 1: Introduction

1.1 Leishmaniasis

Leishmaniasis is a disease caused by infection with the protozoan haemoflagellate of the genus *Leishmania*. In 1885, Cunningham, who observed *Leishmania* amastigotes in skin lesions of patients from India, suggested it was a fungus. However, in 1898, Borovsky recognized it as a protozoal infection and in 1903 Leishman named the parasite and established that *Leishmania* parasites were morphologically related to trypanosomes. A similar observation was made in the same year by Donovan (reviewed by Kobets *et al.*, 2012).

Leishmaniasis is a public health problem; approximately 0.2 to 0.4 million cases of visceral leishmaniasis and 0.7 to 1.2 million cases of cutaneous leishmaniasis occur each year (WHO, 2012). Leishmaniasis is endemic in 88 countries, 22 countries in the New World and in 66 nations in the Old World (Pavli and Maltezou, 2010). *Leishmania* is transmitted by female sandflies of the genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World (Pavli and Maltezou, 2010) and over than 20 *Leishmania* species have been identified, most of which cause disease in animals or humans (Duthie *et al.*, 2012).

There are two main forms of leishmaniasis, cutaneous and visceral leishmaniasis (WHO, 2012). Cutaneous leishmaniasis (CL) is generally divided into four major clinical classifications; localized cutaneous leishmaniasis (LCL), diffuse cutaneous leishmaniasis

(DCL), mucocutaneous leishmaniasis (MCL) and a fourth form of CL is post kala-azar dermal leishmaniasis (PKDL), a sequel of visceral leishmaniasis (Nylen and Eidsmo, 2012). All the types of Leishmaniasis are summarized in Figure 1.1.

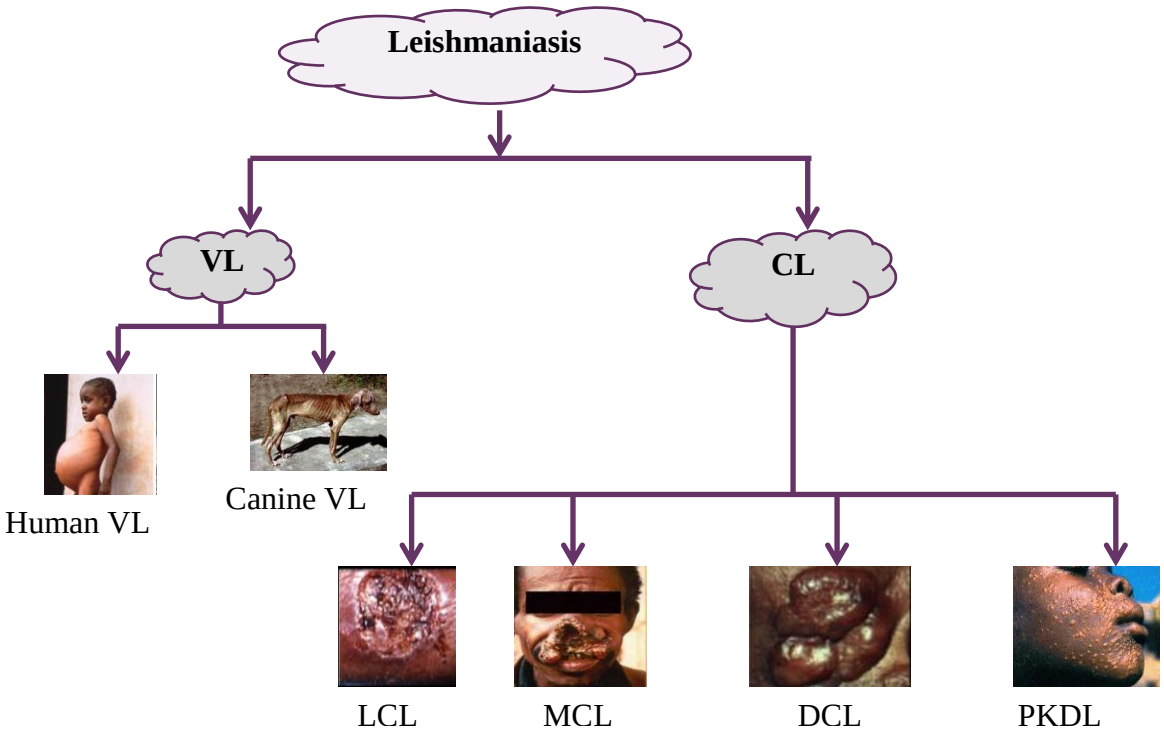


Figure 1.1 Summary of the different types of leishmaniasis. LCL and DCL caused by infection with *L. major*, *L. tropica*, *L. mexicana* complex and *L. braziliensis*. *Leishmania* species of the *Viannia* subgenus caused MCL. *L. chagasi*, *L. infantum* and *L. donovani* caused VL, PKDL. Canine VL caused by infecting dog with *L. infantum*.

In localized cutaneous leishmaniasis (LCL), lesions typically contain organized dermal granulomas with a prominent infiltration of lymphocytes and epithelioid cells, in which tissue destruction is necessary for effective parasite clearance. The ulceration and vigorous immune responses are linked to effective healing, while in diffuse cutaneous leishmaniasis (DCL), the skin pathology is more inert and parasites are not eradicated (Nylen and Eidsmo, 2012). Lesions are often chronic and do not respond to treatment

with anti leishmaniasis drugs or steroids (David and Craft, 2009). The skin lesions may appear weeks to months after infection and the lesions can change in size and appearance and often develop raised edges with a central ulcer, which might be covered by a scab. The lesion is usually painless but can be painful when infected with bacteria and some people develop swollen glands near the sores (Ameen, 2010). If untreated, lesions may heal but the healing process can take months, sometimes years, and typically results in scarring (Banuls *et al.*, 2007). *L. major* and *L. tropica* are the predominant species causing CL in the Old World (Figure 1.2A). *L. mexicana* complex and *L. braziliensis* cause CL in in the New World countries (Figure 1.2B, WHO, 2010). Usually, cutaneous leishmaniasis is diagnosed by visualising amastigotes in biopsies, impression smears or scrapings and also *in vitro* growth of promastigotes from a tissue sample (Llambrich *et al.*, 2009).

Mucocutaneous leishmaniasis (MCL) is potentially life threatening and requires treatment (David and Craft, 2009). It characterised by a lesion or a papule that develops into a painless ulcer. The ulcer rarely heals spontaneously but instead metastasizes to various mucocutaneous regions, including the nasal septum, mouth, nasopharynx, and sometimes the genitalia (Goto and Lindoso, 2010). MCL is caused by infection with *Leishmania* species of the *Viannia* subgenus, typically found in the Americas, *L. (V) braziliensis*, *L. (V) amazonensis, L. (V) panamensis, and L. (V) guyanensis* (David and Craft, 2009).

Visceral leishmaniasis can be classified depending on the transmission characteristics into two types. The zoonotic form, with dogs as the main reservoir, occurs in the

Mediterranean basin, China, the Middle East, and South America. This form is caused by *L. infantum.* At the global level, the anthroponotic form where transmission from human to human, is caused by *L donovani* and is prevalent in East Africa, Bangladesh, India, and Nepal (van Griensven and Diro, 2012). Human visceral leishmaniasis (VL), also known as kala-azar, meaning Black fever (McGwire and Satoskar, 2013) has the symptoms such as fever, weight loss, cough, diarrhoea, dizziness, vomiting, bleeding of gums, pains in the limbs and a grossly enlarged abdomen as a result of hepatomegaly and splenomegaly. In the final stages blood is excreted in the urine, and VL is fatal due to haemorrhage or complications related to anaemia or secondary infection (Neuber, 2008). *L. chagasi* is the main cause of VL in Brazil, *L. infantum* in Mediterranean countries and *L. donovani* in Africa and India, Figure 1.2C (WHO, 2010).

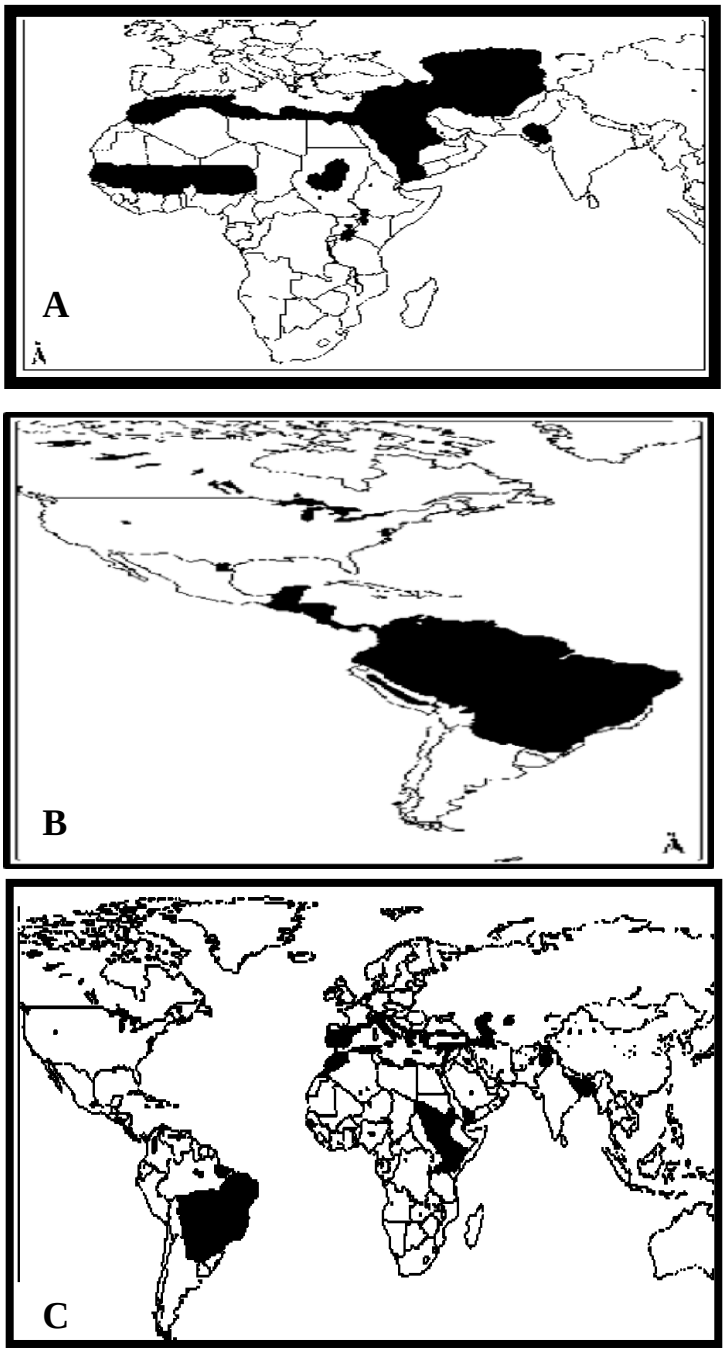


Figure 1.2 Geographical distribution of leishmaniasis (A) Old World cutaneous leishmaniasis due to *L. major*; black area shows distribution of CL. (B) cutaneous and mucocutaneous leishmaniasis in the New World, black area shows distribution of MCL. (C)Geographical distribution of visceral leishmaniasis in the Old and New world, black areas show the distribution of VL (adapted from WHO 2010).

Post kala-azar dermal leishmaniasis is a dermal sequel of VL. Although it is not life-threating, it is considered a public health problem since patients remain reservoirs of the disease (Ganguly *et al.*, 2010). Post kala-azar dermal leishmaniasis (PKDL) is mainly seen in the Indian subcontinent and East Africa (Singh *et al.*, 2011). The disease is mainly caused by *L. donovani* in India and Sudan, with a few cases reported to be caused by *L. infantum*, *L. chagasi*, or *L. tropica* (Singh *et al.*, 2011). The disease begins with hypopigmented macules, papules or nodules on the face and then spreads to other regions of the body (Ganguly *et al.* 2010). Clinically a nodular rash, in people who are otherwise well and recovering is observed, although more serious signs of facial ulcers can occur (Adams *et al.*, 2013).

Visceral leishmaniasis and human immunodeficiency virus co-infection (VL-HIV) is a public health problem. Over 34 countries have reported cases of VL-HIV and the number is increasing. Individuals co-infected with HIV develop atypical symptoms, and increased severity of VL symptoms (Hurissa *et al.*, 2010). This is feasibly due to the dysregulation effects of the virus or parasite on the immune system of the host (Okwor and Uzonna, 2013).

Canine visceral leishmaniasis (CVL) is present in approximately 50 countries, mainly in South America and the Mediterranean region. CVL infection rate depends on several factors including the length of the transmission season, the vector density and the susceptibility of the dog population (Dantas-Torres *et al.*, 2012). Infected dogs are the

primary reservoir for zoonotic VL in endemic regions, and are the most significant risk factor for infecting humans. Dogs have a wide range of clinical presentations due to infection with *L. infantum*, ranging from asymptomatic to a fatal disease (Petersen, 2009).

The life cycle of *Leishmania* occurs in two hosts and is shown in Figure 1.3. The life cycle involves an extracellular flagellated promastigote in the alimentary tract of its insect vector and an intracellular amastigote within a vertebrate host (Banuls *et al.*, 2007). The motile promastigote measures 15-20 μm in length and has a large central nucleus and kinetoplast (Figure 1.4). The parasites initially attach to the epithelial cells lining the midgut of the sandfly and multiply as an avirulent form. In a process called metacyclogenesis, the promastigotes eventually stop dividing, detach from the epithelial cells, and migrate to the mouthparts of the insect and develop into the virulent metacyclic promastigote form (Barbieri, 2006). Transformation from the procyclic to metacyclic promastigote is associated with specific changes that enable the parasite to enter and survive successfully within the mammalian host e.g. upregulation of the glycolipid lipophosphoglycan (LPG) and the glycoprotein (gp63) on its surface, and changes in its enzyme content (Dominguez *et al.,* 2003). Metacyclic promastigotes are deposited into human skin or another suitable vertebrate when a female sandfly feeds. Promastigotes activate the complement system resulting in opsonisation by C3b and C3bi, which allows parasite uptake via the macrophage type 1 complement receptor (CR1) and/or type 3 complement receptor (CR3). By entering macrophages via CR1 and/or CR3, the promastigotes do not to trigger a respiratory burst (Dominguez *et al.,* 2003; Sibley, 2011). LPG and gp63 protect the parasite from complement-mediated lysis by shedding the

membrane attack complex (MAC) from the parasite's surface membrane (Mukhopadhyay, 2005; Moradin and Descoteaux, 2012). Upon entry into the phagolysosome of the macrophage, promastigotes differentiate into the non-flagellated amastigote (Figure 1.5). Amastigotes are non-motile, round or oval in shape, with a diameter of 2-5μm and have a large nucleus and kinetoplast. They are colourless and have a homogenous cytoplasm. In infected macrophages, amastigotes are present in acidic parasitophorous vacuoles that exhibit most of the features of phagolysosomes including the presence of lysosome markers such as lysosomal-associated membrane protein, LAMP-1 and LAMP-2 (Courret *et al.*, 2002; Dermine *et al.*, 2005). Activation of phagocytic cells causes a respiratory burst which results in the production of antimicrobial agents e.g. reactive oxygen species (ROS) such as superoxide, hydrogen peroxide and reactive nitrogen species (RNS) such as nitric oxide, which are toxic to *Leishmania* amastigotes (Birnbaum and Craft, 2011). *Leishmania* have evolved several mechanisms to avoid degradation in the hostile parasitophorous vacuoles (Duclos and Desjardins, 2000; Van Assche *et al.*, 2011). The amastigotes proliferate within this usually hostile environment until the infected macrophage lyses, releasing amastigotes into the surrounding environment, where they can infect other macrophages. *Leishmania* infected macrophages are taken up when a sandfly takes a blood meal from an infected host and parasites are released into the fly's gut during digestion. LPG and gp63 on the surface of procyclic promastigotes protect them from the hydrolytic enzymes within the sandfly gut. LPG also aids in attachment to the insect gut epithelium (Dostalova and Volf, 2012).

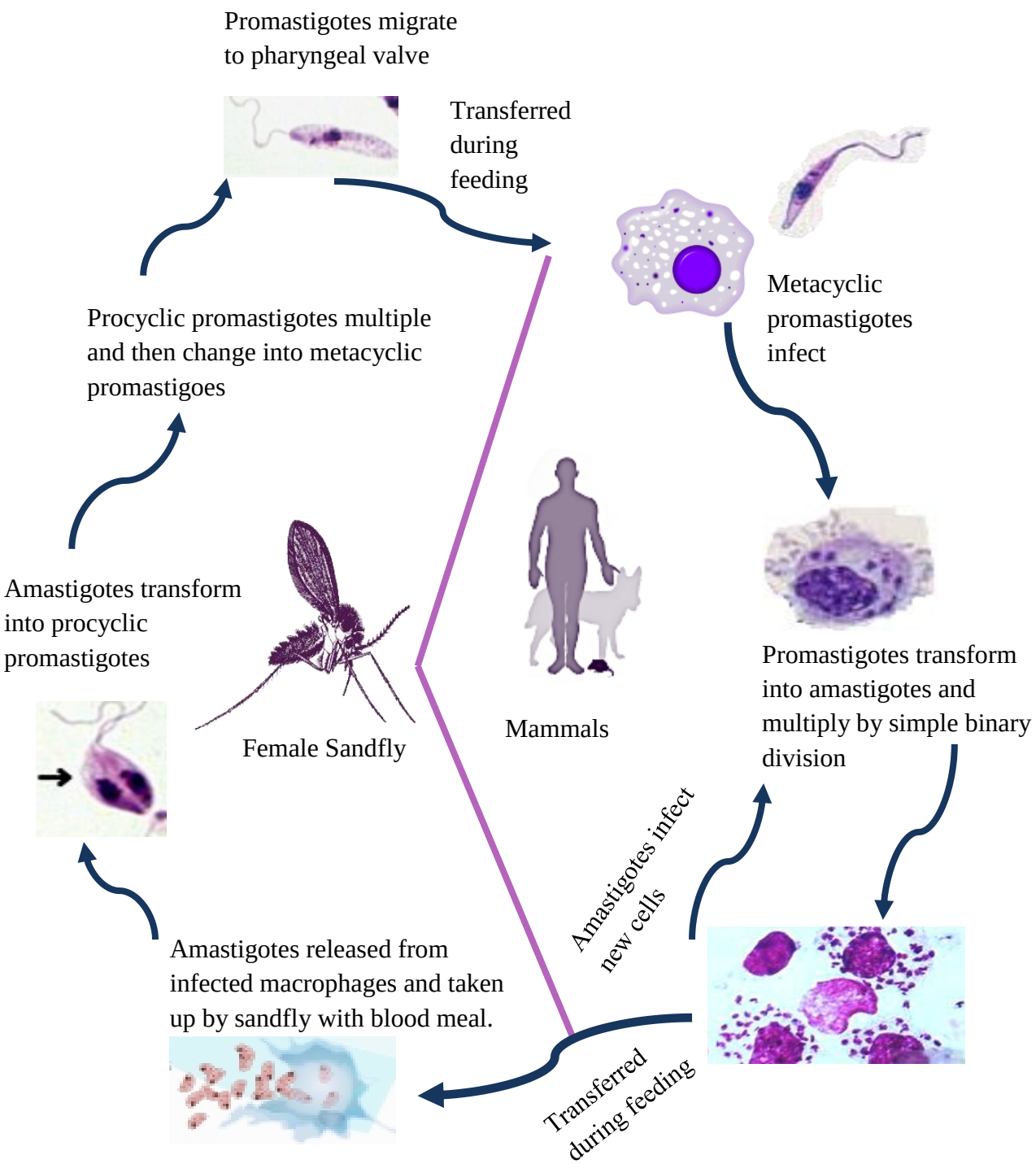


Figure 1.3 Diagram of the life cycle of *Leishmania* (adapted from Banuls *et al*., 2007).

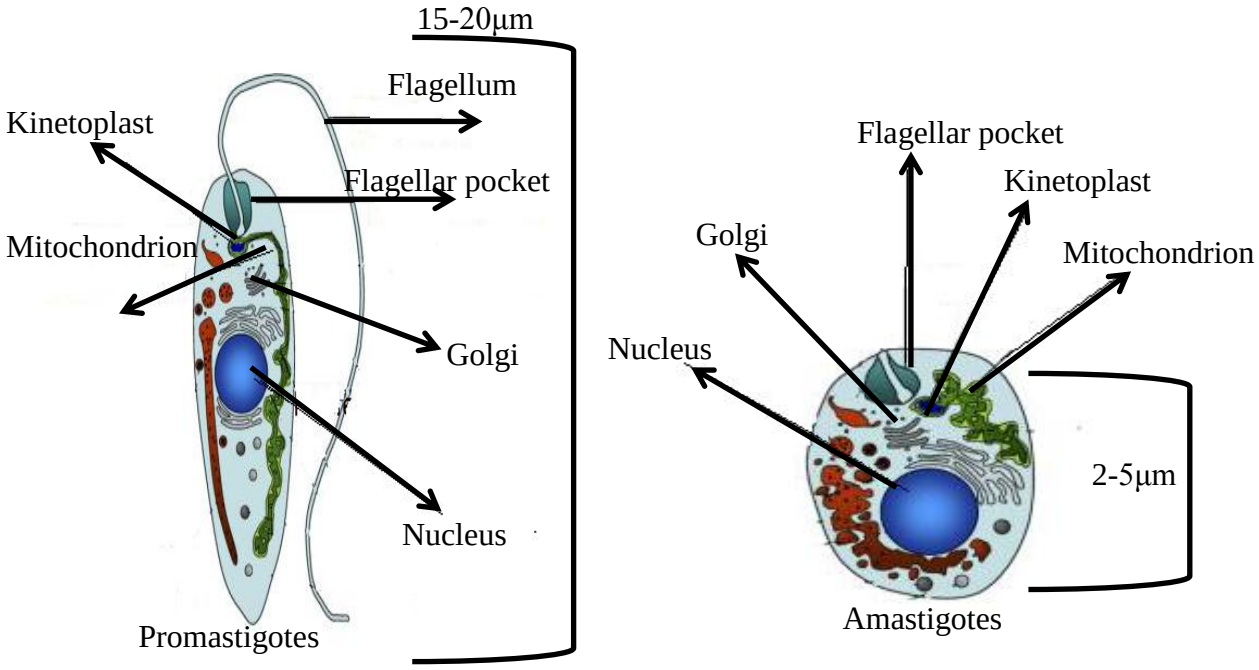


Figure 1.4 Diagram showing the shape of *Leishmania* promastigotes and amastigotes (adapted from Besteiro *et al.*, 2007).

1.2 The Immune response to *Leishmania* infection

The immune response against *Leishmania* infection is mediated by innate immunity and adaptive immunity (Kedzierski, 2010).

The role of innate immune cells in the skin in leishmaniasis is summarized in Table 1.1. Wounding caused by the sand fly bite induces a local inflammatory response leading to the recruitment of neutrophils and monocytes (Mougneau *et al.*, 2011). Activation of phagocytic cells causes a respiratory burst, which is toxic to *Leishmania* amastigotes (Birnbaum and Craft, 2011). The type of cells that involved in uptake of *Leishmania*

parasite is seen in Figure 1.5. *Leishmania* elimination is dependent on the interaction between neutrophils and macrophages and is associated with TNF-α and superoxide production (Mougneau *et al.*, 2011).

The cellular components of anti-leishmanial immune response are seen in Figure 1.6. Monocytes infiltrate the site of infection and differentiate into dendritic cells (DCs). DCs become infected but fail to become activated, whereas local uninfected DCs upregulate major histocompatibility complex class II. Macrophages are also infected by the parasites. Uninfected DCs may pick up dead parasites or leishmanial antigen and become the critical antigen-presenting cells (APCs). CD4+ T cells are then activated and differentiate into Th1 cells, which produce IFN-γ, and this promotes parasite killing by infected cells and also further promotes the development of Th1 cells. Some CD4+ T cells fail to become Th1 cells, and adopt a central memory T cell phenotype. CD8+ T cells recognizing leishmanial antigens are also activated and also produce IFNγ. Control of the response is largely mediated by the production of IL-10, which can come from several different cell types, including regulatory T cells, Th1 cells, CD8+ cells, NK cells, B cells, macrophages and DCs (review by Kaye and Scott, 2011).

Table 1.1 The role of innate immune cells in the skin in leishmaniasis.

Location	Cell Type	Function	References
Epidermis	Keratinocytes	Sensors of injury and infection	Ehrchen *et al.*, 2010
	Langerhans cells	Antigen presentation and induced Th2.	Pavelka and Roth 2010
Dermis	Neutrophils	The first cell to arrive at the site of *Leishmania* infection. Uptake and destruction of parasite.	Filardy *et al*., 2011
	Macrophages	Antimicrobial activity	Filardy *et al*., 2011
	Mast cells	Regulating inflammatory response by neutrophils	Romao *et al*., 2009
	DC	Antigen presentation cells.	Nautz Neu *et al*., 2012
	NK cells	Early resistance against *Leishmania*	Bogdan 2012
	Monocytes	Inflammatory cell	Sheel and Engwerda, 2012

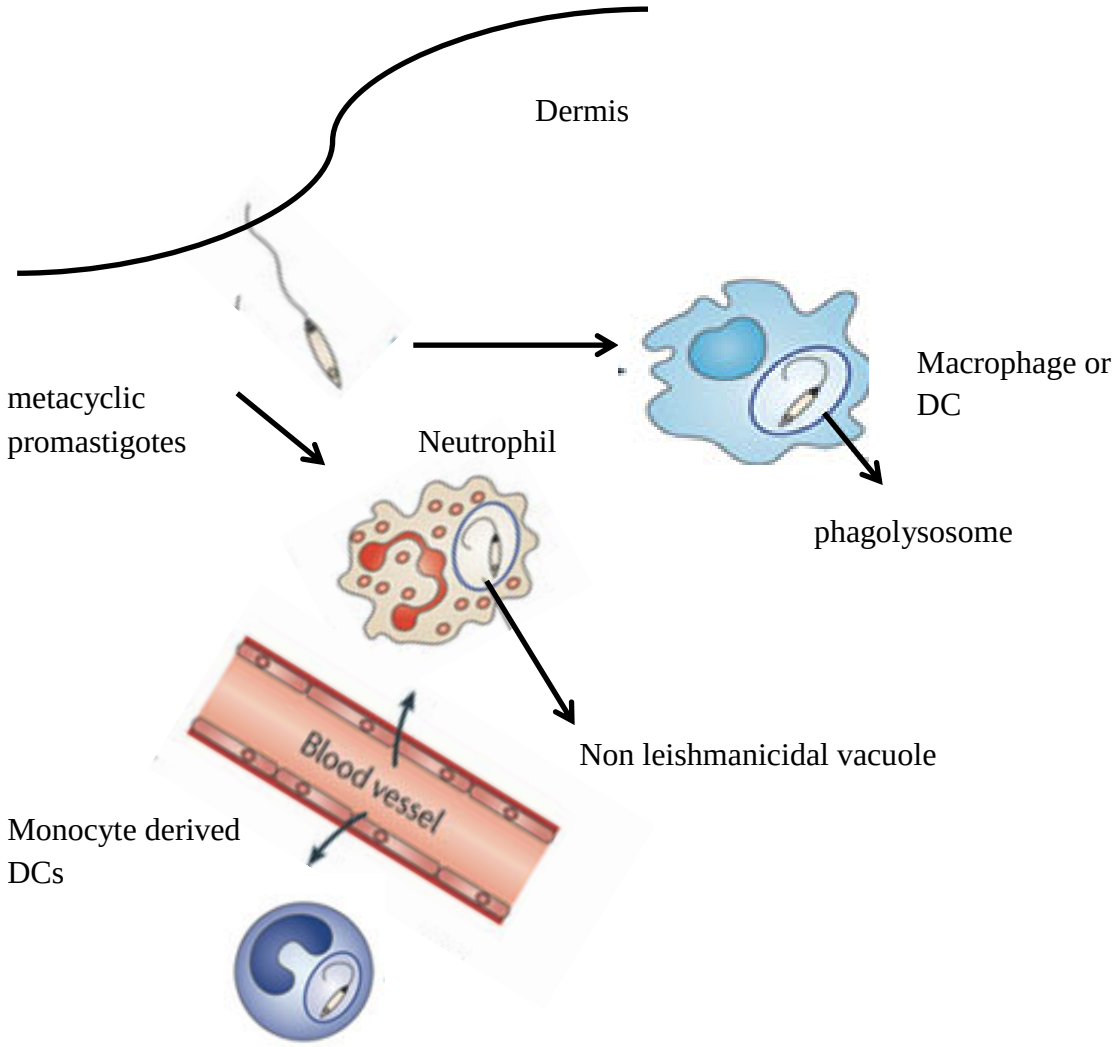


Figure 1.5 The cell types involved in the uptake of *Leishmania* parasites (adapted from Kaye and Scott, 2011).

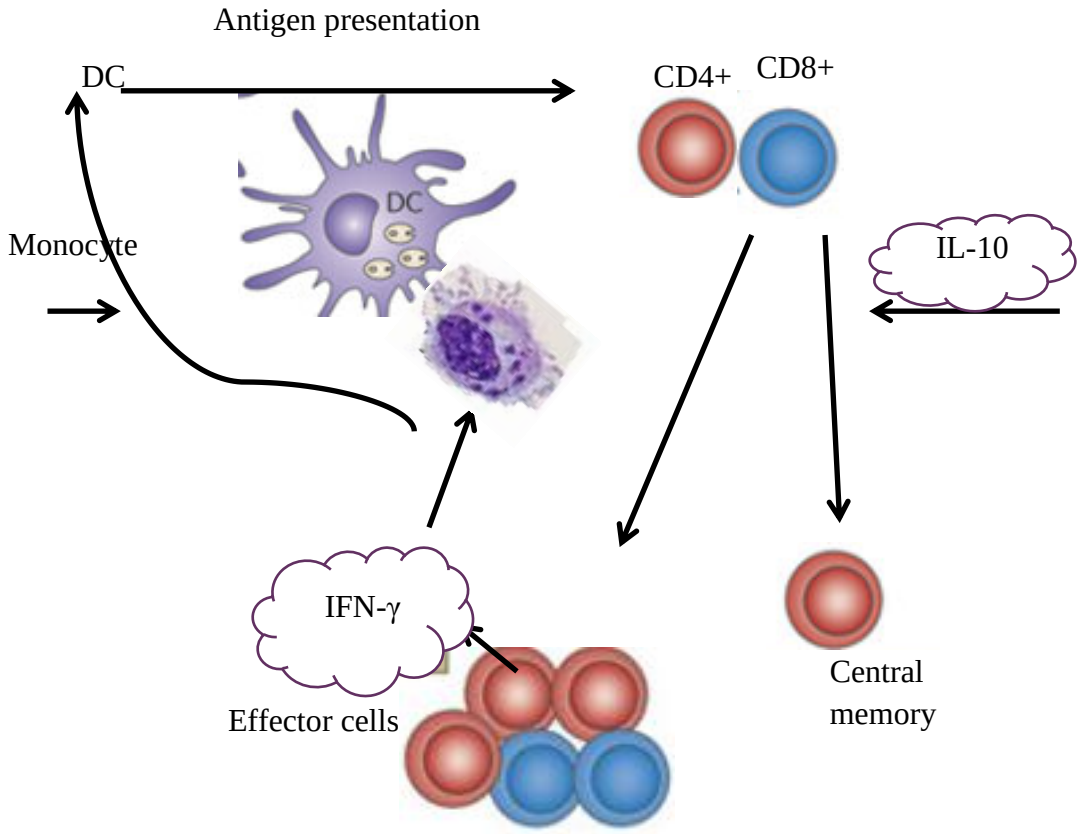


Figure 1.6 The cellular immunity components of the anti-leishmanial response (adapted from Kaye and Scott, 2011).

The presence of promastigotes rapidly induces epithelial cells to secrete cytokines such as IL-12, IL-1β, IL-4, and IL-6. Stimulated macrophages produce IL-12, which then acts on $CD4^+$ T cells through the activation of signal transducer and activator of transcription 4 (STAT 4) pathways, promoting differentiation of antigen-stimulated cells into Th1 cells. IL-12 is also necessary for the production of IFN-γ by NK cells and production of the anti-leishmanial cytokine TNF-α by macrophages (Prajeeth *et al.*, 2011).

A variety of cytokines are induced during *Leishmania* infection and these are summarised in Table 1.2a and 1.2b. IL-10, produced by macrophages, mast cells, and T regulatory cells, preferentially stimulates a Th2 response which suppresses the production of pro-inflammatory mediators such as TNF-α, ROS, IL-12 and RNS by macrophages and dendritic cells (Bogdan, 2012).

IL-10 is believed that has an important role in the immunologic defects in VL (Nyen and Sacks, 2007). IL-10 is a regulatory cytokine that has primarily suppressive effects on immune function, targeting multiple activation and Antigen presentation pathways of macrophages and dendritic cells. Although induction of IL-10 by host cells during chronic infection is considered a homeostatic mechanism to limit the tissue damage caused by excessive inflammation, effective clearance of *Leishmania* can also be compromised. In mice, treatment with anti–IL-10R Ab promotes clinical cure of *L. donovani* infection (review by Kaye and Scott, 2011). The findings suggest that IL-10 producing adaptive T regulatory cells, some of which may coexpress IFN-γ, are important in suppression of anti-leishmanial immunity in human VL. Similar cells have

been shown to be an important source of IL-10 in *L. donovani* infected mice and in C57BL/6 mice infected with a nonhealing strain of *L. major* (Ansar *et al*., 2011).

Antibodies also have a protective role and specific antibodies can bind to *Leishmania* during its extracellular phases and promote promastigote uptake by phagocytic cells (Mougneau *et al.*, 2011). A high antibody titre correlated to parasite burden in blood of patient with VL (Halstead *et al.*, 2010). Antibodies can also exacerbate infection since bound Fc-receptors can deactivate macrophages if the pathogen is internalised through inhibitory Fc receptors (Nylen and Eidsmo, 2012).

Table 1.2a The cytokines associated with immunity to *Leishmania* species.

Cytokine	Sources	Function	References
IL-12	Dendritic cells and macrophages	IL-12 enhances innate NK response to induce differentiation of $CD4^+$ naïve T cells to Th1 cell.	Torti and Feldman, 2007
IL-4	$CD4^+$ T cells, Th2 cells, mast cells	IL-4 can have protective role or exacerbation role.	Alexander and Bryson, 2005, Cummings *et al.*, 2010
IL-5	Th2 cells	Promotes antibody production and IgE class switching by B cells.	Cummings *et al.*, 2010
IL-13	Th2 cells	Promotes antibody production by B cells. Inhibits IL-12 function.	Solbach and Laskay, 2000, Cummings *et al.*, 2010
TNF-α	Produced by antigen stimulated T cells, NK cells and mast cells.	Proinflammatory cytokine Active parasite killing by macrophages	Watts, 2005, Ansari *et al.*, 2006, Nylen and Eidsmo, 2012
IFN-γ	Th1 cells, NK cells, and cytotoxic T lymphocytes	Stimulate the production of Th1 cells, activation of NK cells and can cause direct activation of macrophage.	Ansari *et al.*, 2006, Kima and Soong, 2013,

Table 1.2b The cytokines associated with immunity to *Leishmania* species.

Cytokines	Sources	Function	References
IL-10	Macrophage, mast cell, T regulatory cells	Induce non-healing disease, maintaining latent infection and concomitant immunity.	Alexander and Bryson, 2005, Ansari *et al.*, 2011
IL-18	Macrophage, dendritic cells and Kupffer cells	Induces IL-12 production, promotes disease progression during CL while inhibiting the development of VL BALB/c mice.	Mullen *et al.*, 2006, Bryson *et al.*, 2008 , Moravej *et al.*,2013
IL-21	Produced by antigen presenting cells or T cells	Drive T cell IL-10 secretion	Ansari *et al.*, 2011
IL-23	Dendritic cells	Induce production of IL-17	Mudigonda *et al.*, 2012
IL-33	Th2 cells, Macrophage, NK cells.	It plays opposing roles. Involved Th1/Th2 response	Rostan *et al.*, 2013
IL-6	Endothelial cells, DCs,	Regulating the balance between IL-17 producing Th17 cells and regulatory T cells.	Kimura and Kishimoto, 2010, Goodman *et al.*, 2009
IL-17	T helper 17 cells	Stimulates endothelial cells, neutrophils, macrophage, and epithelial cells to produce IL-1, IL-6, and TNFα.	Bacellar *et al.*, 2009

Figure 1.7 summarises the healing and non-healing immune response in *Leishmania* infection (Alexander and Brombacher, 2012; Nylen and Eidsmo, 2012). Clinical studies have shown that healing of cutaneous leishmaniasis and immunity in *L. major* infection correlates with development of a Th1 immune response and productions of IL-12 and resulted in healing disease. In contrast, susceptibility to infection is characterized by the development of a Th2 immune response and production of IL-4 and IL-13 cytokines (Alexander and Brombacher, 2012; Nylen and Eidsmo, 2012). IL-12 production from infected cells induces NK cell activation and CD4+ T helper 1 differentiation and IFN-γ production. IFN-γ stimulates iNOS expression and NO production in the macrophage, which mediates parasite killing and therefore a healing response. Failure to produce IL-12 or alternatively IL-4/IL-13 production results in unregulated parasite replication within the infected cells facilitated by host cell IL-10 production. IL-10 production by CD4+ CD25+ T regulatory cells can both facilitate non-healing disease as well as maintaining latent infection and concomitant immunity (Alexander and Brombacher, 2012).

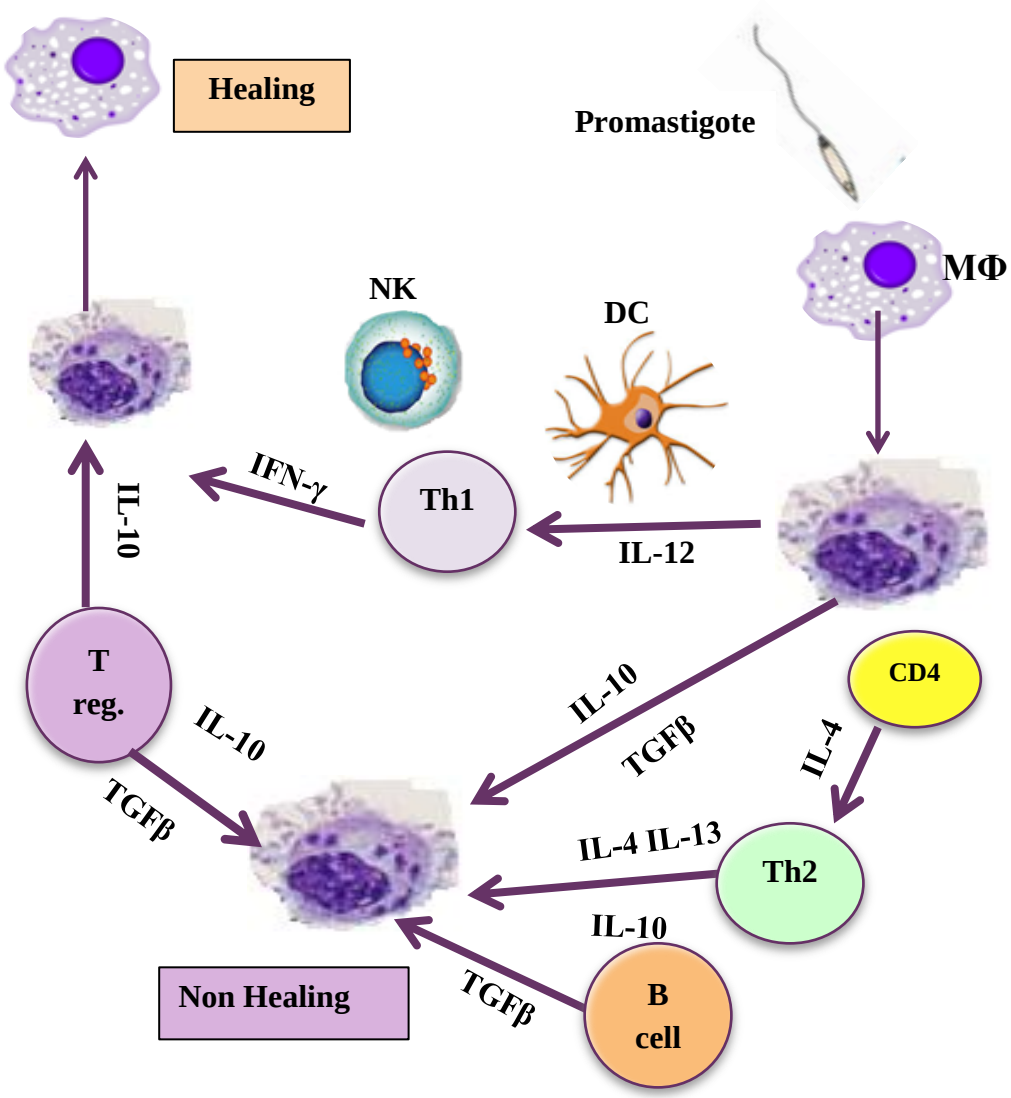


Figure 1.7 Immunological responses to *L. major* during healing and non-healing infection (adapted from Alexander and Bryson, 2005).

Leishmaniasis is a disease caused by different species of *Leishmania*, where each species can induce different immune and/or pathological responses (Kedzierski, 2010). However, protective immunity for both cutaneous leishmaniasis, caused by *L. major*, *L. mexicana* infection and visceral leishmaniasis, caused by *L. donovani* or *L. infantum* infection, is dependent on the development of Th1 immunity and IFN-γ production (Alexander and Brombacher, 2012; Nylen and Eidsmo, 2012).

The immune response to *L. mexicana* is complex and the parasite is highly resistant to neutrophil or macrophage killing. There is evidence that infection with *L. mexicana* suppresses IL-12 production by macrophages and dendritic cells. This suggests that failure to produce IL-12 may limit the Th1 response, resulting in the observed susceptibility to *L. mexicana* (Soong *et al.*, 2012). Studies suggest that during *L. mexicana* infection reduced migration of monocytes and dendritic cells to the draining lymph nodes may result in the insufficient priming of a Th1 response (Petritus *et al.*, 2012).

Clinical studies have shown that human VL has complex immunology characterized by mixed Th1/Th2 cells, where a suppressed Th1 response along with an elevated Th2 is the active disease form. Conversely, protective immunity is achieved by up regulation of a Th1 response after successful chemotherapy treatment (Das and Ali, 2012). Immunity against VL is organ specific and in an acute infection there is minimal damage to the liver. However, resolution of *L. donovani* infection depends on the production of hepatic granulomas, which is initiated and driven by the production

of IL-12 and IFNγ. A hepatic granuloma consists of a core of parasitized Kupffer cells, which are surrounded by monocytes and $CD8^+$ and $CD4^+$ T cells. Within the granuloma, parasites are killed by ROS and RNS. Mice that are unable to produce nitric oxide synthase (iNOS), and thus unable to make nitric oxide, have a reduced ability to attract mononuclear cells early on in infection and a reduced ability to produce mature granulomas. These mice are unable to kill the parasites within Kupffer cells, demonstrating that nitric oxide is an important anti-leishmanial agent (Moore *et al.*, 2013). The use of IL-4 and IL-4 receptor alpha gene deficient mice has demonstrated that both IL-4 and IL-13 are important in controlling the development of hepatic granulomas (McFarlane *et al.*, 2011). Studies with IL-12 gene deficient mice highlighted Th1 cytokines importance in the immune response against *Leishmania.* These mice had significantly increased parasite burdens, reduced inflammatory responses, and impaired hepatic granuloma formation compared to experimental controls. This demonstrated that IL-12 is not only necessary for the resolution of infection but is also responsible for inflammatory pathology (Cummings *et al.*, 2010). During *L donovani* infection both IFN-γ and IL-10 are produced, and it is thought that the balance between the two has a major influence on the outcome of infection. IL-10 gene deficient mice or mice treated with anti-IL-10 receptor antibodies are resistant to *L. donovani*, and in both groups granuloma formulation was accelerated when parasite killing was augmented (Deak *et al.*, 2010).

1.3 Control and treatment of leishmaniasis

Leishmaniasis is the third most important vector borne disease after malaria and African trypanosomiasis (Stockdale and Newton, 2013). The first World Health

Organisation meeting to improve research on prevention, control and management of leishmaniasis was held in 2010. The resulting technical report ‘‘Control of the Leishmaniasis’’(WHO, 2010a) suggested a range of measures which could be used to reduce the incidence of leishmaniasis; control of vector infections, drug treatment and vaccination were their main targets (Stockdale and Newton, 2013).

The control of infection vectors reduces the transmission rates and this can be done by spraying houses with insecticides. Spraying was started in 2005 in India to help reduce the incidence of VL (Picado *et al.*, 2012) but the complex ecology of vectors makes reducing their numbers difficult. Insecticide application generally only has a temporary effect as spraying is difficult to maintain for technical and economic reasons (Otranto and Dantas-Torres, 2013). Insecticide treated bed nets have been used as an alternative (Picado *et al.*, 2012). A study in Bangladesh evaluated the effect of a community-based intervention with insecticide impregnated bed nets. The study showed that this intervention reduced VL by 66.5% (Mondal *et al.*, 2013). However, studies in India and Nepal which tested the effectiveness of large scale distribution of bed nets impregnated with slow release insecticide, showed there was no significant additional protection against VL compared with existing control practices (Picado *et al.*, 2010).

At present, the main method used to control VL is treatment of clinical infections as there is no effective vaccine against leishmaniasis (Santos *et al.*, 2012). The typical treatment of leishmaniasis involves the administration of toxic and poorly tolerated

drugs (Tiuman *et al.*, 2011). Pentavalent antimonials were the first line treatment of leishmaniasis; the main drugs presently used for treatment of leishmaniasis are shown in Table 1.3. The treatment has been unsatisfactory because of drug toxicity, inadequate responses, complicated disease syndromes, and drug resistance (Tiuman *et al.*, 2011). Combination drug treatment would improve treatment and using the course of treatment shorter (Pavli and Maltezou, 2010). However, a recent study showed that *L. donovani* can develop resistance to drug combinations, such as miltefosine /paromomycin or pentavalent antimonials/paromomycin (Garcia-Hernandez *et al.*, 2012). As there are few drugs to treat leishmaniasis, drug resistance is a major problem as its limits the number of drugs, which can be used. Even if a skin lesion responds to treatment the lesion can causes disfigurement and scarring. Therefore, a vaccine to prevent leishmaniasis is the most attractive option.

Table 1.3 Drugs used in treatment of leishmaniasis.

Drug	Route of administration	Dose	Mode of Action	References
Antimonials **e.g. sodium** **stibogluconate**	Intramuscular or intravenous injections	20 mg / kg for 28 days	Inhibition of ADP phosphorylation	Sundar & Chatterjee 2006
Amphotericin B **Liposomal Amphotericin B**	Intravenous Intravenous	0.75-1 mg/ kg for 20 infusions 5 mg/ kg, 4–10 doses over 10–20 days	Impairs permeability of plasma membrane	Sundar & Chatterjee 2006 Van Griensven *et al.*, 2010, Word *et al.*, 2012 Solomon *et al.*, 2013
Paromomycin	Parenteral for VL. Topical for CL.	11mg/ kg /21 days/im Topical up to 20 days.	Induces respiratory dysfunction	Sundar & Chatterjee 2006 Sundar *et al.*, 2007
Pentamidine	Parenteral administration	Intravenous 12 mg/ kg daily for 20 days	Interferes with DNA synthesis.	Amato *et al.*, 2008 Murray *et al.*, 2005
Miltefosine	Oral treatment for VL	100 mg daily for 28 days	Inhibits membrane signalling pathways	Murray *et al.*, 2005 Sundar & Chatterjee 2006, Burza *et al.*, 2013

1.4 Vaccination

A vaccine would be the most effective strategy to control leishmaniasis (Mutiso *et al.*, 2013). The main advantages of vaccines compared to chemotherapy is that they induce long lasting protection and can be administered both in prophylactic and therapeutic modes. In addition the vaccine would be effective against drug resistance parasites (Nagill and Kaur, 2011).

Vaccines can be defined as a special class of drugs, which do not have a direct effect on a pathogen. The protective efficacy of a vaccine depends on the induction of appropriate and effective immune responses. Vaccine development is a lengthy process, which includes expensive laboratory experiments to determine their effectiveness and safety (Pappalardo *et al.*, 2010). A successful *Leishmania* vaccine should be safe to use, induce a high level of long-lived efficacy, be cost effective and easy to store and administer. The vaccine should have the ability to stimulate cell mediated immunity against *Leishmania* (Nagill and Kaur, 2011). There are hundreds of potential vaccine candidates but issues regarding cost, antigenic complexity, variability of organisms and the mixed type of responses produced in the host, are limiting progress of vaccine development (Singh and Sundar, 2012). For example, the parasites causing cutaneous disease in the Old World e.g. *L. major* and in the New World e.g. *L. mexicana* or *L. amazonensis,* are genetically different (McMahon-Pratt and Alexander, 2004). There are also differences in virulence factors between species and the type of immune responses induced. For example, LPG is a virulence factor for

L. major (Spath *et al.*, 2000) but not for *L. mexicana* (Ilg *et al.*, 2001). Different types of *Leishmania* vaccine candidates have been tested (Figure 1.8). The main ones are *Leishmania* antigens from whole parasites as live attenuated or killed vaccines, subunit vaccines, recombinant vaccines and DNA vaccines (Nagill and Kaur, 2011). Recently, sandfly salivary components have been suggested as potential vaccine candidates (Collin *et al.*, 2009; Gomes *et al.*, 2012) and a combination vaccine using *Leishmania* antigen and sandfly candidates has been tested (Alvar *et al.*, 2013).

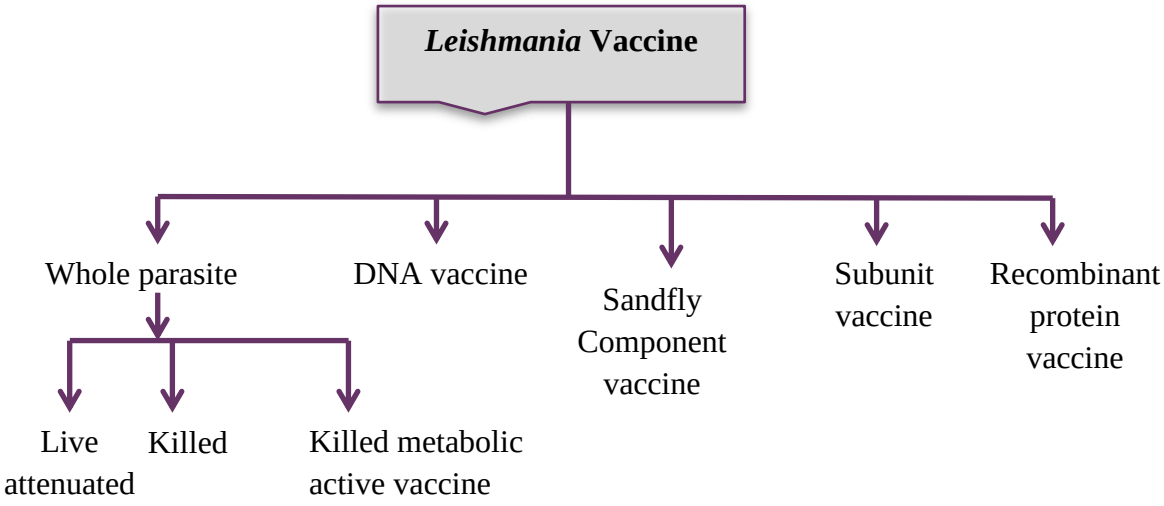


Figure 1.8 Summary of the types of vaccine used against leishmaniasis.

The first vaccine tested against *Leishmania* was 'Leishmanization', where individuals were inoculated with live *Leishmania* parasites to protect against CL (Evans and Kedzierski, 2012). This type of vaccine has been used prophylactically against CL in Kurdistan tribal societies and people in the Middle East. People still vaccinate their babies with CL by transferring infectious material from a lesion to

areas where the lesion would not cause disfigurement. This would then protect the babies against future infection. Live *L. major* promastigotes grown from infected lesion exudates, were used as a prophylactic vaccine in Iran and vaccination caused a decrease in the incidence of CL. A mixture of live virulent *L. major* parasites and killed parasites was used in Uzbekistan (Coler and Reed, 2005; Khamesipour *et al.*, 2006), and in 1937, a live promastigote *L. major* vaccine was used in Russia (Coler and Reed, 2005). Several thousand people were vaccinated with live *L. major* in Turkmenistan (Kellina and Strelkova, 2010). However, this type of vaccine is not suitable as its use is associated with a high risk of parasite latency after cure so its use is not approved because of ethical and safety reasons (Kedzierski, 2011).

An attenuated vaccine is a vaccine which the virulence of a pathogen is reduced so that the vaccine can induce a protective immune response without causing the disease. The parasite can be attenuated by irradiation, chemical mutagenic treatment or through genome alteration (Kobets *et al.*, 2012). Live attenuated parasites can deliver complete antigens to the antigen presenting cells (Kedzierski, 2011). Vaccination using attenuated lines of *L. mexicana* or *L. major* to protect susceptible BALB/c mice from infection with wild-type *L. mexicana* or wild-type *L. major* has been tested. The attenuated parasite was prepared by culturing promastigotes of *L. mexicana* in the presence of gentamicin which resulted in the development of longer promastigotes, although the growth rate remained the same. After 20 passages of *L. mexicana* in complete HOMEM medium supplemented with 20 μg/mL of gentamicin, the parasites showed reduced virulence towards macrophages and mice. The lesion developed slowly in vaccinated mice, and the mean lesion size at 18

weeks after infection was about 500 mm^3 and thereafter declined (Daneshvar *et al.*, 2003).

Centrin, a calcium-binding cytoskeletal protein, is required for duplication of centrosomes. A deficiency in centrin affects parasite viability. A study using centrin null mutants of *L. donovani* ($LdCEN^{-/-}$) showed that these mutants were unable to survive *in vitro* in human macrophages or in animals (Selvapandiyan *et al.*, 2009). Mice immunised with $LdCEN^{-/-}$ parasites cured a challenge with *L. donovani* wild-type parasites by week 10 week of infection, demonstrated by a significant reduction in *L. donovani* parasite burden in the spleen and eradication of parasites in the liver. Protection was associated with a significant increase in Th1 type associated cytokines, IFNγ, IL-2, and TNF-α (Selvapandiyan *et al.*, 2012). An older study used *L. mexicana* parasite lacking cysteine proteinases to vaccinate BALB/c mice. Vaccinated mice exhibited a delay in lesion development when the animals were challenged with virulent parasites (Alexander *et al.*, 1998). However there is always the possibility of the parasite change to the virulent form so studies using killed parasites have been carried out (Reviwed by Mutiso *et al.*, 2013).

A killed vaccine is safe to use and should be low cost. The pathogen may be killed by heat e.g. autoclaving (Nagill *et al.*, 2009), formaldehyde (Mutiso *et al.*, 2010), or by repeated cycles of freezing and thawing (Okwor *et al.*, 2010). A study in CL, using a killed *Leishmania* vaccine, was carried out in BALB/c mice. The whole killed formalin-fixed *L. major* promastigotes (KLM) in combination with alum, BCG or montanide ISA 720 (MISA) as adjuvants to enhance the immunogenicity of the

vaccine, showed that significant protective type 1 immune responses occurred in mice immunized with BCG and KLM or MISA and KLM. However, adverse reactions did occur following immunization with the BCG vaccine as inflammation (Mutiso *et al.*, 2009). In Ecuador, vaccination with two doses of a vaccine composed of *L. amazonensis* and *L. mexicana* promastigotes mixed with BCG, induced 73% protection in school children (Armijos *et al.*, 1998). A clinical trial using autoclaved *L. major* promastigotes was tested in Iran. The vaccine had passed phase I-II safety and dose-response trials and using BCG as an adjuvant to increase immunogenicity would make it possible for a killed *Leishmania* vaccine with BCG to be incorporated into national vaccination programme in endemic areas (Dowlati *et al.*, 1996; Modabber, 2010). This vaccine should be easy to produce at a low cost in endemic countries; however, standardization of cultured parasite-derived vaccines is a drawback in the way to their registration. In general, the whole-cell, killed vaccines have been rather poorly defined and variable in potency, hence they have rendered inconclusive results (Evans and Kedzierski, 2012).

A new type of killed vaccine that is being used is killed but metabolically active (KBMA) vaccine, where *L. infantum* and *L. chagasi* promastigotes are treated with the psoralen compound amotosalen, and low doses of UV radiation. The KBMA technology is a potentially safe and effective vaccine strategy that protected mice against the intracellular protozoan via subcutaneous vaccination (Bruhn *et al.*, 2012).

Wolff and co-workers established the feasibility of a nucleic acid or DNA vaccine to protect against infection in 1990. In this case the gene encoding for the target protein is cloned into a mammalian expression vector, and the purified DNA vaccine is directly injected intradermal or intramuscular (Coban *et al.*, 2011). DNA vaccines can have single or multiple gene sequences where plasmids that encode for various antigens are used. These vaccines are extremely safe as they do not contain pathogens that could revert to virulent forms (Kobets *et al.*, 2012). Once a DNA vaccine is delivered to its target cell, the plasmid-encoded antigen is translated and subsequently degraded in the cytoplasm of the host cell. The degraded fragments are processed for antigen presentation and displayed in conjunction with MHC class-1 molecules to antigen specific $CD8^+$ T cells. In some cases, the antigen may become secreted instead of degraded, and this allows the antigen to be taken up by antigen presenting cells by pinocytosis, and presented via MHC class-II. Cells can also die as a result of DNA transfection and once the dead cell is phagocytosed, and depending on the phagocytosing cell, the antigen may be processed and presented to $CD4^+$ T cells via MHC class-II molecules or undergo a process of cross-presentation via MHC class-I (Coban *et al.*, 2013). There have been several studies conducted using DNA vaccines against *Leishmania* For example, a plasmid vaccine containing gene sequences for *L. major* gp63 protein induced strong Th1 responses as well as significant resistance to *L. major* infection in BALB/c mice (Xu and Liew, 1995). A recent study found that higher protection was achieved using *L. mexicana* gp63 cDNA compared to soluble *Leishmania* antigen (SLA), and protection was associated with a higher Th1 immune response (Rezvan *et al.*, 2011). Carter *et al.* (2007) found that intramuscular DNA vaccination using the gene sequences for *L.*

donovani gamma glutamyl cysteine synthetase gave protection against *L. donovani* in BALB/c mice. Combination immunisation has been tested to improve vaccine efficiency. This DNA cocktail vaccine which contained plasmids encoding for *Leishmania* homologue of receptor for Activated C Kinase (LACK) and thiol specific antioxidant (TSA) from *L. major*, was tested in BALB/c mice. The cocktail DNA vaccine increased the cellular immune response against *Leishmania* and induced protection against infection (Ghaffarifar *et al.*, 2013). Despite efficiency in animal studies the immune responses measured in humans in preclinical trials were not considered to be sufficient to induce strong protection (Bins *et al.*, 2013).

Subunit vaccine studies have focused primarily on using protein antigens as they are easy to identify and isolate (Mutiso *et al.*, 2013). In Brazil the first purified *Leishmania* subfraction vaccine, purified *L. donovani* promastigote glycoprotein fraction, was named Fructose mannose ligand (FML) and its efficacy with the adjuvant *Quillaja saponaria* (saponin) was tested. This vaccine passed Phase I–III trials and is licensed as Leishmune® vaccine for vaccination against canine VL. Protection induced by the FML-*Quillaja* saponin vaccine lasted up to 3.5 years after vaccination and it induced strong protective response against canine kala-azar in field studies (Saraiva *et al.*, 2006). In 2011, the first European canine *Leishmania* vaccine CanLeish® was approved. It contains proteins excreted secreted proteins from *L. infantum* and the dominant antigen is the promastigote surface antigen (Day, 2011). However this vaccine has not been tested in humans.

The development of vaccine using whole sand fly saliva or components of saliva was tested (Kobets *et al*., 2012). The reason for the ability of saliva, to induce an immune response is that immunomodulatory molecules in the saliva when deposited with parasites into skin, induce species-specific humoral and cellular response in the host (Drahota *et al.,* 2009). The exposed mice, long or short term to sand fly bites, followed by *L. major* infection, either immediately or after a sand fly-free period was studied. They observed that protection against leishmaniasis is limited to short-term exposure to sand flies immediately before infection (Rohousova *et al.*, 2011). Saliva components were isolated from sand flies and their prophylactic ability has been tested. Vaccination with sand fly salivary protein maxadilan partially protected CBA mice from *L. major* infection. The protective role of a yellow protein from saliva of *Lutzomyia* sandfly was tested by immunization of C57BL/6 mice with peptide-encoding plasmids and subsequent infection with *L. major* (Xu *et al*., 2011).

Recombinant protein vaccines have a number of advantages. However, the purified vaccine may contain co-purified undesired contaminants, and it may be difficult to obtain sufficient quantities for vaccine programs (Nascimento and Leite, 2012). Varieties of methods are available and most recombinant proteins are produced using *Escherichia coli.* However, expressed proteins may be produced in an insoluble form in inclusion bodies. It is possible to refold protein recovered as a soluble protein form from inclusion bodies but the method involved is time consuming, and sometimes refolding to an active protein is not possible (Chan *et al.*, 2010).

Basically, any parasite protein might function as an antigen regardless of its location in the parasite as T cells can recognize cytosolic proteins if they are presented with MHC class I or MHC class II molecules on the surface of antigen-presenting cells. Therefore *Leishmania* vaccines consisting of recombinant proteins or poly-proteins have been tested (Mutiso *et al.*, 2013). Various *Leishmania* recombinant proteins have been produced for vaccination, For example, surface expressed glycoprotein leishmaniolysin (gp63) (Olobo *et al.*, 1995, Mazumder *et al.*, 2011), *Leishmania* activated C kinase (LACK) (Hugentobler *et al.*, 2012), *Leishmania* derived recombinant polyprotein (Leish-111f) (Skeiky *et al.*, 2002; Coler *et al.*, 2007; Trigo *et al.*, 2010) and recombinant *L. donovani* γGCS protein (Henriquez *et al.*, 2010). Various studies have shown that recombinant proteins can protect against leishmaniasis in animal models (Kobets *et al.*, 2012). The surface expressed glycoprotein leishmaniolysin, gp63, was tested against *L. major* (Abdelhak *et al.*, 1995; Olobo *et al.*, 1995; Jaafari *et al.*, 2006; Olivier *et al.*, 2012), *L. mexicana* (Connell *et al.*, 1993) and *L. donovani* infection. It induced protection against *L. donovani* in BALB/c mice and significantly reduced the parasite load in immunized animals compared to infected controls (Sachdeva *et al.*, 2009; Kaur *et al.*, 2011, Hezarjaribi *et al.*, 2013). Immunization against *L. major* infection using LACK recombinant protein delayed footpad swelling and induced partial protection against *L. major* infection (Hugentobler *et al.*, 2012) but failed to protect against experimental VL (reviewed by Duthie *et al.*, 2012). Cells from BALB/c mice vaccinated with parasite ribosomal proteins purified from *L. infantum* with saponin, produced significant amounts of IFN-γ, IL-12 after *in vitro* stimulation with *L. infantum* ribosomal proteins. The vaccine also induced a significant reduction in the liver and

spleen parasite burdens in vaccinated mice challenged with *L. chagasi*. After *L. amazonensis* challenge, vaccinated mice showed a decrease in dermal pathology and a reduction in the parasite load in the footpad and spleen (Chavez-Fumagalli *et al.*, 2010). Another example of a recombinant protein vaccine used against leishmaniasis is polyprotein Leish-111f, which is composed of three proteins, thiol-specific antioxidant (TSA), *L. major* stress-inducible protein 1 (LmSTI1), and *Leishmania* elongation initiation factor (LeIF). Immunization trials in mice demonstrated that Leish-111f was able to protect BALB/c mice against *L. major* and *L. amazonensis* infection (Skeiky *et al.*, 2002) and to induce partial protection against VL in C57BL/6 mice and golden Syrian hamsters (Coler *et al.*, 2007). The first clinical trial using a recombinant vaccine was against CL in healthy Colombian adult volunteers who had no history of *Leishmania* infection. People were vaccinated with three injections of LEISH-F1 MPL-SE vaccine, which is composed of Leish-111f antigen with MPL-SE adjuvant. Clinical studies showed that the LEISH-F1 with MPL-SE vaccine was safe and immunogenic in healthy subjects (Sachdeva *et al.*, 2009). In a subsequent trial targeting prevention of VL, healthy Indian adult volunteers were evaluated for indication of previous infection with *L. donovani* based on the direct agglutination test. This clinical trial shows that the LEISH-F1 with MPL-SE vaccine was safe and immunogenic in healthy subjects with or without a history of previous infection with *L. donovani* (Chakravarty *et al.*, 2011).

Most vaccines using purified or recombinant subunit vaccines are poorly immunogenic and require additional components to help boost immune responses, and adjuvants have been shown to increase the immunogenicity of vaccine antigens. They enhance T and B cell responses by having a direct effect on lymphocytes (Mutiso *et*

al., 2010). Adjuvants can create an antigenic reservoir for slow clearance of the antigen from the body, facilitate targeting of the antigen to immune cells, enhance phagocytosis and modulate and enhance the type of immune response induced by the antigen alone (Wilson-Welder *et al.*, 2009). The main properties required for an adjuvant is that it is non-toxic or has a negligible toxicity at the used dose, it is non-mutagenic, non-carcinogenic, non-teratogenic, non-pyrogenic, can stimulate a strong humoral and cellular immune response, can induce a good immunological memory or long-term immunity, and is stable under a broad range of storage times, temperature, and pH (Reed *et al.*, 2009). Currently licensed adjuvants include Alum (aluminum hydroxide or phosphate), MF59 which consists of <250 nm droplets of two surfactants, polyoxyethylene sorbitan monooleate and sorbitan trioleate, and AS04 which contains aluminum and the bacterial lipid, monophosporyl lipid A (Pignon *et al.*, 2008). Common adjuvants that have been used experimentally with *Leishmania* vaccines include IL-12, granuolocyte macrophage-colony stimulating factor (GM-CSF), Bacille Calmette Guerin (BCG), monophosphoryl lipid A, aluminium salts, liposomes (Mutiso *et al.*, 2010) and non ionic surfactant vesicles (NIVs) (Henriquez *et al.*, 2010). NIVs are formed from a non ionic surfactant and cholesterol (Kazi *et al.*, 2010). The constituents form closed bilayer structures when agitated or heated, separating the hydrophobic parts of the molecule from the external solution. These closed vesicles structures are analogous to liposomes and can be small unilamellar or large multilamellar structures (Kumar, 2011). NIV are produced by similar techniques to liposomes and have the distinct advantage in they are stable in air and do not require special storage or handling conditions (Junyaprasert *et al.*, 2008).

Despite the wide range of studies carried out over a prolonged period of time, there is no reliable vaccine against leishmaniasis at the present moment (Nagill and Kaur, 2011; Mutiso *et al.*, 2013).

1.5 The route of vaccine administration

In order to be used in a vaccine programme, a vaccine must be efficient, easy to administer and stable. Studies have shown that vaccination reduces disease transmission in most of the World's countries (Kristensen and Chen, 2013). Most of the approved vaccines are given by one of five main administration routes. The most common route is intramuscular injection; a route used for hepatitis A and B, rabies, influenza and diphtheria–tetanus–pertussis-based combination vaccines. Subcutaneous injection is used for vaccination against measles, mumps and rubella, and yellow fever and intradermal injection is used for BCG vaccination. Intranasal inoculation is used for the live attenuated influenza vaccine and oral vaccination is used for poliomyelitis, cholera, rotavirus and typhoid fever (Lambert and Laurent, 2008). Invasive routes such as subcutaneous injection and intramuscular injection are used to ensure that a sufficiently high bioavailability of vaccine is administered. However, they can create a potential public health problem as it increases the risk of transmission of blood borne pathogens, such as hepatitis B or HIV. Annually it is an estimated 3 million healthcare workers over the World are infected with hepatitis C virus, hepatitis B virus or HIV by accidental injection with a contaminated needle (Pruss-Ustun *et al.*, 2005). These risks can be reduced using proper disposal of apparatus or safer devices, which prevent needle re-use or completely eliminated if a non-invasive administration routes is used instead (Sullivan *et al.*, 2006). Non-invasive vaccine treatment is an efficient and cost effective immunisation method against most diseases. This route would also eliminate

the requirement of using trained personnel and sterile equipment for dosing and give better patient compliance, especially in children (Gupta, 2004; Giudice and Campbell, 2006). Non-invasive vaccination can involve transcutaneous immunization or mucosal immunization. To date, mucosal delivery is the only non invasive immunisation that has been used frequently or effectively (Giudice and Campbell, 2006).

Pulmonary vaccination has a considerable potential as a route of vaccine administration (Sullivan *et al.*, 2006). It offers several advantages over parenteral and other mucosal routes of immunization. The large surface area of the mucosa in the lung, about 140 m^2 in humans, allows rapid and efficient uptake of the delivered agent (Vujanic *et al.*, 2012). That could facilitate efficient systemic delivery of antigens and induce protective immunity. Pulmonary administrations should allow rapid and efficient immunization of mass populations during pandemics (Sou *et al.*, 2011), as inhalation devices such as nebuliser or personal inhalers are routinely used. Moreover, the pulmonary route allows 10–200 times greater bioavailability compared to other non-invasive routes (Patton and Byron, 2007). In the lung the enzymatic activities are much lower than that associated with the oral route, and therefore the metabolism of peptides and proteins in the lungs is relatively mild. However, the epithelial lining fluid, epithelial cell layer and endothelial membrane of capillaries form major barriers to the transport of protein through the alveoli to the bloodstream. But macrophages in the lung can also take up and degrade the therapeutic peptides and proteins and so local responses may facilitate systemic immunity as antigen presentation could occur locally in systemic sites e.g. spleen or lymph nodes (Patton and Byron, 2007).

Pulmonary vaccination induces mucosal and systemic immunity against an invading pathogen. Many pathogens infect the host via mucosal membranes so this route of vaccination provides more local and direct protection at the site of infection (Holmgren and Czerkinsky, 2005). The IgG and mucosal IgA, which mediate immune protection, are produced in the mucosal area (Meitin *et al.,* 1994). Mucosal IgA inhibits surface colonization of microorganisms and reduces penetration of potentially dangerous exogenous proteins (Brandtzaeg, 2010). It provides a first line of defence that reduces the need for elimination of penetrating exogenous antigens by proinflammatory systemic immunity. Pulmonary vaccination can stimulate pulmonary dendritic cells, which, once activated, can transport antigens via the draining lymphatics to the local lymph nodes where they instruct naive T and B cells (Bivas-Benita *et al.,* 2005). It is the effective priming and mobilization of antigen specific T and B cells that ultimately gives rise to immunity. T-cell and B-cell activity was shown to also present in inducible bronchus-associated lymphoid tissue within the lung (Bivas-Benita *et al.*, 2005).

Pulmonary vaccines can be administered as liquids or dry powder aerosols. In the 1950s, the first pulmonary vaccine was tested in poultry to protect against influenza. Since then, many different types of pulmonary vaccines have been tested in other models including mice, rats, guinea pigs, and macaques and humans (Tonnis *et al.*, 2012). Clinical studies using formulation for the pulmonary route that it could be used as an alternative to needle-based delivery of hepatitis B vaccine, with increased

immune response after pulmonary administration (Thomas *et al.*, 2008) with higher systemic, local, and cellular immune responses than a vaccine administered by the nasal route (Minne *et al.*, 2007).

1.6 Gamma glutamylcysteine synthetase

Gamma glutamylcysteine synthetase (L-glutamate, L-cysteine, Gamma ligase, γGCS) catalyses the rate limiting step of glutathione biosynthesis, and its activity is controlled by non allosteric feedback by glutathione, the availability of cysteine, and factors which control the transcription and post translational processing of the enzyme (Lu, 2009). In mammals, glutathione (L-gamma-glutamyl-L-cysteinylglycine, GSH) is a tripeptide thiol. It is an essential component for cells, and it performs a number of vital functions such as maintaining intracellular redox balance and protecting against potential toxic agents which cause chemical or oxidative stress (Manta *et al.*, 2013). Early studies established that when GSH synthesis was inhibited this to a significant apoptotic cell death in multiple tissues. The use of gamma glutamylcysteine synthetase knockout mice resulted in the embryonic mice dying at day 8 of foetal growth. Therefore, maintenance of GSH level appears to be an important mediator of signal transduction in cell survival (Dalton *et al.*, 2000; Hamilton *et al.*, 2003, Dalton *et al.*, 2004).

Trypanothione (TSH) and trypanothione reductase are important parts of the *Leishmania* antioxidant defence system, whereas mammalian cells depend on GSH and glutathione reductase to control their intracellular thiol redox state. The oxidized cell components can be efficiently reduced by GSH, forming oxidized glutathione

disulphide. However *Leishmania* its redox metabolism relies on the GSH conjugate sperimidine is TSH as shown in Figure 1.9 (Van Assche *et al.*, 2011). It is a major regulatory mechanism operating in the trypanothione biosynthetic pathway of these parasites (Olin-Sandoval *et al.*, 2012). γGCS enzyme is the first step in the biosynthesis of GSH and thereby TSH by catalysing the ATP dependent ligation of L-cysteine and L-glutamate to produce gamma glutamylcysteine and TSH. Trypanosomatids contain varying levels of four major low molecular mass thiols: GSH, mono-glutathionyl spermidine, TSH and ovothiol A. The type and amount of each molecule depend on the species, life stage and growth phase of parasite. Trypanosomes and *Leishmania* have trypanothione reductase instead of glutathione reductase (Krauth-Siegel and Comini, 2008). A study investigated the importance of γGCS in *Leishmania* by generating the GSH1 null mutants in *L. infantum.* They observed that promastigotes were less able to survive inside activated macrophages and concluded that the γGCS gene of *Leishmania* is protects against oxidants (Mukherjee e*t al*., 2009). It has been concluded that γGCS is a drug target (Frearson *et al*., 2007). Moreover, Carter and her research group concluded that γGCS is essential to the survival of *L. donovani* (Carter *et al.*, 2003; Carter *et al.*, 2005) and that γGCS is a potential vaccine target despite its intracellular location (Carter *et al*., 2007).

Studies have shown that alteration in thiol levels correlates with resistance to antimonial drugs (Decuypere *et al.*, 2005; Mukherjee *et al.*, 2007). This resistance could relate to a change in the expression of γGCS and was identified as an antimony resistance associated gene (Kumar *et al.*, 2012).

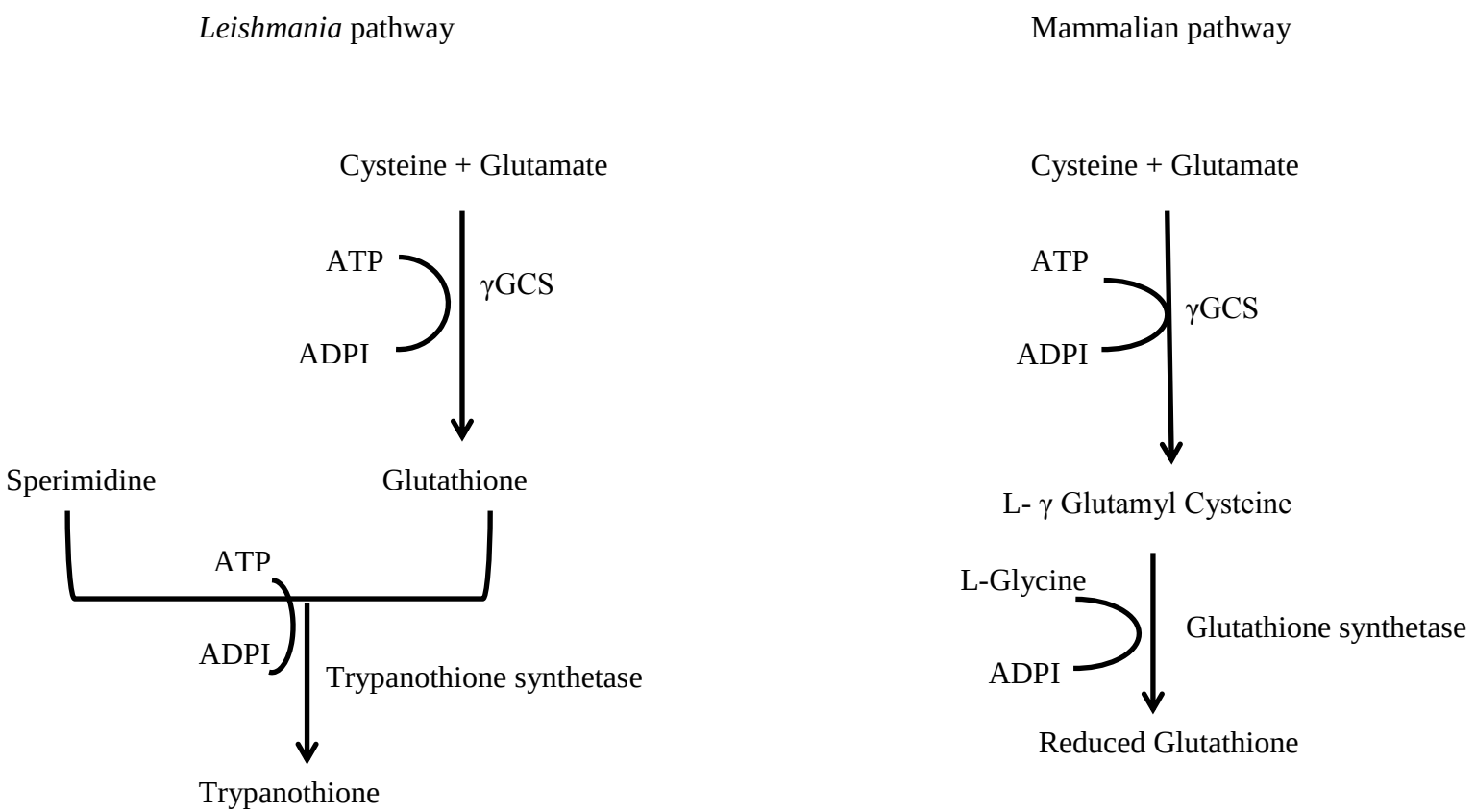


Figure 1.9 The trypanothione and glutathione synthesis pathways (adapted from Van Assche *et al.*, 2011).

1.7 L-buthionine sulphoximine

L-buthionine sulphoximine, *S*-(3-amino-3-carboxypropyl)-*S*-butylsulfoximine; *S*-(*n*-butyl) homocysteine sulfoximine; ($C_8H_{18}N_2O_3S$), Figure 1.10, is a specific irreversible inhibitor of γGCS. It blocks the biosynthesis of GSH as well as the glutathionyl spermidine derivatives. BSO strongly impairs multiplication of *L. donovani* in macrophages (Krauth-Siegel and Comini, 2008). A study also concluded that BSO, which lowers GSH levels, could also cause cytochrome P450 dependent oxidative injuries in cells (Gong *et al.*, 2004).

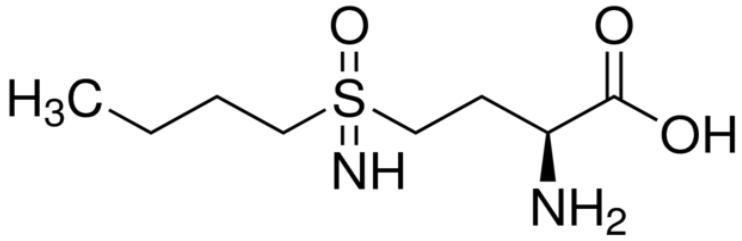


Figure 1.10 The chemical structure of L-buthionine sulphoximine.

BSO acts by inhibiting the reaction of L-glutamate with MgATP to form γ-glutamyl phosphate as an enzyme bound intermediate. Reaction of gamma glutamyl phosphate with the α-amino group of L-cysteine completes the catalytic cycle, where ATP phosphorylates the sulfoximine nitrogen of L-methionine-S-sulfoximine forming the products, MgADP and methionine sulfoximine phosphate which are tightly bound in the active site of the enzyme. This enzyme inhibition is mediated by the initial formation of an enzyme-BSO complex but the inhibitor does not bind covalently to the enzyme (Griffith, 1982).

In 1981, Arrick *et al*. first used BSO to study its effect on thiol metabolism for *Trypanosoma* spices parasites. They observed BSO as an irreversible inhibitor of GSH1 and that trypanocidal activity (Arrick *et al.*, 1981; Krauth-Siegel and Comini, 2008). Also there were studies on *L. donovani, T. brucei* and *T cruzi* parasites that assessed BSO blocks the biosynthesis of GSH (Weldrick *et al.*, 1999; Huynh *et al.*, 2003) (Faundez *et al.*, 2005). Such studies demonstrated that inhibiting the production of GSH in *Trypanosoma* species. by administration of BSO can improve the survival time of mice infected with *T. brucei* and with longer duration treatments reduce *T. brucei* to undetectable blood levels. Also, BSO treatment allows for the administration of lower doses of anti-trypanosomal drugs with the same outcomes as higher doses. As anti-trypanosomal drugs are associated with severe side effects, the ability to lower dosages may improve patient outcomes (reviewed by Morris *et al.*, 2013). *In vitro* studies on *L. donovani* show that BSO has a strong effect on growing amastigotes and depleted more than 95% of GSH and $T(SH)_2$ (Weldrick *et al.*, 1999). BSO strongly inhibit the growth of *Leishmania* parasites in macrophages (Kapoor *et al.*, 2000). The effects of BSO on DNA have been studied. In the rabbit treated subcutaneously with BSO, the level of GSH in heart, brain and liver was significantly reduced but not in the kidney. They found that depletion of GSH due to BSO treatment induced DNA damage in the isolated tissues (Gokce *et al.*, 2009).

In humans, *in vitro* and *in vivo* studies, concluded that BSO is a potent inhibitor of GSH biosynthesis and sensitizes tumour cells for apoptosis by several chemotherapeutic agents in cancer therapy (Lewis-Wambi *et al.*, 2009). In the 1990s clinical phase I trials of BSO at doses resulting in both peripheral and tumor GSH

depletion, showed that BSO can be safely administered to patients with refractory disease (O'Dwyer *et al.*, 1996).

1.8 Luciferase-expressing *Leishmania*

In 1885, French physiologist Raphael Dubois carried out the first study demonstrating luminescence (Reviwed by Lew, 2008). Then Harvey studied several bioluminescence systems and showed that within each system there was specificity between the luciferins and the luciferases (Reviwed by Jing *et al*., 2013). In 1947 McElroy confirmed the results of previous studies. He observed that light produced luminescence, and that ATP had a role in its production (Fraga, 2008).

Bioluminescence naturally occurs in different organisms, such as bacteria, fungi, fish, and insects. It results from the oxidation of a luciferin substrate by luciferase, an enzymatic reaction, which usually requires energy and oxygen (Andreu *et al.*, 2011). This phenomenon is useful in monitoring the course of infection in animals infected with luciferase expressing parasites rather than using animals at each time points to assess parasite burden during the course of the infection (Claes *et al.*, 2009). All animal bioluminescent imaging is widely used, as it has a relatively low cost, high throughput, and relative ease of operation in visualizing a wide variety of *in vivo* cellular events. As the method can be used to continually monitor a single individual it reduces the amount of inter-animal variation and can reduce error, leading (Baker, 2010).

There are different types of luciferase enzymes; For example; firefly luciferase, Renilla luciferase, Gaussia luciferase, Metridia luciferase, Vargula luciferase, and bacterial luciferase. However, the firefly, Renilla, and bacterial luciferases, are the most popular for optical imaging (Close *et al.*, 2011). Firefly luciferase is the best studied (Brogan *et al.*, 2012) and has the advantages of high sensitivity, quantitative correlation between signal strength and cell numbers and low background in animal tissues. However, it requires the addition of exogenous luciferin. Fast consumption of luciferin can lead to an unstable signal and its dependence on ATP and oxygen, currently, cannot makes it practical for large animal models (Close *et al.*, 2011). Firefly luciferase generates bioluminescence in a two-step reaction. The first step involves the converion of luciferin plus ATP to luciferyl adenylate, and the production of pyrophosphate. The second step requires oxygen to enable the forward reaction to occur with the reactant luciferyl adenylate, yielding the products oxyluciferin, adenosine monophosphate (AMP), and light (Figure 1.11). The original chemical reaction without luciferase is extremely slow, but once the enzyme is introduced, the catalysed reaction can be turned into a usable assay, where photon production is measured (Brogan *et al.*, 2012). The reaction is usually measured in terms of total flux per second (Millington *et al.*, 2010).

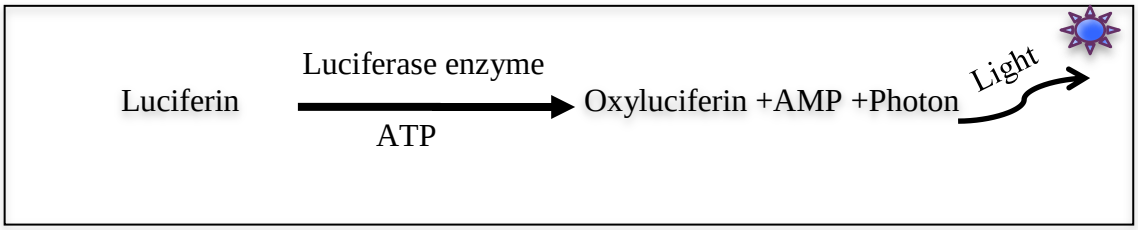


Figure 1.11 The reaction of luciferin with luceriferase enzyme in light production.

The typical method for monitoring leishmaniasis in the mouse model is based on the estimation of parasite loads in target organs such as liver, spleen, or lymph nodes by microscope examination or real time PCR to quantify parasite DNA. However these techniques require large groups of mice to be euthanized (Michel *et al.*, 2011). Therefore real time monitoring methods, using reporter genes encoding firefly luciferase, have been developed for *in vitro* drug screening or *in vivo* determination of parasite infection level (Dube *et al.*, 2009). The reporter gene should be absent from the host; and should not affect the physiology of the parasite cell and should represent a simple, sensitive, and inexpensive assay for quantification of reporter expression (van Rossum *et al.*, 2013). The luciferase reportor gene has been expressed in several parasite species such as *Leishmania* (Ramamoorthy *et al.*, 1996), *Trypanosoma* (Sommer *et al.*, 1992) and *Plasmodium* (Goonewardene *et al*., 1993). Luciferase expressing *Leishmania* are generated by cloning a firefly luciferase-coding region into a suitable *Leishmania* expression vector. The gene mainly can be integrated into 18 s rRNA locus of the nuclear DNA of *Leishmania* (Lang *et al.*, 2005) or introduced as a episomal gene (Ashutosh *et al*., 2005).

1.9 Aims of this study

There is currently a tremendous research effort directed at the production of a vaccine against leishmaniasis. Previous studies have shown that intramuscular immunization with a plasmid containing the gene sequence for *L. donovani* gamma glutamylcysteine synthetase (γGCS) protected BALB/c mice against infection (Carter *et al.,* 2007). More recent studies have shown that immunisation with recombinant γGCS protein gave significant protection against *L. donovani* (Henriquez *et al.,* 2010), *L. major* and *L. mexicana* infection (Campbell *et al.*, 2012). However, in these studies, there was a problem with recombinant γGCS protein expression and purification, as the protein was poorly soluble and truncated forms were obtained after purification. Ideally a *Leishmania* vaccine to protect against all *Leishmania* species is required, which can be given by a non-invasive route. Therefore, the aims of this project are to:

1. Amplify and clone the complete gene of *Leishmania* γGCS from *L. major*, *L. mexicana* and *L. donovani* into pET24-a plasmid to produce C terminal His-tag to get pure full length recombinant protein.
2. Optimize the expression conditions for *Leishmania* γGCS to increase protein yield and produce an active enzyme for *L. major*, *L. mexicana* and *L. donovani*.
3. Compare the importance of γGCS in the survival of all three *Leishmania* species, using luciferase-expressing *Leishmania* and BSO. Compare the effect of BSO inhibition on the enzyme activity of recombinant *Leishmania* γGCS from different *Leishmania* species.

4. Identify the most suitable site of infection for a CL by comparing in footpad versing rump infection in *L. major* and *L. mexicana* infected BALB/c mice.
5. Compare the ability of different recombinant *Leishmania* γGCS formulations to protect against *Leishmania* infection and determine if pulmonary administration is as effective as subcutaneous administration.
6. Determine if protection against infection is associated with a specific type of immune response.

Chapter 2: Materials and Methods

2.1 Materials

Tris, potassium chloride, sodium chloride, EDTA, lithium chloride, luminol (sodium salt), L-broth medium, and, antibiotics (ampicillin, kanamycin, tetracyclines and chloramphenicol), Ponceau S solution and resazurin were supplied by Sigma Aldrich, Irvine, Scotland, UK. RPMI 1640 medium, penicillin-streptomycin, and L-glutamine were obtained from Gibco BRL, Paisley UK. D-luciferin potassium salt was obtained from Caliper Life Sciencee, Massachusetts, USA. Bacterial strains**,** Rosetta blue *E. coli* BL21 and DH5α *E. coli* were supplied by Novagen, London UK. The pET-24a plasmid and Trizol® reagent, Primers, TOPO® TA cloning® kit, PureLink™ Quick Plasmid Miniprep Kit, Platinum® Taq DNA Polymerase High Fidelity and 1 X High Fidelity PCR Buffer reagents for molecular studies were all obtained from Invitrogen, Paisley, UK. Reverse Transcriptase kit obtained from Promega Southampton, UK. SYBR Green, Absolute QPCR, Abgene was supplied by Thermo Scientific Analytical, Hemel Hempstead, UK. Anti-His Tag Mouse monoclonal and polyclonal antibodies and anti-mouse IgG antibody, anti-mouse IgG HRP conjugated secondary antibody, the restriction enzymes *Pvuii, NdeI*, and *Avai, Not1* and *BamH1*, 1 Kb DNA Ladder and 100 bp DNA Ladder were obtained from New England Biolabs, Hitchin, UK. Merck Chemicals Ltd., Nottingham, UK supplied The Perfect protein marker (15-150 kDa). Pharmingen capture and detection anti-cytokines antibodies, cytokines standard and alkaline phosphatase conjugate were obtained from BD Biosciences, Oxford, UK. Horseradish peroxidase (HRP) conjugated goat antimouse IgG1, horseradish peroxidise conjugated goat anti-mouse IgG2a and Mouse anti-Hamster IgG HRP were obtained from Southern Biotechnology Associates Inc, Birmingham, USA. The

QIAprep Spin Miniprep Kit, Qiagen gel extraction kit, T4 DNA Ligase kit were all obtained from Qiagen, Crawley, UK. HisTrap® Column was purchased from GE Healthcare, Amersham, UK and cOmplete™ was obtained from Roche Diagnostics, Burgess Hill, UK. Fisher Scientific, Loughborough UK provided the ProteoSpin Endotoxin Removal Maxi kit, and Pierce LAL Chromogenic Endotoxin Quantitation Kit used in studies. Multipurpose agarose was obtained from Bioline Reagents Ltd. London UK. Bio-Rad protein assay reagent was obtained from Bio-Rad Laboratories Ltd, Hemel Hempstead, Hertfordshire, UK and the Supersignal System®, used in chemiluminescence studies, was obtained from Pierce Chemical, Rockford, USA. IPTG (isopropyl-β-D-thiogalactopyranoside was obtained from Apollo Scientific Ltd., Bredbury, Stockport UK and Prosieve®50 gel solution was obtained from Cambrex Bio Science, Wokingham, Ltd. UK. Nytex was obtained from Tetko Inc, Kansas City, USA. All other reagents were of analytical grade.

2.2 Animals and Parasites

Male and female age matched BALB/c (20-25g) in-house inbred mice from the Strathclyde University were used in these studies. Commercially obtained Golden Syrian hamsters (*Mesocricetus auratus*) were purchased from Harlan Olac, Bicester, UK). Wild-type *L. donovani* (strains 200016), wild-type *L. major* (strain WHOM/IR/-/173) and wild type *L. mexicana* (strain MNYC/BZ/ M379) were used for RNA and genomic DNA extractions. Wild-type *L. donovani* (MHOM/ET/67:LV82) for vaccine studies. Luciferase-expressing strains of *L. donovani* (Ldonluc) were supplied by Dr Carter and *L. mexicana* (strain Lmexluc), and *L. major* (Lmajluc) were supplied by Dr Wiese. Studies were carried out in accordance with local ethical requirements and United Kingdom Home Office approval.

Dr Williams at Strathclyde University generated luciferase-expressing *Leishmania* cell lines LmexWT, LmajWT and LdonWT. These were used for generating transgenic luciferase-expressing *Leishmania* lines using conventional methods and were designated Ldonluc, Lmexluc), and Lmajluc. The integrative construct (a gift from Dr D.F. Smith), designated pGL1313, contained pSSU-int fragments to facilitate integration into the ribosomal DNA locus of *Leishmania* (Misslitz *et al*., 2000). The vector (45μg) was prepared for transfection by enzymatic digestion using the restriction enzymes, *PmeI* and *AseI*. The plasmid cassette was purified using the Qiagen gel extraction kit after separation by DNA electrophoresis. The digested cassette was precipitated with ethanol in a sterile class two cabinet. The precipitated DNA was suspended in sterile water and used to transfect 2.5 x 10^7 *Leishmania* promastigotes in 100 μl of nucleofector solution from the Amaxa Human T cell nucleofector kit (Amaxa AG Cologne, Germany). The program V-033 programme was used to electroporate the cells using an Amaxa nuclefector device. Transfectants were selected with hygromycin and the parasite diluted down and plated in three 96 well plates, to give an average of 0.1 parasite per well in order to generate clones. Genes integrated in this locus are constitutively expressed in both promastigote and amastigote life cycle forms. All these parasites were investigated their infectivity *in vitro* using bone marrow macrophages (Chapter 5), and *In vivo* using BALB/c mice for *L. mexicana* and *L. major* (Chapter 6), where the *L. donovani* was tested before with Dr Carter (Alsaadi *et al*. 2012).

2.3 Molecular biology methods

2.3.1 *Leishmania* RNA extraction

Total RNA was isolated using Trizol® reagent, using a protocol based upon the single-step RNA isolation method (Chomczynski and Sacchi, 1987). A stationary phase *Leishmania* promastigote culture (approximately 1 ml per gram) was pelleted and resuspended in 1 ml of Trizol® reagent. The parasite suspension was passed through a 23 gauge needle attached to a 1 ml syringe 10 times and then incubated at room temperature for 5 minutes with 0.5 ml of chloroform. The suspension was then shaken vigorously for 15 seconds and pelleted by centrifugation at 10,000 g for 15 minutes at 4°C. The resulting aqueous layer was carefully removed and 0.6 ml of chilled isopropanol added to precipitate any RNA present. After vigorous mixing, the sample was incubated at room temperature for 10 minutes and then centrifuged at 10,000 g for 10 minutes at 4°C. The supernatant was removed and the RNA pellet was washed with 1 ml of 70% v/v aqueous ethanol by centrifuging at 7000 g. The RNA pellet was air dried briefly and dissolved in 50 µl RNAase-free water. The concentration of RNA was determined using a NanoDrop2000® (Thermo Scientific, Wilmington, USA). The sample was loaded on to a 1% w/v agarose gel containing TBE buffer (0.09 M Tris base, 0.09 M boric acid and 2 M EDTA pH 8.0) with 0.3 µg /ml ethidium bromide and run at 100 V for 1 hour to check the purity of the RNA. The RNA was stored at -80°C until needed.

2.3.2 Isolation of Genomic DNA from *Leishmania*

Genomic DNA was prepared from *Leishmania* promastigotes using the method of Medina-Acosta and Cross (1993). Three ml of a stationary phase culture of

Leishmania promastigotes (approximately 10^7 parasites) was centrifuged at 15,800 g for 30 seconds and sedimented cells were resuspended in 400 µl fresh TELT buffer (50 mM Tris-HCl pH 8, 62.6 mM EDTA pH 8, 2.5 M LiCl and 4%v/v Triton X-100). After incubation at room temperature for 5 minutes, 400 µl of ice cold phenol were added to the suspension, and the mixture was end-over-end rotated at 4°C for 5 minutes. The mixture was then centrifuged at 15,800 g at 4°C for 10 minutes and the resulting aqueous upper layer transferred to a fresh micro centrifuge tube and 400 µl chloroform/ isoamylalcohol (24:1) added. The mixture was end over end rotated at room temperature and then centrifuged as described above. The aqueous upper phase was transferred to a fresh micro centrifuge tube and 1 ml of ice cold 100% ethanol was added to precipitate any genomic DNA present. After incubation on ice for 5 minutes the tube was centrifuged at 15,800 g at 4°C for 10 min. The resulting DNA pellet was washed with 400 µl ice cold 70 % v/v aqueous ethanol and then allowed to air dry at room temperature. The genomic DNA pellet was resuspend in 100 µl $T_{10}E_{0.1}$ buffer (10 mMTris-HCL pH 8.0, 0.1 mM EDTA pH 8.0) with gentle mixing to avoid shearing of the genomic DNA, and stored at 4°C until required. The concentration of genomic DNA was determined using a NanoDrop2000® (Thermo Scientific, Wilmington USA).

2.3.3 Polymerase Chain Reaction (PCR)

Genomic DNA (gDNA) isolated from each *Leishmania* species was used to amplify the relevant γGCS gene sequence, The primers were used in table 2.1, containing a *BamH1* site (underlined); reverse, containing a *Not1* site (underlined). The PCR amplification reaction contained a final concentration of 0.2 mM deoxynucleoside triphosphate mixture (dNTPs), 0.2 mM $MgSO_4$, 0.2–0.4 µ M of each primer 1 unit of

Platinum® Taq DNA Polymerase High Fidelity and High Fidelity PCR Buffer (600 mM Tris-SO_4, pH 8.9, 180 mM $(NH_4)_2SO_4$ in a final volume of 50 µl with 1 µg gDNA as the template (prepared as described in 2.3.2). The PCR conditions consisted of an initial denaturation at 94ºC for 30 seconds, followed by 35 cycles of denaturation at 94ºC for 30 seconds and annealing at 56°C for 30 seconds. A final extension was carried out at 68ºC for 2 minute (1 minute per kb of PCR product), gradient PCR machine (Eppendrof Mastercycle Gradient, Hamburg, Germany). The amplification products were analysed by agarose gel electrophoresis.

Table 2.1 Primer sequences used to amplify the relevant γGCS gene sequence.

Gene	Sequence
γGCS *L. donovani*	Forward GTCGGATCCATGGGGCTCTTGACGACTGGC Reverse CTAGCGGCCGCCTTGGTGCTCTCTACTGTATC
γGCS *L. mexicana*	Forward GTCG GATCCATGGTATTCTTGACGGATGGC Reverse CTAGCGGCC GCCTTGGTGCTTTCTACTGAATC,
γGCS *L. major*	Forward GTCGGATCCATGGGGCT CTTGACGACTGGC Reverse CTAGCGGCCGCCTTGACAC CTCTCTTGTGGT

2.3.4 Agarose gel electrophoresis

DNA and RNA were separated by gel electrophoresis using 0.8% (w/v) agarose gels containing 0.3 µg/ml ethidium bromide and one Kb DNA ladder marker. 0.8% w/v agarose was prepared with Tris-borate-EDTA buffer pH 8.0 (TBE) containing 0.09 M Tris base, 0.09 M H_3BO_3 and 2 M EDTA. A 1:10 volume of 10x DNA loading buffer (0.5x TBE buffer, 0.1 M EDTA pH 8.0, 0.1% w/v Bromophenol blue, 0.1% w/v xylenecyanol and 50% v/v glycerol) was added to DNA samples, and the mixtures were loaded onto the gel. Gels were run at 90 V for 60 minutes and separated DNA or RNA bands were visualized under illumination UV and photographed for analysis.

2.3.5 Cloning Reaction

A Taq polymerase-amplified PCR product was directly inserted into a plasmid vector using TOPO® TA cloning® kit (plasmid map in Appendix1). The TOPO® cloning reaction was set up by mixing 0.5-4 µl of a fresh PCR product of amplified *Leishmania* γGCS using specific primers (prepared as described in section 2.3.3) with 1 µl salt solution provided with the kit, 1 µl TOPO® vector and enough water give a final volume of 6 µl. The reaction mixture was mixed gently and incubated for 5 minutes at room temperature and then kept on ice. Two µl of the TOPO® cloning reaction were added to a vial of One Shot® chemically competent *E. coli* and mixed gently. The mixture was incubated on ice for 30 minutes and then the cells were heat-shocked for 30 seconds at 42°C without shaking. Immediately, tubes were placed on ice and 250 µl of Super Optimal Broth media (supplied within the kit, containing 2% w/v Tryptone, 0.5% w/v Yeast Extract, 10 mM NaCl, 2.5 mM KCl,

10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose), which was kept at in room temperature, were added to the tube. The tube was shaken horizontally at 200 rpm at 37°C for 1 hour. 50 µl-100 µl from each transformation were spread onto a sterile pre-warmed selective plate LB agar plates (1% w/v bacto tryptone, 0.5% w/v yeast extract, 1% w/v NaCl and 1.5% w/v agar), which contained 50 µg/ml aqueous ampicillin and 40 mg/ml X-gal dissolved in dimethylformamide. The plate was incubated overnight at 37°C to allow colony growth. Several hundred blue/white colonies grew and 10 white or light blue colonies were picked for analysis. Each colony picked was inoculated into 5 ml sterile LB medium (1% w/v bacto tryptone, 0.5% w/v yeast extract, 1% w/v NaCl) containing 50 µg/ml aqueous ampicillin. The culture was grown overnight at 37°C on a horizontal Shaker at 200 rpm. Plasmid DNA was prepared from each culture using a PureLink™ Quick Plasmid Miniprep Kit or using a Plasmid DNA mini-preparation, following the method described by Zhou and Meyer (1990). Briefly, 1.5 ml of an overnight culture were harvested at 15800 × g for 30 s, the supernatant was decanted, and the sedimented cells were resuspended to homogeneity in the residual supernatant (approximately 100 µl). Three hundred µl of TENS (10 mM Tris/HCl pH 8, 1 mM EDTA pH 8, 100 mM NaOH, 0.5% w/v SDS) were added, and the cell suspension was vortexed at medium speed for 4 sec. and immediately placed on ice. 150 µl of 3 M sodium acetate pH 5.2 were added, and the cell lysate was vortexed at medium speed for 3 sec. and placed on ice again. Cell debris was sedimented at 15800 × g at 4°C for 10 min, and the supernatant was transferred to a new 1.5 ml tube. 900 µl of ice-cold 100% ethanol were added to the particle-free solution to precipitate any plasmid DNA present, and the mixture was centrifuged at 15800 × g at 4°C for 15 min. The DNA pellet was

washed with ice-cold 70% v/v aqueous ethanol and centrifuged under the same conditions as before for 10 min. The supernatant was removed completely, and the pellet was allowed to air dry (10-15 min) before it was dissolved in 40 µl double distilled water.

Each stock of plasmid DNA was stored at -20°C until required. The plasmids were analysed by gel electrophoresis after digestion with restriction enzymes to confirm the presence and correct orientation of the *Leishmania* γGCS gene sequence PCR insert. The reaction mixture which contained 3 µl plasmid, 1.5 µl appropriate buffers and 0.5 U of each enzyme was added to a sterile DNA/RNA free tube and molecular grade water added to give a 15 µl final volume. Digestion reactions were incubated at 37°C for 2 hours and then loaded onto a 0.8% w/v agarose gel and subjected to gel electrophoresis for 60 minute at 90V. Once a clone with the correct insertion and right orientation had been identified, a glycerol stock for long term storage was prepared by adding 0.5 ml of the LB bacteria stock to 0.5 ml of sterile glycerol and stored at -80°C.

Leishmania γGCS TOPO® plasmid of each *Leishmania spp.* and pET-24a plasmid were isolated using a Qiagen QIAprep Spin Miniprep Kit. Both plasmids (5-20 µg DNA) were cut using *Not1* and *BamH1* (30-50 U) according to the manufacturer's recommendation and the buffers supplied in a final volume of 100 µL. After 4 hr of incubation at 37ºC, 10 µl of each mixture were analysed using gel electrophoresis to ensure that complete digestion of the samples had occurred. Calf intestinal alkaline phosphatase (CIAP) was used to dephosphorylation pET-24a vector by incubating at 37ºC for 1 hr. The CIAP enzyme was then inactivated and the sample subjected to gel

electrophoresis for 2 hr at 90 V. The band of interest (γGCS 2000 bp) and the pET-24a plasmid were excised from the gel and purified using a Qiagen gel extraction kit. The two samples were ligated using a T4 DNA Ligase kit. The ligation reaction was incubated overnight at 13ºC and a 5 µl ligation reaction was added to a sample of DH5-α of *E.coli* bacteria and the mixture was kept on ice for 20 min, and then incubated at 42ºC for 90s. The suspension was kept on ice for 2 min and then incubated for 1 hr at 37ºC in a shaking incubator after adding 250 µl of Luria-Bertani medium (LB broth) pH 7.4 (1% w/v tryptone, 1% w/v NacL and 0.5% w/v yeast extract). The bacteria suspension was plated out on LB agar (1% w/v tryptone, 1% w/v NacL, 0.5% w/v yeast extract and 1.5% w/v agar) containing 50 µg/ml kanamycin, and incubated overnight at 37ºC to allow colonies to develop. Plasmid was isolated from a single colony grown overnight using a Qiagen QIAprep Spin Miniprep Kit and the purity and amount of DNA present quantified as before. A sample of each isolated plasmid was digested with relevant selected restriction enzymes (*Pvuii, NdeI,* or *Avai)* to ensure it contained an insert of correct size and that the insert was in the correct orientation. The map for pET-24a with each *Leishmania* γGCS insert is shown in Figure 2.1, 2.2 and 2.3. A commercial laboratory sequenced the selected plasmids using T7 forward and T7 reverse primers and internal forward primers (5'-GAGAGAAAACGGCAGGA-3'), and an internal reverse primer (5'-CATCTTGTC GTACATCACAA-3').

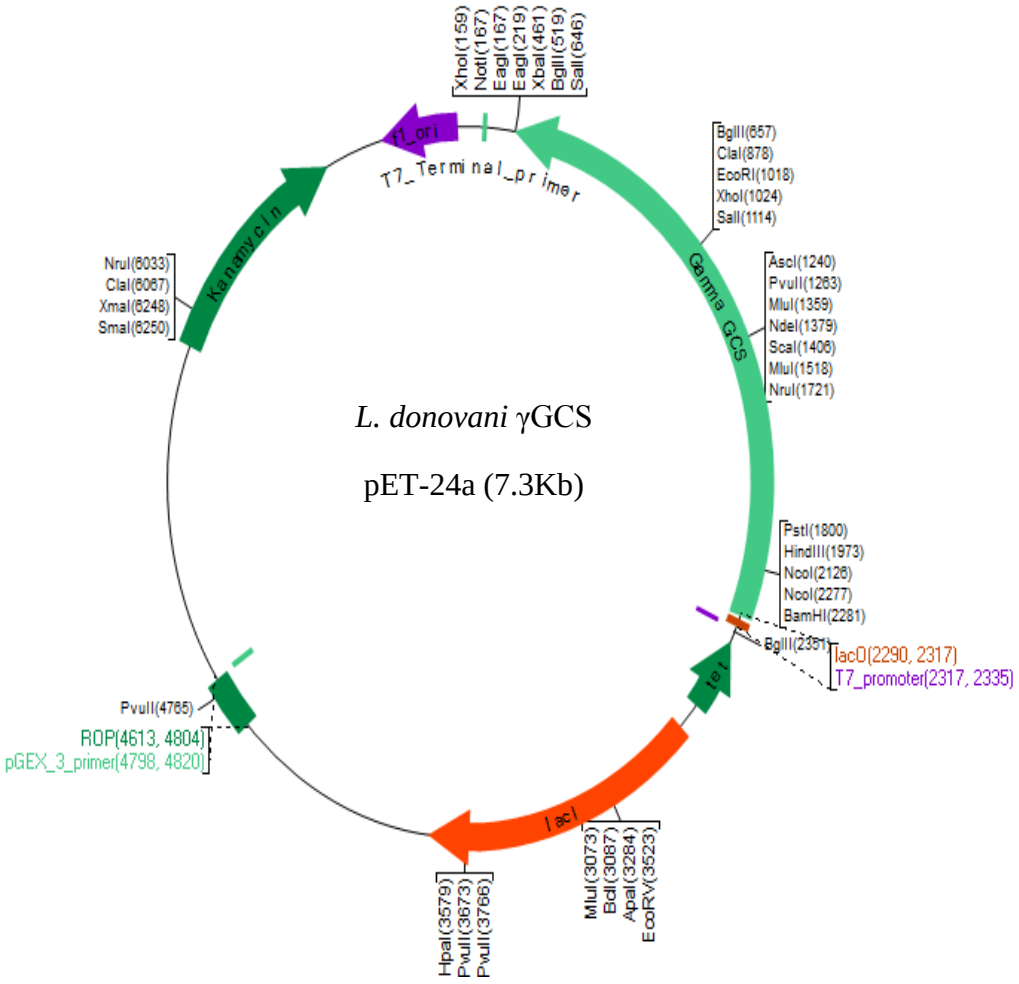


Figure 2.1 Plasmid map to show restriction enzyme sites on *L. donovani* on the γGCS pET-24a plasmid.

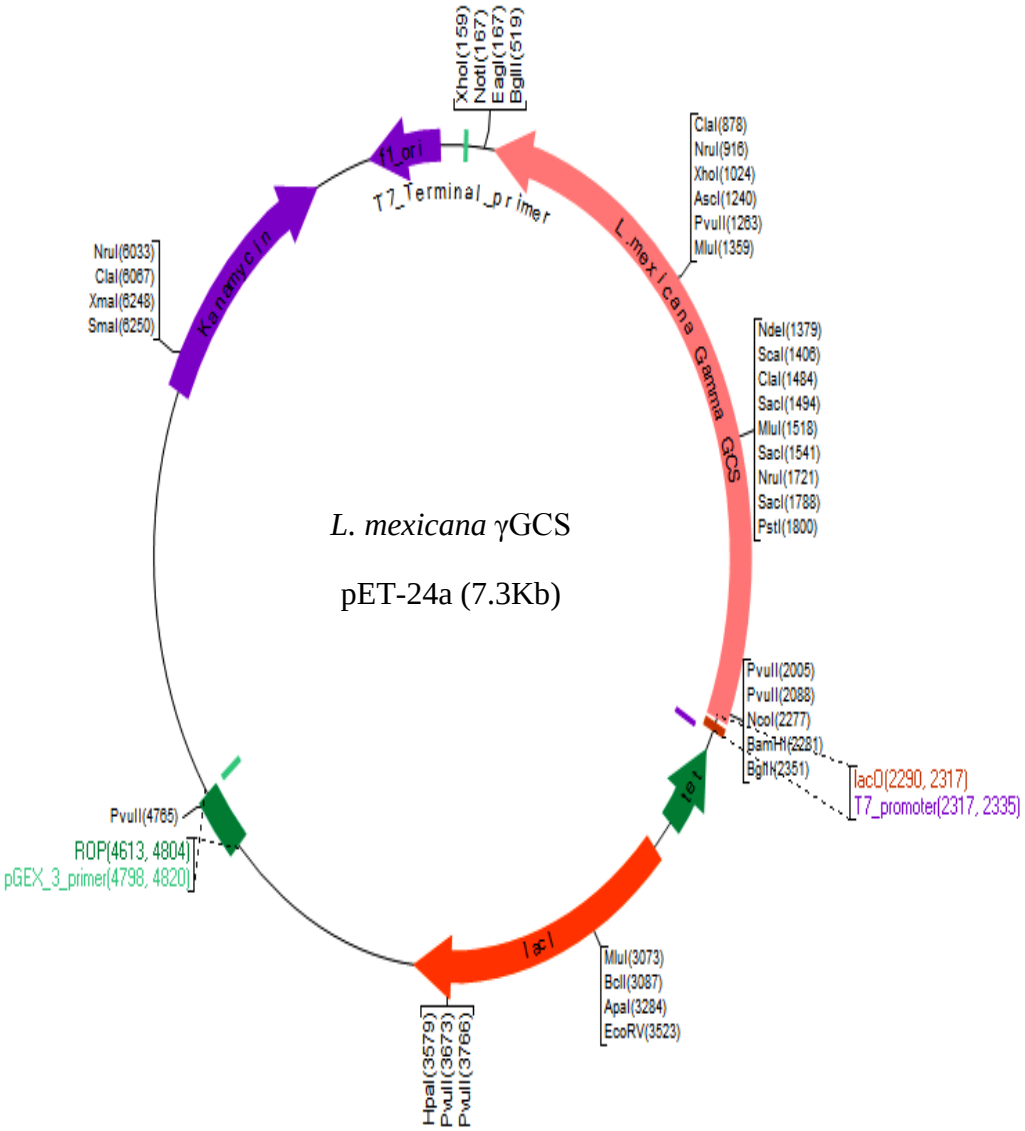


Figure 2.2 Plasmid map to show restriction enzyme sites on *L. mexicana* on the γGCS pET-24a plasmid.

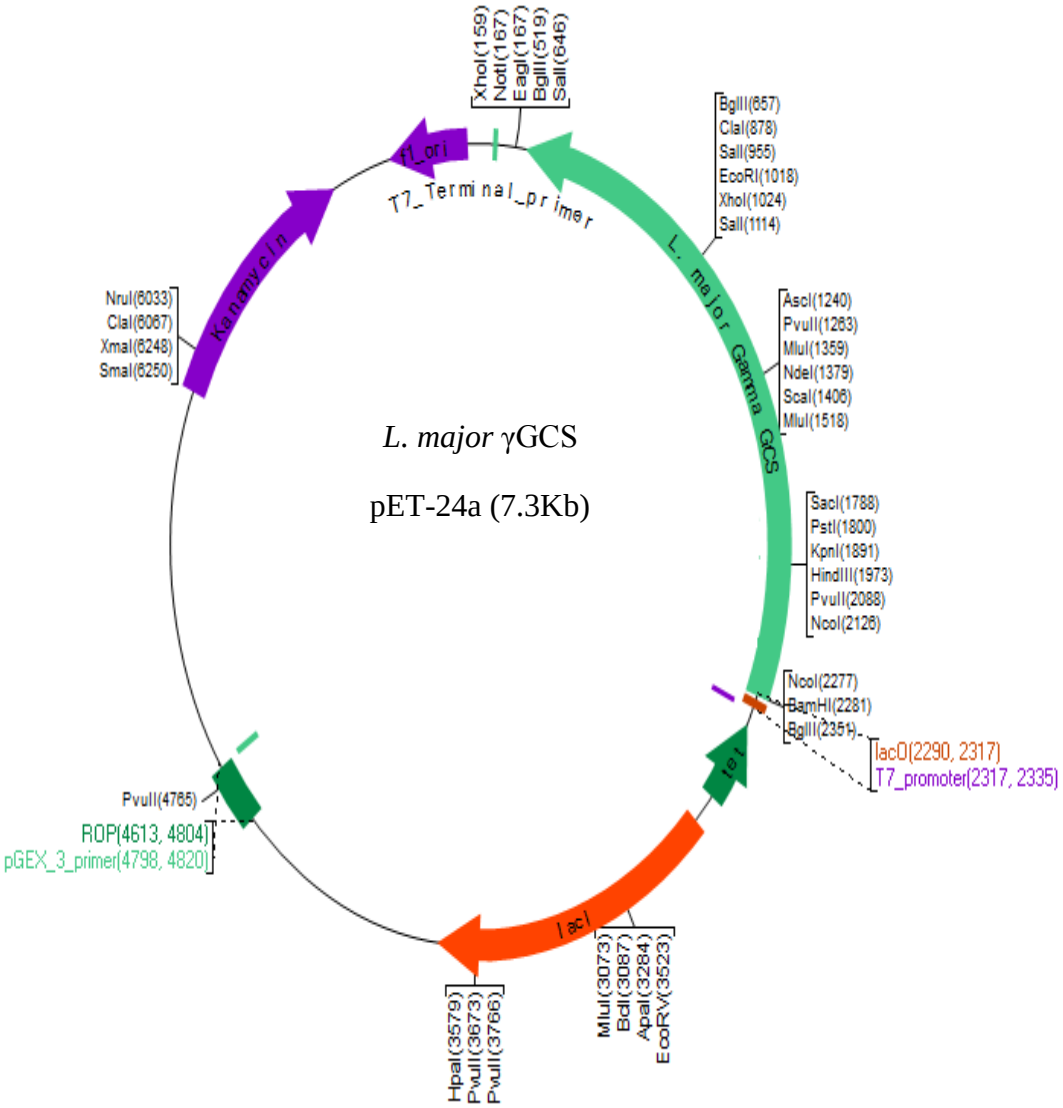


Figure 2.3 Plasmid map to show restriction enzyme sites on *L. major* on the γGCS pET-24a plasmid.

2.3.6 Competent cells

Competent cells were prepared using the method of Hanahan (1983). A single *E. coli* colony of the parental cells was picked from an LB agar plate (1% w/v tryptone, 1% w/v NaCl, 0.5% w/v yeast extract and 1.5% w/v agar) and used to inoculate 3 ml of LB medium (1% w/v tryptone, 1% w/v NaCl and 0.5% w/v yeast extract). The culture was grown in a shaking incubator at 37°C overnight and 500 µl of the culture were added to 100 ml of fresh LB medium. The culture was grown until an optical density of 0.2 at a wavelength of 600 nm (OD_{600}) was obtained. The culture was maintained on ice for 15 min, divided into two 50 ml tubes and centrifuged at 3500 × g at 4°C for 15 min. The pelleted cells were carefully resuspended in 16 ml sterile RF1 solution (100 mM RbCl, 50 mM $MnCl_2$, 10 mM $CaCl_2$**,** 30 mM CH_3CO_2Kpo, 15% v/v glycerol, adjusted to pH 5.8), pooled and incubated on ice for 90 min. The cells were sedimented under the same conditions as before, carefully resuspended in 8 ml sterile RF2 solution (10 mM RbCl, 75 mM $CaCl_2$, 10 mM 3-(N-morpholino) propane sulfonic acid, 15% v/v glycerol, adjusted to pH 6.8) and incubated on ice for 15 min. Competent cells were aliquoted in 200 µl volumes into 1.5 ml sterile eppendorf tubes, quick-frozen in liquid nitrogen and stored at -80°C until required.

2.3.7 cDNA synthesis

Complementary DNA (cDNA) was synthesised from the RNA using reverse transcriptase. This reaction required 2 µg of RNA, 1 µl of random primers and enough molecular grade water to give a final volume of 14.2 µl. The samples were incubated at 65°C for 5 minutes, left to cool to room temperature for 10 minutes and then 2 µl of 10X Affinity Script RT buffer, 2 µl of 100 mM DDT, 0.8 µl of 100 mM of deoxynucleotide triphosphate (dNTPs) mix (10 mM each of dATP, dGTP, dCTP,

dTTP at neutral pH) and 1 µl of RT enzyme were added to the samples and enough molecular grade water to give a final volume of 20 µl. The sample was then incubated at 25°C for 10 minutes, 55°C for 1 hour and 70°C for 15 minutes in order to inactivate the reaction. Complementary DNA samples were stored at -20°C until required.

2.3.8 Quantitative Real Time PCR (qRT-PCR)

The Stratagene Mx3000p Real Time PCR thermo cycler (Stratagene, Agilent Technologies UK Limited) was used in all qRT-PCR assays. Each sample was analysed in duplicate. SYBR green (6.25 µl), 25 pmol of forward and reverse oligonucleotide primers (see Table 2.2 and 2.3 for primers used in studies) 1 µl of appropriate cDNA template and enough molecular grade water added to give a final volume of 12.5 µl. PCR reactions were carried out using the following protocol: 1 cycle of denaturation at 95°C for 10 minutes, 40 cycles of denaturation at 95°C for 30 seconds, annealing at 64°C for 45 seconds and extension at 72°C for 1 min followed by 1 cycle at 95°C for 1 min and 55°C for 30 seconds (Carter *et al.*, 2006). The C_T value of each sample was calculated using the Stratagene MX3000 Pro QPCR software (2007 Stratagene© version 4.10) and relative gene expression was calculated by using the $2^{-\Delta\Delta C}{}_T$ method (Livak and Schmittgen, 2001). This method compares test samples to a comparator sample and results are expressed as the degrees of difference in ΔC_T values between the test and comparator sample was calculated as following steps:

- Step 1 Normalisation to housekeeping gene:
  Δ Ct = Ct target gene – Ct housekeeping gene.
- Step 2: Δ Ct sample- Δ Ct calibration (control) = $\Delta\ \Delta$ Ct
- Step 3: relative gene fold change = $2^{-\Delta\ \Delta\ Ct}$

Table 2.2 Primer sequences used in *in vitro* studies.

Gene	Type	Sequence
γGCS	*L. donovani* *L. mexicana* *L. major*	Forward 5′CCGTGCATCTACATGGACTGCATGGCCTTTGGCAT3′ Reverse 5′ ATAGTCAGCCAGCGCACATCGGTGTCGC3′
alpha-tubulin	*L. donovani* *L. mexicana* *L. major*	Forward 5′ AGCTGTCCGTCGCGGACATCACGAACTCGGTGTTT3′ Reverse 5′CGAACTGAATTGTGCGCTTCGTCTTGATCGTCGCAAT3′
γGCS	Mouse	Forward 5′ AGAACAATCGCTTTAGGATCA3′ Reverse 5′ AGAAGATGATCGATGCCTTC3′
GAPDH (glyceraldehyde-3-phosphate dehydrogenase)	Mouse	Forward 5′ AGATTGTTGCCATCAACGAC 3′ Reverse 5′ ATGACAAGCTTCCCATTCTC3′

Table 2.3 Primers sequences for expression studies in golden hamster experiments.

Primer	Sequences
HPRT forward*	5′ GATAGATCCACTCCCATAACTG 3′
HPRT reverse*	5′ TACCTTCAACAATCAAGACATTC 3
IFN- γ forward	5′ GCTTAGATGTCGTGAATGG 3′
IFN- γ reverse	5′ GCTGCTGTTGAAGAAGTTAG 3′
IL-12 forward	5′ TATGTTGTAGAGGTGGACTG 3′
IL-12 reverse	5′ TTGTGGCAGGTGTATTGG 3′
IL-4 forward	5′ GCCATCCTGCTCTGCCTTC 3′
IL-4 reverse	5′ TCCGTGGAGTTCTTCCTTGC 3′

*HPRT is hamster housekeeping gene Hypoxanthine-guanine phosphoro ribosyltransferase.

2.4 Protein expression and purification

2.4.1 Protein induction and cell lysis

The protein expression and purification methods used were similar to those described by Henriquez *et al.* (2010). Five microliter of the pET-24a plasmid containing the gene sequence for his-tagged *Leishmania* γGCS were used to transfect 50 µl Rosetta blue *E. coli* bacteria using the method described in 2.3.5. Transformed bacteria were plated on LB agar plates (1% w/v tryptone, 1% w/v NacL, 0.5% w/v yeast extract and 1.5% w/v agar) supplemented with 50 µg/ml kanamycin, 12.5 µg/ml tetracycline and 34 µg/ml chloramphenicol. A single colony was selected and used to inoculate 10 mls

LB broth supplemented with the same antibiotics and the culture was grown overnight at 37ºC in a shaking incubator. This culture was then used to inoculate 100 ml of similarly supplemented LB broth and grown in a shaking incubator at 37ºC. 1 ml aliquots of the culture were removed at various time points so that the absorbance could be determined. Once the culture had reached an absorbance of 0.6-0.8 at OD_{600}, 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was added to the culture. The bacteria were grown at various temperatures and various protocols were evaluated to optimise recombinant protein expression. At the end of the incubation period the bacteria present were pelleted by centrifugation at 6000 g for 10 min at 4ºC (Sorvall RC-5B Refrigerated Superspeed centrifuge, Du Pont, Connecticut, USA). The resulting pellet was washed with 10 ml of phosphate buffer pH 7.4 (PBS) and centrifuged as before. The pellet was then resuspended in buffer (0.05 M Tris, 0.2 M KCl pH 7.4) containing 40 mM imidazole and 1% v/v Triton X-100. The bacteria were kept on ice where possible throughout processing to minimise protein degradation. Soluble protein was released from the bacterial cells by sonicating using a Branson sonifier S250 (G Heinemann Ultraschall Laborte Chnik, Schwabisch, Germany) fitted with a 10 mm tip, using 4-10 pulses of 20 seconds with increasing intensities, (intensity 2 three times, intensity 3 three times, intensity 4 three times) with 2 minute breaks between pulses to allow the probe to cool down. The resulting suspension was centrifuged at 18,000 g at 4ºC for 20 min and the resulting supernatant collected and processed for column purification under native condition. The pellet was then processed to extract the insoluble protein present.

Insoluble protein was extracted using a modified protocol described by Henriquez *et al.* (2010). The pellet was resuspended in 20 ml extraction buffer (50 mM Na_2HPO_4,

0.3 M NaCl, 6 M urea pH 7.5), or buffer containing 0.05 M Tris-HCl, 0.2 M KCl, 40 mM imidazole and 6M urea, pH 7.4). The mixture was incubated for an hour at 30°C and resulting suspension centrifuged at 18,000 rpm for 20 minutes. The supernatant was then used in purification studies.

2.4.2 Recombinant protein purification

GE Pharmacia FPLC (Fast Protein Liquid Chromatography) system was used for protein purification and placed in a cold room to perform purification at 4°C. The following solutions were used in soluble protein purification studies: distilled water (sterilized by filtration and pH was adjusted to 7.4), start buffer (0.05 M Tris 0.2 M KCl pH 7.4 containing 40 mM imidazole), and elution buffer (0.05 M Tris, 0.2 M KCl pH 7.4 containing 500 mM imidazole). The extracted bacterial supernatant containing soluble protein was loaded onto a 1 ml HisTrap nickel column using a flow rate of 0.33 ml/min. The column was washed extensively (20 x column volume) with start buffer. Recombinant his-tag protein was eluted using a elution buffer. The collected fractions were assayed at 280 nm to detect which fractions contained protein and the purity of the eluted protein was checked by SDS polyacrylamide gel electrophoresis. The protein concentration of the eluted samples was determined using the Bio-Rad protein assay reagent (assay method section 2.4.3).

Two methods of refolding of protein extracted from insoluble fraction using urea were tested. The samples of insoluble protein extract were either dialyzed against PBS pH 7.4 or refolded on the column. In dialysis, the supernant of the extract obtained after incubation with urea was dialyzed against 1 litre PBS pH 7.4 for 24 hours. The PBS was changed at least three times over this period before the extract was added to a 1

ml HisTrap nickel column, wash column using PBS pH 7.4 as start buffer then eluted using PBS pH 7.4 with 250 mM imidazole. The protein eluted was dialysed against PBS pH 7.4 before use in vaccine studies to ensure there was no imidazole present.

The second method tested was refolding the protein on the column using a urea gradient to elute it from the column. Then protein was eluted using an imidazole gradient, using the method described by GH Life Sciences (1999) and the following buffers, buffer 1 (50 mM Na2HPO4, 300 mM NaCl, 1mM β- mercaptoethanol and 6 M urea, pH 7.4), buffer 2 (20 Mm Tris base, 100 mM NaCl, 6 M urea, 1 mM β-mercaptoethanol and 250 mM imidazole, pH 7.5), refolding buffer (20 mM Tris buffer pH 8, 100 mM NaCl and 1 mM β-mercaptoethanol), and elution buffer (20 mM Tris buffer pH 8, 100 mM NaCL, 1 mM β-mercaptoethanol and 500 mM imidazole). Soluble protein was loaded on to the 1 ml HisTrap nickel column and washed using 10 mM imidazole in 96% of the start buffer and 4% of second buffer. A gradient of urea was applied to the column using start buffer and refolding buffer by moving from 100% start buffer containing 6 M urea to 100% refolding buffer (no urea). The protein was eluted using a gradient of imidazole, moving from refolding buffer (no imidazole) to 100 % elution buffer (500 mM imidazole).

2.4.3 Bio-Rad protein assay reagent

The protein concentration of the purified γGCS preparation was determined using the Bio-Rad protein assay reagent before storage at -20ºC. Briefly, 10 µl of protein standards (BSA 0.1-1 mg/ml) or 10 µl of the γGCS protein sample were added to appropriate wells of a 96 well ELISA plate. 200 µl of Bio-Rad protein assay reagent (diluted 1:5 with distilled water) were added to each sample. The absorbance of the

samples was measured at O.D 595 nm using Softmax Molecular Device (Molecular Devices Corporation, Sunnyvale. USA). The concentration of the unknown sample was determined from the standard curve plotted using the protein standards by linear regression. In all cases, a correlation coefficient of > 0.97 was obtained.

2.4.4 Protein separation studies

Samples were separated using a 12% w/v resolving gel and 5% w/v stacking gel prepared using Prosieve®50 gel solution. Protein samples were diluted in an appropriate amount of sample buffer (25% v/v 0.5 M Tris-HC pH 6.8, 20% glycerol, 4% of 10% w/v SDS solution, 5% v/v of 2-mercaptoethenol, 5% v/v from the solution of 1% v/v bromophenol blue), were boiled for five minutes, cooled on ice and then a 15 µl aliquot added to the appropriate lane of the gel. A protein marker (5 µl, 15-150 KDa) was also loaded into one of the lanes of the gel and running buffer (0.29% w/v Tris base, 1.44% w/v glycine, 0.1% w/v SDS, pH 8) added. The electrophoresis was run for 60-90 min at using a 200 V, 40 mA. The gel was either stained to visualize protein or processed for western blot studies. If stained, the gel was fixed using 40% aqueous ethanol: 10% v/v aqueous acetic acid for one hour, washed with water twice for 10 min, and then stained overnight with freshly prepared colloidal dye solution (80% v/v of colloidal Coomassie dye stock and 20% aqueous methanol). The colloidal Coomassie dye stock was prepared using 5% w/v ammonium sulphate, 1.2% v/v 85% aqueous phosphoric acid, 2% v/v Coomassie stock (5 % w/v aqueous Coomassie Brilliant Blue G250). A picture of the gel was taken to provide a permanent record of the result obtained.

2.4.5 Western blotting studies

Blotting pads and filter paper were soaked in blotting buffer (12 mM Tris base, 96 mM glycine, 20% v/v ethanol, distilled water up to 1 liter). The gel nitrocellulose paper sandwich was prepared as described by the manufacturer's instructions. Electrophoresis was carried out at a voltage of 40-60 V for 2 hrs. Ponceau S solution was used to demonstrate that proteins had successfully been transferred on to the membrane from the gel and the position of the molecular weight markers noted. The membrane was incubated in PBS pH 7.4 containing 10% v/v Fetal calf serum (FCS) for one hour at room temperature on a rocker. The membrane was then washed three times by incubating with 15 ml PBS pH 7.4 for 5 min/wash and then incubated for 1 hour with 10 mls of the primary antibody (mouse anti-His tag polyclonal antibody, diluted 1:4000 in PBS pH 7.4 containing 10 % v/v FCS). The membrane was washed three times as before and then incubated for 1 hour with 10 mls of the secondary antibody (anti-mouse IgG HRP conjugated antibody diluted 1:10000 in PBS pH 7.4 containing 10% v/v FCS). The membrane was washed three times as before then incubated with 10 ml substrate solution (1 mg/ml aqueous Sigma Fast™ of BCIP/NBT). His-tagged proteins were visualized as black lines on the membrane and a picture was taken to record the results obtained. In some studies, the proteins were visualized using chemiluminescence detection using the Supersignal system. The chemiluminescence substrate for horseradish peroxidase (HRP) is a two components system consisting of a peroxide solution and luminol/enhancer solution. Equal volumes of the two components are mixed and then incubated with the blot membrane. The interaction between the HPR-conjugated antibodies and the substrate produce a

chemical reaction that results light release that can detected by film (CEA medical X ray film screen, AgFa healthcare NV, Mortsel, Belgium).

2.4.6 MALDI Studies

The major protein bands separated by gel electrophoresis were analysed by mass spectrometry. The relevant protein bands were cut out of the gel and placed in a microcentrifuge tube and sent to Proteomic Department, Polyomics Centre, Glasgow University. The Mascot search engine was used to interrogate protein sequences of *L. donovani*, *L. major*, *L. mexicana* and *E. coli* genome datasets.

2.4.7 Endotoxin test

Endotoxin contamination from the bacteria used to produce the recombinant *Leishmania* γGCS may occur. Therefore endotoxin was removed using ProteoSpin Endotoxin Removal Maxi kit (Fisher Scientific, Loughborough UK). All recombinant proteins then had their endotoxin levels assessed before use as vaccine using Pierce LAL Chromogenic Endotoxin Quantitation Kit from Fisher Scientific, Loughborough UK. The test is sensitive to detect as little as 0.1 EU/ml of endotoxin. That use 10 µl of protein was diluted with 50 µL of endotoxin-free water to 50 µl then mixed with 50 µl Limulus Amebocyte Lysate solution. After that incubated 10 min at 37ºC. After exactly 10 minutes, 100 µL of substrate solution was added to each well. Then the plate was covered with lid and gently shakes on a plate mixer for 10 seconds and incubated the plate at 37ºC for 6 minutes. The 50 µL of Stop reagent (25% acetic acid) was added, then the absorbance measured at 405nm using ELISA plate reader (Softmax Molecular Device, Molecular Devices Corporation, California. USA). The

standard curve was created using *E. coli* endotoxin standard calculate endotoxine levels.

2.4.8 Preparation of cell extract for γGCS enzyme assay

A crude promastigote parasite extract was prepared using a freeze-thaw protocol following the method of Williams *et al.* (2009). Cells ($1x10^8$/tube) from promastigotes parasite culture were washed with PBS pH 7.4 and harvested by centrifugation at 13,000 rpm for 30 min. Parasite pellets were resuspended in lysis buffer [0.25 M sucrose, 0.25% v/v Triton X-100, 10 mM EDTA, 10 μM E-64 protease inhibitor, 2 mM 1,10-phenanthroline, 4 μM pepstatin A and 1 mM phenylmethylsulfonyl fluoride]. Lysates were centrifuged at 13000 g for 30 min at 4°C. The amount of protein in the supernatant was measured as described in section 2.4.3.

2.4.9 Enzyme activity

The activity of γGCS was determined spectrophotometrically using the method described by Misra and Griffith (1998). Briefly, 396 µl of buffer (150 mM Tris–HCl, pH 8.2, 100 mM KCl, 0.3 mM EDTA and 40 mM $MgCl_2$) was added to the substrates were 15 mM L-4-aminobutyrate, 20 mM L-glutamate and 10 mM phosphoenolpyruvate then added 0.3 mM NADH, 14 IU of pyruvate kinase, and 38 IU of lactate dehydrogenase and 5 mM ATP. The mixture was then added to 100 µl of soluble *Leishmania* γGCS to give a final volume of 1 ml. The oxidation of NADH was monitored at 340 nm and NADH oxidation rate was assumed to equal the rate of ADP formation. In this assay one unit of γGCS activity is defined as the amount that catalyses the formation of one mol of ADP per hour. The specific activity was

expressed as units/mg protein (Appendix 8). Assays were carried out in the presence and absence of L-glutamate. The effect of BSO treatment on γGCS activity was determined by pre incubating γGCS with different concentrations of BSO for 60 min at 4°C. Absorbance of samples at 340 nm measured using UV 2550, UV Vis spectrophotometer (Shimadzu corporation, Kyoto, Japan). Studies were also carried out without L-glutamate as a negative control. An additional control used was purified protein isolated from the pET-24a plasmid, which did not contain the *Leishmania* γGCS insert to ensure that activity obtained was related to *Leishmania* γGCS enzyme.

2.5 *In vitro* promastigote studies

Leishmania promastigotes ($5x10^6$/ml) were added to the wells of 96 well tissue culture plate at a volume of 100 μL/ well and incubated with medium alone (control, n=4) or doubling dilutions of BSO (starting from 20 mM BSO, n=4) in HOMEM medium supplemented with 10% v/v heat-inactivated FCS for *L. mexicana* and *L. major*, or 20% v/v heat-inactivated FCS for *L. donovani*. The plate was incubated for 72 hours at 25°C and plates were wrapped in parafilm to avoid evaporation. Parasite growth was measured by adding 50 μL luciferin solution (150 μg/ml) using the method described by Alssadi *et al*. (2012). The amount of bioluminescence emitted/well was measured as total flux (p/s). Results were expressed as percentage reduction in parasite viability compared to controls and concentration that gave the 50% inhibition (IC_{50}) was calculated by Probit analysis (Vermeersch *et al.*, 2009). The mean value was calculated from three separate experiments.

2.6 *In vitro* amastigotes studies

Experiments were carried out using 24 well or 96 well tissue culture plats using bone marrow macrophages. The bone marrow was harvested from the femur of individual mice by injecting 10 ml of bone marrow medium [Dulbecco's medium supplemented with 20% v/v heat-inactivated fetal calf serum, 30% v/v L-cell supernatant (supplied by Dr Carter), 100 μg/ml penicillin/streptomycin and L-glutamine] into one of the cut ends of the femur. The resulting cell suspension obtained was incubated in a Petri dish for 7–10 days at 37°C in an atmosphere of 5% CO_2 95% air. Cells were harvested from the plates, pooled, pelleted by centrifugation and resuspended in complete medium [RPMI 1640 supplemented with 10% v/v FCS and 100 μg/ml penicillin/streptomycin and L-glutamine]. Cell viability was determined microscopically by Trypan Blue exclusion and was always >95%.

Bone marrow macrophages cells ($1–2\times10^5$) in 0.2 ml complete medium were added to individual wells of a 24-well tissue culture plate, which contained a 13 mm^2 diameter circular glass cover-slip or 0.1 ml of cell suspension was added to a 96 well plate without circular glass cover-slip. Plates were incubated overnight at 37°C in an atmosphere of 5% CO_2 /95% air to allow macrophages to adhere. The medium was then removed from each well and replaced with 0.2 ml of complete medium (uninfected controls) or 0.2 ml of complete medium containing *Leishmania* promastigotes at various parasite : host cell ratio. The cells were incubated for further 18-24 hours at 37°C in an atmosphere of 5% CO_2 /95% air and then the well contents were removed to remove any unattached parasites. Cells were incubated with 0.1 ml (96 wells plates, n=6/treatment) or 0.5 ml (24 wells plates, n=4/treatment) complete medium (control) or complete medium containing BSO double dilution (starting from

0.5 mM BSO). The cells were incubated as before for a further 72 hours. In studies using luciferase-expressing parasites, the well contents were removed and 0.1 ml luciferin solution (150 µg/ml in complete medium) was added. The amount of bioluminescence emitted / well was measured as total flux (p/s).

Alternately the well contents were removed (24 well plates) and 0.2 ml of methanol was added to fix the cells. After 2–3 min. incubation at room temperature the methanol was removed and 0.5 ml of aqueous 10% v/v Giemsa solution was added. After 20 min incubation at room temperature, the Giemsa was removed and the wells were washed with tap water. The cover-slips from individual wells were removed, air dried, and mounted on to glass slides (4 coverslips per slide). The percentage of cells infected from 200 randomly selected cells, and the mean number of parasites/host cell from 20 randomly selected cells, was determined microscopically at 100x magnification (Carter *et al.,* 2005). The reduction in the parasite burdens for treated cells compared to the mean control values was determined and the IC_{50} was calculated by Probit analysis (Vermeersch *et al.*, 2009). The mean value was calculated from three separate experiments. Also the data represent in a single value that takes into account both the number of parasite/host cells and the percentage of cells infected, which are shown for the directly counted results. The infection rate was culculated by multiplying number of parasite/host cells and percentage of infection.

2.7 *In vivo* imaging studies

BALB/c mice were infected in the footpad or rump with luciferase-expressing *L. mexicana* or *L. major* promastigotes or amastigotes 10 µl of $1x10^7$ parasites in incomplete medium (RPMI 1640 supplemented with 100 µg/ml

penicillin/streptomycin and L-glutamine) was inoculated into mice subcutaneously. The light emission was monitored during the course of infection using the method described by Alsaadi *et al*. (2012). In some studies the presence of neutrophils in the infection site was determined using the method of Gross *et al.*, (2009) were carried out not on the same time of luciferin injection. Mice were imaging 5 min. after injection of luminol solution (i.p, 50 mg/ml PBS pH 7.4, at dose of 200 mg/Kg body weight). The amount of bioluminescence emitted in each region of interest (ROI) was determined using the Living Image software, and the results were recorded as photons/sec emitted (Appendix 9).

2.8 Vaccine studies

BALB/c mice (n = 8/treatment) were injected subcutaneously with PBS pH 7.4 alone (control), triple vaccine combination of γGCS proteins from *L. donovani*, *L. mexicana* and *L. major* 20 µg/protein or treated by inhalation using Buxco© Inhalation System (Buxco® nebulization system, Buxco Research Systems, Wilmington, USA) with 50 µg/protein either alone, as two proteins or all three simultaneously on days -28 and -14. On day 0 three mice from each treatment group were sacrificed and under aseptic conditions blood was collected from each mouse for ELISA assays and the spleen removed for lymphocyte proliferation assays. The blood was allowed to clot at 4°C for up to 12 hours and the resultant serum collected and stored at -20°C until required. On day 0 the remaining mice (n=5/treatment) were infected with 10 µl of $1x10^7$ *L. mexicana* luciferase-expressing promastigotes (LmexLuc) or *L. major* luciferase-expressing promastigotes (LmajLuc) by subcutaneous injection into the footpad (10 µl, Figure 2.4). Parasite growth was monitored by measuring footpad thickness using a pocket thickness gauge range 9 mm (Mitutoyo Corporation, Tokyo, Japan) and

measuring the amount of bioluminescence over the course of infection (section 2.7). At the end of the experiment the footpad was removed from each mouse and disrupted in 5 mls incomplete medium using the end of a 2 ml syringe. The number of parasites present in the homogenate was determined by viewing a sample loaded into a haemocytometer (x 400 magnification). In addition, the amount of bioluminescence (BLI) emitted by the homogenate, (diluted 1:1 with luciferin solution, 300 µg/ml) in incomplete medium was determined. The mean total flux for control and vaccinated mice was determined. Each experiment was repeated.

In some studies the ability of the triple *Leishmania* γGCS recombinant vaccine to protect against wild type *L. donovani* (MHOM/ET/67:LV82) by subcutaneous or inhalation route was determined. The vaccinated and control mice (n=5/treatment) were infected with *L. donovani* by i.v inoculation of $1x10^7$ amastigotes /0.2 ml incomplete medium without anaesthesia (Figure 2.5).

In the inhalation studies, golden Syrian hamsters (n=6 /treatment), Figure 2.6, day 0 hamsters were infected with *L. donovani*, 1 x 10^7 amastigotes, 0.2 ml incomplete medium, the tongue vein by Dr Carter. Hamsters and mice were sacrificed on day 14. Spleen and liver of the hamsters were removed and stored at -70°C in cryotubes until cytokine levels could be assesses by comparing relative expression of cytokine RNA for cytokine levels determined using real time PCR (described in section 2.3.8) and parasite burdens in the spleen and liver determined by preparing impression smears of the relevant organ on a labelled glass slide. Bone marrow smears from the same animals were also smeared on to the same slide using a 25G needle. The smears were fixed in methanol for 2–3 min, stained with 10% aqueous Giemsa's stain for 20 min,

washed in tap water, and then allowed to dry. The slides were viewed under a 1000× magnification and the number of parasites present/1000 host cell nuclei assessed.

Blood was collected from fromtail of mice and from tongue vein of hamsters at various intervals during experiments or at sacrifice. Samples were incubated overnight or for a minimum of 12 hours at 4ºC, and then centrifuged at 13,000 rpm for 10 minutes at 4ºC. The resulting serum was stored at -20 ºC until required for antibody detection using ELISA.

2.8.1 Specific antibody responses

Serum IgG1 and IgG2a (mouse studies) and IgG (hamster studies) antibody titres against *Leishmania* γGCS were determined by ELISA (Carter *et. al*, 2007). Briefly, a 96 well micro-titre plate (Greiner Bio-One GmbH, Frickenhausen, Germany) was coated with 100 µl of a *Leishmania* γGCS solution (0.1 µg/ml / PBS pH 9.0) overnight at 4ºC. The plates were then washed three times with wash buffer (PBS pH 7.4/0.05% v/v Tween-20). The plates were blocked by adding 150 µl of Marvel® solution (4% w/v in PBS pH 7.4) to the appropriate wells of the plate, and the plate was incubated for 1 hour at 37ºC. The plate was washed three times in wash buffer and then 100 µl of the relevant serum sample, serially diluted in PBS buffer, from 1:100 (mice) or 1:40 (hamster) were added to the appropriate wells of the plate. The plate was incubated as before for 1 hour, washed three times in wash buffer, and 100 µl/well of horseradish peroxidase (HRP) conjugated goat antimouse IgG1 or IgG2a or mouse anti-hamster HRP IgG all used of 1:4000 dilution /PBS pH 7.4 /10 % v/v FCS were added to the appropriate wells of the plate. The plate was incubated for 1 hour as before, washed three times with wash buffer, and 100 µl/well of

substrate (prepared by adding 250 µl of tetramethylbenzidine [6 mg/ml dimethyl sulfoxide] to 25 ml of sodium acetate buffer pH 5.5, containing 7 µl hydrogen peroxide were added. The reaction was stopped after 20 minutes by the addition 50 µl/well of 10% aqueous sulphuric acid. The absorbance of the wells was measured at 450 nm using a Softmax Molecular Device (Molecular Devices Corporation, California. USA) and the mean endpoint ± standard error (SE) for each group were determined**.**

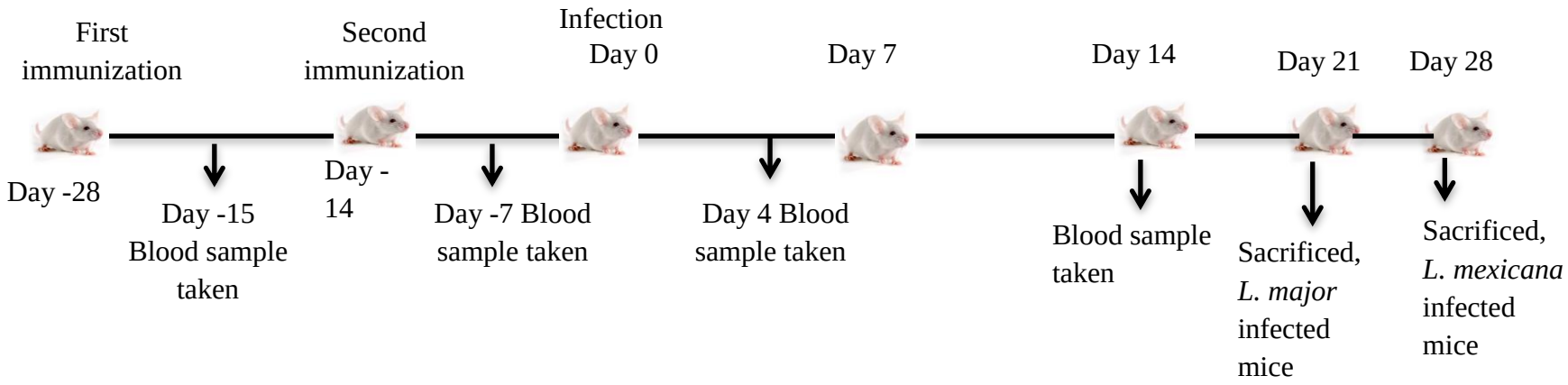


Figure 2.4 The immunization protocol used for *L. mexicana* and *L. major* experiments. BALB/c mice (n=8/treatment) were immunized on day -28 and -14 with *Leishmania* γGCS (from *L. donovani*, *L. mexicana* or *L. major* using single, double or triple protein) by subcutaneous (20 μg/each protein) or inhalation route (50 μg/each protein). On day 0, 3 mice were sacrificed to determine the immune response before infection. The remaining 5 mice were infected by subcutaneous injection into the footpad with $1x10^7$ *L. mexicana* (LmexLuc) or *L. major* (LmajLuc) promastigotes. The blood samples were taken at different time points to measure IgG1 and IgG2a antibody titre. The effect of vaccination on disease progression was determined by monitoring BLI and footpad thickness on days 7, 14, 21 and 28 post infections. *L. major* experiments were terminated on day 21 and *L. mexicana* on day 28, when footpad size controls had reached the maximum size allowed.

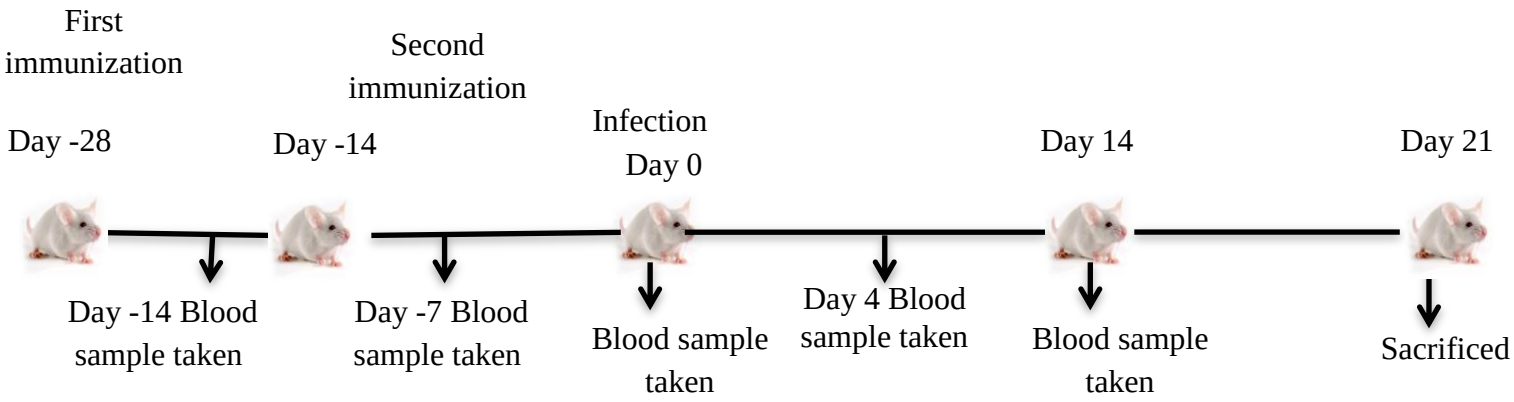


Figure 2.5 The immunization protocol used for *L. donovani* experiments. The BALB/c mice (n=5/treatment) were immunized on day -28 and -14 with triple *Leishmania* γGCS (from *L. donovani, L. mexicana* or *L. major*) by subcutaneous injuction (20 μg/each protein). On day 0 mice were infected by intravenous injection into a tail vein with $1x10^7$ *L. donovani* amastigotes. The blood samples were taken at different time points to determine IgG1 and IgG2a antibody titres. The experiments were terminated on day 21 and the effect of vaccination determined by measuring parasite burden in liver, spleen and bone marrow.

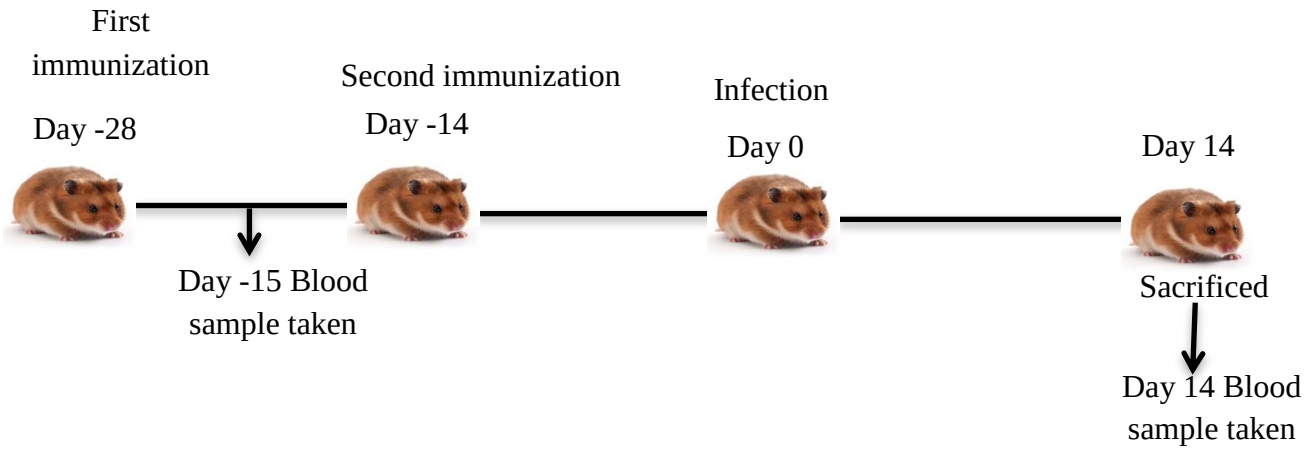


Figure 2.6 The immunization protocol used for *L. donovani* experiments. The Golden Syrian hamsters (n=6/treatment) were immunized on day -28 and -14 with triple *Leishmania* γGCS (from *L. donovani*, *L. mexicana* or *L. major*) by inhalation (50 µg/each protein). On day 0 the hamsters were infected by intravenous injection with $1x10^7$ *L. donovani* amastigotes. The blood samples were taken at different time points to determine IgG antibody titres. The experiments were terminated on day 14 and the effect of vaccination determined by measuring parasite burden in liver, spleen and bone marrow.

2.8.2 *In vitro* proliferation assays

Spleens were removed at sacrifice under aseptic condition and single cell suspensions prepared in incomplete RPMI-1640 medium. The spleen was passed through Nitex filter using the end of a 2.5 ml syringe and the resulting cell suspension was transferred to a labelled universal tube. The cells were pelleted by centrifuging at 300 g (BioFuge Fresco, Heraeus instruments, supplied by Thermo Scientific, Hemel Hempstead, UK) for 5 minutes at 4ºC, and the resulting cell pellet was resuspended in 3 mls Boyle's solution (0.007 M NH_4Cl, 0.0085 M Tris, pH 7.2), which was used to lyse red blood cells. The suspension was incubated for 5 minutes at room temperature and the sample centrifuged as before for 5 minutes and the cells resuspended in 5 mls incomplete medium. This process was repeated to ensure that all the Boyle's solution had been removed. The cells were then resuspended in 1 ml RPMI-1640 complete medium, and the cell concentration determined mixing 15 µl of the cell suspension 1:1 with Trypan blue solution. The cell suspension was loaded into a haemocytometer and the cells were viewed at x400 magnification on a microscope (Nikon Eclipse E400 Microscope, Nikon UK limited, London, UK). Dead cells stain blue and the number of viable cells/ml was determined. In all cases cell viability was > 97%. Live cells, $5x10^5$ /well, were added to the appropriate wells of a 96 well tissue culture plate and incubated with medium alone (un-stimulated controls), *Leishmania* γGCS recombinant protein (12.5 µg /ml, PBS pH 7.4) or concanavalin A (5 µg /ml, positive control) in a final volume of 200 µl (Carter *et al*., 2007). Plates were incubated for 72 hours at 37ºC in an atmosphere of 5% carbon dioxide /95% air. After 72 hours the plates were stored at -20ºC until cytokine or nitrite levels could be determined. In some studies, resazurin solution was added to make 20 µl /well of a stock solution with concentration of 0.125

mg/ml, to measure splenocytes proliferation. This was added to cells before the end of the experiment. The plates were incubated, as before, for 18 hr. and the absorbance at wavelength 570 nm of each well was read using a Softmax Molecular Device (Molecular Devices Corporation, California. USA).

2.8.3 Cytokine determination

Cytokine levels in the cell supernatants were determined by ELISA assay using anti-mouse cytokine antibodies and cytokine standards (Carter *et al*., 2007). Briefly a 96 well ELISA plate was coated with 50 µl/well of the appropriate rat anti-mouse anti-cytokine antibody (IL-10, IL-12, IL-6, IL-4, IL-5, TNFα and IFNγ), 2 µg/ml, in coating buffer (PBS pH 9). Plates were incubated overnight at 4ºC and then washed three times in wash buffer (PBS pH 7.4 containing 0.05% v/v tween-20). Plates were then blocked by adding 150 µl PBS pH 7.4 containing 10% v/v FCS to the appropriate wells of the plate and plates were incubated for 1 hour at 37ºC. Plates were washed as before and then 30 µl of cell supernatant or cytokine standard (serially diluted from 20 ng/ml with 10% v/v FCS in PBS pH 7.4) were added to the appropriate wells of the plate before incubating as before for 2 hours. Plates were washed as before and then 100 µl of the appropriate rat anti-mouse biotin anti-cytokine antibody (1 µg/ml, 10% v/v FCS in PBS pH 7.4) were added to the appropriate wells of the plate. Plates were incubated for 1 hour as before and then washed three times. Streptavidin alkaline phosphate conjugate (100 µl, 1:4000 in 10% v/v FCS in PBS pH 7.4) was added to the appropriate wells of the plate before incubation for an hour at 37ºC. Plates were washed as above and then 100 µl of p-nitrophenyl phosphate disodium salt hexahydrate substrate (1 mg/ml in glycine buffer [0.1 M glycerin, 2 mM magnesium dichloride, 1 mM Zinc, pH 10.4]) were added to the appropriate wells of the plate,

before incubating at room temperature in the dark for 20-60 minutes. The absorbance of the samples at 405nm was measured and the amount of cytokine present (ng/ml) in the cell supernatants was determined from the standard curve plotted from standards run on the same plate. In each case linear regression analysis of the standards gave a correlation coefficient of > 0.97. The mean cytokine production (ng/ml ± SE) for each treatment was determined.

2.8.4 Nitrite determination

Nitrite levels in cell supernatants were determined using the Greiss reagent (Carter *et al*., 2005). A sample (50 µl) of the cell supernatant or the nitrite standards (doubling dilution from 100 µM in PBS pH 7.4) was added to the appropriate wells of a 96 well ELISA plates. Greiss reagent (50 µl 1:1 mixture of 2 % w/v sulphanilamide in 5% v/v orthophosphoric acid: 0.2% w/v naphthylene diamide hydrogen chloride) was added to the appropriate wells of the plate and the plates were incubated at room temperature for five minutes. The absorbance of the samples at 540 nm was determined and the nitrite concentrations (µM) for the samples were determined from the standard curve plotted using the standards run on the same plate. In each case linear regression of the standards gave a correlation coefficient > 0.97. The mean nitrite concentration (µM ± SE) for each treatment was determined.

2.9 Statistical analysis of data

The data was tested for normality using a QC Analyses/K-S Normality Test. Normally distributed data was analysed by student's t-test or one-way analysis of variance (ANOVA) combined with Fisher's LSD test post-hoc. Non parametric data from the *in vitro* and *in vivo* studies were analysed using a Mann Whitney U test to

compare two treatments, or a Kruskal Wallis test followed by Dunn's ad hoc test for three or more treatments, using the Statview® version 5.0.1 software package (SAS Institute Inc, Abacus Concept, *Inc.*, Berkeley, CA, USA). A p value of < 0.05 was considered significant.

Chapter 3: Cloning of *Leishmania* γGCS into the expression vector.

3.1 Introduction

Recombinant DNA technology is the most efficient way of producing protein; it provides a high amount of pure protein that is not available naturally. The starting point for recombinant protein production is isolation of the gene of interest and cloning it into a suitable expression vector (Li, 2011). In these studies the gene of interest is moved from a large and complex genome to a smaller, simpler one that facilitates manipulation. Recombination technology makes it possible to cut DNA with the relevant restriction enzymes and join or recombine the excised DNA fraction using complementary base pairing (Chen *et al.*, 2013). Restriction endonucleases cut DNA at recognition sites, which are specific to the particular of enzyme site. Therefore, it is possible to predict the size and number of DNA fragments, that would be obtained by cutting a DNA sequence of known size. The resulting DNA fragments purified after separation using gel electrophoresis, can then be recombined into an expression vector using DNA ligase e.g. T4, which in the presence of ATP, catalyses the formation of phosphodiester bonds at the ends of DNA strands (Lee *et al.*, 2012). This process is shown in Figure 3.1. *Leishmania* recombinant proteins have been prepared by a number of researchers (Fernandez-Robledo and Vasta, 2010).

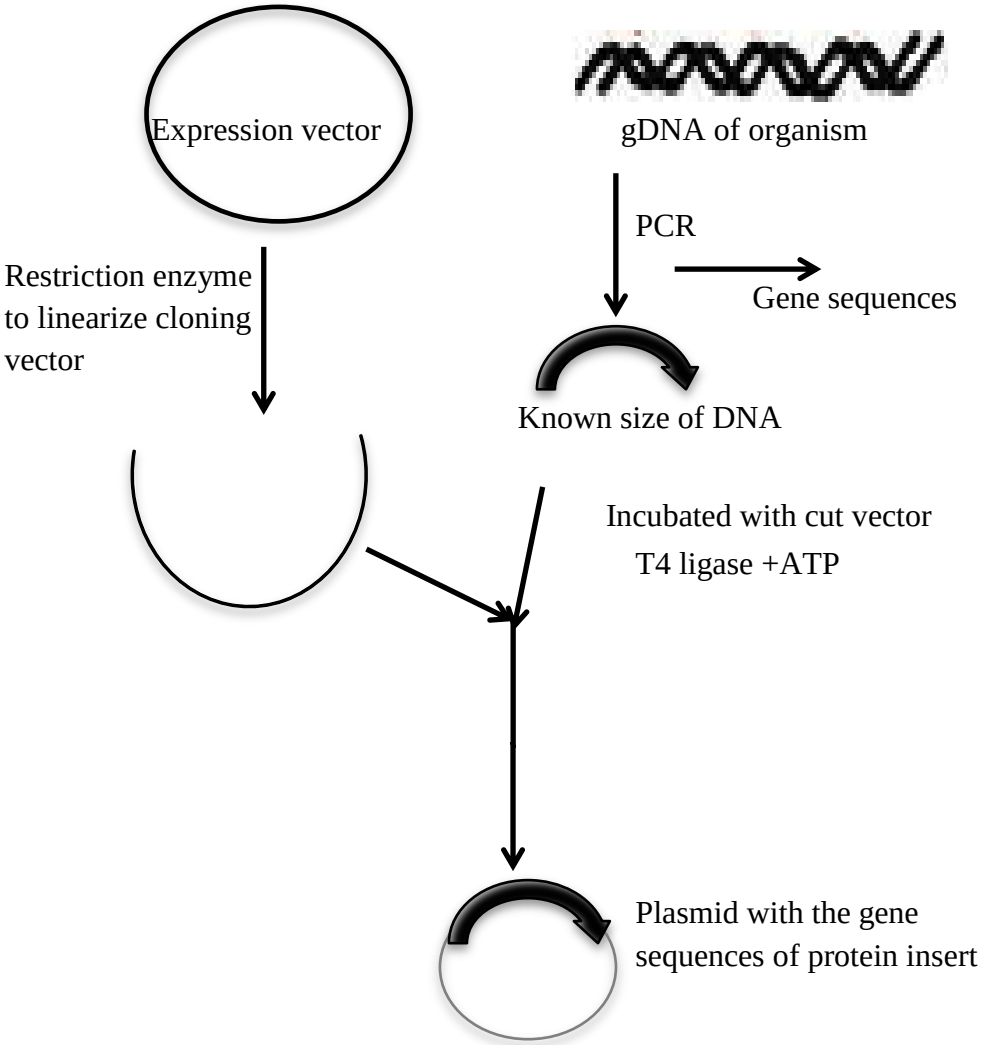


Figure 3.1 The procedures in inserting gene sequence into an expression vector.

Expression vectors used in recombinant protein production are extrachromosomal genetic elements (plasmids) that can replicate autonomously, usually within bacterial cells (Claeys Bouuaert *et al.*, 2013). In this study TOPO® cloning was used to insert *Leishmania* γGCS gene in the expression vector. TOPO® cloning uses the *Vaccinia* virus enzyme topoisomerase and it is a cloning method which provides a high cloning efficiency after incubation for just 5 minutes at room temperature (Liu *et al.*, 2012). The amplified PCR product of *Leishmania* γGCS gene sequences from gDNA was cloned into the TOPO® vector (vector map in Appendix1). The TOPO® cloning vector has ampicillin and kanamycin resistance markers, lacZ reporter gene, and *EcoR* I sites at the sides of the insertion site. The ampicillin and kanamycin resistance inserts in the plasmid allow for quick selection of bacterial colonies that take up plasmid containing *Leishmania* γGCS gene sequences during transformation. Expression of the lacZ gene product in bacteria causes colonies to have a blue colour. If the PCR product of *Leishmania* γGCS gene sequences is inserted in the cloning vector it will then insert in the middle of the lacZ gene causing white colonies instead of blue colonies to grow. Single white colonies are then grown to produce large quantities of the plasmid. The plasmid is then extracted to ensure that the γGCS gene sequence is incorporated in the correct orientation by restriction enzyme digest to ensure products of the correct size are obtained (Shuman, 1994).

At each stage, sequencing of the γGCS gene in the relevant plasmid is carried out to ensure that it contains the gene insert. This is facilitated by the fact that the *Leishmania* genome has been published and is available online via the gene bank

database (http://www.ncbi.nlm.nih.gov). There are differences in the gene sequence of different *Leishmania* species For example, *L. donovani* and *L. major* have 36 chromosome pairs (Wincker *et al.*, 1996). Whereas *L. mexicana* has 34 or 35 pairs (Britto *et al.*, 1998). *Leishmania* parasites are genetically unusual, as they lack transcriptional control, so genes transcribed in polycistronic pre-mRNAs are subsequently processed into mature mRNAs by trans splicing (LeBowitz *et al.*, 1993).

The main objectives of this study are to:

- Amplify and clone the γGCS gene sequence of *L. mexicana*, *L. major* and *L. donovani*.
- Clone the γGCS gene sequence of each *Leishmania* species into the TOPO® cloning vector.
- Clone the γGCS *Leishmania* gene sequences from the TOPO® vector into the pET-24a expression vector. This would allow production of recombinant *Leishmania* γGCS protein for expression studies.

3.2. Results

3.2.1 Amplification and cloning of *Leishmania* γGCS gene

Genomic DNA (gDNA) from *L. donovani* (strain 200016), *L. major* (strain WHOM/IR/-/173), and *L. mexicana* (strain MNYC/BZ/ M379), was isolated from promastigote parasites grown *in vitro.* Table 3.1 shows the amount of gDNA obtained for the three *Leishmania* species, and the purity of the DNA sample based

on ratio absorbance of reading at 260nm and 280nm. A ratio of >1.8 showed that the samples contained DNA of good quality and purity. The gDNA samples were separated by agarose gel electrophoresis (Figure 3.2).

Table 3.1 The concentration and A260/280 ratio for gDNA isolated from the three *Leishmania spp.*

Parasite	Concentration of gDNA (µg/µl)	Ratio of A260/A280
L. donovani	0.0422	1.82
L. mexicana	0.0583	1.83
L. major	0.0466	1.84

The gDNA samples of *L. major*, *L. mexicana* and *L. donovani* were used as a template to amplify γGCS gene sequences by PCR. The γGCS PCR products were analysed by agarose gel electrophoresis (Figure 3.3) and gave the predicted size of 2064 bp for each *Leishmania* species.

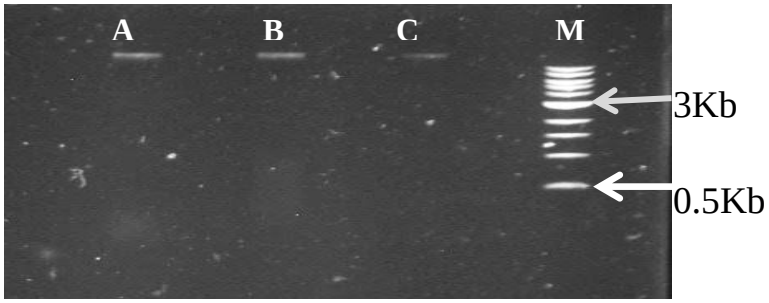


Figure 3.2 The size of gDNA samples isolated from *L. major* (A*), L. mexicana* (B) and *L. donovani* (C). The molecular weight marker (M) is also shown. The gDNA of three parasites has the large size over 3kb. Agarose gel was stained with ethidium bromide to show the bands using UV light.

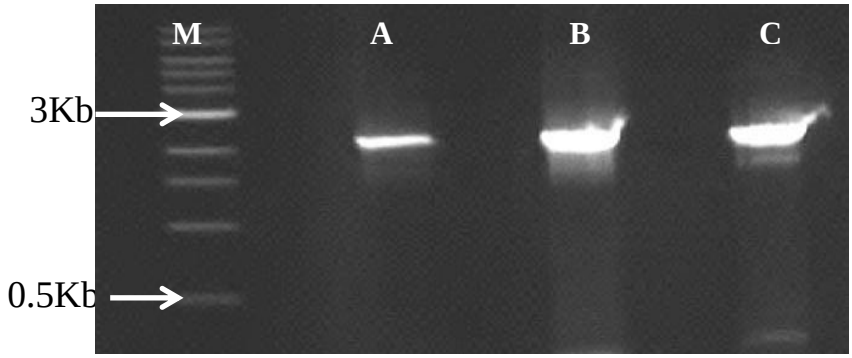


Figure 3.3 Picture of PCR amplified products for the three *Leishmania* species. The agarose gel was stained with ethidium bromide to show DNA PCR amplified gene products from *Leishmania* γGCS gene from *L. donovani* (A), *L. mexicana* (B) and *L. major* (C), that each amplification product has size 2064 bp. M is marker.

3.2.2 Cloning into PCR TOPO® cloning vector

The PCR product for each γGCS gene sequence of the three *Leishmania* species was cloned into the TOPO® cloning vector. The plasmid containing the γGCS gene sequence was used to transform *E. coli* bacteria to grow up a large quantity of the plasmid. The plasmid was extracted from three individual colonies for each *Leishmania* species and used in digests with the restriction enzyme. *EcoR*I sites are at each side of the PCR insertion site, which allows the inserted gene sequence to be extracted from the plasmid. The amplified PCR of *Leishmania* γGCS gene plasmid was analysed by agarose gel electrophoresis to ensure the ligation between TOPO® vector and amplified PCR of *Leishmania* γGCS gene was successful. The transfected TOPO® cloning plasmid was digested using the enzymes shown in Table 3.2. If the gene had inserted in the correct orientation in the vector, the gel picture for samples

after digestion with *EcoR 1* (Figure 3.4), *HindIII* (Figure 3.5) and *PvuII* (Figure 3.6).

All resultant samples gave the predicted fragments as shown in Table 3.2.

Table 3.2 Shows the size of fragments obtained after digestion for the three TOPO® cloning vector containing *Leishmania* γGCS gene.

Species	**Predicted size of fragments (Kb) for *Leishmania* γGCS gene sequences.**		
	EcoR1	*HindIII*	*PvuII*
L. donovani	3.912, 1.257 and 0.845	4.166 and 1.848	2.433, 1.260, 1.244, 0.717 and 0.360
L. mexicana	3.912 and 2.102	6.014	2.433, 1.260, 0.742, 0.717, 0.419, 0.360 and 0.083
L. major	3.912, 1.257 and 0.845	6.014	2.433, 1.260, 0.825, 0.717, 0.419 and 0.360

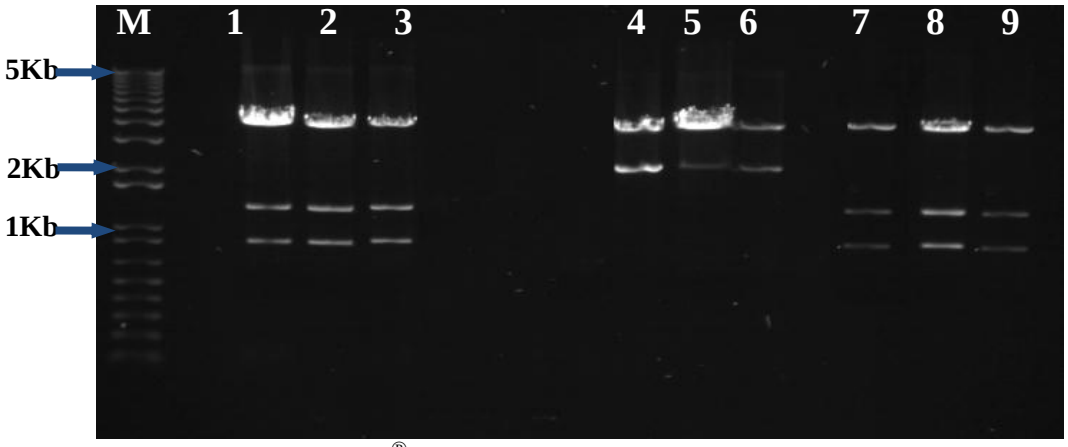


Figure 3.4 Picture of TOPO® γGCS samples after digestion with *EcoR1*. The gel was stained with ethidium bromide and viewed under UV light. The TOPO® γGCS plasmid of *L. donovani* (1, 2 and 3), *L. mexicana* (4, 5 and 6) and *L. major* (7,8 and 9). Marker (M). TOPO® γGCS plasmid was incubated with *EcoR1* restriction enzyme for 2 hours at 37ºC. The ligation was successful and contained the sequence of the predicted nucleotide of γGCS as described in table 3.2.

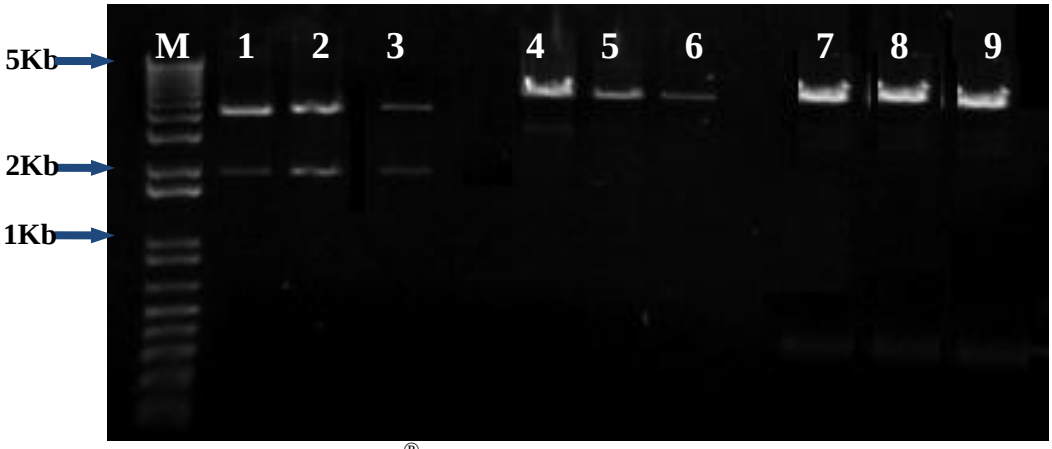


Figure 3.5 Picture of TOPO® γGCS samples after digestion with *HindIII*. The gel was stained with ethidium bromide and viewed under UV light. The TOPO® γGCS plasmid was incubated with *HindIII* restriction enzyme for 2 hours at 37ºC. Marker (M). The TOPO® γGCS plasmid of *L. donovani* (1,2 and 3), *L. mexicana* (4, 5 and 6) and *L. major* (7, 8 and 9). The ligation was successful and contained the sequence of the predicted nucleotide size as described in table 3.2.

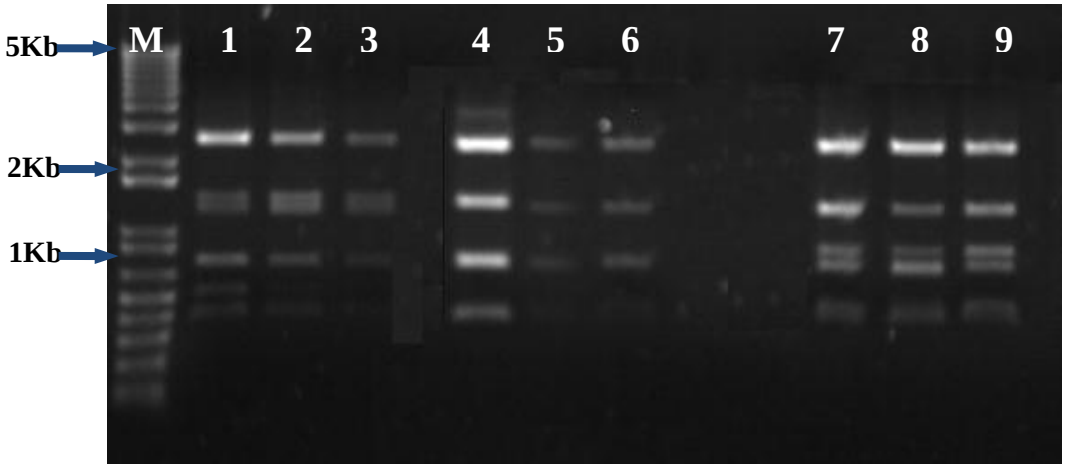


Figure 3.6 Picture of TOPO® γGCS samples after digestion with *PvuII*. The gel was stained with ethidium bromide and viewed under UV light. The TOPO® γGCS plasmid was incubated with *PvuII* restriction enzyme for 2 hours at 37ºC. Marker (M). The TOPO® γGCS plasmid of *L. donovani* (1, 2 and 3), *L. mexicana* (4, 5 and 6). *L. major* (7, 8 and 9). The ligation was successful and contained the sequence of the predicted nucleotide size as described in table 3.2.

After confirming that the TOPO® cloning vector had the *Leishmania* γGCS gene sequences in the correct orientation, it was important to shown that the genes were identical to the *Leishmania* γGCS gene sequence. TOPO® PCR 2.1- γ GCS plasmids were sent for sequencing, three samples for each *Leishmania* species. Sequences results for the samples were compared to *L. mexicana* (Gene bank code LmxM.18.1660.1.pep), *L. major* (Gene bank LmjF.18.1660.pep), and *L. donovani* (Gene bank AY371486.2) γGCS gene sequences. Only one sample for each *Leishmania* parasite gave 100% homology to the original γGCS gene sequence for the specific *Leishmania* species (Appendix 2 shown the gene sequences).

3.2.3 Cloning *Leishmania* γGCS into pET-24a vector

The TOPO® cloning vectors containing the *Leishmania* γGCS gene and pET-24a expression plasmids were digested using *NotI* and *BamHI*. The resulting products were separated using gel electrophoresis (Figure 3.7) and the required products extracted. The samples were then incubated with T4 ligase to allow insertion of the γGCS gene sequence into the expression vector. After ligation the resulting product was transfected into *E.coli* DH5α and the plasmid was extracted from bacteria grown from a single selected colony. The γGCS with expression plasmid was subjected to enzyme digestion using *NdeI*. This should give two products of 6428 bp and 921 bp. A picture of the gel after digestion of the *Leishmania* γGCS pET-24a plasmid is shown in Figure 3.8.

Samples of the γGCS after cloning into pET-24a expression plasmids for *L. donovani, L. mexicana* and *L. major* were sent for sequencing using T7 forward and T7 reverse primers. The sequencing data obtained was compared to *L. mexicana*

(Gene bank code LmxM.18.1660.1.pep), *L. major* (Gene bank LmjF.18.1660.pep), and *L. donovani* (Gene bank AY371486.2). Only one of three samples in which was sent for each species had 100% homology sequence in the gene bank plasmids. These were used for protein expression study. The sequencing data obtained from *L. donovani*, *L. mexicana* and *L. major* are shown in the Appendix (2). Where another two samples had less than 90% homology sequence in the gene bank plasmids.

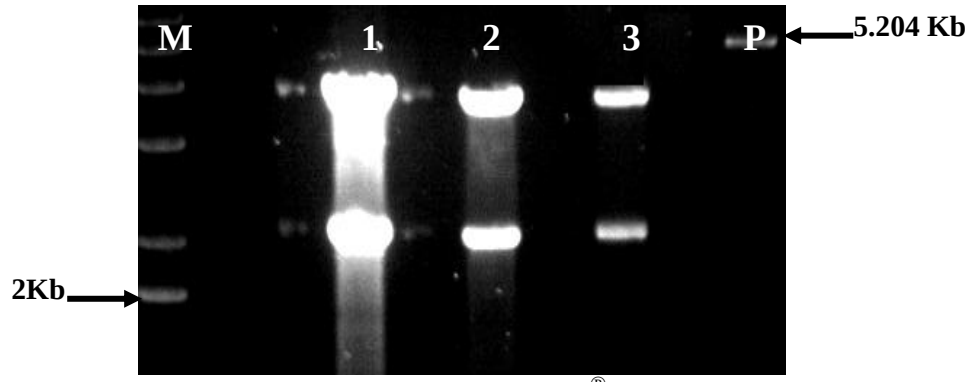


Figure 3.7 Picture of the agarose gel from the TOPO® cloning plasmid containing the gene sequences γGCS and pET24-a after digestion with *Not1 & BamH1* enzyme. The gel was stained with ethidium bromide and viewed under UV light. Samples were incubated with restriction enzyme for 2 hours at 37ºC. Marker (M). *L. donovani* (1), *L. mexicana* (2) and *L. major* (3) and pET24-a (P).

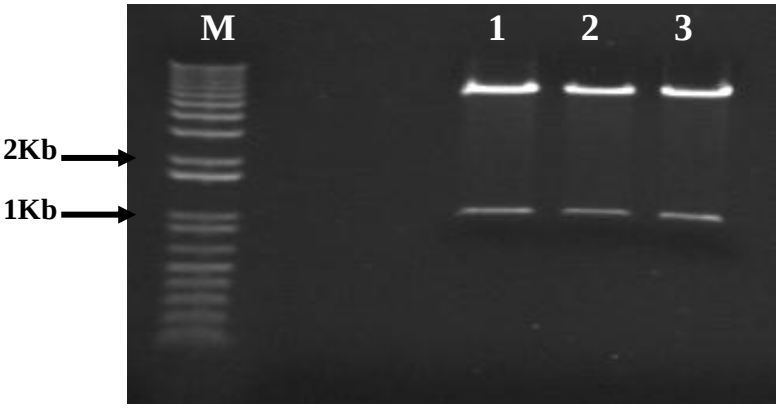


Figure 3.8 Picture of gel pET-24a plasmid containing *Leishmania* γGCS after digestion with *NdeI*. Plasmids were incubated at 37ºC for 2 hours. Two bands of size 6428 bp and 921 bp. *L. donovani* (1), *L. mexicana* (2), and *L. major* (3). M is marker.

3.3 Discussion

The relevant gene sequence of γGCS for the three *Leishmania* species was cloned into an expression vector for protein expression studies. In this study genomic DNA was used for γGCS amplification rather than cDNA as *Leishmania* mature mRNAs result from coordinated polyadenylation and *trans*-splicing of a polycistronic RNA (Perry and Agabian, 1991) and the genes lack individual transcriptional regulation, since they are transcribed as long polycistronic units (LeBowitz *et al.*, 1993). The protein-coding genes are organized into large polycistronic gene clusters where many genes are situated on the same DNA strand (Myler and Stuart, 2000; Martinez-Calvillo *et al.*, 2010).

The fragments of a particular size range are amplified using PCR. It is difficult to control the composition of the amplified sets of fragments and therefore PCR amplification conditions must be optimised (Dahl *et al.*, 2005). The γGCS gene is relatively large with a nucleotide size of 2064 bp. Therefore, *Taq* polymerase was used to amplify the γGCS gene, as it reduces the frequency of new mutations occurring (Gilje, 2008). Sequencing of the γGCS PCR products showed the γGCS gene were successfully amplified after just one PCR reaction.

Not all selected colonies obtained after insertion of the γGCS gene sequence into the TOPO® cloning vector contained the correct γGCS gene insert. Therefore it is

essential that insertion is confirmed using enzyme digestion followed by gene sequencing studies. Carrying out restriction enzyme digestion first reduces the costs associated with sequences studies, as these were carried out using a commercial sequencing company (Yang *et al.*, 2013).

It took approximately three months to get the correct TOPO® cloning plasmid and over 20 TOPO® γGCS plasmids from each plasmid from a single selected white colony were analysed for each *Leishmania* species. This low efficiency may be related to the large size of the γGCS gene sequence. In other studies, from 60 samples tested, only 30 gave an insert of a positive TOPO® cloning plasmid for a gene insert of 1000 bp, and 18 positive colonies for an insert of 1500 bp. It was suggested that cloning efficiency depends on the size of the insert gene sequences into the TOPO® cloning plasmid (Geng *et al.*, 2006). Only one of the three γGCS TOPO® plasmids for each *Leishmania* species had a gene insert with 100% homology to the original γGCS gene sequences. The main problem during cloning was a mutation, where a guanine (G) was replaced with a cytosine (C). This mutation would have altered the protein expression but the reason for the occurrence of this common mutation is unknown (Downing *et al.*, 2011).

Alignment of the γGCS gene sequence for the three *Leishmania* species showed there was considerable homology (87.6%) for all three species. There was 95% identity and 97% similarity between *L. donovani* and *L. major*, 91% identity and 95% similarity between *L. donovani* and *L. mexicana* and 89% identity and 94%

similarity between *L. major* and *L. mexicana*, as see in Appendix 4 (Henriquez *et al.*, 2010)

Thus all the aims of the study were achieved; i.e. the γGCS sequences of all three *Leishmania* species were successfully cloned into the pET-24a expression vector. Sequencing studies showed that the γGCS gene sequences for all three *Leishmania* were in the correct orientation for expression, and sequences had 100% homology to published γGCS sequences for the three *Leishmania* species (Appendix 2 gene sequences of γGCS).

Chapter 4: Optimisation of expression and purification of *Leishmania* recombinant γGCS protein.

4.1 Introduction

Advances in genomics, proteomics and bioinformatics have allowed that recombinant proteins to be successfully produced in bulk. These proteins can be used for therapeutic, diagnostic or industrial applications (Korf *et al.*, 2005; Su *et al.*, 2013). The expression vector can be grown in different expression systems using *E. coli*, *S. cerevisiae*, *P. pastoris*, insect and mammalian cell lines which are used for expression of heterologous proteins (Prinz *et al.*, 2004; Meyer *et al.*, 2013). The most often used host is *E. coli*, as it has a short generation time, it can be produced in high volume at low cost and it is easy to use (Bernaudat *et al.*, 2011). It is important to choose the most appropriate strain of *E. coli*, expression vector and growth conditions. Studies have shown that most recombinant proteins expressed at high levels in *E. coli* (Zheng *et al.*, 2011). However, it is possible that polypeptide proteins can accumulate as insoluble aggregates that lack functional activity. The bacteria have to be chemically treated to induce expression using agents such as isopropyl β-D-1-thiogalactopyranoside (IPTG), a synthetic analogue of lactose, which can cause cell toxicity, protein instability, inappropriate processing, post-translational modification or inefficient translation of the recombinant protein (Makino *et al.*, 2011; Zheng *et al.*, 2011). Therefore, it is important that the optimal expression conditions be identified so that a soluble, functionally active protein is produced. This is particularly import if the protein is going to be used a vaccine or for biochemical application as its 3-D conformation structure may be critical to its activity (Gnoth *et al.*, 2010).

The pET expression system (Novagen, 2003) is often used for expression studies as it allows a high quantity of protein produced. It uses the T7 promoter, which is not recognized by the *E. coli* RNA polymerase, so expression of the recombinant protein only occurs when the T7 RNA polymerase is present. The expression vector, transfected the gene sequences of the specific recombinant protein, in this case *Leishmania* γGCS, is transformed into an *E. coli* strain that has the gene encoding for the T7 RNA polymerase under the control of the *Lac* promoter. Gene expression for the recombinant protein is activated by the addition of IPTG. This initiates transcription of the T7 RNA polymerase, which subsequently leads to the transcription of the recombinant protein gene sequence (Figure 4.1).

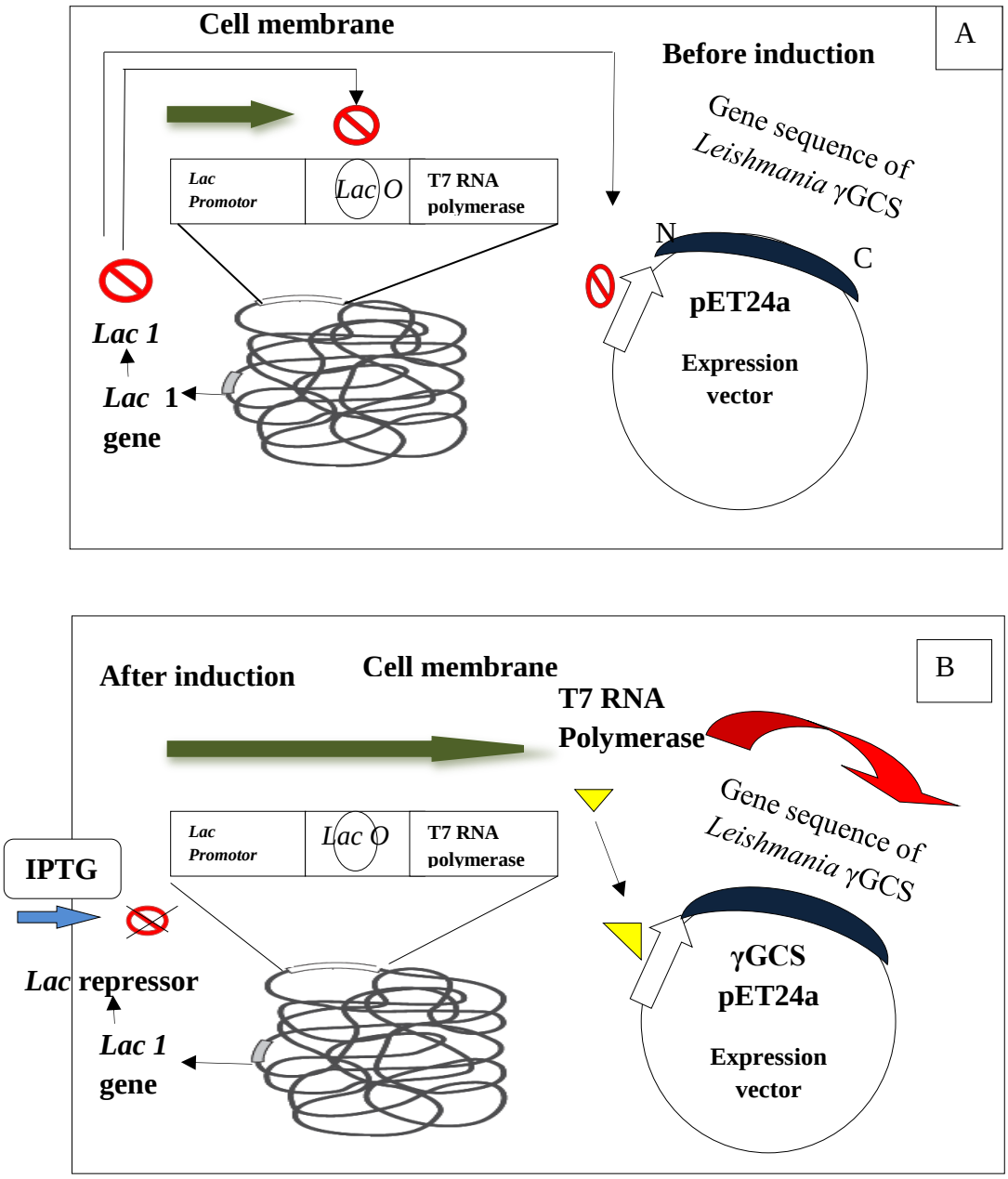


Figure 4.1 Mechanism for protein expression of γGCS recombinant protein in *E.coli* BL21. (A) Before induction, the *Lac*I repressor binds to the *Lac* operator and inhibits expression of the T7 RNA polymerase. (B) After induction, IPTG binds to the *Lac*I repressor and allows the transcription of the T7 RNA polymerase. The T7 RNA polymerase activates the T7 promoter in the pET expression vector and thus the protein of interest is expressed (adapted from Novagen, 2003).

The next stage involves recombinant protein purification. The affinity tags are also used to allow purification of virtually any protein without requiring knowledge of the biochemical properties of the induced protein (Arnau *et al.*, 2006). Affinity tags are exogenous amino acid sequences with a high affinity for a specific biological or chemical ligand (Fassina *et al.*, 2001). The pET-24a expression plasmid ensures that the recombinant protein is expressed as fusion protein with a histidine tag (his-tag) which allows purification using metal affinity chromatography as the histidine binds to nickel (Harris *et al.*, 2010). The his-tag consists of six histidine residues and since histidine is not present in many proteins, the recombinant fusion proteins have the his-tag after purification. Nickel is immobilized on an ion affinity column using an agent present on an agarose resin. When a mixture of proteins recovered from an *E.coli* culture is passed through the column, the his-tagged recombinant protein is retained on the column whilst other proteins pass through in the effluent. This separates the target protein from the mixture. The protein is then eluted from the column using imidazole which has a stronger binding affinity for nickel than histidine, so it replace histidine on the column`s binding sites (Tropea *et al.*, 2009).

In previous studies, *L. donovani* γGCS was expressed and purified as a his–tagged recombinant protein (Henriquez *et al.*, 2010 and Campbell *et al.*, 2012) but it was mainly expressed in inclusion bodies, are nuclear or cytoplasmic aggregates of proteins (Bernaudat *et al.*, 2011). Urea had to be used to extract the recombinant protein and that truncated as well as full length proteins were produced. All the proteins present were identified as *L. donovani* γGCS, using MALDI (Matrix-assisted

laser desorption/ionization mass spectrometry) but it was difficult to purify these truncated proteins from the full-length protein.

The main purpose of this study was to optimise the expression, purification and refolding of the recombinant γGCS enzyme of the three *Leishmania* species *L. donovani*, *L. mexicana* and *L. major*, using a different gene construct. This time the his-tag was incorporated into the C-terminus instead of N-terminus of the protein, as it was thought that this would help in the production of full-length protein without degradation of protein during protein synthsis in bacteria as his-tag at the end of protein sequences (Hartinger *et al.,* 2010). Recombinant *Leishmania* γGCS protein was expressed under the control of the T7 promoter in *E. coli.* The aim was to obtain recombinant γGCS from each *Leishmania* species sufficient to allow enzyme studies and for vaccine studies.

4.2 Results

Expression studies showed that the three γGCS *Leishmania* recombinant proteins were expressed as full and partial length proteins, rather than just one full-length protein. The main bands present after separation using gel electrophoresis were extracted and examined in MALDI studies. These confirmed that the expressed proteins were *Leishmania* γGCS (Appendix 3). In this study we investigated the effect of changing the experimental conditions, i.e. the incubation temperature and concentration of IPTG on expression of *Leishmania* γGCS protein in an attempt to increase expression and improve expression of the soluble protein without obtaining truncated protein in the preparation.

The effect of different incubation temperatures on induced protein production was determined. In all studies IPTG was added when the *E. coli* culture had an absorbance of 0.6-0.9. The amount of *L. donovani* γGCS protein produced was higher if the bacteria were incubated at 25°C after induction with 1 mM IPTG (Figure 4.2A) and similar results were found for expression of *L. mexicana* γGCS (Figure 4.2B) and *L. major* γGCS (Figure 4.2C).

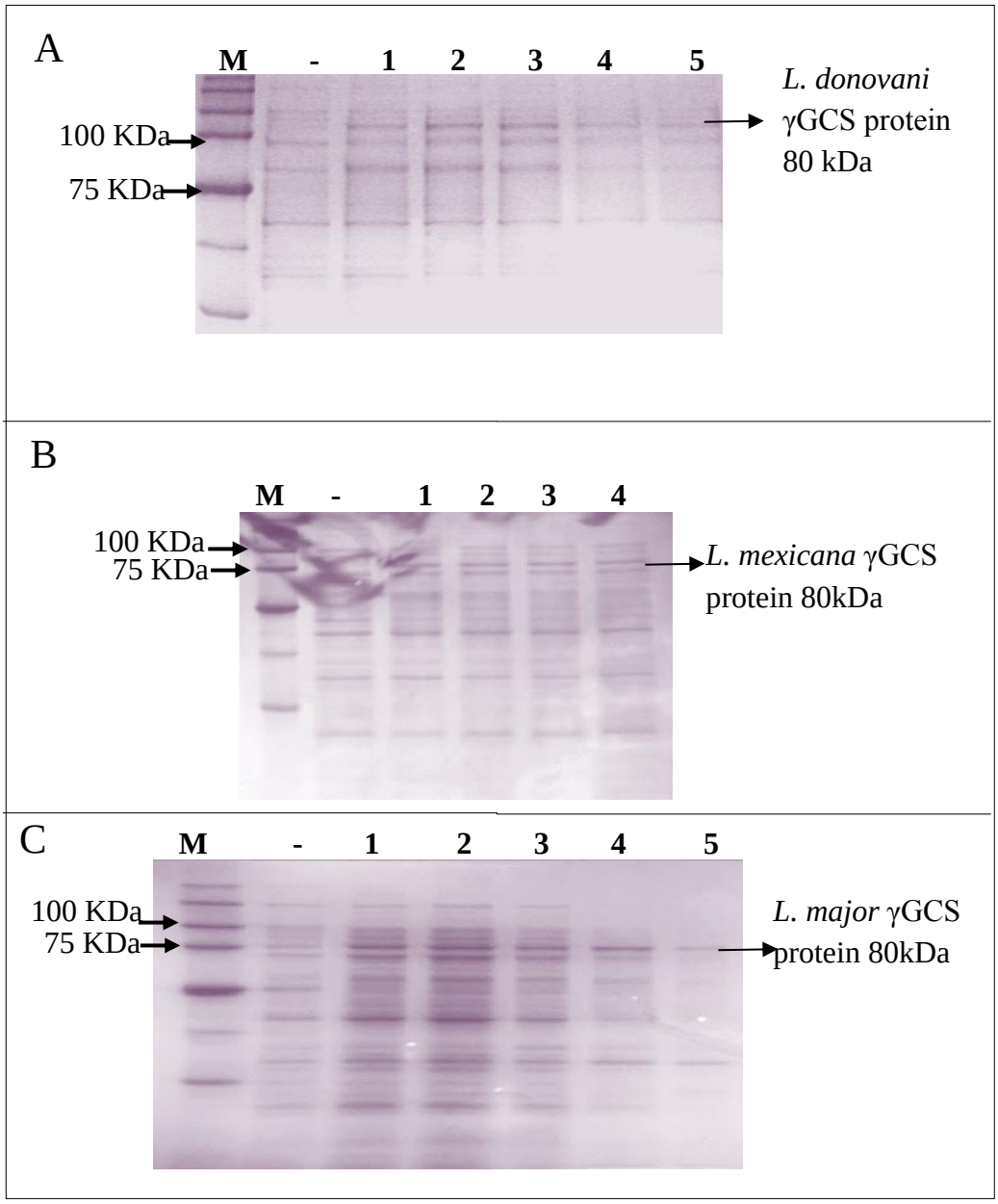


Figure 4.2 The effect of incubation temperature on expression of *Leishmania* γGCS recombinant protein. *E. coli* was transfected with the *L. donovani* γGCS (A), *L. mexicana* γGCS (B) and *L. major* γGCS (C) pET-24a expression plasmid. The *E.coli* culture was incubated at 37°C until it has an absorbance of 0.6 and then expression was induced by adding 1mM IPTG. Cultures were then incubated at, 37°C (lane 1), 30°C (lane 2), 25°C (lane3), 20°C (lane4), 15°C (lane 5), the uninduced (-). The bacteria pellet was separated by gel electrophoresis and the proteins produced visualised using Coomassie blue stain. M (protein marker).

Expression studies when IPTG the concentration was altered (0.1, 0.25, 0.5, 1, 2, 4 and 5 mM) showed that a higher amount of protein was produced using the lowest IPTG concentration (Figure 4.3). These finding were similar for all three *Leishmania* species. The effect of altering the incubation temperature on protein expression for *L. donovani* and *L. mexicana* γGCS was determined using a temperature of either 25ºC or 30ºC. Induction using 0.1mM IPTG and an incubation temperature of 25ºC gave the highest insoluble protein expression for both the recombinant proteins (Figure 4.4).

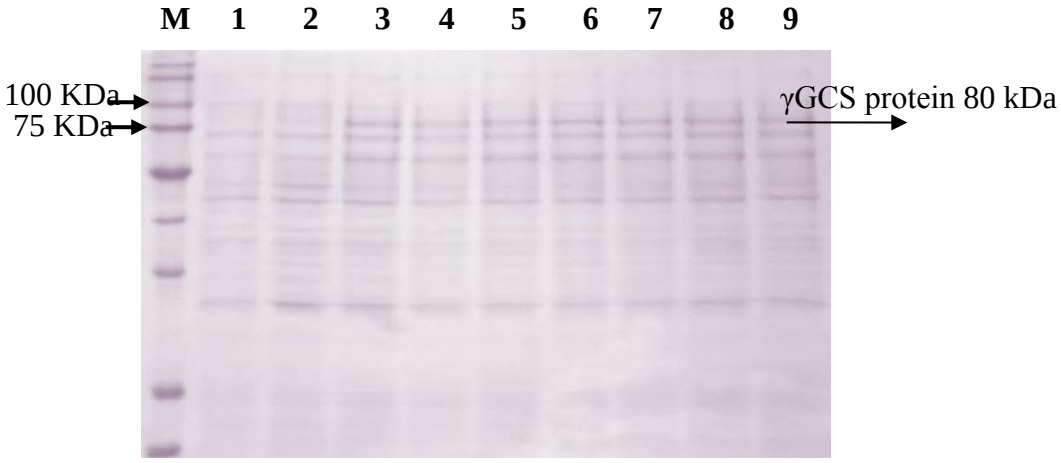


Figure 4.3 The effect of IPTG concentration on *L. donovani* γGCS protein at 25ºC. *E. coli* was transfected with the *L. donovani* γGCS pET-24a expression plasmid. The *E. coli* culture was incubated at 37°C until it has an absorbance of 0.6 and then induced by adding IPTG. Uninduced sample (lane1), no IPTG add (lane 2), induced samples with 0.1, 0.25, 0.5, 1,2,4, and 5 mM (lanes 3, 4, 5, 6, 7, 8 and 9 respectively). The bacteria pellet were separated by gel electrophoresis and protein produced visualised using Coomassie blue stain. M is protein marker.

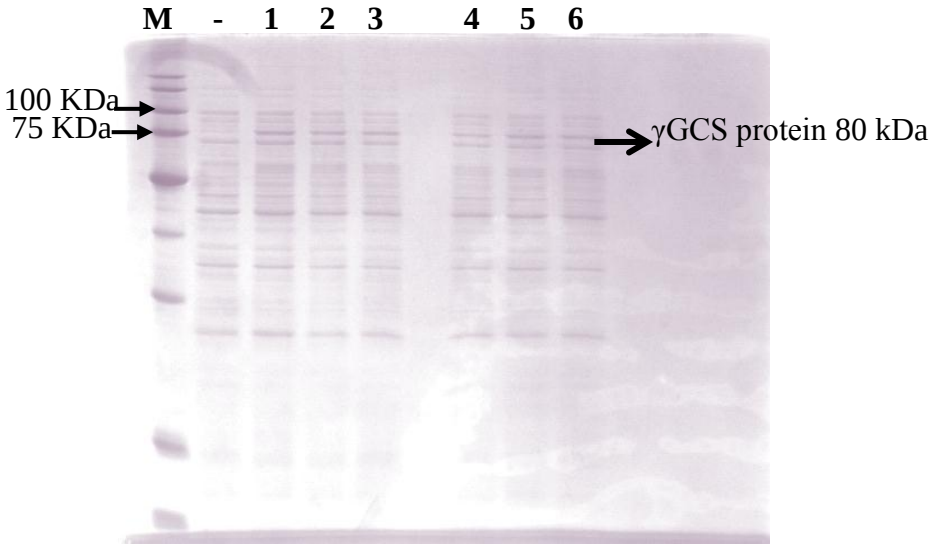


Figure 4.4 The effect of IPTG concentration and the temperature on *L. donovani* γGCS protein at 25ºC (1, 2, 3) and 30ºC (4, 5, 6). *E. coli* was transfected with the *L. donovani* γGCS pET-24a expression plasmid. The *E. coli* culture was incubated at 37°C until it has an absorbance of 0.6 and then induced by adding IPTG. Uninduced sample (-), IPTG induced samples with 0.1 (lane 1 and 4), 0.5 (lane 2 and 5), and 1 mM (lane 3, and 6). The bacteria pellet were separated by gel electrophoresis and protein produced visualised using Coomassie blue stain. M is protein marker.

Previous studies showed that most of the γGCS *L. donovani* recombinant protein was expressed in inclusion bodies (Henriquez *et al.*, 2010). Therefore the temperature used after induction was reduced to slow down protein production and hopefully reduce aggregation of protein into inclusion bodies. Studies showed that the solubility of the expressed γGCS protein increased as the incubation temperature was reduced. However, most protein was still inside inclusion bodies as see in Figure 4.5 that the expected protein size 80KDa was more in pellet than in supernatant after the bacteria cell lysed. The results indicated that 18ºC was the best temperature to use (Figure 4.5) as soluble protein was produced for the three *Leishmania* γGCS

recombinant proteins, using IPTG at a concentration of 0.1mM. Also this result was confirmed using western blotting for detect the His tag protein from pellet and supernatant (Figure 4.6), That indicated more proteins in pellet as insoluble form than supernatant.

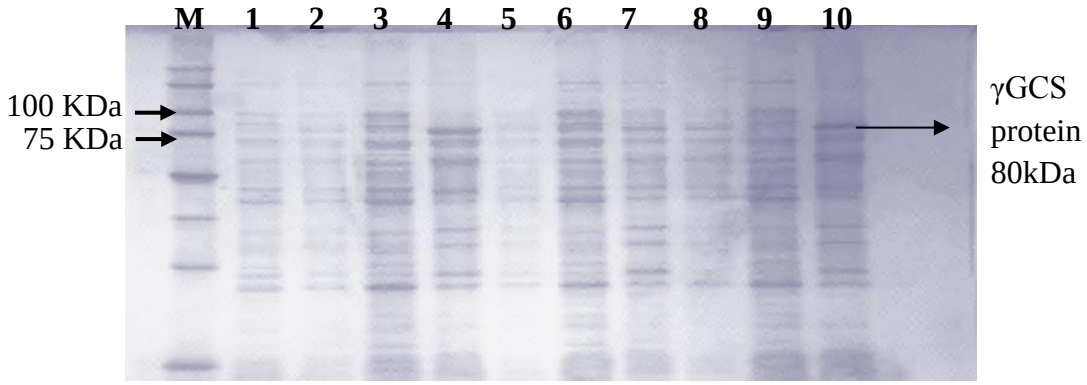


Figure 4.5 The effect of temperature on the solubility of the recombinant *Leishmania* γGCS protein produced. Molecular weight marker (M). lane 1 (uninduced), 2, 3, and 4 incubated at 37 ºC and then at 15 ºC after inducing. Lane 5, 6, 7 are incubated at 37ºC then 18 ºC after inducing. Lane 8, 9, 10 are incubated at 25ºC. Lane 2, 5 and 8 (inducing sample), Lane 3, 6 and 9 (pellet sample). Lanes 4, 7 and 10 (supernatant). Bacteria pellets were separated by gel electrophoresis and protein produced visualised using Coomassie blue stain.

Western blotting studies were carried out to confirm that target protein produced had a his-tag. The eluted recombinant protein of *L. donovani* (LdγGCS), *L. mexicana* (LmγGCS) or *L. major* (LjγGCS) were purified using a his Trap® column. In Figure 4.6, it is clear that each had a his-tag but more than one protein band was obtained. The other his-tag protein bands could be degraded products from the expressed protein or proteins from the expression host. Therefore the effect of different purification methods on protein recovery was determined. Gradient elution using different concentration of imidazole was tested but this did not improve recovery into gave the same result.

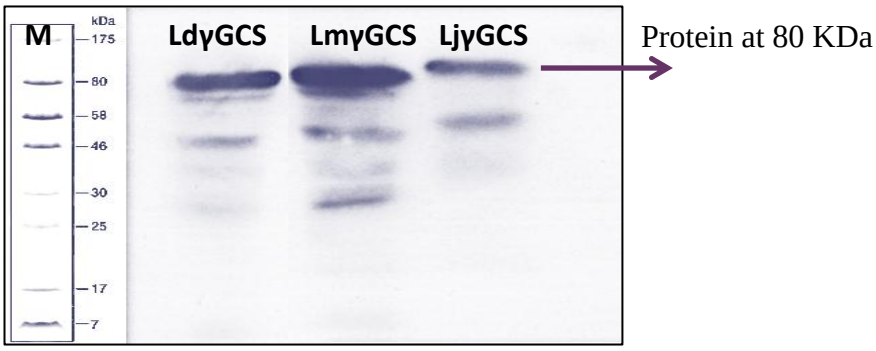


Figure 4.6 His-tag proteins present in LdγGCS, LmγGCS and LjγGCS recombinant protein samples. The samples of final elute were incubated with a primary anti-His tag mouse polyclonal antibody and then a secondary anti-mouse IgG HRP conjugated antibody. The presence of labelled proteins was determined be measuring the amount of chemiluminescence produced. The position of the molecular weight markers (M) and the major bands present for the three recombinant γGCS *Leishmania* proteins are clearly seen.

The different bands produced for each recombinant γGCS prepared under native or denaturation conditions were separated by electrophoresis and the band was sequenced by the Proteomics centre in the University of Glasgow by Mass Spectrometry after trypsin digestion, and the data were analyzed by Mascot software (Matrix Science). The threshold ion score was calculated to be above 42 for 5% confidence. The analysis is based on the mass of the peptides identified and bioinformatics compared to the protein sequences, using multiple sequence alignment of identified proteins. That was homology based to those of *L. donovani*, *L. major*, *L. mexicana* and *E. coli* genome datasets. MALDI studies identified 80 KDa MW proteins as *Leishmania* γGCS proteins for *L. donovani*, *L. mexicana* and *L. major* and the smaller bands were truncated forms of the same protein. The total mascot score needed for significance is

above 100. The result indicated that, all four bands have a high ion score of more than 100, and a high percentage of coverage for preparation under denaturing or native conditions. The largest 80 KDa band had a score of 606, 500, and 901 for *L. donovani*, *L. mexicana* and *L. major* samples respectively. Another band with size 40 and 30 KDa also had a higher score of more than 150 (Appendix 4 mascot result).

The effect of altering the buffer used in protein purification and using ion exchange chromatography on protein recovery was investigated. Studies under native conditions showed that high amounts of protein were recovered if Tris buffer pH 7.4 was used (0.553 mg/ml) instead of PBS pH 7.4 (0.05 mg/ml) from 1L *E.coli* broth. Therefore in subsequent studies Tris buffer pH 7.4 was used with γGCS under native conditions.

The effect of altering the urea concentration on purification under denaturation conditions on protein recovery was determined. A lower amount of protein was obtained if 2 or 4 M urea was used compared to extraction using 6 M urea (Figure 4.7). The effect of the altering the incubation temperature during extraction was also determined and the highest protein yield was obtained if extraction was carried out for one hour at 30°C instead of 4°C or 25°C.

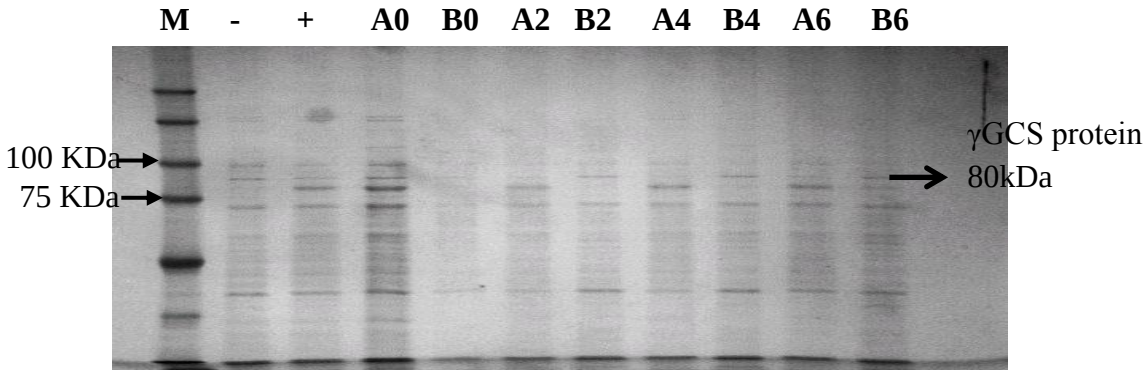


Figure 4.7 The effect of urea concentration on the solubilisation of the recombinant *Leishmania* γGCS protein from inclusion bodies in the pellet. M is molecular mass, uninduced (-), induced (+), incubated at 37ºC and then at 18ºC after inducing. Pellet samples (A), supernatant (B). The A 0, B 0 samples without urea, A 2, B 2 samples with 2 M urea, A 4, B 4 samples with 4 M urea, A 6, B 6 samples with 6 M urea. Bacteria pellets were separated by gel electrophoresis and the protein produced visualised using Coomassie blue stain.

In the case of protein purified under denaturing conditions (Figure 4.8), lower amounts of protein were produced if the purified protein was dialysed after purification on the column. Therefore the procedure was changed so that pelleted bacterial extract was incubated one hour at 30°C with 6M urea solution containing a cocktail of protease inhibitors and then dialysed against PBS buffer for 24 hours, and then purified in the column. This increased the protein yield to 1 mg/ml from 100 ml bacteria broth. Protein production under denaturing conditions also gave a higher protein yield if PBS buffer was used during purification using nickel affinity column.

Figure 4.9 shows the protein eluted under native condition using tris buffer. That protein was produced from a supernatant from a bacteria extract which was purified using a nickel column. The main problem of this purification was that a lower amount of protein yield was collected, also purification with truncated protein (Appendix 7 show a picture of protein).

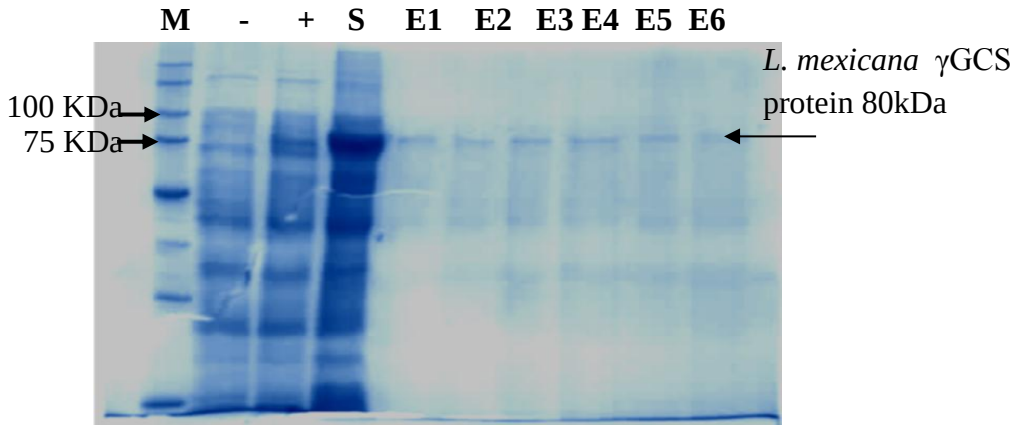


Figure 4.8 Coomassie stained SDS-PAGE showing *L. mexicana* γGCS protein purified under denaturing condition. Protein expression in *E. coli* was induced by adding 0.1mM IPTG and bacteria were incubated at 18ºC overnight, M (molecular mass), uninduced (-), induced(+), supernatant (S), supernatant after adding 6M urea. Eluted protein fraction E1-E6.

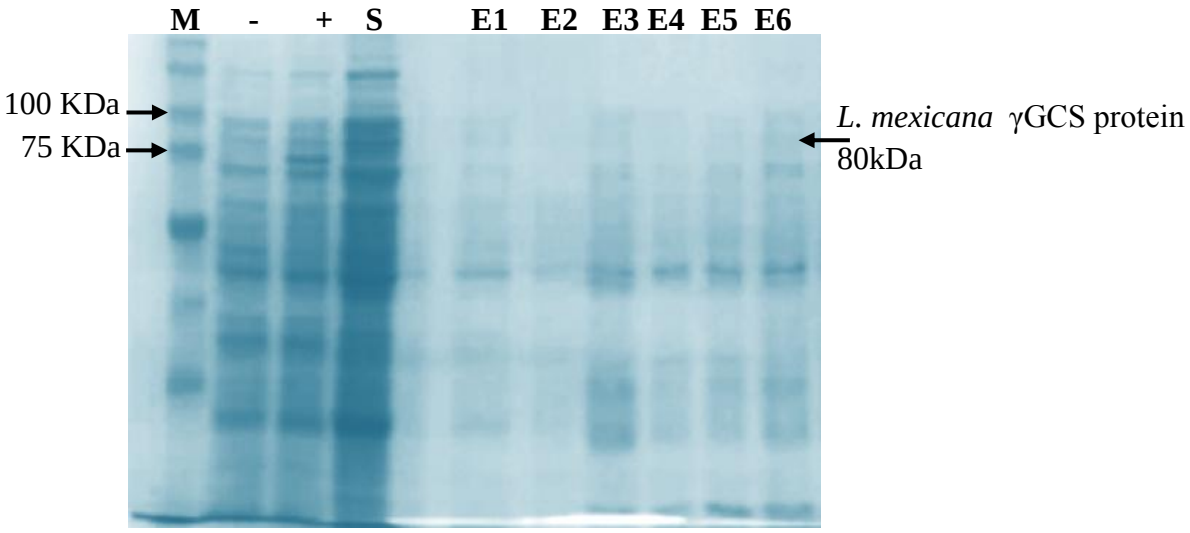


Figure 4.9 Coomassie stained SDS-PAGE showing *L. mexicana* γGCS protein purified under native condition. Protein expression in *E. coli* was induced by adding 0.1mM IPTG and bacteria were incubated at 18ºC overnight, M (molecular mass), uninduced (-), induced (+), supernatant (S), Eluted protein fraction E1-E6.

4.3 Discussion

In previous studies recombinant *L. donovani* γGCS protein was mainly expressed in inclusion bodies and both full length and truncated forms of the protein were produced (Henriquez *et al.,* 2010 ; Campbell *et al.,* 2012). This present study used a different expression system and a γGCS gene construct with a C-terminus his-tag instead of N-terminus his-tag used. Previous studies suggested that the location of his-tag can influence the expression, solubility, and bioactivity of a recombinant aminotransferase protein from fungi (Hartinger *et al.*, 2010). In their study the purified aminotransferase produced had an N-terminal his-tag and the protein was present in both the soluble and insoluble lysate fraction. In addition, in their study truncated versions of the aminotransferase were also detectable. However, when purified aminotransferase was produced with a C-terminal his-tag no truncated versions were formed (Hartinger *et al.*, 2010). In this study, we had similar problems to those that occurred before, we had truncated bands for each expressed γGCS protein, and most of the expressed protein was in the insoluble bacteria lysate. Previous studies in this laboratory carried out to determine the best host cell for *Leishmania* γGCS protein expression (unpublished data). It was concluded that Rosetta blue was the best strain for expression and so in this study this bacteria strain was used. In fact the Rosetta blue strain of *E. coli* supplies tRNAs for the rarely used codons AUA, AGG, AGA, CUA, CCC and GGA that improved expression of recombinant protein (Bessette *et al.*, 1999) but their presence did not improve solubility of the expressed protein in this study.

The temperature used during expression has been known to be a key factor in determining the solubility of recombinant proteins in the cytoplasm of *E. coli* (Schein, 1989) but the nature of the target protein, the level of expression, growth conditions, length of induction and inducer levels can also have an effect (Peternel *et al.*, 2008). Reducing the temperature during protein production in *E. coli* after adding IPTG inducing to 18ºC increased the amount of soluble recombinant γGCS protein produced by the bacteria. This funding is similar to previous studies; For example, expression of Vaccinia virus a large subunit ribonucleotide reductase (87kDa) is completely insoluble if *E. coli* are grown at 37ºC after induction but is partly soluble if the bacteria are grown at 15ºC after protein expression is induced (Slabaugh *et al.*, 1993). The incubation of the induced bacteria culture at reduced temperature that could slow down transcription and translation in *E. coli* so that proteins have more time to refold into their native structures resulting in less aggregation (Vera *et al.*, 2007).

In this study, lowering the concentration of IPTG used to induce γGCS protein production in *E. coli* increased the expression of soluble *Leishmania* γGCS protein. This finding is similar to that reported in previous studies, where better yields of soluble protein were obtained if the IPTG concentration was reduced from its usual 1 mM concentration (Pedelacq *et al.*, 2011). Other studies also reported that inclusion body formation is reduced when IPTG is used at a lower concentration (Upadhyay *et al.*, 2012). In this study most of the *Leishmania* γGCS protein is expressed in inclusion bodies even at the lower IPTG concentration. The previous study the researchers, using a different protein expression in *E. coli*, found that no inclusion bodies formed

using IPTG 0.2 mM but inclusion bodies containing protein were produced if IPTG was used at 0.4 or 0.6 mM IPTG (Tegel *et al.*, 2010). Inclusion body formation is a common problem for eukaryotic proteins expressed in *E. coIi* (Sauerborn *et al.*, 2010). A strong detergent such as urea is required to extract recombinant proteins from inclusion bodies and this can make the protein unsuitable for enzymatic studies. However the insoluble recombinant protein can be used in vaccine studies. In vaccine studies the T cells are important in mediating the immune response. The T cell receptor only interacts with peptide-major histocompatibility complex, so the solubility of protein and full length form of protein is not important in cellular immunity (Aleksic *et al.*, 2010; Sauerborn *et al.*, 2010). In contrast, if antibodies mediate immunity, the 3D structure of a protein is important (Davies and Cohen, 1996). In *Leishmania,* studies have indicated that immunity is dependent on the T cell rather than the B cells response, especially for *L. donovani* (Nylen and Gautam, 2010).

Different strategies have been used to refold proteins extracted using urea, with dialysis being the traditional method. Although it is relatively simple to carry out it is time consuming and requires large volumes of buffers. In this study, purified *Leishmania* γGCS protein obtained by refolding using dialysis does not have any enzyme activity, which is similar to the finding in previously reported study (Hartinger *et al.*, 2010). Other researchers reviewer reported expression of *Penicillium amagasakiense* glucose oxidase recombinant protein as an insoluble form and after refolding it by dialysis it had enzyme activity after purification using 8 M urea (Raghava *et al.*, 2008). The amino acid composition of *Leishmania* γGCS is different from the *P. amagasakiense* protein and this may explain why this approach was

unsuccessful. An alternative method is to refold the protein on an affinity column. This can save time and is often more effective than dialysis but it can give low protein yield. Overall, the best method to produce recombinant *Leishmania* γGCS from the insoluble fraction is dialysis after urea treatment and then purification on an affinity column using PBS buffer pH 7.4 rather than Tris buffer pH 7.4. This procedure increased protein yield to 1mg/ml from just 100 ml bacteria broth. This improvement in protein yield after urea removal before applying extract to affinity column could be related to the effect of urea on protein bonding on column (Liu *et al.*, 2012).

In conclusion, recombinant *Leishmania* γGCS for all the three species *L. donovani*, *L. mexicana* and *L. major* were obtained but the protein contained full and partial length proteins. The full length and truncated bands in the protein eluted were identified as *Leishmania* γGCS by MALDI. Western blot studies showed that the full length and truncated form had a his-tag.

Chapter 5: The effect of L-buthionine sulfoximine treatment on the survival of *Leishmania* and the enzymatic activity of recombinant γGCS.

5.1 Introduction

All living organisms need to maintain an adequate intracellular redox environment and GSH is involved in protection against oxidative stress. γGCS catalyses the rate limiting step in GSH biosynthesis (Penninckx, 2002). *Leishmania* maintains redox balance by synthesizing TSH, a conjugate of GSH and spermidine. *Leishmania* parasites maintain significant levels of GSH by recycling TSH disulphide by the enzyme Trypanothione reductase (Romao *et al.*, 2006). The concentration of Trypanothione (TSH) does not change significantly during the different logarithmic growth phases but decreases when the cells enter the stationary phase. TSH is necessary for the parasites survival since it is used by several enzymes to neutralize the ROS produced by the macrophages during infection (Colotti and Ilari, 2011). One of the major biological functions of the trypanothione pathway is to regulate oxidative and, probably, nitrosative stress by shuttling reducing equivalents from NADPH to hydroperoxides and peroxynitrites (Fiorillo *et al.*, 2012).

Mammalian γGCS consists of a catalytic or so called heavy subunit of about 70 KDa and a regulatory or so called light subunit of about 30 KDa (Hibi *et al.*, 2004). The regulatory subunit modulates the activity of the catalytic subunit by increasing the affinity of the enzyme for L-glutamate (L-Glu) and decreasing its affinity for GSH. Bacterial, fungal and γGCS from protozoan parasites such as *Trypanosoma brucei* and *Plasmodium falciparum* only have the catalytic subunit. The catalytic subunit of γGCS has been cloned in humans (Gipp *et al.*, 1992), *Saccharomyces cerevisiae* (Ohtake and

Yabuuchi, 1991), *E. coli* (Kelly *et al.*, 2002), *Trypanosoma brucei* (Lueder and Phillips, 1996) and *Leishmania* (Henriquez *et al.*, 2010).

BSO is an irreversible inhibitor of γGCS and results in decreased GSH levels. Previous studies have shown that BSO can inhibit the survival of *L. donovani* both *in vitro* and *in vivo* (Carter *et al.*, 2003). BSO treatment is also lethal for *P. falciparum* inside erythrocytes, implying that the parasites rely on GSH biosynthesis (Luersen *et al.*, 2000).

The action of enzymes on cell function is a dynamic process that involves constant changes in chemical composition. Enzyme assays have many applications in enzyme kinetics. Understanding the rates of reactions can help imply the mechanism that the reaction follows, a single-substrate or multiple-substrate mechanism (Wang, 2013).

Determination of enzyme activity is among the most important analytical procedures in biochemistry. The rate at which enzymes catalyse chemical reactions are traditionally measured with a variety of methods including spectroscopy, potentiometric measurements and chromatography (Olsen, 2006). Enzyme specific activity measure micromoles of substrate converted to product per minute (μmol P/min), or International Units (IU); these are commonly the used units to express reaction rates (Vynohradova, 1999).

The γGCS activity was determined using spectrophotometry on the basis of ADP formation using a pyruvate kinase and lactate dehydrogenase-based coupled assay system (Figure 5.1). The reaction was initiated by addition of γGCS, and NADH oxidation, which is equal to ADP formation, was monitored at 340 nm. One unit of

γGCS activity is the amount necessary to form one mol of ADP per hour under the conditions of the assay. Specific activity is expressed as units per milligram of protein (Misra and Griffith, 1998).

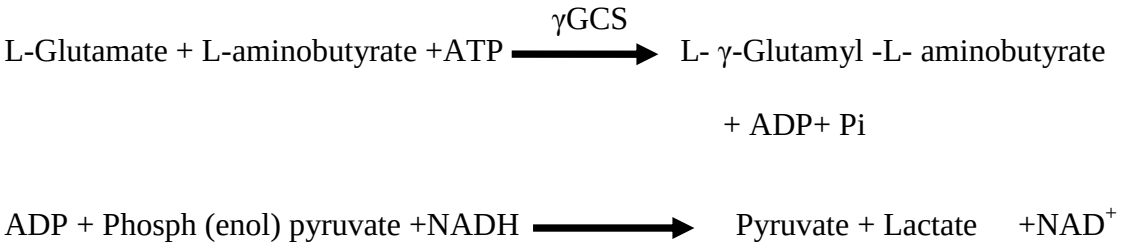


Figure 5.1 The reaction of γGCS enzyme used in this study (Misra and Griffith, 1998).

Gene expression studies are extremely sensitive to the biological activity study. It provides unique information on the parasite at the time of interaction with the host cell (Decuypere *et al.*, 2008). Using quantitative real-time PCR (Q-PCR), for rapid and sensitive analysis of a specific set of genes in large sample collections. It is considered an emerging technology for the detection and quantification of genetic characterization for parasites. Real time quantitative PCR has been the used for parasite DNA study in *L. infantum*, and *P. falciparum* (Antinori *et al.*, 2009). PCR was used to quantify the number of copies of the target gene compare to one or more housekeeping genes. Several studies have investigated gene amplification among different *Leishmania* strains exposed to drug pressure to determine the role of specific genes in drug resistance mechanisms (Beverley, 1991). The *Leishmania* γGCS gene was used to investigate antimony drug resistance (do Monte-Neto *et al.*, 2011), as expression of the gene is related to antimony resistance (Mukherjee e*t al*., 2009).

The main aim of this studies to:

- Compare the enzyme activity of recombinant γGCS enzyme (prepared in chapter 4 from native condition) for *L. donovani*, *L. mexicana* and *L. major*.
- Compare the effect of BSO treatment on the survival of extracellular promastigotes and intracellular amastigotes of *L. donovani*, *L. mexicana* and *L. major* using luciferase expressing parasites.
- Compare effect of BSO on expression of γGCS in promastigotes and amastigotes of *L. donovani*, *L. mexicana* and *L. major*.

5.2 Results

5.2.1 The activity of recombinant γGCS from the three *Leishmania* species

Perviouse studies showed that the majority of the γGCS enzyme activity was present in the soluble fraction of the γGCS protein produced from *L. donovani*, *L. mexicana* and *L. major* prepared under native conditions. There was little or no activity if the enzyme was purified under denaturing condition (Chapter 4).

Therefore the studies described here concentrated on using the soluble protein prepared under native conditions. In this case the protein preparation contained truncated protein bands, presumably because the extraction method was less harsh than that used for denatured protein. An example of the results obtained in enzyme studies for each *Leishmania* species is shown in Figure 5.2. There was no significant difference in the specific activity (Table 5.1) of *L. mexicana* γGCS (0.2 µmol/min/mg), *L. major* (0.15 µmol/min/mg) but there was a significant difference in

activity for *L. mexicana* and *L. donovani* γGCS (0.08 μmol/min/mg). There was no activity in the absence of glutamate indicating that the γGCS in the protein solution is responsible for the reaction. Data from the enzyme assays were used to determine the specific activity for γGCS from each species.

Recombinant *L. mexicana* γGCS was significantly more active ($p < 0.001$) compared to the recombinant γGCS for the other two *Leishmania* species and this enzyme was also the least susceptible to BSO inhibition. It had also the highest IC_{50} value (Table 5.1). In contrast, there was no significant difference in the enzymatic activity or susceptibility to BSO treatment between *L. donovani* and *L. major* recombinant γGCS (Table 5.1).

In order to confirm that enzyme activity in the recombinant proteins was related to the presence of *Leishmania* γGCS and not to bacterial contamination, expression-using bacteria transfected with an empty expression plasmid which carried out. Protein production was completed using bacteria transfected with a plasmid without the γGCS gene sequence. The purified product had no enzyme activity in the extract and no protein band was present after affinity column purification of the extract (Figure 5.3, appendix 7).

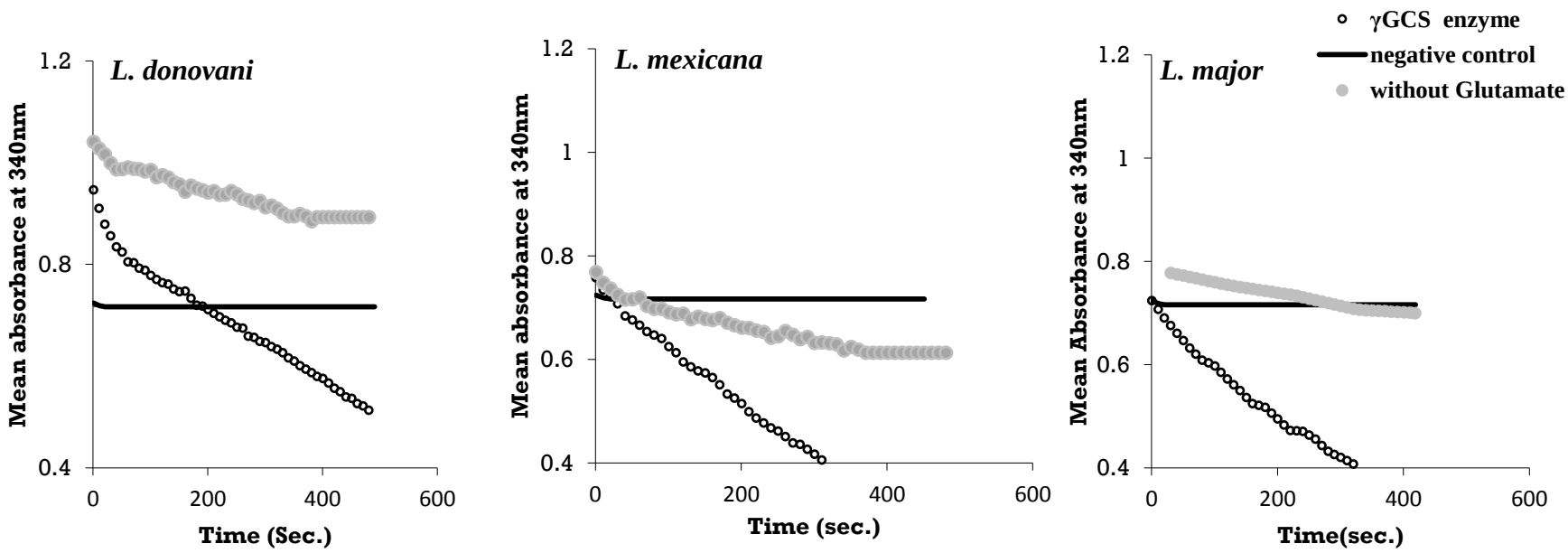


Figure 5.2 The enzymatic activity of recombinant γGCS soluble recombinant protein obtained from *L. donovani*, *L. mexicana* and *L. major*. It was spectrophotometrically determined by coupling the release of ADP by γGCS to oxidation of NADH through pyruvate kinase and lactate dehydrogenase. γGCS was active and the detected activity was proportional to the amount of enzyme. The activity of γGCS was dependent on the presence of the substrate glutamate. The absence of glutamate is a negative control. There was no activity of the enzyme if glutamate is absent. The reaction at 37°C of the mixture in a final volume of 1.0 ml containing 15 mM L-α-aminobutyrate, 20 mM L-glutamate. The oxidation of NADH was monitored at 340 nm over time (second), n=4/ treatment.

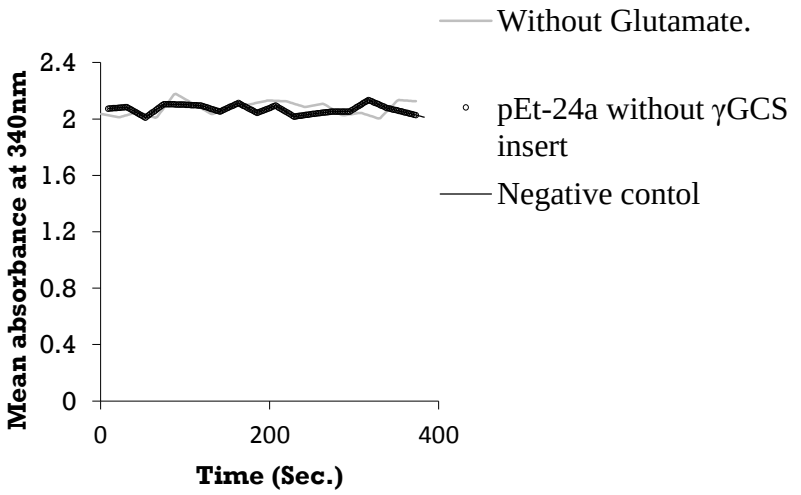


Figure 5.3 The enzymatic activity of pET24-a plasmid without the γGCS gene sequence was formed and the purified product had no enzyme activity. It was spectrophotometrically determined by coupling the release of ADP, oxidation of NADH through pyruvate kinase and lactate dehydrogenase. Reactions were carried out in the absence of glutamate and negative control without purified product. The reaction at 37°C mixture in a final volume of 1.0 ml contained 15 mM L-α-aminobutyrate and 20 mM L-glutamate. The oxidation of NADH was monitored at 340 nm over time (second), n=4/ treatment.

Table 5.1 Comparison of the specific activity of recombinant protein of *L. donovani*, *L. mexicana* and *L. major* γGCS and then IC_{50} value for BSO treatment (n=4/treatment), [a]p <0.0001 comparing *L. donovani* and *L. mexicana*, [b]p<0.001 comparing *L. mexicana* and *L. major*. Data were determined using results of studies in Figure 5.1. The values are from four separated experiments. Statistical analysis was performed using using ANOVA combined with Fisher's LSD test post-hoc as data normal distribution.

***Leishmania* γGCS protein**	**Mean specific enzyme activity (µmol/min/mg ± SE)**	**Mean IC_{50} (mM± SE)**
L. donovani γGCS	0.08 ± 0.008	0.42 ± 0.05
L. mexicana γGCS	0.24 ± 0.01**[a,b]**	1.23 ± 0.08 **[a, b]**
L. major γGCS	0.12 ± 0.01	0.65 ± 0.16

The effect of using PBS pH 7.4 or tris buffer pH 7.4 during affinity column purification showed that the best activity was obtained when Tris buffer at pH 7.4 was used (Table 5.2). The enzyme was still active on day 5 after storage in 25% glycerol at -80 °C but it was reduced compared to fresh protein and completely non active after being stored for one month. If the protein was stored at 4 °C or -20°C the enzyme lost its activity completely by day 5 (Table 5.3). Therefore, all the enzyme activity studies were done on the day of purification.

Table 5.2 Comparison of the effect of buffer on specific activity of recombinant γGCS proteins of *L. donovani*, *L. mexicana* and *L. major* (n=4/treatment).

***Leishmania* γGCS protein**	**Mean specific enzyme activity (µmol/min/mg ± SE)**	
	Tris buffer	**PBS buffer**
L. donovani γGCS	0.08 ± 0.006	0.02 ± 0.005
L. mexicana γGCS	0.24 ± 0.02	0.18 ± 0.01
L. major γGCS	0.12 ± 0.008	0.09 ± 0.01

Table 5.3 Comparison of the effect of storage condition on specific activity of recombinant γGCS of *L. donovani*, *L. mexicana* and *L. major* (n=4/treatment).

		Mean specific enzyme activity (µmol/min/mg ± SE)		
***Leishmania* γGCS protein**		*L. donovani* γGCS	*L. mexicana* γGCS	*L. major* γGCS
Fresh protein		0.08 ± 0.006	0.24 ± 0.02	0.12 ± 0.008
24 hours	4 °C	0.04 ±0.001	0.1±0.01	0.07±0.01
	-20°C	0.06±0.002	0.12±0.02	0.09±0.009
	-80°C	0.079±0.008	0.19±0.02	0.1±0.01
5 Days	4 °C	No activity	No activity	No activity
	-20°C	No activity	No activity	No activity
	-80°C	0.06±0.02	0.16±0.03	0.08±0.01

5.2.2 The effect of BSO treatment on the *in vitro* survival of *Leishmania spp.*

(i) Promastigotes studies

In vitro studies showed there were a good linear correlation between bioluminescence (BLI) by *Leishmania* promastigotes and the number of parasites (Figure 5.4); correlation coefficient was obtained for a linear fit to the data 0.99. The amount of BLI produced was stable (Figure 5.5), and calculation of the BLI produced/parasite for each week. Table 5.4 showed similar levels of BLI per parasite ratio for seven passages. There was no significant different in the BLI for all three *Leishmania* species.

Table 5.4 The amount of BLI/parasite ratio for the three *Leishmania* species promastigotes during 7 passages.

Species	(BLI / parasite ratio) ± SE						
	Week						
	1	2	3	4	5	6	7
L. donovani	2.26 ± 0.3	2.8 ± 0.1	2.8 ± 0.12	2.2 ± 0.1	2.5 ± 0.2	2 ± 0.3	2.9 ± 0.01
L. mexicana	2.7 ± 0.1	3 ± 0.01	2.4 ± 0.04	2.6 ± 0.3	2.8 ± 0.1	2.6 ± 0.3	2.7 ± 0.3
L. major	2.25 ± 0.3	2.6 ± 0.04	2.4 ± 0.2	2.5 ± 0.1	2.6 ± 0.02	2.7 ± 0.2	2.6 ± 0.1

The effect of time on BLI of promastigotes of different *Leishmania spp*. over 7 weeks of culture was calculated in term of BLI/parasite ratio. The light emitted by $2x10^6$ parasites /ml determined after incubated of cells in 150 µg/ml luciferin solution (n=4/treatment).

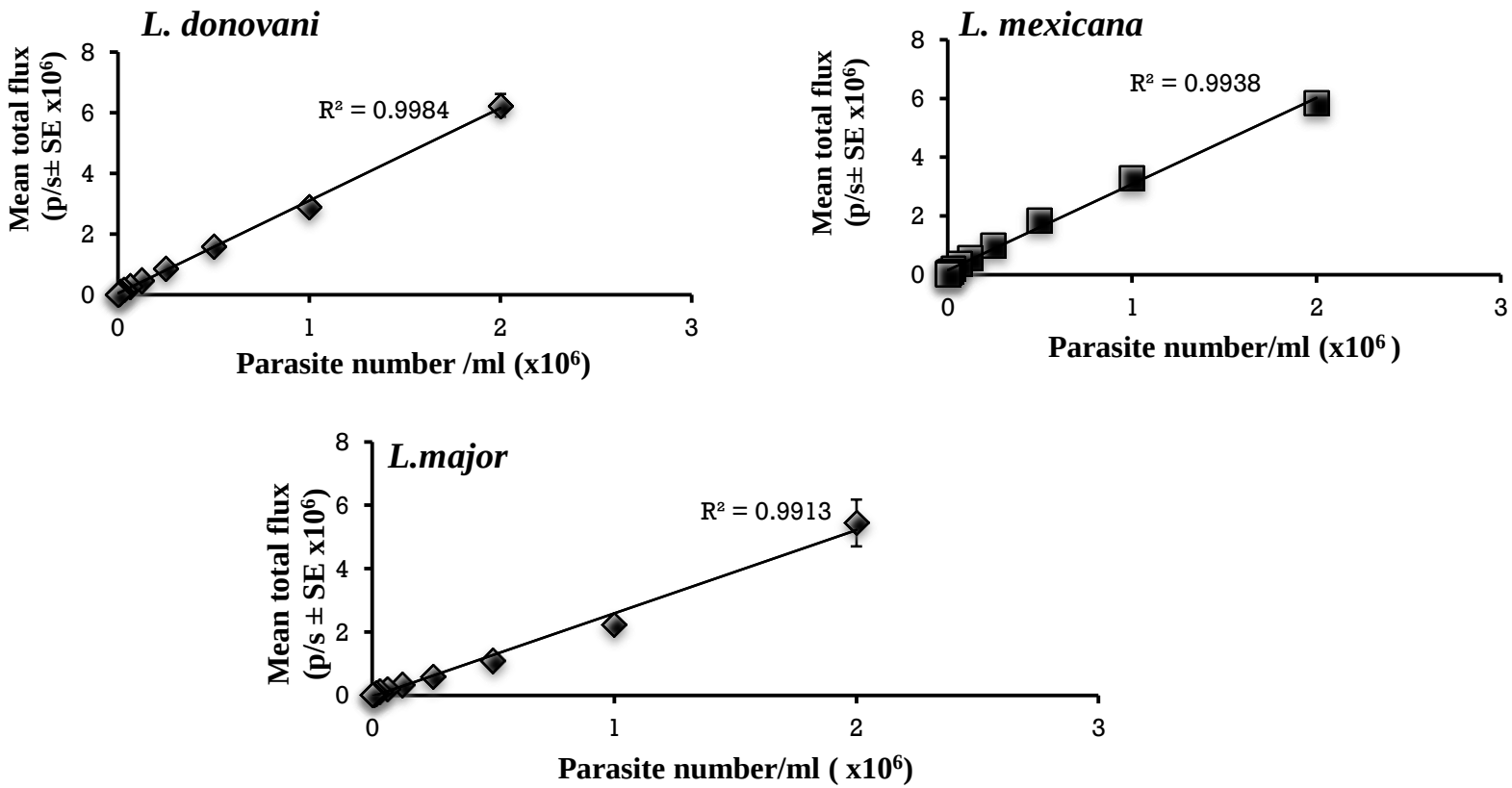


Figure 5.4 The linear correlation between light production and promastigotes parasite number from promastigotes of different *Leishmania spp*. The light emitted starting with $2x10^6$ parasite /ml following by double dilution, determined after incubated of cell in 150 µg/ml luciferin solution. n=4/ treatment.

The BSO treatment significantly inhibited the survival of promastigotes of all three *Leishmania spp*. with *L. mexicana* parasites exhibiting the greatest resistance to treatment, ($p < 0.01$, Figure 5.5). However, *L. donovani* and *L. major* had a similar susceptibility to BSO treatment.

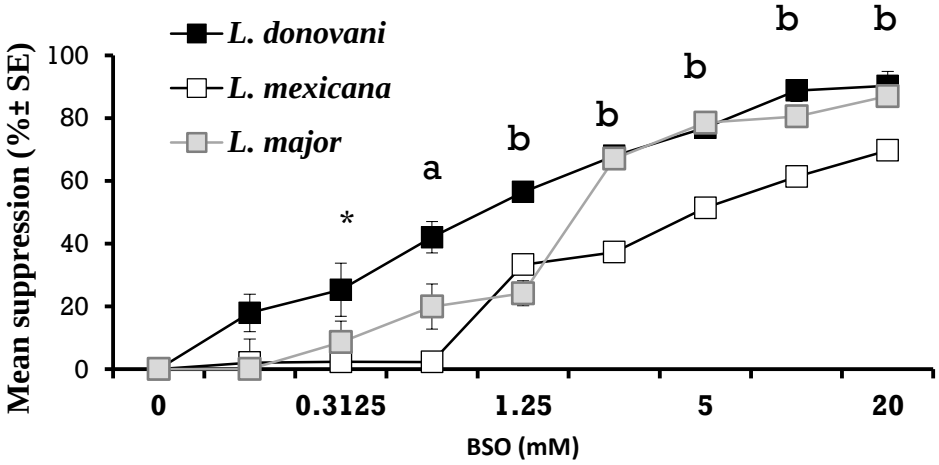


Figure 5.5 The effect of BSO treatment on the survival of *Leishmania* promastigotes. The 100 µl luciferin solution (150 µg/ml) was added/ well and the amount of bioluminescence emitted/well determined for control and BSO treated cells. The mean suppression in bioluminescence for BSO treated cells was determined by comparing each experiment's value with the mean control value. $^{*}p<0.05$ between *L. donovani* and *L. mexicana*. $^{a}p<0.05$ *L. donovani, L. mexicana and L. major*. $^{b}p<0.01$ *L. mexicana* compare to *L. major and L. donovani*, n=4 /treatment. Statistical analysis was performed using using ANOVA combined with Fisher's LSD test post-hoc as data had a normal distribution.

(ii) Intracellular amastigotes

Initial studies were carried out to show if BLI data correlated linear with intracellular amastigotes number. The studies showed that BLI and direct counting result in Figure 5.6. *L. mexicana* promastigotes were more infective than *L. major* or *L. donovani* promastigotes parasites and the parasites could be ranked on their infectivity as *L. mexicana* > *L. major* > *L. donovani*, using both methods to determine parasite numbers. *L. mexicana* gave a significantly higher infection rate ($p<0.001$) compared to *L. donovani* and *L. major*. Based on this data it was decided to use a parasite: host cell ratio of 40:1 for *L. donovani*, 10:1 (*L. mexicana)*, and 20:1 (*L. major*) for *in vitro* studies. Treatment with BSO had no significant effect on the intracellular survival of *L. mexicana*, as even the highest dose tested did not inhibit parasite survival. BSO was not tested above 0.5 mM (Figure 5.7). In contrast, BSO treatment significantly inhibited the survival of *L. donovani* parasites ($p < 0.05$). At the concentrations tested it was only possible to determine an IC_{50} value for *L. donovani* (Table 5.5). The IC_{50} value for the intracellular amastigote stage of *L. donovani* is much lower than that of promastigote stage (Table 5.5) and this could reflect stage-specific differences or the influence of the host cell on the outcome of treatment. This observation could also apply to the other two *Leishmania* species, as it was impossible to identify an IC_{50} value for the amastigotes stage.

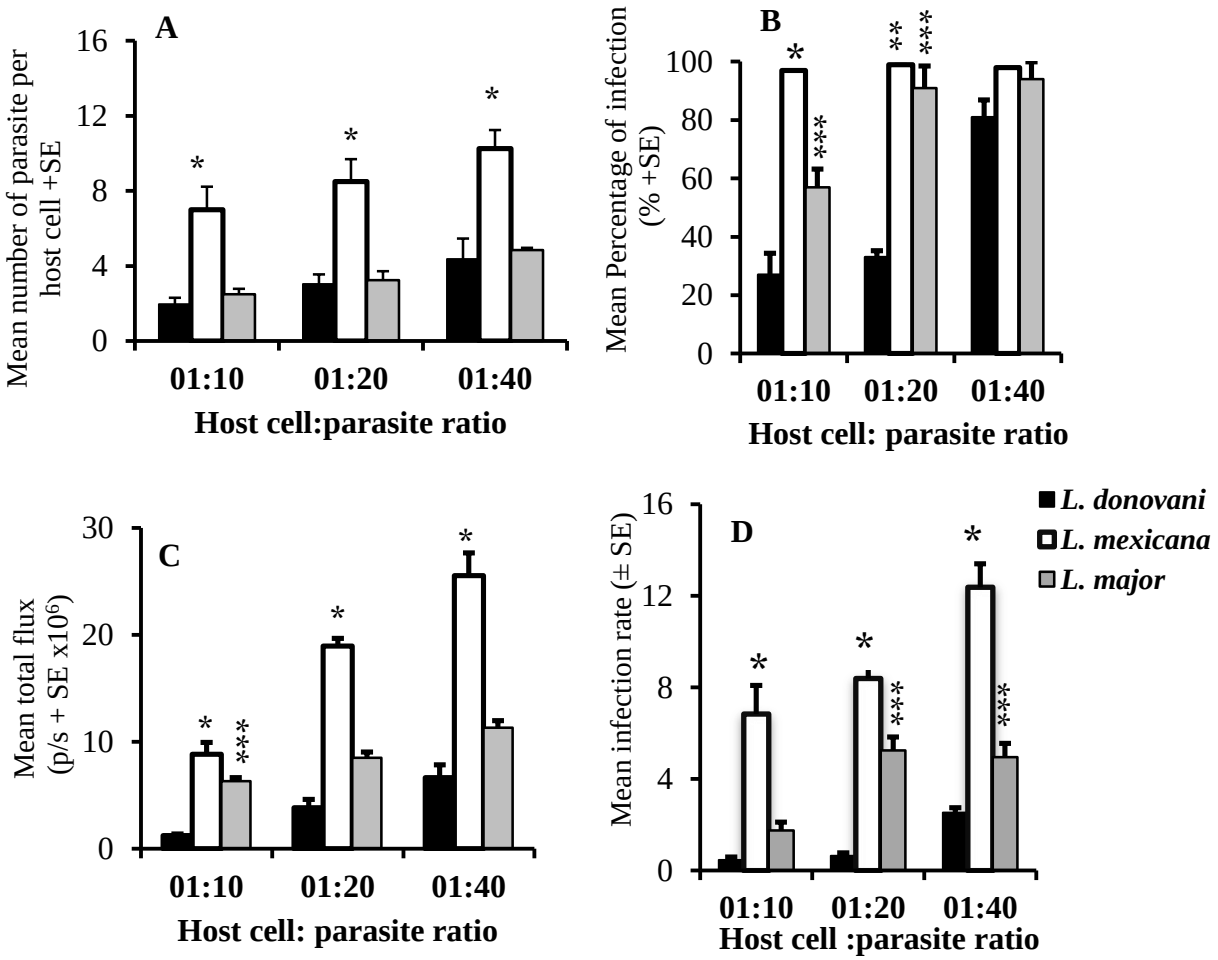


Figure 5.6 The effect of altering host: parasite ratio on infectivity of three *Leishmania* species. Infectivity was based on the number of parasites per host cell (A) mean percentage of cells infected (B) and BLI emitted by infected cells (C), mean infection rate (D). Bone marrow derived macrophages were infected with luciferase *Leishmania* promastigotes at host cell: parasite ratios of 01:10, 01:20 or 01:40. The amount of BLI emitted from cell and the percentage of cell infected was determined 72 h after infection, after incubated of cell in 150 µg/ml luciferin solution, which normalized to negative background of non-infected macrophage. $^{*}p<0.001$ between *L. mexicana* compare to *L. donovani* and *L. major*. $^{**}p<0.01$ *L. mexicana* compare to *L. donovani*, $^{***}p<0.01$ *L. major* compare to *L. donovani.* n=4 / treatment. Non-parametric data analysed using Kruskal Wallis test followed by Dunn's ad hoc test.

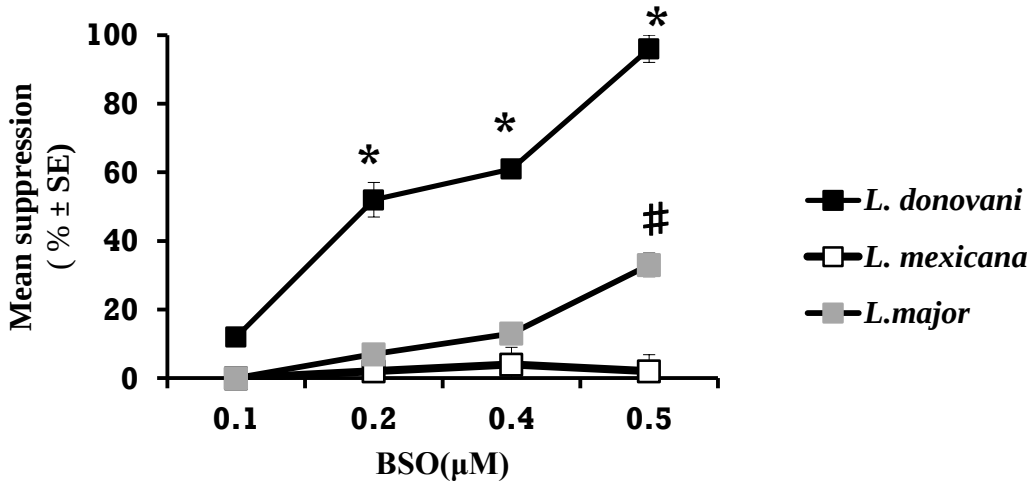


Figure 5.7 The effect of BSO treatment on the intramacrophage survival of three *Leishmania* species. Macrophages ($4x10^6$ cell/ml) were infected with *Leishmania* promastigotes at a host: parasite ratio of 40:1 (*L. donovani*), 20:1 (*L. major*), or 10:1 (*L. mexicana*) for 24 hours. The medium was changed and infected cells were treated for 72 hours with medium alone (controls) or BSO (0.1-0.5 mM, n=4/ treatment). At the end of the study, the medium was removed and 100 µl luciferin solution (150 µg/ml medium) was added/ well and the amount of BLI emitted/well determined for control and BSO treated cells. The mean suppression in BLI caused by BSO treatment was determined by comparing each experiment value with the mean control value. $^{*}p<$ 0.01 *L. donovani* compared to *L. mexicana* or *L. major*, $^{\#}p <0.01$ for *L. major* compared to *L. mexicana*. Statistical analysis was performed using using ANOVA combined with Fisher's LSD test post-hoc as data had a normal distribution.

Table 5.5. The effect of BSO treatment on survival of *Leishmania* parasites. The data for studies recorded in Figure 5.5 for promastigote stage and 5.7 for intracellular amastigotes, were used to calculate IC_{50} values for three separated experiments. [a]$p < 0.01$ comparing *L. mexicana* and *L. major*, [b]$p < 0.001$ comparing *L. mexicana* and *L. donovani*. The non-parametric data was analysed using a Kruskal Wallis test followed by Dunn's ad hoc test.

Parasite	**Mean IC_{50} (mM ± SE)**	
	Promastigote stage	**Amastigote stage**
L. donovani	1.3 ± 0.29	0.19 ± 0.02
L. mexicana	3.5 ± 0.53**[a,b]**	> 0.5 mM
L. major	1.79 ± 0.01	> 0.5 mM

5.2.3 The effect of BSO on expression of γGCS

The difference in susceptibility to BSO could be related to the amount of γGCS produced by each parasite. Therefore the effect of BSO treatment on γGCS expression was determined for promastigotes and intracellular amastigotes. There was no significant difference in the expression of *Leishmania* γGCS mRNA for the three *Leishmania* species as promastigotes (Figure 5.8) or intracellular amastigotes (Figure 5.9). However, treatment with BSO identified species- and stage-specific differences. Treatment of *L. mexicana* promastigotes with BSO induced a significant up regulation in γGCS expression compared to controls ($p < 0.05$). In contrast, BSO treatment caused a significant reduction in γGCS expression compared to its control and the expression was significantly lower than that of *L. major* or *L. donovani* parasites treated with the same concentration of BSO. BSO treatment only inhibited γGCS of *L.*

major at the highest dose tested. BSO treatment of infected macrophages had no influence on γGCS expression for *L. mexicana* amastigotes but significantly inhibited gene γGCS expression for *L. donovani* amastigotes at both dose levels, and suppressed gene γGCS expression at the highest BSO dose tested for *L. major*.

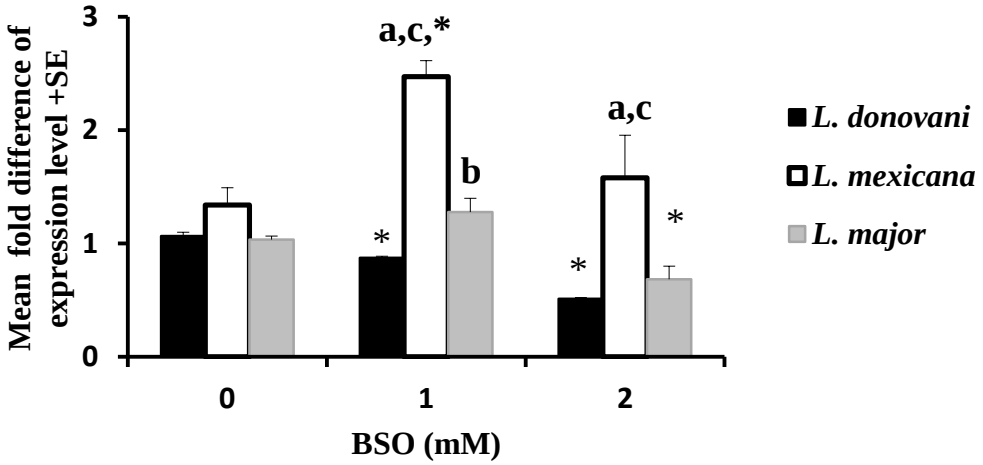


Figure 5.8 The effect of BSO treatment on the expression of *Leishmania* γGCS in promastigotes of three *Leishmania* species. The levels of γGCS normalize to *Leishmania* tubulin mRNA and then the fold gene expression relative to control in parasites was determined using RT-PCR. Promastigotes ($5x10^5$) were incubated in the presence of medium alone (control) or BSO (1mM, or 2mM) for 72 hours. The effect of BSO treatment on γGCS gene expression was calculated relative to control value (without BSO) using the $2^{-\Delta\Delta C}{}_T$ method. The data are representative of three separate experiments. $^a p<0.05$ comparing *L. mexicana* and *L. donovani*, $^b p<0.05$ comparing *L. major* and *L. donovani*, $^c p<0.05$ comparing *L. major* and *L. mexicana*. $^* p<0.05$ comparing to relevant control group, n=3 / treatment. Data was analysed using a Kruskal Wallis test followed by Dunn's ad hoc test.

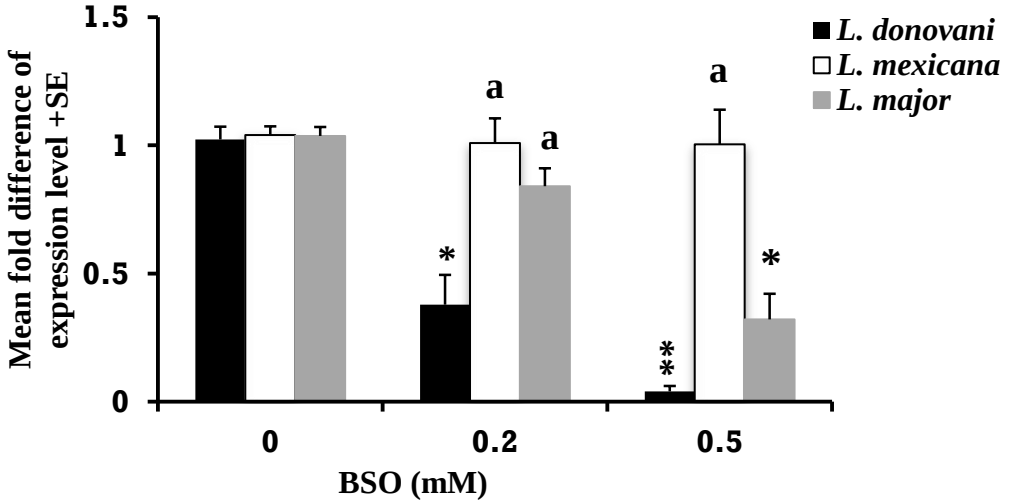


Figure 5.9 The effect of BSO treatment on *Leishmania* γGCS mRNA expression levels in bone marrow macrophages infected with *Leishmania*. *Leishmania* γGCS mRNA normalize to *Leishmania* tubulin mRNA and then the fold gene expression relative to control was quantified using RT-PCR. The three treatments were 0 (control), 200, and 500 µM BSO (for 72 hours treatment). The data are representative of three separate experiments; the fold gene expression was calculated relative to control (without BSO for each species) using the $2^{-\Delta\Delta C}{}_{T}$ method. $^{a}p<0.05$ *L. mexicana* compared to *L. donovani*, $^{a}p<0.05$ *L. major* comparing to *L. donovani.* $^{*}p<0.05$, $^{**}p<0.01$ comparing to relevant control, n=3 / treatment. Data was analysed using a Kruskal Wallis test followed by Dunn's ad hoc test.

Infection with *Leishmania* up regulated the expression of mouse γGCS compared to uninfected cells for all *Leishmania* species. The highest increase in expression of mouse γGCS was caused by *L. donovani* infection; $p<0.05$ compared to *L. major* or *L. mexicana.* Also there were no significant differences between macrophages infected with the three different species treated with BSO (Figure 5.10).

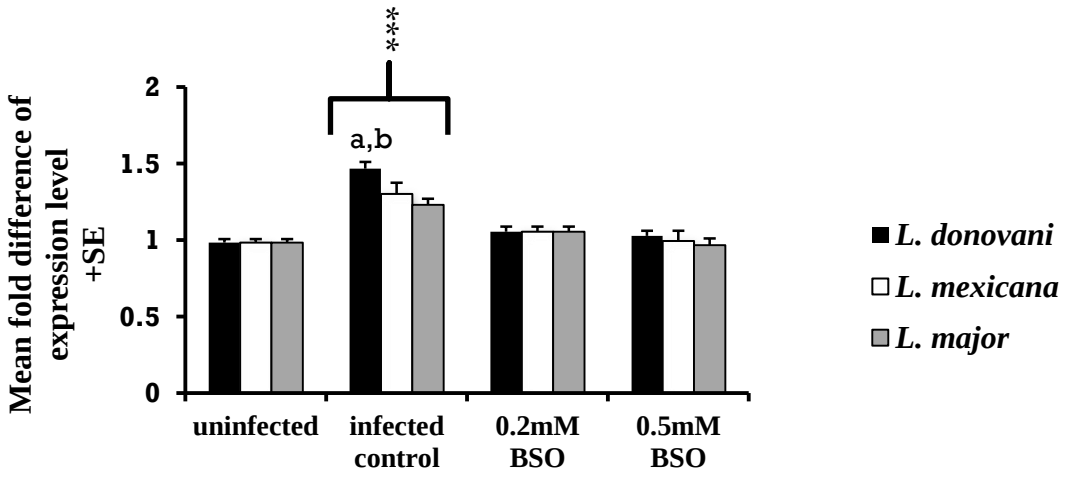


Figure 5.10 The effect of BSO treatment on the expression of mouse macrophages γGCS mRNA levels. Bone marrow macrophages, taken from Figure 5.8, were infected with three *Leishmania* species, mouse γGCS mRNA expression was normalized to mouse housekeeping gene and mouse γGCS mRNA fold expression relative to un infected control was quantified using RT-PCR. The three treatments were 0 (control), 200, and 500 µM BSO (for 72 hours treatment). The data are representative of three separate experiments; the fold gene expression was calculated relative to control (without BSO for each species) using the $2^{-\Delta\Delta C}{}_{T}$ methods. In infected macrophage with *L. donovani* $^{a}p<0.05$ compare to *L. mexicana.* In infected macrophage with *L. donovani* $^{b}p<0.01$ compare to *L. major,* $^{***}p<0.001$ macrophage infected with *L. donovani , L. mexicana* and *L. major* compare to uninfected , 0.2mM and 0.5 mM BSO. n=3 / treatment. Statistical analysis was performed using using ANOVA combined with Fisher's LSD test post-hoc as data had a normal distribution.

5.3 Discussion

In the present study the purified enzyme was unstable in most storage conditions. This finding is similar to previous studies reporting that γGCS recombinant enzymes were highly unstable under various storage conditions (Olin-Sandoval *et al.*, 2012). In this study the best activity was obtained when Tris buffer at pH 7.4 was used for purification of the native recombinant *Leishmania* γGCS. The enzyme remained active for at least 5 days when stored in 25% glycerol at -80°C but storage at 4°C and -20°C resulted in complete loss of activity after one day of storage. These results are similar to those reported for human γGCS which was found to be stable when stored at -80°C in 25% glycerol (Rathbun *et al.*, 1993). Whereas in another study purified human γGCS and rat kidney γGCS enzyme were stable for at least 7 days in buffer at 4°C, and stable for one year when stored at -20 °C in 25% glycerol. The glycerol allows the enzyme to be stored without freezing as some enzymes lose their activity when frozen (Misra and Griffith, 1998). The decreased activity of γGCS prepared in the present study could be related to denaturation of protein. It has been reported that some proteins undergo cold denaturation when stored below the freezing point of water (Manning *et al.*, 2010). Therefore, there most enzyme studies were done directly after protein purification.

The specific activity of *L. mexicana* γGCS (0.2 µmol/min/mg), *L. major* (0.15 µmol/min/mg), and *L. donovani* (0.08 µmol/min/mg) obtained in this study, is lower than that reported for *Trypanosoma brucei* γGCS (7.6 µmol/min/mg (Lueder and Phillips, 1996), *Ascaris suum* (18 µmol/min/mg, Hussein and Walter, 1995), *Plasmodium berghei* (0.454±0.031 µmol/min /mg, Sharma and Banyal, 2010), rat

catalytic subunit (16 µmol/min/mg, Oppenheimer *et al.*, 1979) and *E. coli* (19 µmol/min/mg, Huang *et al.*, 1988). There are no clear reasons for the differences in γGCS enzyme activity between organisms but it has been suggested that it could be related to amino acid substitutions in the enzyme structure, as this would preserve or weaken the hydrogen-bonding network and effect binding affinity of the substrate (Hibi *et al.*, 2004). In addition, it may be related to differences in the reaction conditions used.

Treatment with 1 mM BSO inhibited the specific activity of *L. donovani* γGCS by 86%, whereas the enzyme from *L. mexicana* (45%) or *L. major* (63%) was less affected. Previous studies have shown that treatment with 1 mM BSO inhibited specific activity of *Trypanosoma brucei* γGCS by 66% (Lueder and Phillips, 1996). Treatment with 2 mM BSO inhibited rat kidney γGCS enzyme activity by 84% and the activity of wild type *E. coli* γGCS by 93% (Huang *et al.*, 1988). Alignment of the N-terminal and central variable arm sequences for prokaryotic and eukaryotic γGCS was carried out for human, *E coli*, *T. brucei* and *T. cruzi*. This showed that there are differences in the binding site of sulfoxime inhibitors; the *E. coli* γGCS enzyme has a high specificity for cysteine and a low affinity for BSO, which does not contain a carboxylate (Appendix 5, Hibi *et al.*, 2004). That difference in enzyme structure at the substrate and inhibitor binding site could explain the difference between mammalian, *E. coli* and other organism γGCS enzymes in susceptibility to BSO inhibitor (Griffith, 1982; Kelly *et al.*, 2002).

The use of luciferase-expressing parasites rapidly performed with higher accuratly than counting method. It also allows to the direct evaluation of the toxicity of new compounds against different life cycle stages in 96-well format for high throughput screening of potential drugs. The luciferase method measures the total number of parasites present, while the counting methods provide an approximation of the macrophages that were counted (Sereno *et al.*, 2001). The result of this study showed that there was a linear correlation between parasite concentration and BLI. Also the BLI/parasite ratio was stable during the study with no difference in the ratio for all three *Leishmania* species. A study by Lang and his group (2005), using luciferase-transfected *L. amazonensis*, assessed the feasibility of using BLI as a quantitative indicator of the viability and the multiplication of parasites. They compared BLI to the number of viable parasites, using the wild type *L. amazonensis* strain as a control. They found that during log phase growth the luminescence signal was proportional to the number of bioluminescent parasites but at the stationary phase the bioluminescence decreased, suggesting that a decrease in growth correlated with a decrease in metabolic activity of the population (Lang *et al.*, 2005). Another study using *L. donovani* expressing firefly luciferase, found that luciferase activity correlated well with the number of parasites. There was an excellent linear correlation between the number of transgenic promastigotes and the luciferase activity and this assay had very high sensitivity as it was possible to detect as few as ten promastigotes (Ashutosh *et al.*, 2005). In the present study BLI emission was stable over time of passage. Also there was a correlation between the infectivity and the amount of BLI emitted. Another study that used luciferase expressing *L. donovani* found that the estimated

number of intracellular amastigote parasites by BLI and microscopic examination correlated well (Roy *et al.*, 2000).

In this study *L. mexicana* had the highest infectivity rate compared to *L. major* or *L. donovani*, where 99% of macrophages were infected using *L. mexicana* at a parasite: host cell ratio of 10:1, whereas higher parasite: host cell ratios were required for the other two *Leishmania spp*. (*L. major*, 20:1; *L. donovani* 40:1). Researchers have suggested that infectivity and virulence in *Leishmania* species is dependent on the parasite species and the type of medium used (Allahverdiyev *et al.*, 2011). In the present study only parasites below seven passages were used as loss of virulence is often associated with the number of passages *in vitro* (Moreira *et al.*, 2012).

Studies have shown that BSO treatment of rats infected with *T. brucei* resulted in a gradual decline in the GSH levels in parasites recovered from treated rats compared to untreated controls (Arrick *et al.*, 1981). In a previous *in vitro* study, BSO was found to be an effective anti-leishmanial agent against *L. donovani* promastigotes as it inhibited intracellular amastigote multiplication (Kapoor *et al.*, 2000). In this study, BSO susceptibility varied between the three species. *L. donovani* had the lowest IC_{50} compared with that obtained for *L. major and L. mexicana*, which was higher than the maximum concentration tested, 0.5 mM BSO. The previous study concluded that the differences between parasite BSO susceptibility is related to their GSH utilization in their cell life cycle, which differed between *Leishmania* species (Romao *et al.*, 1999). Researchers have reported that the GSH concentration in *L. donovani*, *L. major*, and *L. braziliensis* was 0.10, 0.08, and 0.04 nmol/10^7 parasites, respectively (Romao *et al.*,

2006). In addition, studies have shown that *in vitro* macrophage microbicidal activity for *Leishmania* correlates with NO production. A study using NO donor, S-nitroso-N-acetyl-D,L-penicillamine (SNAP), which is able to kill *Leishmania* parasites, compared GSH levels and SNAP sensitivity of *L. amazonensis*, *L. braziliensis*, *L. donovani*, and *L. major*. These studies showed that the sensitivity of the *Leishmania* species to SNAP was inversely correlated with their GSH concentration. *L. amazonensis*, with a higher level of GSH, was less susceptible to SNAP (30 and 100 μM). The IC_{50} values of *L. amazonensis*, *L. donovani*, *L. major*, and *L. braziliensis* to SNAP were 207.8, 188.5, 160.9, and 83 μM, respectively (Romao *et al.*, 2006).

The difference in susceptibility of *Leishmania* species to BSO inhibition could be dependent on variable intracellular ovothiol concentrations. Trypanosomatids also produce significant amounts of ovothiol, which has recently been shown to catalytically accelerate formation of NO, which a significant oxidative burst during macrophage invasion (Ariyanayagam and Fairlamb, 2001). A study found that the total thiol content of each mammalian stage is less than the corresponding insect stage. The ratio of ovothiol between the promastigote and amastigote stages is nine-fold in *L. major* whereas in *L. donovani* it is >250-fold. Nonetheless, amastigotes from both species contain equivalent amounts (Ariyanayagam and Fairlamb, 2001). This could explain why in this study a higher concentration of BSO is needed in promastigotes compared to amastigotes. Also there were differences in ovothiol content in the three *Leishmania* species *L. donovani* (15%), *L. mexicana* (58%) and *L. major* (50%) whereas *L. donovani* has the highest percentage of TSH and GSH compared to other species (Weldrick *et al.*, 1999; Ariyanayagam and Fairlamb, 2001). A previous study to investigate resistance of *L. donovani* to sodium stibogluconate, showed the parasite

down-regulated γGCS expression in infected macrophages and increased expression of its own γGCS, which would result in a reduction in intramacrophage GSH levels and would promote an oxidative intramacrophage environment (Carter *et al.*, 2006).

In other studies PCR results indicated that the expression of macrophage γGCS increased in macrophages infected with any type of *Leishmania* species (Lueder and Phillips, 1996) and that treatment with 0.3 mM BSO decreased macrophage GSH levels but had no effect on parasite GSH levels (Romao *et al.*, 1999). These studies showed there was no difference in macrophage γGCS expression for cells treated with 0.2 or 0.5 mM BSO. Overall these results indicate that *Leishmania* γGCS enzyme has a different *in vitro* kinetic compared to the mammalian γGCS enzyme (Lueder and Phillips, 1996). *L. mexicana* was more resistant to BSO inhibition at both promastigote and the intracellular amastigote stage and resistance could be related to overexpression of γGCS. Resistance of new and old World *Leishmania* to antimony, has previously been found and all resistant strains overexpress the thiol gene that correlated to increased GSH and TSH levels (do Monte-Neto *et al.*, 2011).

In summary, the results of this study showed that there were differences between three *Leishmania* species in their susceptibilities to BSO treatment in both the promastigote and intracellular amastigotes stages. *L. mexicana* was the most resistant species to BSO. There are species stage specific differences in the susceptibility to BSO and this could be correlated to differences in γGCS expression. Moreover, the results of the enzyme assays suggested that the enzyme activity was different between species as *L. mexicana* and *L. major* had a high specific activity than *L. donovani* γGCS.

Chapter 6: *In vivo* imaging of cutaneous leishmaniasis in BALB/c mice.

6.1 Introduction

Alsaadi *et al.,* (2012) showed that it is possible to monitor *Leishmania* infection in mice by measuring the bioluminescent signal from mice given parasites expressing the luciferase gene. The method is simple, reliable and rapidly provides quantification of *Leishmania* amastigote burdens in infected cells (Lang *et al.,* 2009) and has been used *in vivo* to follow *L. major* and *L. mexicana* infections (Thalhofer *et al.,* 2010). The method depends on transfecting parasites with luciferase, an enzyme that works on a substrate to emit light. This makes it possible to monitor the *in vivo* progression of *Leishmania* infection by determining light production and determining its correlation to lesion size/footpad thickness in cutaneous leishmaniasis (Reimao *et al.*, 2013). The luciferase expression *L. mexicana* and *L. major* were used in this study their ability to infect bone marrow macrophages from BALB/c mice was tested *in vitro* in chapter 5. In this chapter their ability to infect BALB/c mice at rump and footpad will be study. The Llave and his Lab group (2011) monitored of luciferase-expressing *L. major* and mouse tissue transcript abundance in the ear and draining lymph node (DLN). Metacyclic promastigotes of luciferase-expressing *L. major* were inoculated into the ear dermis of C57BL/6 mice. BLI was performed and *L. major* mRNA was quantified by RT-PCR in ears and DLNs. The result indicated that high linearity was maintained over the range of template amastigote numbers added to the tissue. It is significantly that there was a strong correlation between the bioluminescence values and the parasite numbers as determined by RT-PCR (Llave *et al*., 2011).

Moreover the Michel and his groups (2011) assessed the infectivity of the *L. infantum* luciferase parasites (LUC-parasite), as well as the usefulness of bioluminescence for the monitoring of parasite proliferation in target organs, BALB/c mice were inoculated by IV route with various inocula of the stationary phase LUC-parasites or WT parasites. One month following inoculation, when generally both liver and spleen are infected, mice were imaged and sacrificed. The liver or spleen were prepared for parasite quantification by ELISA or bioluminescence analysis *ex vivo* They found that mice inoculated with increasing numbers of the LUC-parasite or WT parasites exhibited increasing and similar spleen and liver parasite burdens. This indicates that the selected LUC-parasite exhibits infectivity identical to that of wild type parasites (Michel *et al*., 2011).

Polymorphonuclear neutrophils are the most abundant white blood cell in mammals and they have an essential role in the host defence against microbial infection. Neutrophils can ingest microbes into intracellular compartments called phagosomes (Atosuo and Lilius, 2011). Neutrophils have a short life span of 10 hours in the circulation as they undergo spontaneous apoptosis (Sarkar et al., 2013). Neutrophils are the first cells recruited to the dermal site of Leishmania infection following injection using needle or from a sand fly bite (Ribeiro-Gomes and Sacks, 2012) and studies have shown neutrophils kill parasites (Von Stebut, 2007). Neutrophils also appear to play a major role in the development of protective immunity as they release proteins such as neutrophil elastase, cathepsin G and myeloperoxidase at inflammation sites which activate macrophages to release cytokines (Filardy *et al*., 2011). Murine studies have shown that neutrophils produce a number of cytokines after infection

with L. major, both in vitro and in vivo including, TNF-α, TGF-β and IL-8 (Safaiyan et al., 2011). The role of neutrophils in Leishmania infection has mainly been studied in mice given neutrophil depleting antibodies. However the results can depend on parasite species and the mouse strain used, the site of challenge, and the timing and specificity of the depleting antibody (Ribeiro-Gomes and Sacks, 2012).

Both neutrophils and macrophages have oxygen-dependent and oxygen-independent microbicidal mechanisms and oxygen-dependent pathways, reactive oxygen species (ROS) are produced by the action of NADPH oxidase complex in a process called oxidative burst (Wright *et al.*, 2010). A neutrophils oxidative burst can be quantified by chemiluminescence, which monitors the oxidation of luminol by neutrophil-generated reactive oxygen species. Luminol is a chemical that emits blue light (λmax = 425 nm) when exposed to an appropriate oxidizing agent (Gross *et al.*, 2009). As a result, luminol bioluminescence is preferentially localized within phagosomes of activated neutrophils and to a lesser extent for monocyte, during acute inflammation. On the other hand, after maturation from circulating monocytes, macrophages in tissue have lost most of their myeloperoxidase activity and, thus, do not actively produce luminol bioluminescence (Tseng and Kung, 2012). It has been used to monitor neutrophils (Atosuo and Lilius, 2011). This method have a number of advantages compared to flow cytometer, or microscopy as it is simple to carry out, requires lower amounts of reagent, is highly sensitive and selective (Yang *et al.*, 2010).

In this research, BALB/c mice infected with luciferase expressing *L. mexicana* or *L. major* were used to determine:

- If bioluminescence obtained *in vitro* or *in vivo* correlated with parasite growth.
- Which site would be better for vaccine studies footpad or rump.
- What stage of the parasite's life cycle would most appropriate for vaccine challenge studies (promastigote/amastigote).
- If the site of infection (footpad or rump) affected local neutrophil levels.

6.2 Results

There was a linear correlation between light emission and traditional measurement of parasite count, footpad thickness and lesion size in mice regardless of the parasite growth stage used to infect mice for *L. mexicana* and *L. major*. The footpad infected mice experiments were terminated earlier compared to rump infected mice as restrictions on the project licences meant that animals must be killed when the footpad swelling is ≥ 3 mm.

In vitro studies showed that there were a linear regression between BLI and *L. mexicana* parasite number (Figure 6.1). The pre-infection results demonstrated that amastigotes and promastigotes emission was the same with constant BLI/ parasite ratio (Table 6.1) and that there was a good linear relationship (correlation coefficient for the fitted line was R^2 >0.99, Figure 6.1). Results for parasites isolated from lesions after sacrifice of infected animals, which showed that the BLI/ parasite ratio for the parasites obtained from the footpad or rump were lower than pre-infected values (Table 6.1).

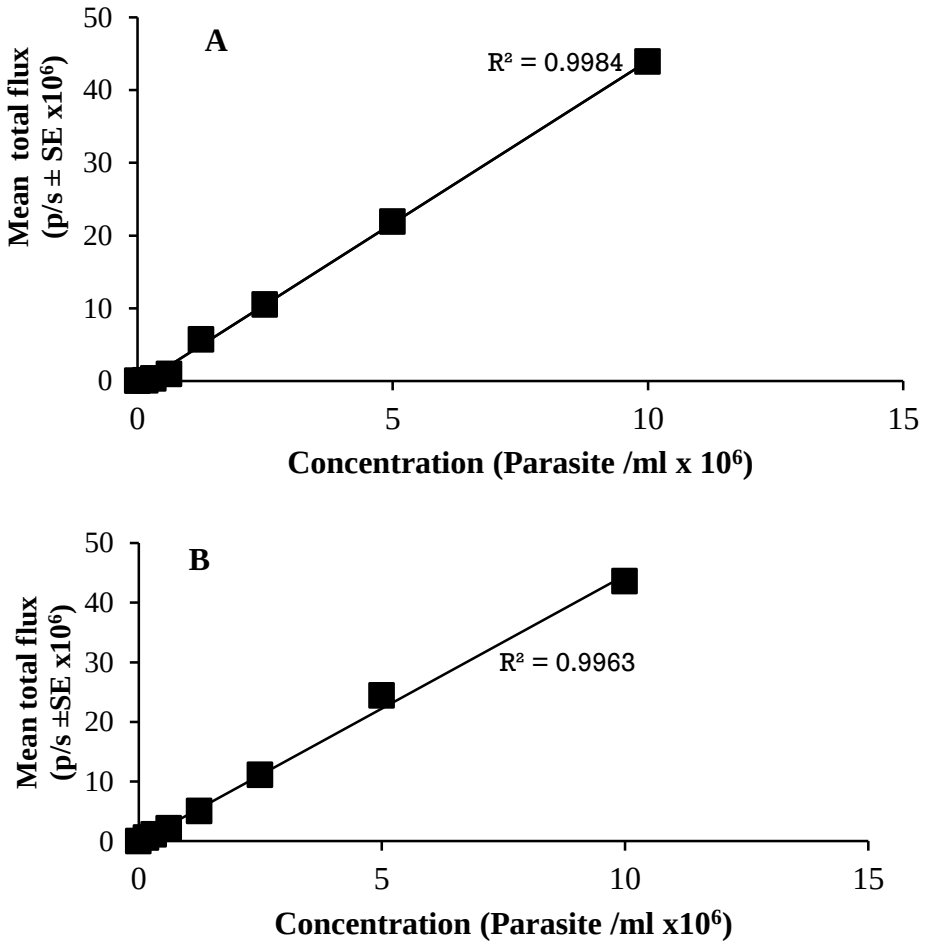


Figure 6.1 The relationship between the BLI and parasite number. The BLI emitted for a 100 µl sample of parasites at different concentrations, after dilution 1:1 with luciferin solution (150 µg/ml), was determined for *L. mexicana* promastigotes (A) and amastigotes (B).

The progress of infection in mice infected on the same day with the same batch of *Leishmania* parasites was determined to allow a direct comparison of parasite growth in the footpad and rump (Figure 6.2). Parasite growth was higher in the footpad infection, shown by the significantly high BLI (total flux/s) and lesion progression (mm) compared to the rump ($p<0.05$). Mice infected in the rump with *L. mexicana* promastigotes had no lesions and no BLI emitted over the course of infection and no parasites were isolated from the lesion site at sacrifice. In contrast, the same batch of *L. mexicana* promastigotes parasite inoculated into the footpad of mice had the ability to cause disease and a high number of parasites were present in the footpad lesion at end of experiment (Table 6.1). Therefore, the footpad was selected for vaccine experiments as it gave better infections than the rump.

Luminol treatment was used in imaging studies to detect the presence of neutrophils in infected mice. There was a significant difference in BLI between rump and footpad for mice infected with *L. mexicana* promastigotes (Figure 6.3 A). Thus indicating that significantly more neutrophils were recruited to the rump site compared to the footpad for *L. mexicana* infected mice ($p < 0.05$). However, there was no significant difference in the total amount of BLI emitted from the whole body of the two groups of infected mice (Figure 6.3B).

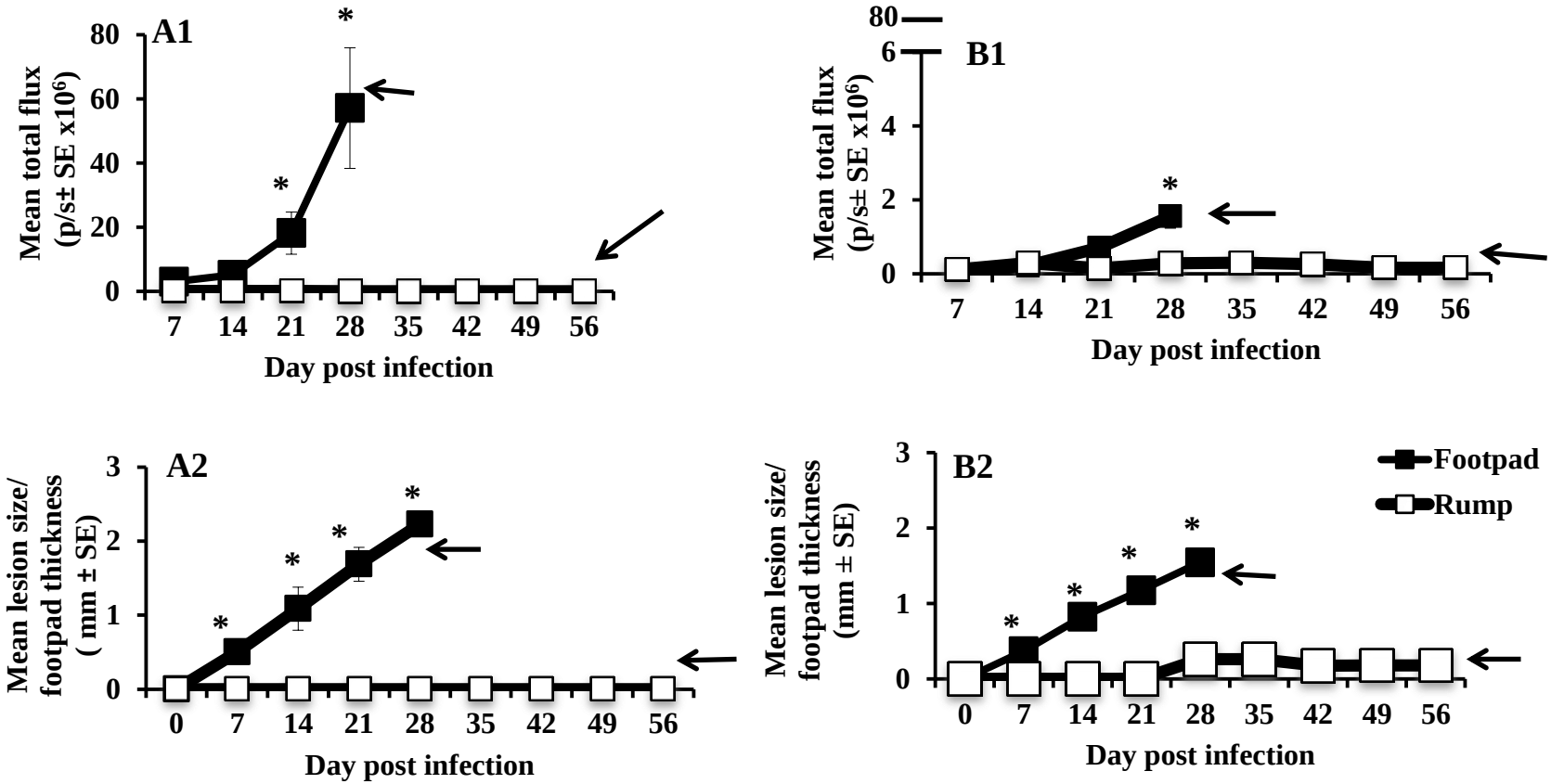


Figure 6.2 Comparison of parasite growth in mice infected with *L. mexicana* promastigotes (A1, A2) or *L. mexicana* amastigotes (B1, B2). Mice were infected with 10^7 *L. mexicana* and progression of infection was determined by measuring light production (A1, B1) or footpad thickness (A2, B2). * $P<0.05$ comparing footpad vs. rump data (n=5/treatment). The arrow indicates the day of mice were sacrificed.

Table 6.1 Comparison of infection route on the number of parasites recovered from the footpad or rump of mice infected with *L. mexicana*. The lesion from rump or footpad from mice shown in Figure 6.2, were disrupted in two mls medium. The parasite number per ml determined by microscopy and used to calculate the amount of BLI emitted/parasite ratio. The light production was determined for a 100 µl sample after dilution of the sample 1:1 with luciferin solution (150 µg/ml) using IVIS. $^{*}p<0.05$ compare footpad to rump infected site. The preinfection ratio was $^{a}p<0.05$ compare to rump site, $^{b}p<0.05$ comparing pre infection data vs. footpad data. $^{*}P<0.05$ comparing footpad vs. rump data (n=5/treatment).

Stage used at infection	**BLI/parasite ratio ± SE**			**Mean total flux (p/s ± SE x 10^6)**		**Mean number of parasite (p/ml ± SE x 10^6)**	
	Preinfection	Rump	Footpad	Rump	Footpad	Rump	Footpad
Amastigotes	$4 \pm 0.1^{a,b}$	1.1 ± 0.2	1.3 ± 0.1	0.17 ± 0.04	6.2 ± 1*	0.15 ± 0.02	5.2 ± 0.6*
Promastigotes	$3.9 \pm 1^{a,b}$	0.0 ± 0.0	1.6 ± 0.1	0.0 ± 0.0	9.9 ± 0.4*	0.0 ± 0.0	6.25 ± 0.4*

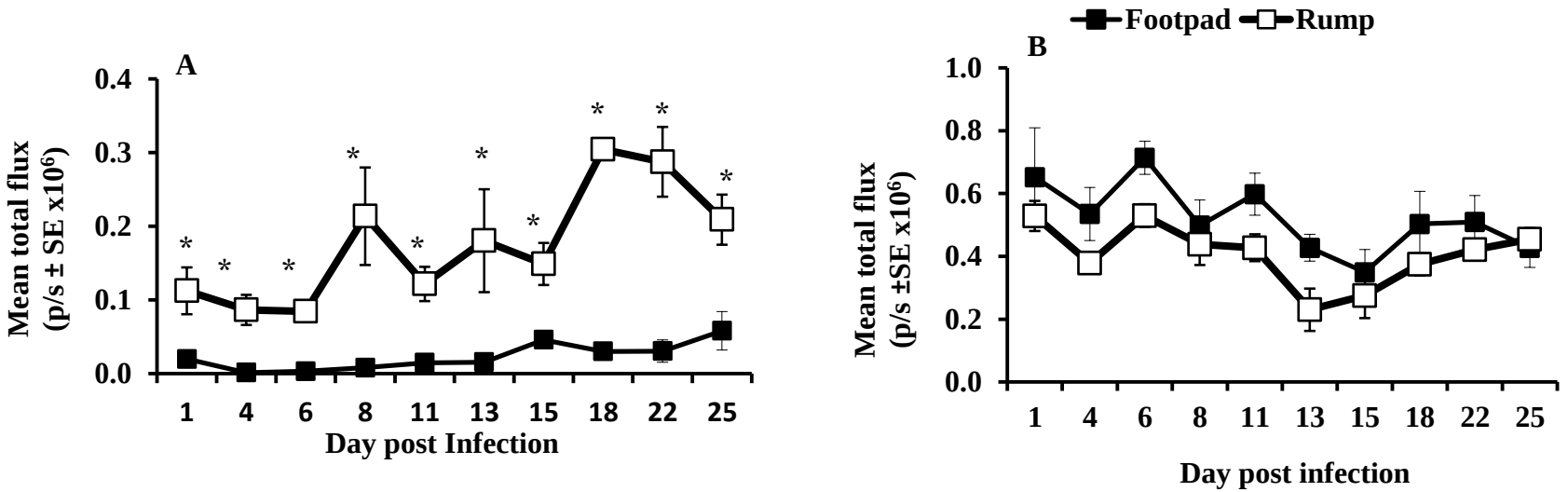


Figure 6.3 Quantification of neutrophils in the footpad /rump of mice infected with *L. mexicana* promastigotes. BALB/C mice from the same group of mice treated in Figure 6.2 were injected IP with luminol solution (dose 200 mg/kg) and imaged for 3 mins from 5 mins after injection (n=5/treatment). BLI was determined at the site of infection (A), whole body (B). * $p<0.05$, using Mann Whitney U test.

In *L. major* studies, the results showed that both promastigote and amastigote stages of *L. major* growth has a linear relationship between parasite number and light emission R^2>0.99 (Figure 6.4). Also in this study, the *L. major* promastigotes emitted more light than amastigotes parasites.

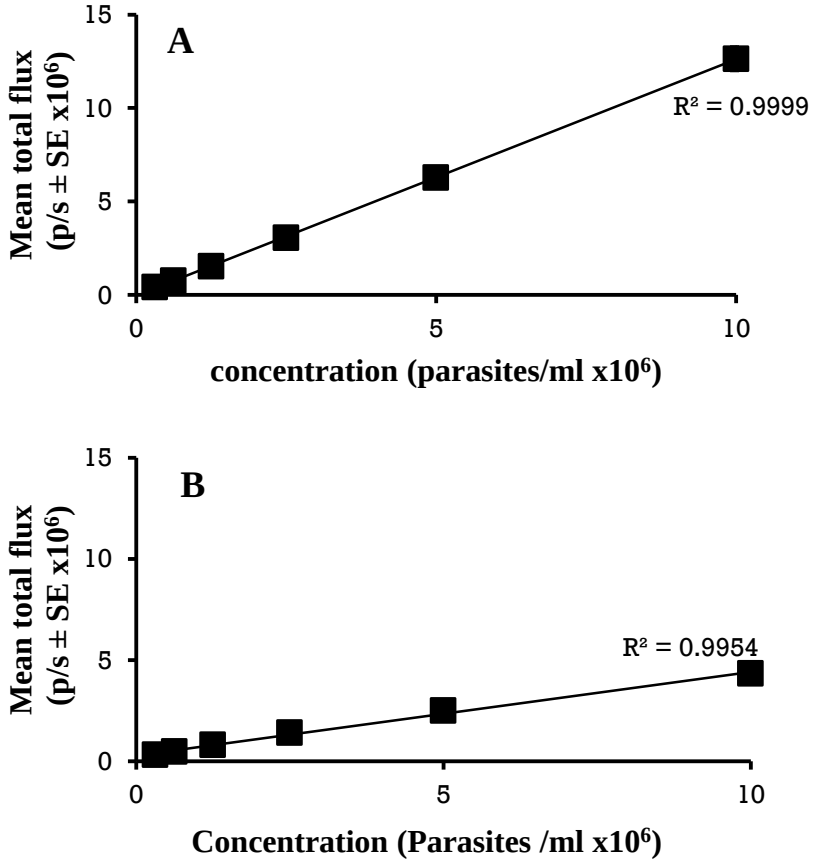


Figure 6.4 The relation between the BLI and parasite number. The BLI emitted for a 100 µl sample of parasites at different concentrations, after dilution 1:1 with luciferin solution (150 µg/ml) was determined for *L. major* promastigotes (A) and amastigotes (B).

These studies also showed that mice infected with *L. major* promastigotes emitted more light than mice infected with *L. major* amastigotes regardless of the site of infection. In addition, similar to the studies for *L. mexicana*, injection of parasites into the footpad gave significantly higher light emission and lesion size than injection of the same batch of parasites into the rump (based on results for day 14 and 21 mice infected with promastigote and day 21 for mice infected with amastigote parasites, Figure 6.5, $p<0.05$). In addition, more parasites were present in the footpad of infected mice compared to the rump, even though the animals were sacrificed a week earlier (day 21 for footpad infected mice, day 28 post infection for rump infected mice, Table 6.2). The bioluminescence/parasite ratio was the same for *L. major* amastigotes preinfection and post infection, whereas promastigote parasites had a higher BLI/parasite.

The study the role of neutrophils in *L. major* infection showed that there was no significant difference in the amounts of light emitted from the whole body and infected area of mice infected in the footpad compared to mice infected in the rump group (Figure 6.6). This indicates no difference in the neutrophils present in footpad of mice infected with *L. major* promastigotes compared to mice infected in the rump area.

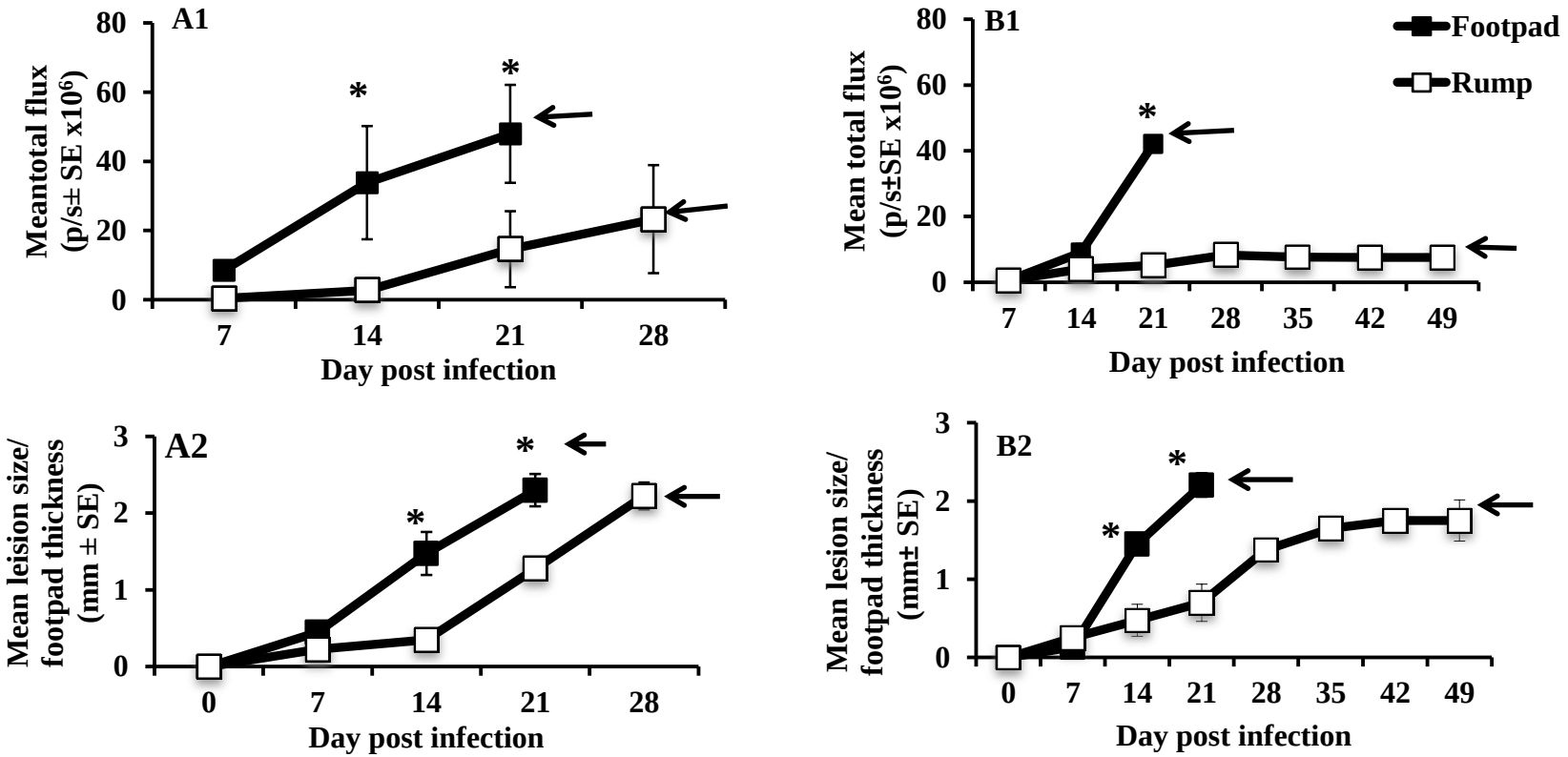


Figure 6.5 Comparison of parasite growth in mice infected with *L. major* promastigotes (A1, A2) or *L. major* amastigotes (B1, B2). Mice were infected with 10^7 *L. major* and progression of infection was determined by measuring light production (A1, B1) or footpad thickness (A2, B2). (n=5/treatment) $^*P<0.05$ comparing footpad vs. rump data. The arrow indicates the day where the mice were sacrificed.

Table 6.2 Comparison of the effect of route of infection on the number of parasites recovered from the footpad and rump following infection with *L. major* parasites on the day of sacrifice. The isolated lesion from the rump or footpad from mice shown in Figure 6.5 was disrupted in two mls medium. The parasite number per ml was determined by microscopy and used to calculate the amount of BLI emitted/parasite ratio. The light production was determined for a 100 µl sample after dilution of the sample 1:1 with luciferin solution (150 µg/ml) using IVIS. [a]p <0.05 comparing preinfection ratio vs. rump ratio, [b]p<0.05 comparing preinfection ratio vs. footpad ratio. [*] p<0.05 for footpad data comparing vs. rump data. (n=5/treatment).

Stage used at infection	**BIL/parasite ratio ± SE**			**Mean total flux (p/s ± SE) x 10^6**		**Mean number of parasite (p/ml ± SE) x 10^6**	
	Preinfection	Rump	Footpad	Rump	Footpad	Rump	Footpad
Amastigotes	0.50 ± 0.10	0.47 ± 0.15	0.55 ± 0.01	1 ± 0.5	5 ± 1*	4 ± 1	10± 1*
Promastigotes	1.2 ± 0.06[a,b]	0.12 ± 0.02	0.50± 0.01	0.5 ± 0.01	13 ± 0.9*	9 ±2	20 ± 2*

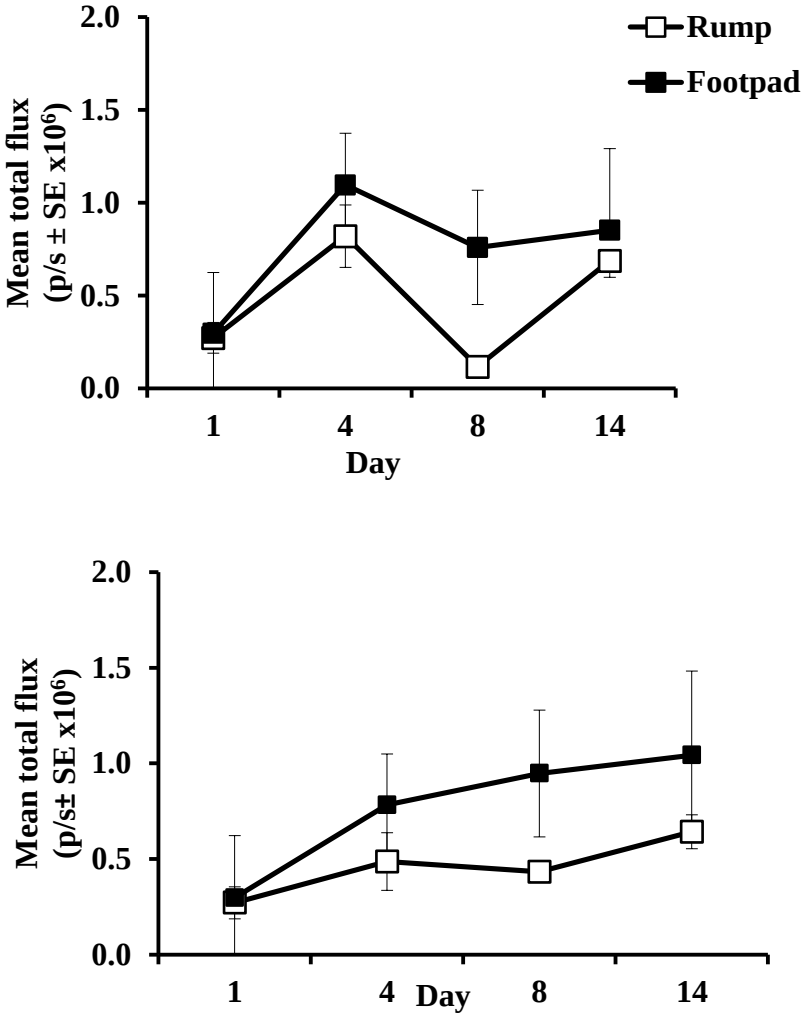


Figure 6.6 Quantification of neutrophils in the footpad /rump of mice infected with *L. major* promastigotes. Mice from the same group of mice treated in figure 6.5 were injected IP with luminol solution (dose 200 mg/kg) and imaged for 3 mins, 5 mins after injection (n=5/treatment). BLI was determined at the site of infection (A), whole body (B).

6.3 Discussion

Using BLI for *in vivo* imaging allows each animal to be used as its own control over time, overcoming the problem of animal variation (Lang *et al.*, 2009). In this study there was a linear correlation between parasite number and bioluminescence (photons per second) for both promastigote and amastigote parasites. However, both *L. mexicana* and *L. major* promastigotes had a higher BLI/parasite than amastigote parasites. The amount of light emitted/parasite for amastigote parasites recovered from rump or footpad lesions were significantly lower than that emitted by promastigote parasites used for the initial infection. In a previous study, Ravinder *et al.*, (2012) found that *L. donovani* axenic amastigotes lost about 20 % luciferase activity compare to preinfection promastigotes. There are many factors that could be responsible for a lower luciferase activity in amastigotes compared to promastigote parasites such as a slower metabolism, lower pH, presence of inhibitors inside the macrophages (Roy *et al.*, 2000) and rate of parasite multiplication (Ravinder *et al.*, 2012). Another study has shown that *L. infantum* luciferase activity of amastigotes was lower than that of promastigotes and that the amastigote emitted lower amounts of BLI (Michel *et al*., 2011). In this study rump infection with amastigotes gave higher infectivity than promastigote infection whereas in footpad both stages produced the same effect. Ravinder *et al.*, (2012) showed that luciferase-expressing *L. donovani* axenic amastigotes are able to enter, survive, and multiply in macrophages better than their promastigote counterparts. They were also more infective to mice as macrophages infected with the axenic amastigotes had a significantly higher parasite load than those exposed to luciferase-expressing

promastigotes. This is similar to the finding in this study, that amastigotes had a higher ability to infect rump and footpad of mice than promastigote parasites. Studies have shown that macrophages produce higher amounts of anti-leishmanial superoxide if infected with promastigotes of different *Leishmania* species compared to infection with amastigotes (Pham *et al.*, 2005). So this could also explain the lower infectivity of promastigotes in rump infections. It also indicates that the rump and footpad present different environments for parasites. Infection progressed quicker in the footpad compared to the rump lesion. Osorio and his research group (2003) compared the foot and snout inoculation site on the clinical evolution and immune responses of hamsters infected with *Leishmania (Viannia) panamensis*. Their results suggested that the site of infection influences the clinical outcome in experimental cutaneous leishmaniasis, and that the expression of macrophage deactivating type 2 cytokines and/or an exaggerated type 1 proinflammatory cytokine response may contribute to lesion severity. Hamsters infected in the snout showed a more rapid and severe lesion evolution at multiple time points, a more extensive inflammatory infiltrate and tissue necrosis, a higher tissue parasite burden, and a higher antibody titre than foot inoculation (Osorio *et al.*, 2003). Studies investigating the role of the site of the initial infection on the subsequent development of disease have concluded that subcutaneous or intradermal infections in the nose, fore or hind footpad, ear, and various sites on the trunk can lead to markedly different degrees of susceptibility (Nabors and Farrell, 1994). They suggested that the site-specific development of resistance could be related to non-immunological factors as differences in the ability of mice to resist infections at different sites on the body are believed to be at least

partially related to local differences in skin temperature, with areas of highest cutaneous temperature being most resistant to infection (Nabors and Farrell, 1994; Kirkpatrick *et al.*, 1987).

In this study, there was a significant difference between rump and footpad infected with *L. mexicana* promastigotes in the chemiluminescence emitted, which was used as a marker of phagocytic cells as neutrophils and macrophages producing an oxidative environment as light expression occurs in response to the luminol given to mice. Rump lesions of mice infected with *L. mexicana* emitted more chemiluminescence than footpad infected mice. Therefore the lower infection in the rump infected mice could be related to neutrophils which can be kill parasite (Ritter *et al,* 2009*).* Otherwise, the local environment in the footpad could favour parasite survival compared to the rump. Previous studies found that depletion of neutrophils in BALB/c mice infected with *L. braziliensis* promastigotes in the ear, enhanced parasite multiplication while co-inoculated with neutrophils enhance parasite death (Novais *et al.*, 2009). The difference in level of neutrophil distribution between rump and footpad could also be related to difference in local luminol levels due to differences in blood flow between mice body organs (Rosas *et al*., 2005). Alternatively the differences in chemoluminscence or bioluminesence for the two sites may be related to tissue properties which can effect light scatter or absorption and the subsequent detection of emitted light (Sadikot and Blackwell, 2005).

In summary, the IVIS technology enables the visualization of luciferase-expressing *Leishmania* species parasites in living anaesthetized BALB/c mice. Once the substrate luciferin distributes through the tissues, the rapid oxidation of D-luciferin by luciferase expressed by the transgenic parasites resulted in the release of bioluminescence at the site of infection. The photons that are not absorbed by the tissue are detected at the surface of the animal by the camera of the IVIS imaging technology. In this study, there was a significant difference between rump and footpad infected with *L. mexicana* promastigotes in phagocytic cells present in the infected site as detected using the chemiluminescence emitted. However there was no significant between rump and footpad for mice infected with *L. major* promastigotes. That could recommend the future studies to investigate the role of phagocytic cells as neutrophils and macrophages in rump and footpad infected with *Leishmania.* Over all, this study concluded that footpad infection could be used to monitor *Leishmania* infection in BALB/c mice as disease progression rapidly, so it was decided to use this in vaccine studies (Chapter 7).

Chapter 7: Development of a recombinant *Leishmania* gamma glutamyl cysteine synthetase vaccine to protect against leishmaniasis

7.1 Introduction

Ideally a *Leishmania* vaccine that protects against all types of leishmaniasis and which could be given by a non-invasive route is required. A 'needle-free' vaccine would be safer to administer, improve patient compliance as vaccination would be 'pain-free', and be environmentally better as no sharps would be required for administration (Misra *et al.*, 1999; Ferro and Carter, 2006; Varmus *et al.*, 2003; Wang *et al.*, 2012). The ability of a vaccine protection against leishmaniasis using components from one *Leishmania* species using non-invasive route has been demonstrated in earlier studies. For example, intranasal vaccination with *L. amazonensis* soluble recombinant gp36/LACK antigen and plasmid DNA coding for the LACK antigen significantly reduced *L. amazonensis* parasite burdens in vaccinated mice compared to control values, and in the protected mice was associated with enhanced IFN-γ and IL-10 production by antigen-stimulated cells obtained from the lymph node at the lesion site (Pinto *et al.*, 2004). The study showed that the vaccine distributed to the local nasal area and peripheral lymphoid organs based on mRNA expression for LACK gene. In addition the same immunisation protocol protected against *L. chagasi* infection in BALB/c mice, based on lower parasite burdens in the liver and spleen compared with controls. LACK mRNA transcripts were detected in the spleen, brain, cervical lymph nodes and popliteal lymph nodes of animals 3 months after vaccination (De Oliveira Gomes *et al.*, 2012).

Visceral leishmaniasis is caused by infection with the parasite *L. donovani* and is fatal if left untreated (Mondal *et al.*, 2010). The murine model of *L. donovani* infection is a good model of early parasite replication followed by immunological control and subclinical infection, but there is no murine model for the progressive disease observed in human VL. That is because a mouse infected with *L. donovani* does not reproduce the features of active human VL. Researchers, studying the differences between murine and hamster models using *L. donovani*, concluded that infection of the Syrian hamster (*Mesocricetus auratus*) with *L. donovani* reproduced the clinical pathological features of human VL. In addition, the immunological mechanism involved in control of VL in the hamster model was completely different from the murine model (Melby *et al.*, 2001). One of the main problems with the hamster model is the lack of available immunological reagents, which makes dissection of the immune responses involved and the determination of cytokine protein levels more problematic. Therefore, the most used technique is quantitative real-time PCR method to determine intracellular Th1/Th2 cytokines (Melby *et al.*, 2001; Gupta *et al.*, 2012).

In other previous studies we have shown that vaccination by an injectable route with either a plasmid containing the gene sequence of *L. donovani* gamma glutamylcysteine synthetase (Carter *et al.*, 2007) or recombinant *L. donovani* γGCS (Henriquez *et al.*, 2010) protected mice against infection with *L. donovani*, *L. major* or *L. mexicana* (Campbell *et al.*, 2011), but sterile immunity was not obtained.

Therefore in this study we:

- Determined if recombinant *Leishmania* γGCS from three species given by inhalation induced similar immune response as that induced by subcutaneous administration of the vaccine.
- Compared the ability of a single and combination *Leishmania* γGCS vaccine to protect against *L. mexicana* infection.
- Determined the ability of a combination vaccine, consisting of recombinant gamma glutamyl cysteine synthetase proteins, from three different *Leishmania* species, to protect against *L. mexicana, L. major* or *L. donovani* infection and compared the efficacy of vaccination by the subcutaneous and pulmonary routes.

7.2 Results

Proteins from *Leishmania* γGCS obtained in Chapter 4 were used as vaccine candidate. Endotoxin contamination from the bacteria used to produce the recombinant *Leishmania* γGCS may occur. Therefore, all recombinant proteins had their endotoxin levels assessed before use as vaccine. Endotoxin contamination was variable for different batches of the recombinant proteins and specific endotoxin levels are shown in Table 7.1. In all cases, processing using commercial endotoxin removal kit resulted in levels that were less than 3 Eu/ml. Therefore, in all vaccine studies mice were given dose with less than 1.5 Eu/dose, even if animals were given a dose of the triple vaccine of 150 µg protein/animal/dose.

Table 7.1 Endotoxin levels in vaccine samples, n=3/treatment (EU/mL ± SE).

Sample No	*L. donovani* γGCS	*L. mexicana* γGCS	*L. major* γGCS
1	1.5 ± 0.5	1.4 ± 0.3	0.7 ± 0.07
2	1.5 ± 0.4	1.9 ± 0.1	1.3 ± 0.5
3	1.5 ±1.2	1.2 ± 0.1	1.6 ± 0.5
4	1.8 ± 0.8	1.3 ± 0.2	1.9 ± 0.6
5	1.5 ± 0.9	1.5 ± 0.3	1.0 ± 1.0

7.2.1 Vaccination with γGCS induced the production of both Th1 and Th2 responses

The initial aim was to vaccinate with all three *Leishmania* γGCS proteins in studies but obtaining recombinant *L. major* γGCS protein was difficult (see Chapter 3). Therefore in the initial studies mice were only vaccinated with *L. mexicana* and *L. donovani* γGCS proteins.

The effect of subcutaneous injection with one or both of the γGCS proteins on cytokine and nitrite production of *in vitro* stimulated spleen cells from immunised mice was determined (Figure. 7.1 and 7.2). Vaccination resulted in a significant increase in nitrite production from cells stimulated with LdγGCS, LmγGCS or ConA (Figure. 7.1). Significantly higher amounts of nitrite were produced by cells from mice

vaccinated with the double vaccine compared to the single vaccine or non vaccinated controls ($p < 0.05$).

IL-5 production can be used as an indicator of a Th2 response (Cummings *et al.*, 2010). Significantly higher level of IL-5 were produced by cells from mice vaccinated with the two γGCS proteins or a single γGCS protein stimulated with LdγGCS, LmγGCS or ConA compared to control value ($p<0.05$). In addition, cells from mice given the double vaccine and stimulated with ConA produced significant higher amounts of IL-5 than cells from mice vaccinated with LdγGCS alone (Figure 7.1, $p < 0.05$).

IL-10 is a regulatory cytokine that has primarily suppressive effects on immune function, targeting multiple activation and Antigen presentation pathways of macrophages and dendritic cells (review by Kaye and Scott, 2011). Cells from mice vaccinated with the double vaccine and stimulated with specific antigen produced a significantly higher amount of IL-10 compared to cells from non vaccinated mice ($p< 0.05$). In addition a significantly higher amount of IL-10 was produced by cells stimulated with LdγGCS or LmγGCS from mice given the double vaccine compared to cells from mice given the single vaccine. Cells from mice given the single vaccine and stimulated with ConA produced significantly higher amount of IL-10 than cells from control mice ($p < 0.05$, Figure. 7.1).

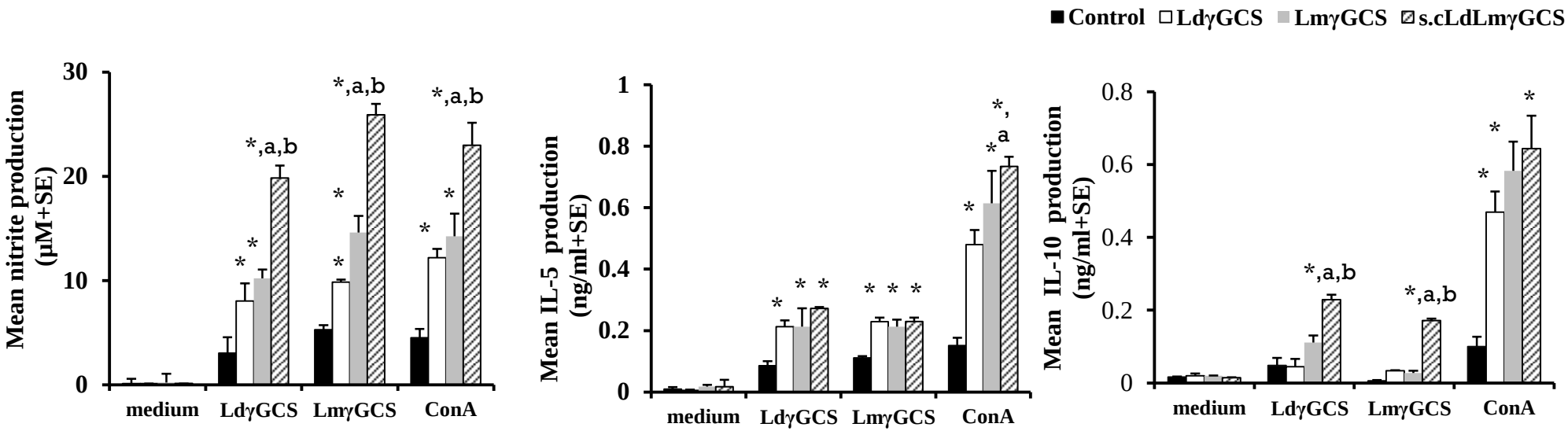


Figure 7.1 Mean nitrite, IL-5 and IL-10 production by splenocytes from vaccinated mice stimulated *in vitro* on day 0 pre infection. Mice were immunised by subcutaneous injection with PBS (control), recombinant γGCS protein *L. donovani* (LdγGCS), *L. mexicana* (LmγGCS), 50 µg/dose, or joint treatment s.c (LdLmγGCS) 50µ g/dose, on day −28 and −14. Mice splenocytes were incubated with medium alone (control), LdγGCS protein, LmγGCS (25 µg/ml), or Con A (5 µg/ml), and 72 hr. later cell supernatants stored until nitrite or cytokine levels could be determined by ELISA. $^{*}p < 0.05$, compare to control, $^{a}p < 0.05$ compare to LdγGCS, $^{b}p < 0.05$ compare to LmγGCS, $p < 0.01$ comparing antigen or treated with its unstimulated control (medium), n=3/ treatment.

TNF-α is an inflammatory cytokine (Mougneau *et al.,* 2011). Only cells from mice vaccinated with the double vaccine (s.cLdLmγGCS) and stimulated with ConA produced significant amounts of TNF-α compared to non vaccinated and unstimulated controls ($p < 0.05$, Figure 7.2). The cells from vaccinated mice produced significant amounts of TNF-α in response to either specific antigen (LdγGCS or LmγGCS) compared to their relevant non vaccinated controls ($p < 0.05$, Figure 7.2).

IFN-γ levels can be used to indicate whether a specific Th1 immune response is being generated and nitrite is one of the products generated by phagocytes stimulated by IFN-γ (Prajeeth *et al.*, 2011). Vaccination with the single vaccine (LmγGCS) and double vaccine (LdLmγGCS) induced significantly higher IFN-γ production by spleen cells incubated with ConA and LdγGCS compared with their relevant non vaccinated controls and cells from mice vaccinated with LdγGCS ($p< 0.05$). Cells from all three groups of vaccinated mice produced significantly higher amounts of IFN-γ in responses to LmγGCS antigenic stimulation compared to non vaccinated controls ($p < 0.05$, Figure. 7.2). The preinfection result indicated that vaccine has ability to induced mixed Th1/Th2 response as induced Th1 cytokine as IL-5, Th2 cytokine as IFN-γ.

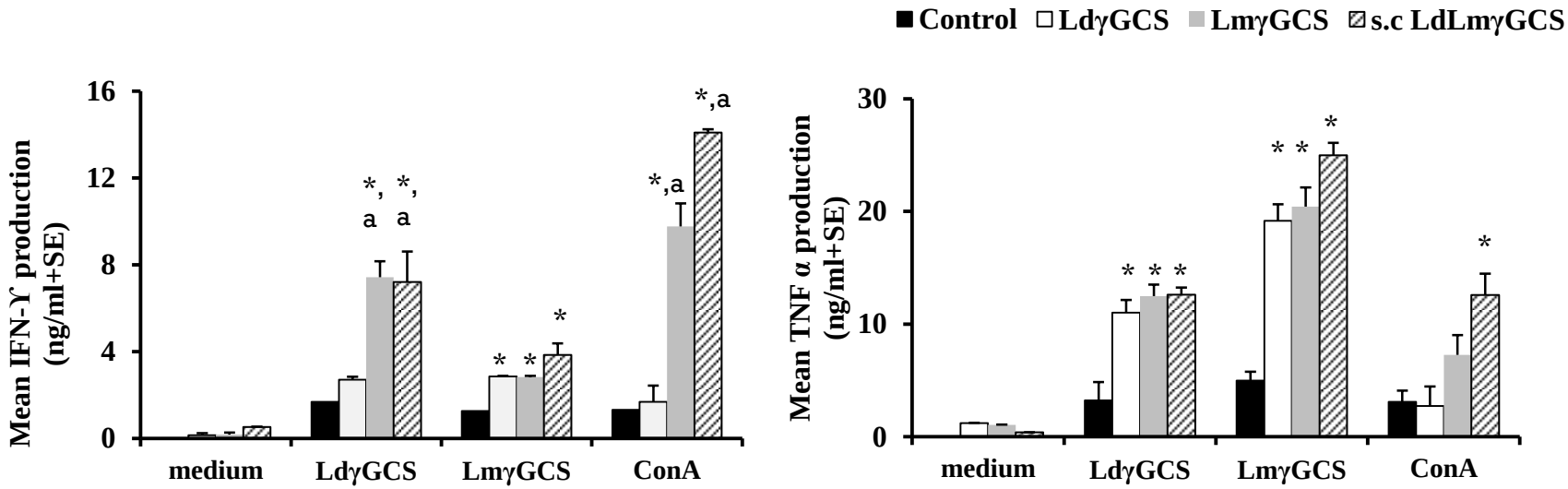


Figure 7.2 Mean IFN-γ and TNF-α production by splenocytes from vaccinated mice stimulated *in vitro* on day 0 pre infection. Mice were immunised by subcutaneous injection with PBS (control), recombinant γGCS protein *L. donovani* (LdγGCS), *L. mexicana* (LmγGCS) 50 µg/dose, or joint treatment s.c (LdLmγGCS) 50 µg/dose on day −28 and −14. Mice were incubated with medium alone (control), LdγGCS protein, LmγGCS (25 µg/ml), or Con A (5 µg/ml), and 72 hr. later cell supernatants stored until nitrite or cytokine levels could be determined by ELISA. $^{*}P < 0.05$, compare to control, $^{a}p < 0.05$ compare to LdγGCS, $^{b}p < 0.05$ compare to LmγGCS. All the treatment significant to relative unstimulated control (medium) $p < 0.01$, n=3/treatment.

7.2.2 Vaccination with the double γGCS vaccine was more effective over the course of infection but resulted in a similar reduction in lesion parasite burdens as mice give a single γGCS vaccine

The efficacy of vaccination with the single or double vaccines (γGCS from *L. donovani*, *L. mexicana* LdLmγGCS, 50 μg/protein) given by the subcutaneous routes on the outcome of subsequent infection with *L. mexicana* (LmexLuc) was determined. Mice vaccinated with double vaccine had a higher level of protection against infection and this was demonstrated as a significant reduction in the both footpad thickness and light emitted from the site of infection compared to both single vaccine groups ($p < 0.05$) and the infection control (Figure. 7.3). Table 7.2 shows the parasite numbers recovered from the footpad of all four groups of mice at sacrifice. All three groups of vaccinated mice had similar parasite numbers and these were significantly lower than infected control values ($p < 0.05$). The amount of BLI/parasite was similar for all four groups of mice (Table 7.2).

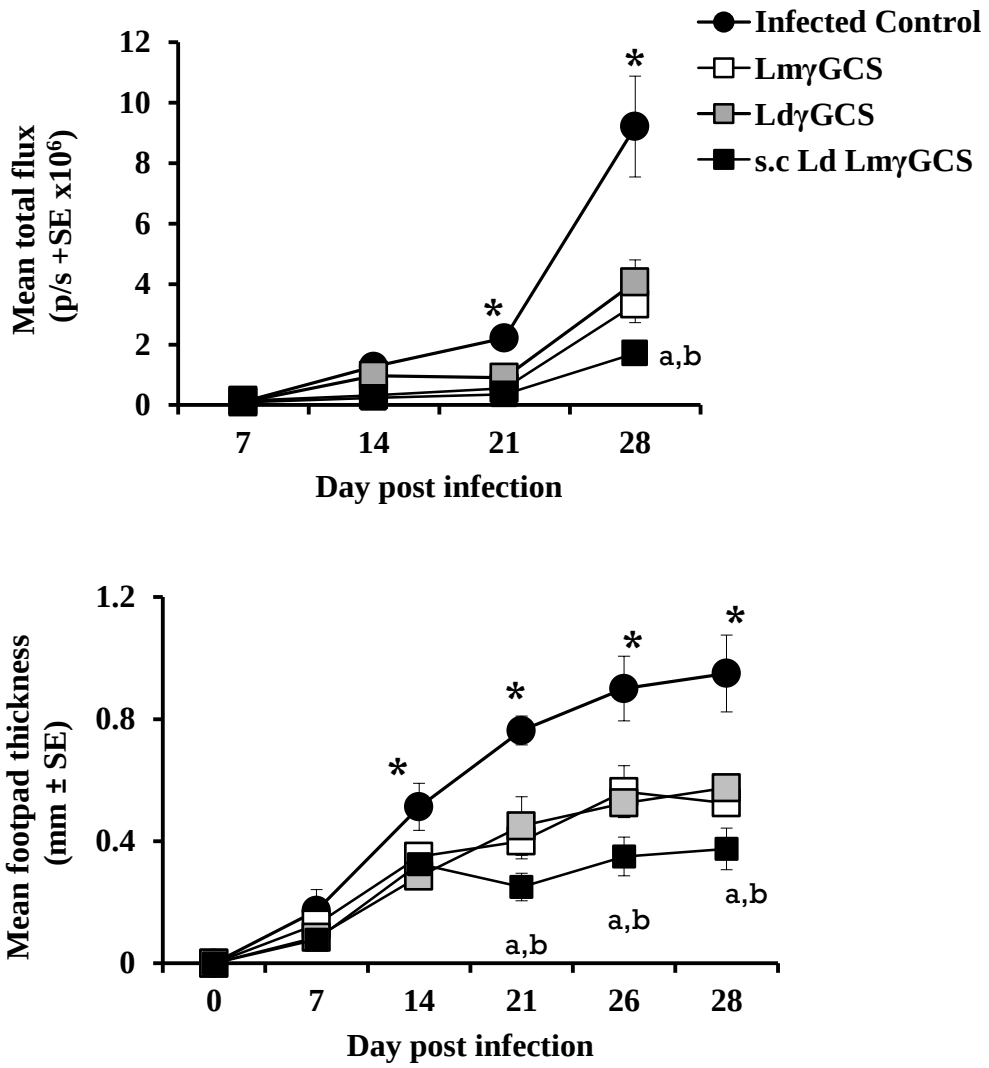


Figure 7.3 Comparing vaccination with different subcutaneous γGCS vaccine on outcome of *L. mexicana* infection. Mice were treated with PBS alone (control), LdγGCS and LmγGCS alone or with both proteins by subcutaneous injection (s.cLdLmγGCS), infected with $1x10^7$ *L. mexicana* (LmexLuc) strain. Determining footpad thickness assessed the progression of infection or bioluminescence in control and immunized mice over the course of infection and mice were sacrificed on day 28. $^{*}p<0.05$ all treatment significant compare to infected control, $^{a}p<0.05$ the s.cLdLmγGCS vaccine compare to LdγGCS, $^{b}p<0.05$ the s.cLdLmγGCS vaccine compare to LmγGCS, n=5/treatment.

.

Table 7.2 The effect of different subcutaneous γGCS vaccines on the outcome of *L. mexicana* infection. The parasite burden of mice from infected control, LdγGCS, LmγGCS and s.cLdLmγGCS groups that amastigotes parasite on day 28 from mice footpad after sacrificed was determined by direct counting of the number of parasite/ml present in footpad lesion homogenate or by determining the amount of bioluminescence emitted for the homogenate sample. $^{*}p < 0.05$ compared to control. The pre infection is the *L. mexicana* promastigotes parasite in medium before inoculation into mice (n=5/treatment).

	Total flux (p/s ± SE×10^6)	**Parasite burden (Number parasite/ml) ×10^6 ± SE**	**BLI/parasite ratio**
Pre infection	23 ± 0.3	10 ± 0.0	2.4 ± 0.3
Infected control	2.5 ± 0.03	6.36 ± 0.52	0.4 ± 0.01
LdγGCS	0.3 ± 0.04*	0.93 ± 0.4*	0.3 ± 0.0.04
LmγGCS	0.3 ± 0.05*	0.9 ± 0.33*	0.3 ± 0.08
s.cLdLmγGCS	0.2 ±0.02*	0.7 ±0.21*	0.31 ± 0.08

Protection with a γGCS vaccine is related to both Th1 and Th2 Specific antibody response

Blood samples were collected for antibody detection as described in Figure 7.4. Immunised mice produced significantly higher amounts of specific IgG1 and IgG2a on 14 day after the first immunisation compared to unvaccinated controls ($p < 0.05$, Figures 7.5 and 7.6). Subcutaneous vaccination with the double vaccine induced the highest specific IgG1 and IgG2a antibody response to all three specific γGCS antigen used in ELISA assays (Figures 7.5 and 7.6). Vaccination with *L. mexicana* γGCS alone induced significant antibodies against both γGCS antigens before infection indicating there is cross-immunity between the two γGCS proteins. Serum IgG2a antibody titres were similar after infection for the three groups of vaccinated mice but on day 28 post infection mice given the double vaccine had significantly higher IgG1 levels compare to mice given either of the single vaccines.

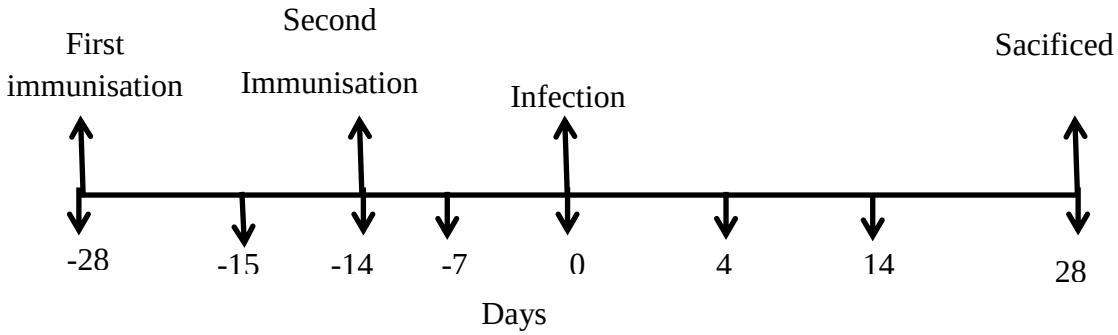


Figure 7.4 The schem described the time point for blood collection during vaccine expermenta to dtected antibody. The mice were immunized on days -28 and -14. The bllod samples were collected on days -15, -7 preinfection, and days 4,14 and 28 postinfection. day 0 is infection day.

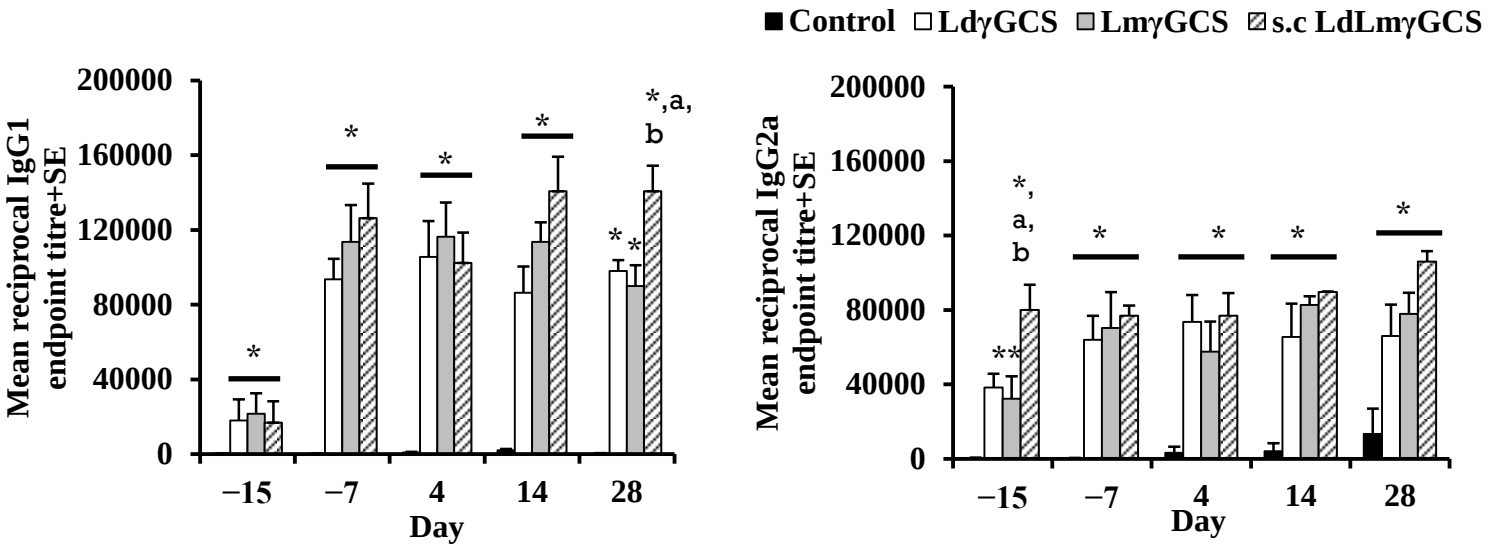


Figure 7.5 The effect of vaccination with γGCS on antibody production in immunized and control mice shown in figure 7.3, against the recombinant *L. donovani* γGCS antigen. Mice were vaccinated by subcutaneous injection with PBS (control), recombinant γGCS protein *L. donovani* (LdγGCS), *L. mexicana* (LmγGCS) 50 µg/dose, or joint treatment s.cLdLmγGCS. On day 0 mice were infected with 1 x 10^7 *L. mexicana* promastigotes by subcutaneous injection into footpad. Mice bleeding on day pre infection (-15, -7) and 4, 14, 28 post infection to detected specific antibody titre. $^{*}p < 0.05$, compare to control, $^{a}p < 0.05$ compare to LdγGCS, $^{b}p < 0.05$ compare to LmγGCS n=5/treatment.

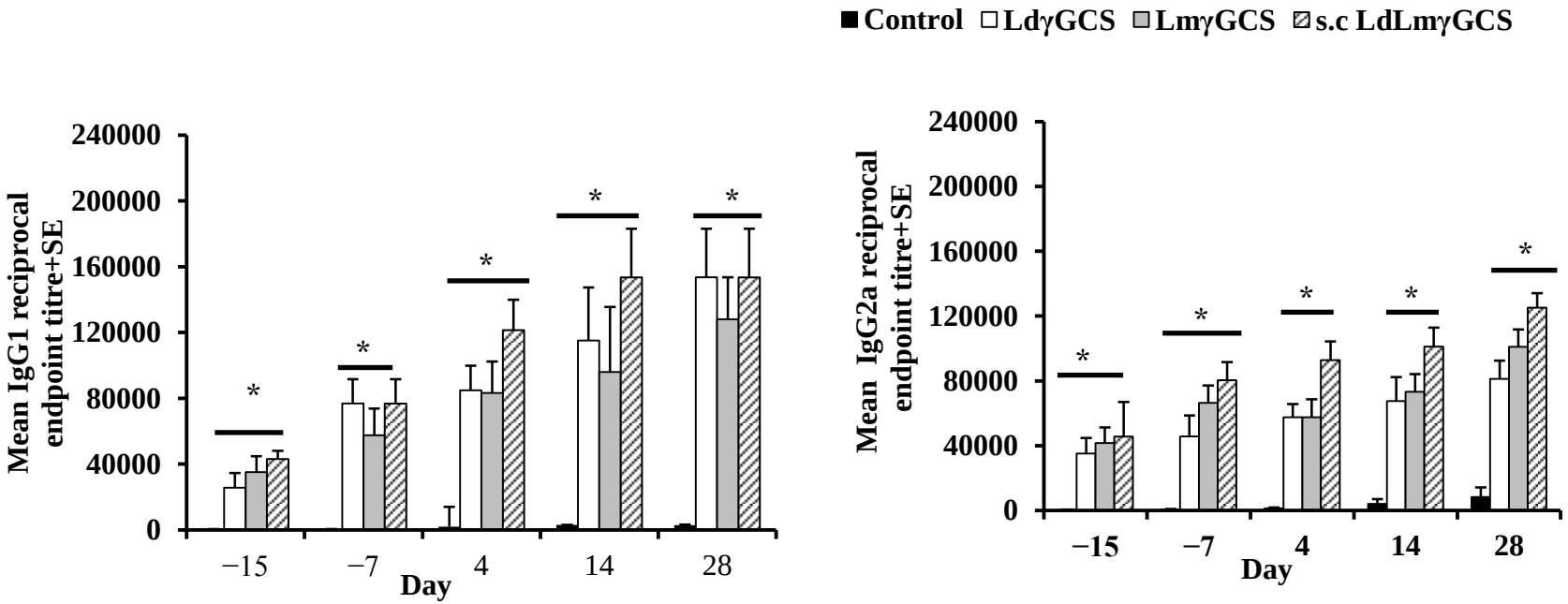


Figure 7.6 The effect of vaccination with γGCS on antibody production in immunized and control mice shown in figure 7.3, against the recombinant *L. mexicana* γGCS antigen. Mice were vaccinated by subcutaneous injection with PBS (control), recombinant γGCS protein *L. donovani* (LdγGCS), *L. mexicana* (LmγGCS) 50 µg/dose, or joint treatment s.cLdLmγGCS. On day 0 mice were infected with 1 x 10^7 *L. mexicana* promastigotes by subcutaneous injection into footpad. Mice bleeding on day pre infection (-15, -7) and 4, 14, 28 post infection to detected specific antibody titre $^*p < 0.05$, compare to control n=5/treatment.

After *L. mexicana* infection there were obvious differences in the IFN-γ production by spleen cells taken on day 28 post-infection for the different treatments (Figure 7.7). Only cells stimulated with ConA and LmγGCS antigen induced significantly higher levels of IFN-γ compared to cells from infected controls and un stimulated control ($p<0.05$).

Nitrite production was elevated in the supernatant of spleen cells taken from all the vaccinated mice and stimulated with *L. mexicana* γGCS and was significant in all vaccinated group compared to the controls (Figure 7.7). There was no difference in the nitrite level produced by spleen cells from all groups of mice stimulated with ConA. Splenocytes from mice subcutaneously vaccinated with the double vaccine produced significantly greater amounts of nitrite in response to *L. donovani* γGCS antigen compared to infected controls and the single vaccine protocol. Splenocytes from vaccinated mice stimulated with LmγGCS produced significantly higher amounts of TNF-α compared to similarly stimulated spleen cells from infected control mice ($p < 0.05$, Figure 7.5).

The IL-5 produced by spleen cells from infected mice stimulated with LdγGCS, LmγGCS or ConA was significantly higher than that of corresponding unstimulated controls (Figure 7.7, $p < 0.05$). Cells from vaccinated mice produced similar levels of IL-5 in response to the two recombinant protein antigens and the levels were significantly higher than that produced by cells from infected control mice.

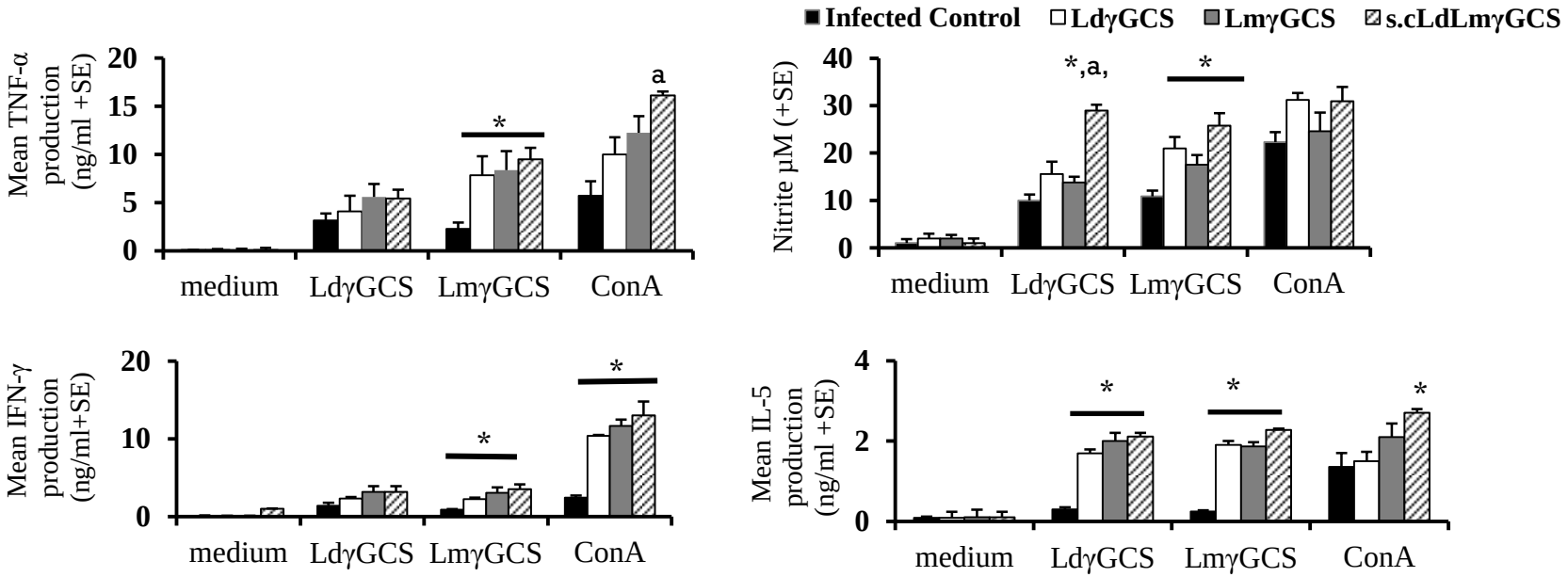


Figure 7.7 Mean IFN-γ, TNFα, nitrite and IL-5 production by splenocytes from mice taken on day 28 post infection from mice shown in figure 7.3 Cells were incubated with medium alone (control), LdγGCS protein or LmγGCS protein (25 µg/ml), or Con A (5 µg/ml) and 72 hr later the cell supernatants were stored until nitrite and cytokine levels could be determined by ELISA. $^{*}p < 0.05$, compare to control, $^{a}p < 0.05$ compare to LdγGCS, $^{b}p < 0.05$ compare to LmγGCS. all treatment significant to un stimulated control (medium) $p<0.01$, n=5/treatment.

7.2.3 Vaccination with γGCS using inhalation delivery compare to subcutaneous

The above studies showed that vaccination with γGCS from *L. donovani* or *L. mexicana* protected against *L. mexicana* infection (section 7.2.2). In the present work, the effectiveness of vaccination by pulmonary delivery was explored.

The effect of immunisation by subcutaneous and inhalation with using different combinations of γGCS recombinant proteins on the outcome of *L. mexicana* infection was determined. The cytokine and nitrite responses were measured from antigen or ConA stimulated spleen cells taken pre-infection (Figure 7.8) and compared with vaccination using a triple antigen vaccine LdLmLjγGCS (20 μg dose of each protein) delivered by subcutaneous and inhalation routes, and inhalation with the double vaccine LdLmγGCS.

IFN-γ levels can be used to indicate whether a specific Th1 immune response and nitrite is one of the products generated by phagocytes stimulated by IFN-γ. Vaccination enhanced IFN-γ production by spleen cells from mice given any of the three vaccine protocols compared to controls given PBS and there was no significant difference in the amount of IFN-γ produced for cells stimulated with LdγGCS, LmγGCS or LjγGCS. However ConA simulation of cells showed that cells taken from all vaccinated groups produced significantly higher levels of IFN-γ than controls ($p < 0.05$, Figure 7.8). There was no difference in the amount of nitrite produced by cells from all vaccinated groups stimulated with LdγGCS, LmγGCS or LjγGCS indicating that vaccination did not alter

this response (Figure 7.8). Cells from mice subcutaneously vaccinated with the triple vaccine produced significantly greater amounts of nitrite in response to ConA antigens compared to non-vaccinated controls, whereas pulmonary administration of the same vaccine did not increase nitrite production. Vaccination with the double vaccine given by the pulmonary route did modulate nitrite production, and in this case significantly greater amount of nitrite were produced in response to ConA and specific compared to controls.

IL-5 and IL-6 production can be used as an indicator of a Th2 response (Cummings *et al.*, 2010). Significantly higher level of IL-5 were produced by cells from mice vaccinated with the γGCS protein stimulated with LdγGCS, LmγGCS, LjγGCS or ConA compared to control value (Figure 7.8, $p < 0.05$). Significantly higher levels of IL-6 were produced by cells from mice vaccinated with γGCS proteins stimulated with LdγGCS or ConA compared to control value ($p<0.05$). However, there was no significant differnce between the vaccinated and control group for spleen cells stimulated with LmγGCS or LjγGCS proteins.

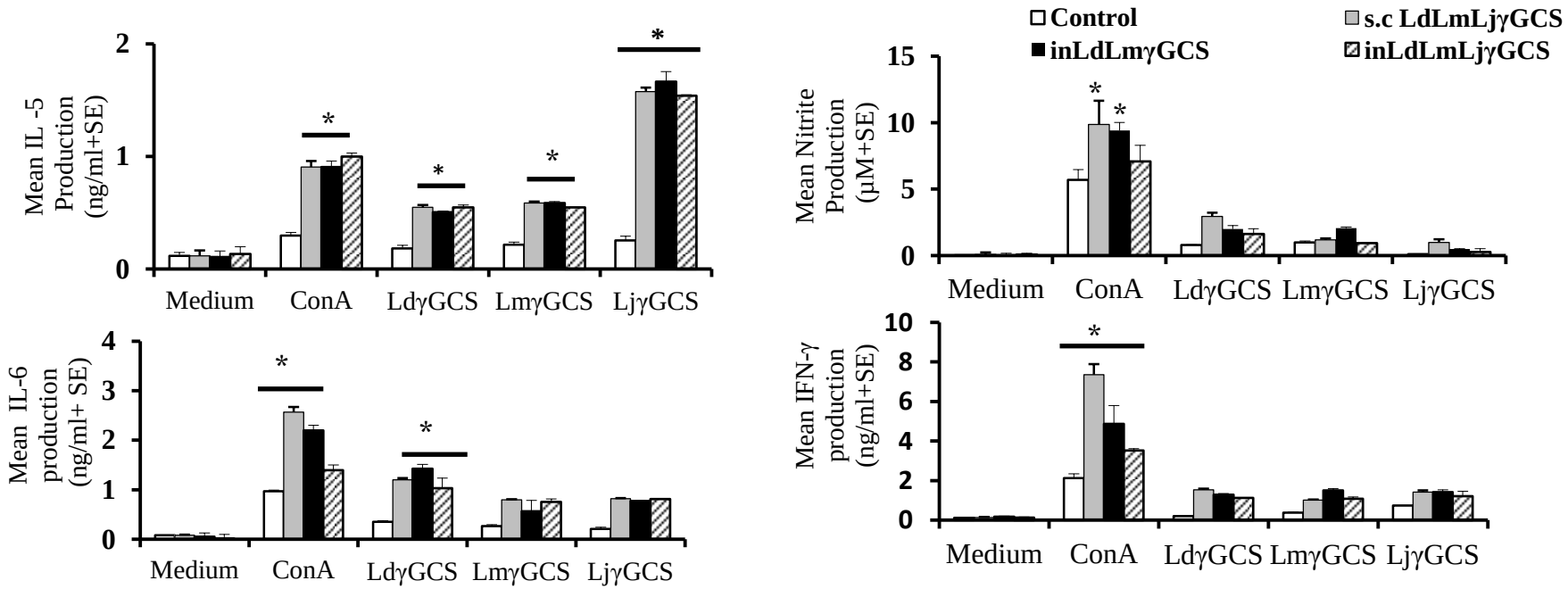


Figure 7.8 The mean IL-5, IL-6, IFN -γ and nitrite production by *in vitro* stimulated splenocytes from vaccinated mice on day 0 pre infection. Mice were immunised by subcutaneous injection with PBS (control), recombinant γGCS protein *L. donovani* (LdγGCS), *L. mexicana* (LmγGCS), *L. major* (LjγGCS) 20 µg/dose, s.cLdLmLjγGCS or inhalation (inLdLmLjγGCS), 50 µg inhalation LmγGCS and LdγGCS (inLdLmγGCS) on days −28 and −14. On day -1 cells were incubated with medium alone (control), LdγGCS protein, LmγGCS or LjγGCS (25 µg/ml), or Con A (5 µg/ml), and 72 hr later cell supernatants stored until nitrite or cytokine levels could be determined by ELISA. $^{*}P < 0.05$, compare to control, all the treatments significantly to different relative unstimulated control (medium) $p< 0.01$, n=3/ treatment.

The effect of immunisation by subcutaneous or inhalation routes using different combinations of γGCS recombinant proteins on the outcome of *L. mexicana* infection was determined. The data showed that vaccination with γGCS recombinant proteins from all three *Leishmania* species (LdLmLjγGCS) at a dose of 20 μg/protein by the pulmonary route reduced the degree of infection in terms of footpad thickness and parasite burden. On day 21 footpads thickness was significant lower for inhalation group compared to control tratment (Figure 7.9).

All four vaccination protocols using the higher vaccine dose induced protection against infection compared to controls, based on the change in footpad thickness and bioluminescence compared to control values ($p < 0.05$, Figure 7.9). Immunisation with subcutaneous triple vaccine gave greater protection than the double vaccine given by inhalation. On day 21 only subcutaneous immunisation with the triple protein vaccine (LdLmLjγGCS) and double inhaled vaccine (inLdLmγGCS) significantly reduced the footpad thickness and light emission compared to infected control values ($p<0.05$). However, there was no significant difference in the footpad thickness or light emission of mice for the three vaccine groups.

It was possible to rank the three types of vaccines based on the parasite burden present in the footpad at the end of the experiment (Table 7.3). Thus, vaccination with the triple vaccine by the subcutaneous route was the most protective followed by the inhaled double protein vaccine, and the inhaled triple protein vaccine. However, the high variability in the data for the triple vaccine may be responsible for the difference observed. There was no difference in BLI/parasite ratio for parasites recovered from the lesion of the different treatment groups (Table 7.3).

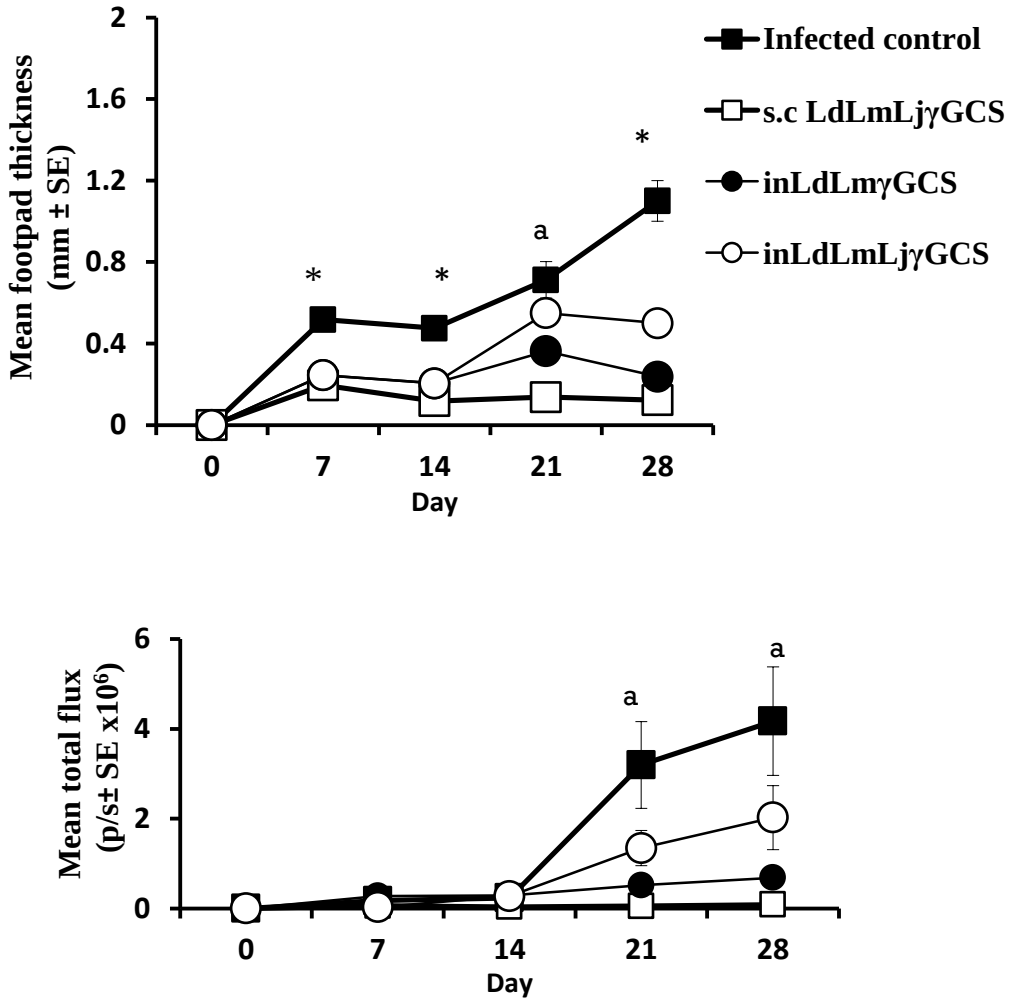


Figure 7.9 The effect of vaccination with γGCS on *L. mexicana* infection. Mice were treated with PBS alone (Infected control), 20 µg each LdγGCS, LjγGCS and LmγGCS proteins by subcutaneous injection (s.cLdLmLjγGCS) or 20 µg inhalation (inLdLmLjγGCS), or 50 µg inhalation LdγGCS & LmγGCS (inLdLmγGCS). Mice were infected with $1x10^7$ *L. mexicana* (LmexLuc strain) promastigotes and treated Determining footpad thickness indicated the progression of infection or BLI in control and immunized mice over the course of infection and mice were sacrificed on day 28. $^*p<0.05$ is all treatment significant to infected control. $^ap<0.05$ the s.cLdLmLjγGCS and inLdLmγGCS comparing to infected control, n=5/ treatment.

Table 7.3 The effect of route of administration on the outcome of vaccination in *L. mexicana* infection. The parasite burden of mice (5/treatment) treated as shown in Figure 7.9 was determined by direct counting of the number of parasite/ml present in footpad lesion homogenate or by determining the amount of bioluminescence emitted for the homogenate sample. $^{*}p < 0.05$ compared to infected control. $^{a}p < 0.05$ compared to inLdLmLjγGCS vaccine. Pre-infection is *L. mexicana* promastigotes parasite in medium before inoculation of mice.

	Parasite burden parasite/ml $\times 10^6 \pm$ SE	**Total flux (p/s $\pm$ SE $\times 10^6$)**	**BLI/parasite ratio**
Infected control	6.19 ± 1.1	2.0 ± 0.58	0.32 ± 0.08
s.cLdLmLjγGCS	$0.25 \pm 0.1^{*a}$	$0.08 \pm 0.037^{*a}$	0.32 ± 0.04
inLdLmγGCS	$0.70 \pm 0.2^{*a}$	$0.21 \pm 0.032^{*a}$	0.33 ± 0.01
inLdLmLjγGCS	3.1 ± 1.5	0.9 ± 0.23	0.31 ± 0.05
Pre infection	10 ± 0.0	5 ± 0.3	0.5 ± 0.1

The type of immune response induced by the different vaccine protocols both before and after infection was assessed to determine if a particular type of immune response was associated with protection against *L. mexicana* infection.

Specific IgG1 and IgG2a antibody responses to all three recombinant antigens was determined as this would also show the amount of cross-immunity induced by the different γGCS proteins. All three vaccines group induced significant IgG1 and IgG2a antibodies against all three γGCS antigens (Figures 7.10-7.12). However, there were differences in the type of antibody response generated by the different vaccine protocols, the route of administration had effect on antibody production. The highest antibody titres were induced by subcutaneous injection with the triple vaccine and the lowest antibody titres were induced by pulmonary vaccination. Vaccination by the pulmonary route induced low antibody titres against all three γGCS antigens and there were differences in the antibody response induced by the double and triple vaccines. Thus the pulmonary double vaccine (LdLmγGCS) induced significant IgG1 antibodies against *L. donovani* but no antibodies against *L. mexicana* γGCS or *L. major* γGCS pre-infection. However after *L. mexicana* infection significant IgG1 and IgG2a antibodies were elicited against all three γGCS proteins in mice immunised with the double vaccine by inhalation. The triple vaccine only induced specific IgG1 antibodies against *L. donovani* and *L. major* γGCS and IgG2a antibodies against *L. donovani* and *L. mexicana* pre-infection. However after infection this group of mice had significant IgG1 antibodies against all three γGCS antigens and IgG2a antibodies against *L. donovani* and *L. mexicana* antigens only (Figure 7.10 -7.12). Subcutaneous

immunisation with the triple vaccine induced significant IgG1 and IgG2a antibody titres to all three antigens both pre and post-infection ($p<0.05$, Figure 7.10-7.12). Pulmonary administration was associated with Th1 responses based on the ratio of IgG2a/IgG1 responses. Vaccination with each γGCS protein resulted in similar Th1 and Th2 responses using IgG2a and IgG1 as proxies. However, in *L. mexicana* infected mice, a polarised Th1 response predominated for the first 14 days but reverted to a mixed Th1/Th2 response by day 28.

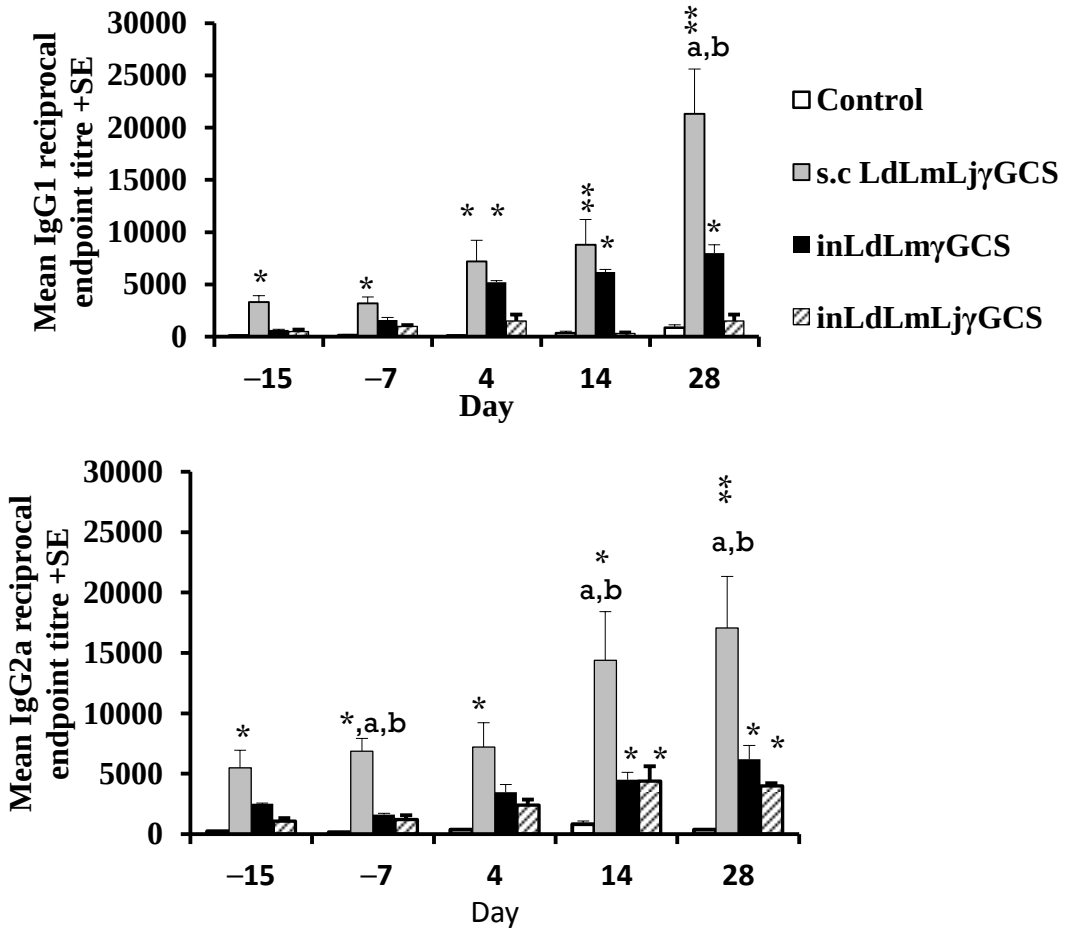


Figure 7.10 The effect of vaccination with γGCS on IgG1 and IgG2a antibody production against the *L. donovani* recombinant γGCS antigen. Mice in figure 7.9 were vaccinated by subcutaneous injection with PBS (control), recombinant γGCS protein joint treatment of *L. donovani* (LdγGCS), *L. mexicana* (LmγGCS) and *L. major* (LjγGCS) 20 µg/dose (s.cLdLmLjγGCS) or inhalation (inLdLmLjγGCS) or 50 µg/dose inhalation (inLdLmγGCS). On day 0 mice were infected with 1 x 10^7 *L. mexicana* promastigotes by subcutaneous injection into footpad. Mice were bled on day pre infection (_15 _7) and 4, 14, 28 post infection to detect specific antibody titre against the recombinant γGCS of *L. donovani* antigen using ELISA. ($^{**}p < 0.01$, $^{*}p < 0.05$ compare to control, $^{a}p < 0.05$ compare to inLdLmγGCS, $^{b}p < 0.05$ compare to inLdLmLjγGCS, n=5/ treatment.

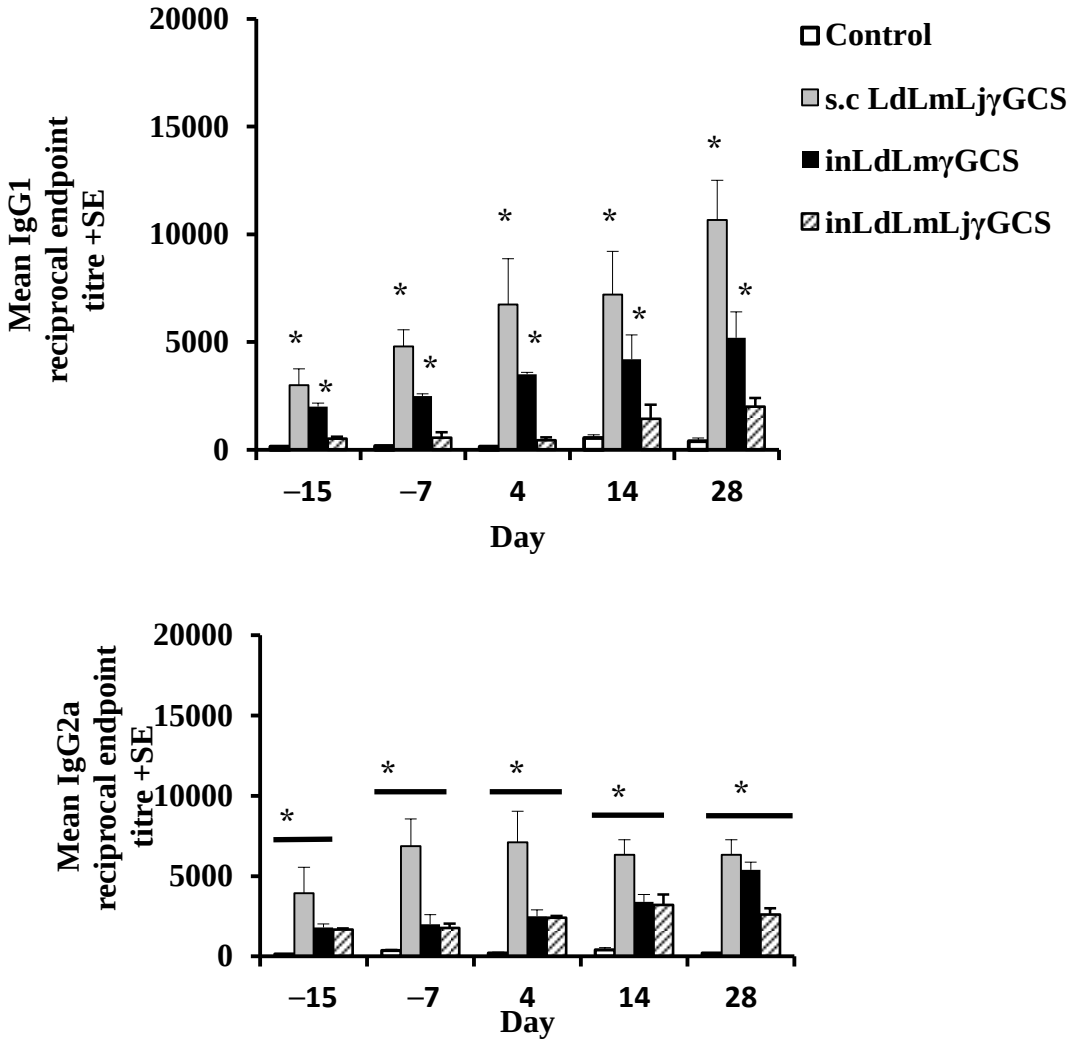


Figure 7.11 The effect of vaccination with γGCS on IgG1 and IgG2a antibody production against the *L. mexicana* recombinant γGCS antigen. Mice in figure 7.9 were vaccinated by subcutaneous injection with PBS (control), recombinant γGCS protein joint treatment of *L. donovani* (LdγGCS), *L. mexicana* (LmγGCS) and *L. major* (LjγGCS) 20 µg/dose (s.cLdLmLjγGCS) or inhalation (inLdLmLjγGCS) or 50 µg/dose inhalation (inLdLmγGCS). On day 0 mice were infected with 1 x 10^7 *L. mexicana* promastigotes by subcutaneous injection into the footpad. Mice bleeding on day pre infection (_15 _7) and 4, 14, 28 post infection to detect specific antibody titre against the recombinant γGCS of *L. mexicana* antigen using ELISA. $^{**}p < 0.01$, $^{*}p < 0.05$ compare to control, n=5/ treatment.

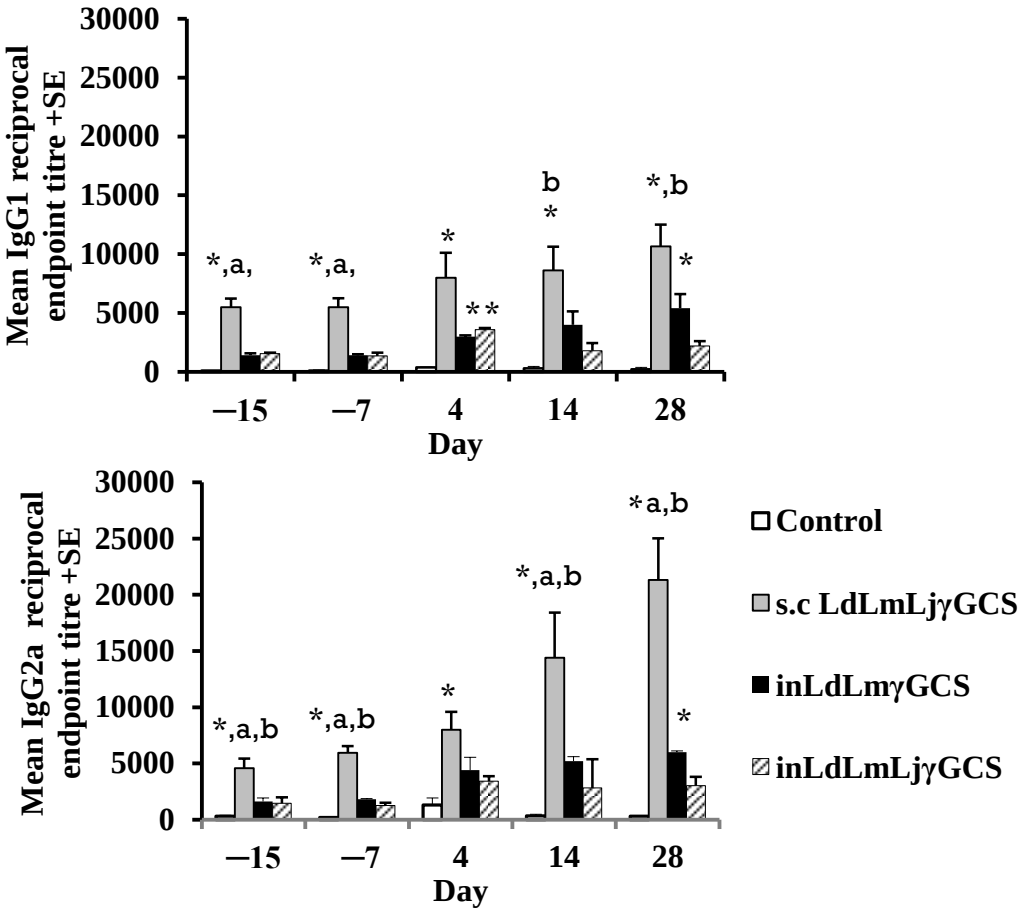


Figure 7.12 The effect of vaccination with γGCS on IgG1 and IgG2a antibody production against the *L. major* recombinant γGCS antigen. Mice in figure 7.9 were vaccinated by subcutaneous injection with PBS (control), recombinant γGCS protein joint treatment of *L. donovani* (LdγGCS), *L. mexicana* (LmγGCS) and *L. major* (LjγGCS) 20 µg/dose (s.cLdLmLjγGCS) or inhalation (inLdLmLjγGCS) or 50 µg/dose inhalation (inLdLmγGCS). On day 0 mice were infected with 1 x 10^7 *L. mexicana* promastigotes by subcutaneous injection into footpad. Mice bleeding on day pre infection (_15 _7) and 4, 14, 28 post infection to detect specific antibody titre against the recombinant γGCS of *L. major* antigen using ELISA $^{**}p < 0.01$, $^{*}p < 0.05$ compare to control, $^{a}p < 0.05$ compare to inLdLmγGCS ,$^{b}p < 0.05$ compare to inLdLmLjγGCS, n=5/treatment.

To more specifically determine how vaccination modulated the host immune responses, cytokine responses were measured from antigen or ConA stimulated spleen cells taken post-infection (Figure 7.13). Vaccination enhanced IFN-γ production in spleen cells from mice given any of the three vaccine protocols compared to controls given PBS and there was no significant difference in the amount of IFN-γ produced between the vaccinated groups. Only vaccination with the triple vaccine given by the subcutaneous route induced significantly higher levels of IFN-γ ($p < 0.05$) compared to cells from infected controls, and this was in response to all antigens. The cells from double inhalation vaccine stimulated significant IFN-γ ($p < 0.05$) compared to infected control. Nitrite production was elevated in the supernatant of cells taken from mice vaccinated with triple subcutaneous vaccine compare to infected control, single vaccine and triple inhalation ($p<0.05$, Figure 7.13). Vaccination with the double vaccine given by the pulmonary route did increase nitrite production, and in this case significantly greater amounts of nitrite were produced in response to ConA and specific antigen stimulation compared to control values.

The results showed post infection IL-5 production, where all vaccinated groups resulted in significantly higher IL-5 production compared to infected controls of same group stimulated with ConA, and three *Leishmania* γGCS and un stimulated control $p < 0.05$ (Figure 7.13). All vaccinated groups produced significantly higher TNF-α compares to unstimulated and stimulated controls ($p < 0.05$, Figure 7.13).

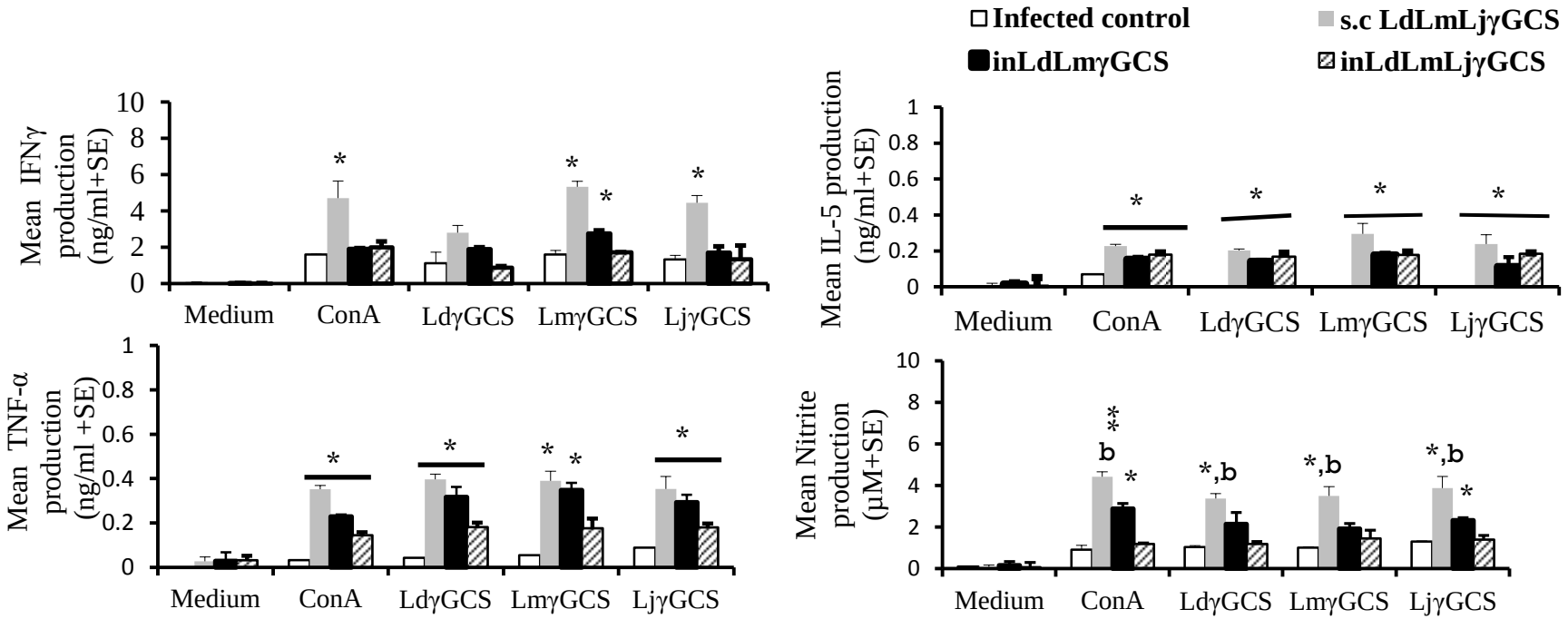


Figure 7.13 The effect of vaccination for mice shown on figure 7.8 on cytokines and nitrite production. Mice were vaccinated On days -28 and day -14 mice by subcutaneous injection with PBS (control), recombinant γGCS protein joint treatment of *L. donovani* (LdγGCS), *L. mexicana* (LmγGCS) and *L. major* (LjγGCS) 20 µg/dose (s.cLdLmLjγGCS) or inhalation (inLdLmLjγGCS) or inhalation both LdγGCS and LmγGCS (inLdLmγGCS) 50 µg/dose. On day 0 mice were infected with 1 x 10^7 *L. mexicana* promastigotes by subcutaneous injection on footpad. On day 28 mice were sacrificed and IFN-γ, IL-5, TNF-α and nitrite, production by splenocytes stimulated with medium alone (un stimulated), γGCS or ConA was determined. $^{**}p < 0.01$, $^{*}p < 0.05$ compare to control, $^{a}p < 0.05$ compare to inLdLmγGCS ,$^{b}p < 0.05$ compare to inLdLmLjγGCS, n=5/treatment.

7.2.4 Vaccination using triple vaccine (LdγGCS, LmγGCS and LjγGCS proteins) by subcutaneous or inhalation routes to protect against *L. mexicana* or *L. major* infection

In this comparative study mice were vaccinated with the triple vaccine by the subcutaneous injection using 20 μg of each γGCS protein or 50 μg of each protein by the pulmonary route. Previously (section 7.2.3) results showed that 20 μg of each protein for the pulmonary route did not induce significant protection against *L. mexicana* infection in terms of parasite burden and footpad thickness.

The *Leishmania* γGCS recombinant proteins had the ability to induce both Th1 and Th2 responses. Spleen cells from mice immunized with the triple vaccine by the subcutaneous or inhalation routes were stimulated *in vitro* with specific antigen to determine their ability to produce cytokines or nitrite before infection. Vaccination by both routes of administration significantly enhanced the production of IFN-γ, IL-4, IL-5, TNF-α and nitrite compared to medium controls and non-infected control ($p<0.05$), indicating that vaccination by both routes of administration induced Th1 and Th2 immune responses.

The subcutaneous vaccines produced significantly higher IFN-γ for cells incubated with ConA, LdγGCS and LmγGCS protein than control ($p< 0.05$) or inhalation vaccine ($p< 0.001$). Cells from mice subcutaneously vaccinated produced significantly higher IL-4 for cells incubated with ConA compare to non-infected control ($p< 0.05$) and inhalation vaccinated mice ($p< 0.01$).

Cells incubated with LmγGCS induced significantly higher IL-4 for inhalation group compare to non-infected control ($p< 0.001$), and to cells from subcutaneous vaccinated mice ($p< 0.01$). Cells from vaccinated mice produced significantly greater amounts of nitrite in response to ConA compared to cells from non-infected control mice ($p< 0.01$).

There were no significant differences in the cytokine or nitrite responses of cells stimulated with ConA, LdγGCS or LmγGCS by both administration routes but vaccination by inhalation induced significantly higher amounts of nitrite ($p < 0.05$) compared to subcutaneous vaccination for cells stimulated with LjγGCS protein (Figure 7.14).

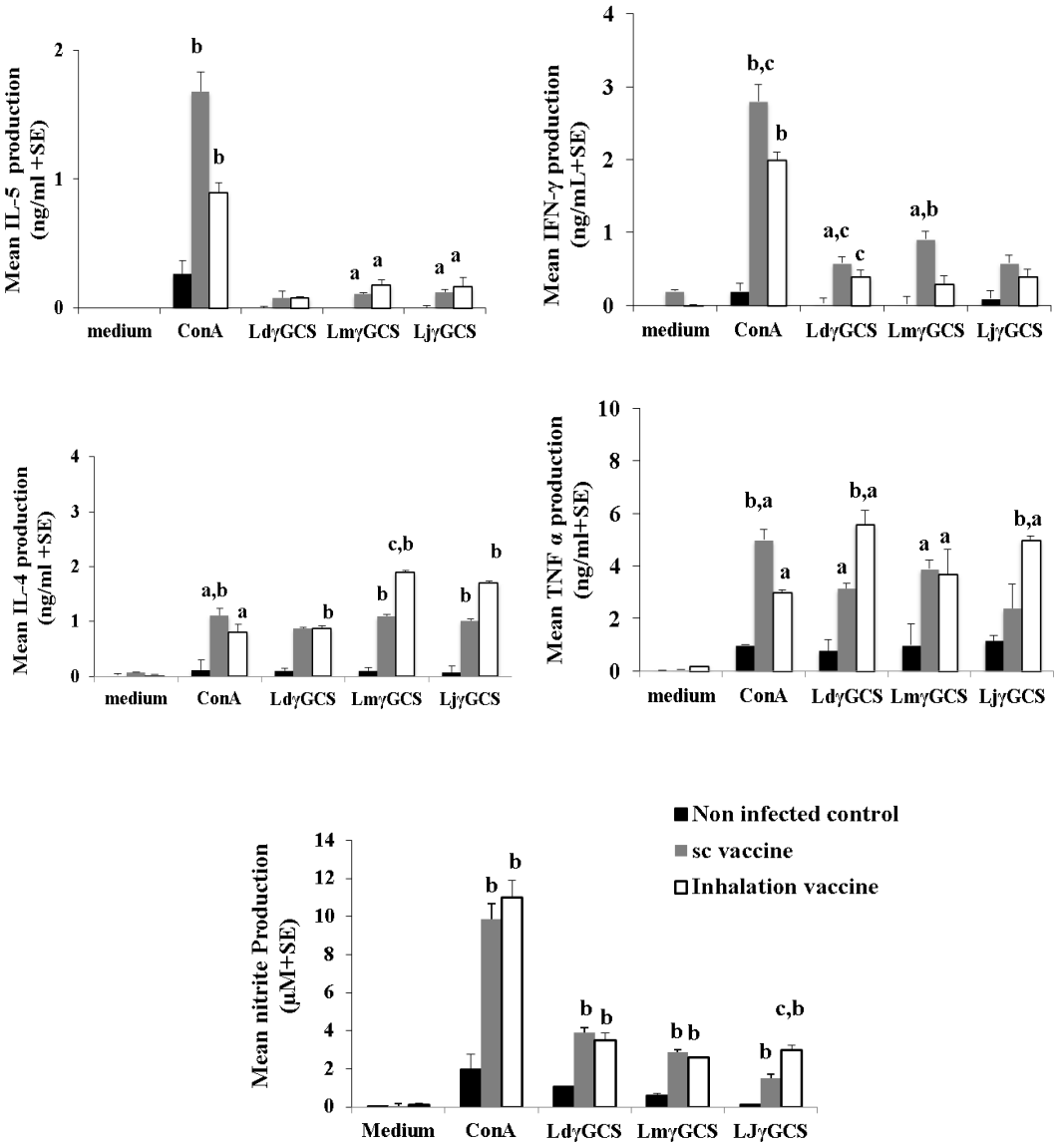


Figure 7.14 The effect of vaccination with the triple vaccine by the subcutaneous or pulmonary routes on the ability of splenocytes to produce IFN-γ, IL-4, IL-5, TNF-α or nitrite. Mice were immunised with the triple vaccine by subcutaneous injection (20 µg/recombinant γGCS protein/dose) or inhalation (50 µg recombinant γGCS protein/dose) on days −28 and −14. On day 0 vaccinated and controls (uninfected untreated mice, non-infected controls) were sacrificed and their splenocytes were incubated with medium alone (unstimulated control, medium), LdγGCS, LmγGCS or LjγGCS recombinant protein (25 µg/ml), or Con A (5 µg/ml) for 72 hours. Nitrite or cytokine levels in cell supernatants were then determined $^{a}p < 0.05$, $^{b}p < 0.01$, $^{c}p < 0.001$, compared to non-infected control, n=4/treatment.

Vaccination with the triple γGCS vaccine protected mice against *L. mexicana* or *L. major* infection regardless of the route of administration based on the reduction in footpad lesion size and the amount of bioluminescence emitted over the course of infection ($p < 0.05$). In addition vaccinated mice had significantly lower number of parasites present in footpad lesions at the end of the experiment based on direct counting of parasite numbers and assessment of parasite numbers using bioluminescence ($p < 0.01$, Figure 7.15 and Table 7.4). There was a difference in the total vaccine dose administered by each route (total protein dose: subcutaneous route, 120 µg protein, inhalation route, 300 µg protein), which would indicate that the subcutaneous route was more effective. However, it is highly likely that the mice only inhaled a fraction of the dose and studies determining delivery of inhaled luciferin solution indicated that only 18% of the dose would have been inhaled by mice (Alsaadi *et al.*, 2012), which would have meant that the mice would have inhaled a total vaccine dose that was lower than that given by the subcutaneous route. The dose used to treat mice is important as the initial studies showed that vaccination using the triple vaccine at a lower dose by pulmonary route (20 µg instead of 50 µg of each recombinant protein at each vaccination) was less protective than subcutaneous immunisation against *L. mexicana*, although vaccination did induce some protection against infection (section 7.2.3).

Table 7.4 The amount of BLI emited from pre infection promastigotes and post infection parasite that from sacrificed vaccinated mice with different routes of administration and infected *L. mexicana* or *L. major*. The parasite burden of mice was determined by direct counting of the number of parasite/ml present in footpad lesion homogenate or by determining the amount of bioluminescence emitted for the homogenate sample. $^{**}p < 0.01$, $^{*}p < 0.05$ compared to control. Pre-infection is *L. mexicana* promastigotes parasite in medium before inoculation of mice.

	Parasite	**Pre infection**	**Infected control**	**S.C vaccine**	**Inhalation vaccine**
Mean no. Parasites/ml ($x10^6$) ± SE	***L. mexicana***	10 ± 0.01	3 ± 0.3	0.6 ± 0.2	0.6 ±0.3
	L. major	10 ± 0.1	9.8 ± 0.2	$3.3 \pm 0.22^{**}$	$3.6 \pm 0.25^{**}$
Mean total flux (p/s) x 10^6 ± SE	***L. mexicana***	21± 1.8	5.0 ± 2.0	$0.55\pm 0.2^{*}$	$0.55 \pm 0.01^{*}$
	L. major	79 ± 2	63 ± 14	$19 \pm 3^{**}$	$22 \pm 2^{**}$
Bioluminescence /parasite ratio	***L. mexicana***	2.3 ± 0.4	1.03 ± 0.1	0.96 ± 0.15	0.96 ±0.2
	L. major	8 ± 0.7	6.4 ± 0.1	5.8 ±0.2	6.1 ± 0.1

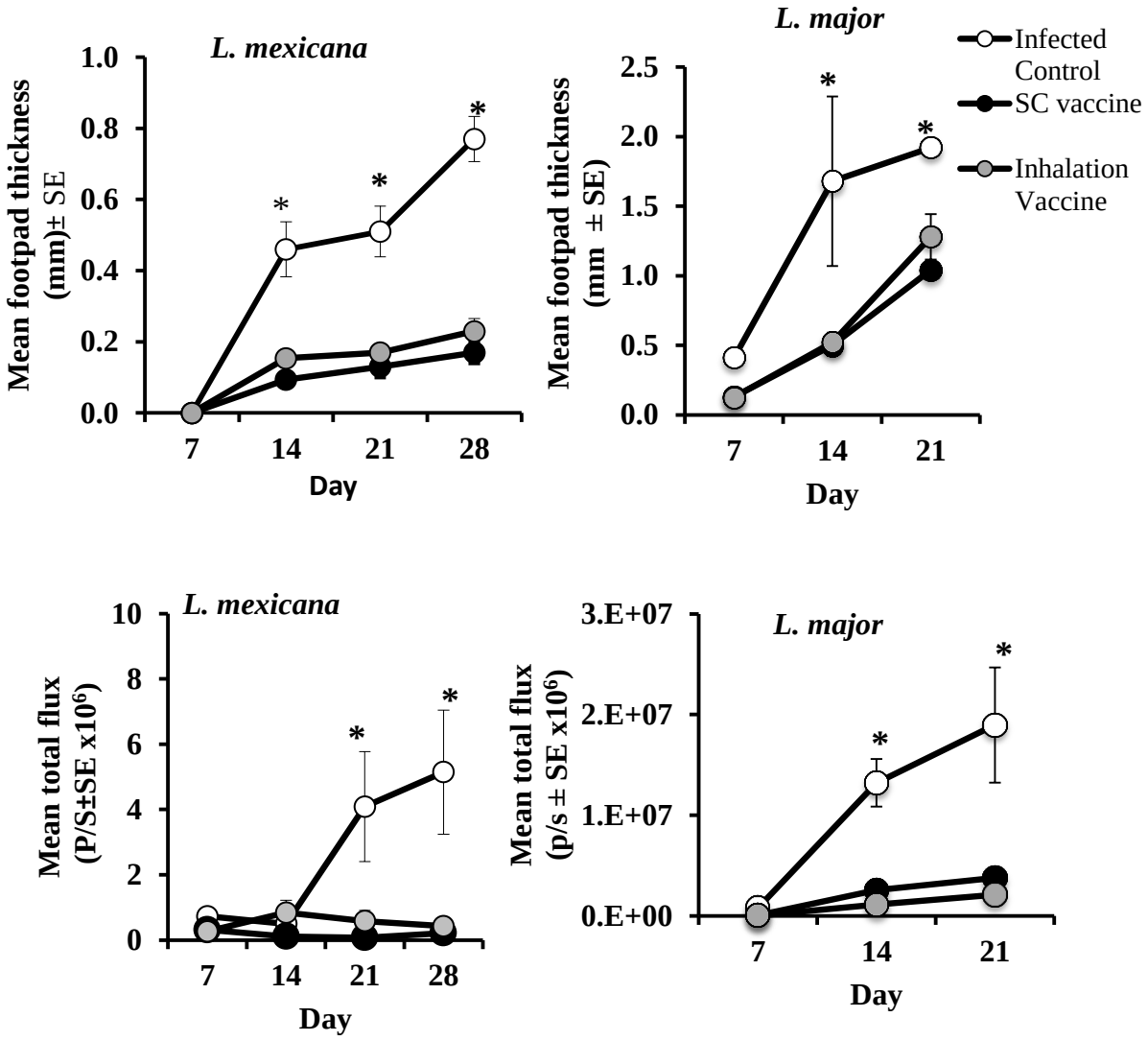


Figure 7.15 The effect of vaccination. Mice were treated with PBS alone (control), 20 µg each LdγGCS, LjγGCS and LmγGCS proteins by subcutaneous injection (s.cLdLmLjγGCS) or 50 µg each inhalation (inLdLmLjγGCS) and infected with $1x10^7$ *L. mexicana* (LmexLuc strain) or *L. major* (LmajLuc strain) promastigotes and the progression of infection was assessed by determining footpad thickness or BLI in control and immunized mice over the course of infection and mice were sacrificed on day 28. Mice were sacrificed on day 28. $^*p<0.01$ compare to control, n=5/treatment.

Protective immunity induced by vaccination with γGCS was associated with both Th1 and Th2 specific immune responses. The specific immune response of mice shown in Figure 7.14 was assessed to determine if protection was associated with a Th1 or Th2 specific immune response. Vaccinated mice had much higher antibody titres to all three antigens after infection compared to infection controls, and infection boosted antibody responses (Figures 7.16-7.18). Subcutaneous vaccination with the triple vaccine induced the highest specific IgG1 and IgG2a antibody response to all three γGCS antigens (Figures 7.16-7.18). The subcutaneous vaccinated group had higher antibody compare to control mice and mice vaccinated by the pulmonary route. However, the inhalation group had significantly higher induced antibody levels compared to infected and non-infected controls. Cross-immunity between the three γGCS proteins was present as mice vaccinated with *L. mexicana* γGCS alone produced enhanced antibody levels to the recombinant γGCS from *L. donovani* before infection (section 7.2.1). Overall, the data indicates that immunity to infection was associated with a mixed Th1/Th2 specific immune response.

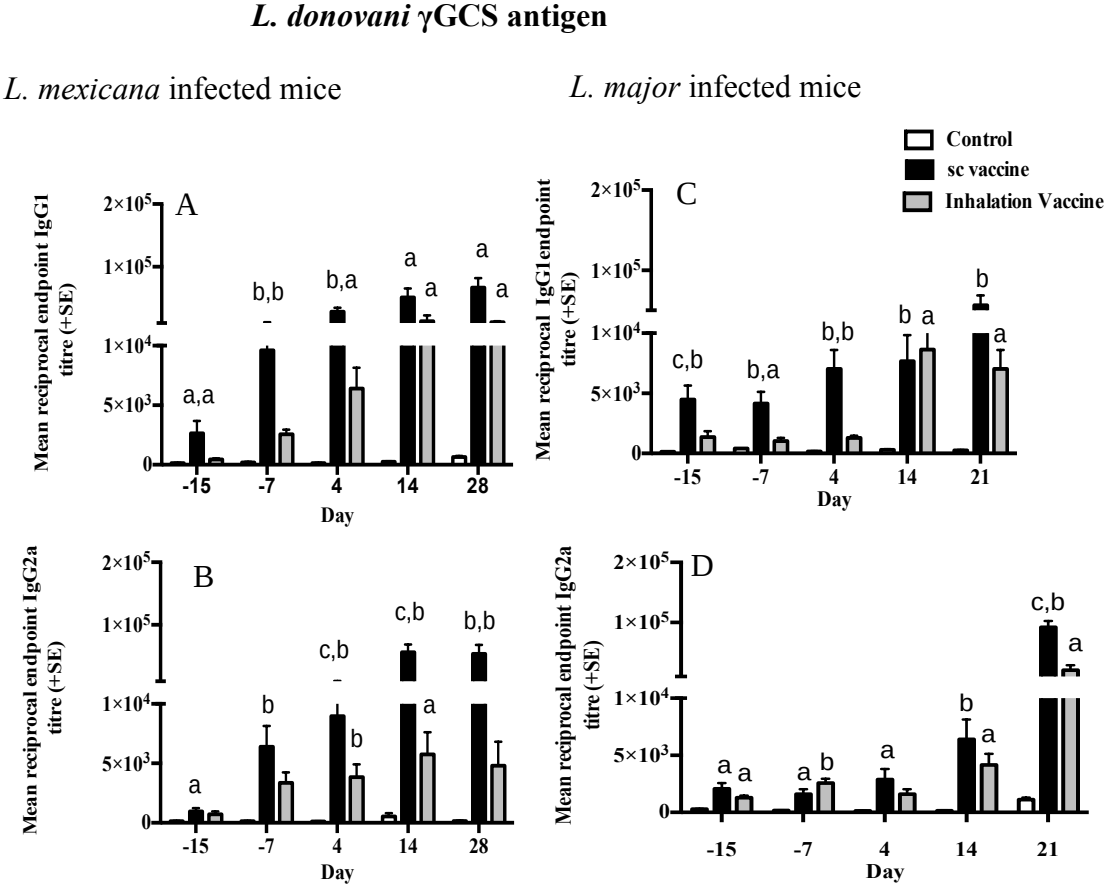


Figure 7.16 The effect of vaccination with γGCS on antibody of immunized and control mice shown in figure 7.15 against recombinant γGCS of *L. donovani* antigen. Mice were vaccinated by subcutaneous injection with PBS (control) recombinant γGCS protein joint treatment of *L. donovani* (LdγGCS), *L. mexicana* (LmγGCS) and *L. major* (LjγGCS) (20µg/dose) (s.cLdLmLjγGCS) or inhalation (inLdLmLjγGCS), 50 µg each protein. On day 0 mice were infected with 1 x 10^7 *L. mexicana* promastigotes (A &B) or infected with 1 x 10^7 *L. major* promastigotes (C &D) by subcutaneous injection into footpad. Mice bleeding on day pre infection (-15, -*7)* and 4, 14, and 28 post infection for *L. mexicana* infected mice or day 21 for *L. major* infected mice to detected Specific IgG1 (A & C) and IgG2a (B & D) antibody titres to LdγGCS were determined by ELISA. Immunization with either route induced significant antibody production compared to control values, $^{a}p < 0.05$, $^{b}p < 0.01$, $^{c}p < 0.001$, n=5/treatment.

***L. mexicana* γGCS antigen**

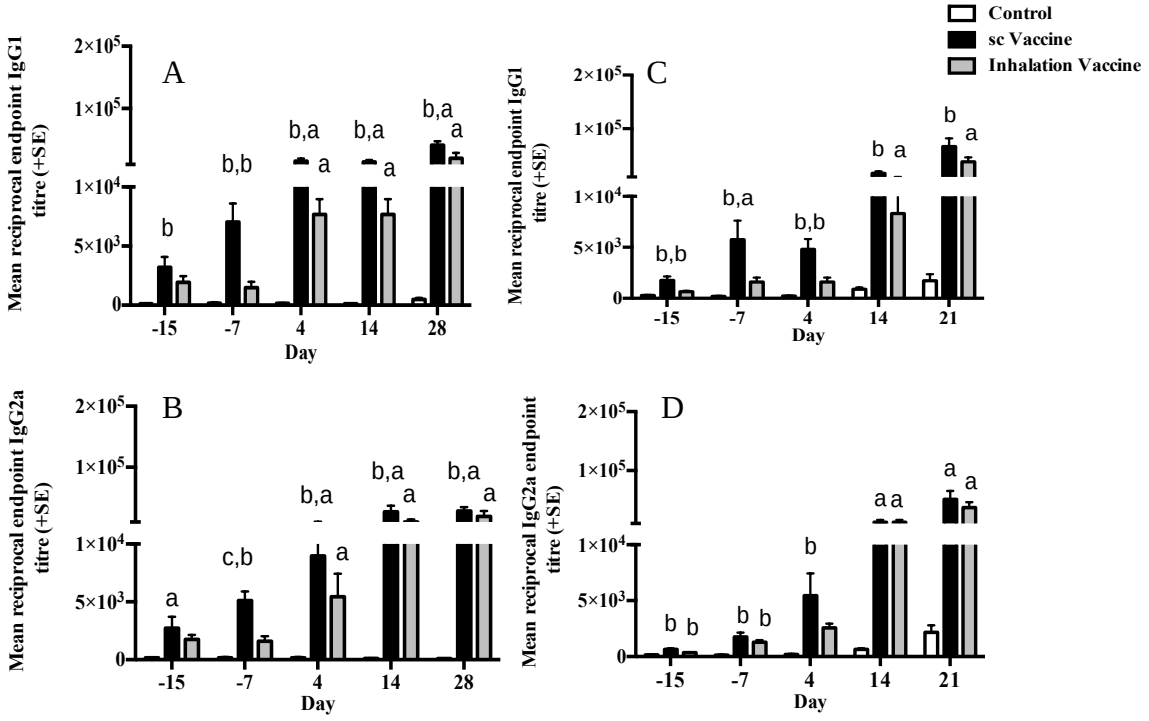


Figure 7.17 The effect of vaccination with γGCS on antibody of immunized and control, mice shown in figure 7.15, against the recombinant γGCS of *L. mexicana* antigen. Mice were vaccinated by subcutaneous injection with PBS (control) recombinant γGCS protein joint treatment of *L. donovani* (LdγGCS), *L. mexicana* (LmγGCS) and *L. major* (LjγGCS) (20 µg/dose) (s.cLdLmLjγGCS) or inhalation (inLdLmLjγGCS), 50 µg each protein. On day 0 mice were infected with 1 x 10^7 *L. mexicana* promastigotes (A &B) or infected with 1 x 10^7 *L. major* promastigotes (C &D) by subcutaneous injection into the footpad. Mice bleeding on day pre infection (-15, -7) and 4, 14, and 28 post infection for *L. mexicana* infected mice or day 21 for *L. major* infected mice to detected Specific IgG1 (A & C) and IgG2a (B & D) antibody titres to LmγGCS were determined by ELISA. Immunization with either route induced significant antibody production compared to control values, $^{a}p < 0.05$, $^{b}p < 0.01$, $^{c}p < 0.001$, n=5/treatment.

***L. major* γGCS antigen**

L. mexicana infected mice

L. major infected mice

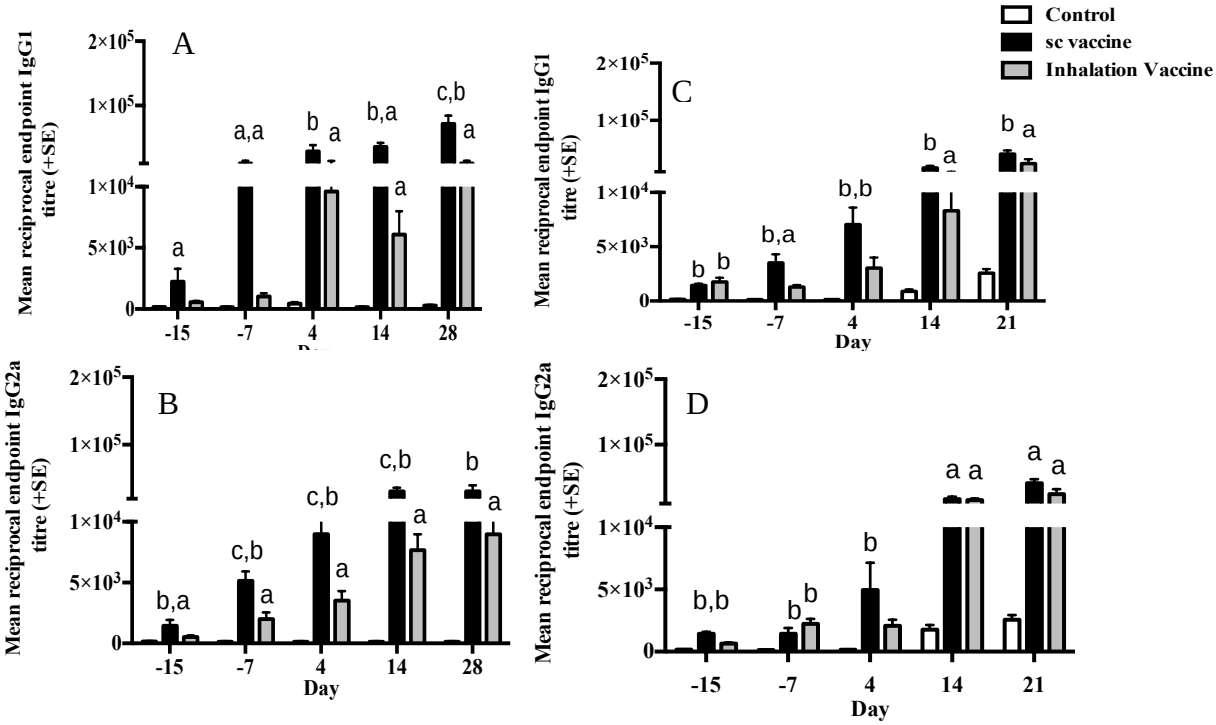


Figure 7.18 The effect of vaccination with γGCS on antibody of immunized and control mice shown in figure 7.15, against the recombinant γGCS of *L. major* antigen. Mice were vaccinated by subcutaneous injection with PBS (control) recombinant γGCS protein joint treatment of *L. donovani* (LdγGCS), *L. mexicana* (LmγGCS) and *L. major* (LjγGCS) 20 µg/dose (s.cLdLmLjγGCS) or inhalation (inLdLmLjγGCS), 50 µg each protein. On day 0 mice were infected with 1 x 10^7 *L. mexicana* promastigotes (A &B) or infected with 1 x 10^7 *L. major* promastigotes (C &D) by subcutaneous injection into the footpad. Mice bleeding on day pre infection (-15, -7) and 4, 14, and 28 post infection for *L. mexicana* infected mice or day 21 for *L. major* infected mice to detected Specific IgG1 (A & C) and IgG2a (B & D) antibody titres to LjγGCS were determined by ELISA. Immunization with either route induced significant antibody production compared to control values; $^{a}p < 0.05$, $^{b}p < 0.01$, $^{c}p < 0.001$, n=5/treatment.

Cytokine and nitrite responses of spleen cells taken from mice shown in Figure 7.14 were also assessed to get an indication the effect of vaccination on cellular immune responses in mice infected with *L. mexicana* (Figure 7.19) or *L. major* (Figure 7.20). IFN-γ levels can be used to indicate whether a specific Th1 immune response was being generated (Spellberg and Edwards, 2001) and nitrite is one of the anti-leishmanial products generated by phagocytes stimulated by IFN-γ. Vaccination enhanced IFN-γ production by antigen stimulated spleen cells from vaccinated mice compared to controls, and there was no significant difference in the amount of IFN-γ produced by cells taken from mice vaccinated by either vaccination route in *L. mexicana* (Figure 7.19) and *L. major* infected mice (Figure 7.20). However, ConA stimulation of cells showed that cells taken from mice vaccinated by inhalation and infected with *L. mexicana* produced higher levels of IFN-γ compared to mice vaccinated by the subcutaneous route (Figure 7.19). Antigen stimulation resulted in similar levels of IFN-γ being produced by cells from vaccinated, infected mice and infected controls given *L. major*, indicating that vaccination did not boost production of IFN-γ production (Figure 7.20). This would explain the similar levels of nitrite produced by cells from all three groups of mice infected with *L. major*. However vaccination did boost IL-4 and IL-5 responses of vaccinated *L. major* infected mice compared to infected control values (IL-4 $p < 0.001$, IL-5 $P < 0.01$), perhaps indicating that vaccination preferentially boosted Th2 cellular immune responses in *L. major* infected mice. TNF-α production was significantly enhanced in infected mice vaccinated by the pulmonary route, and this effect was observed for cells stimulated with ConA or the LdγGCS.

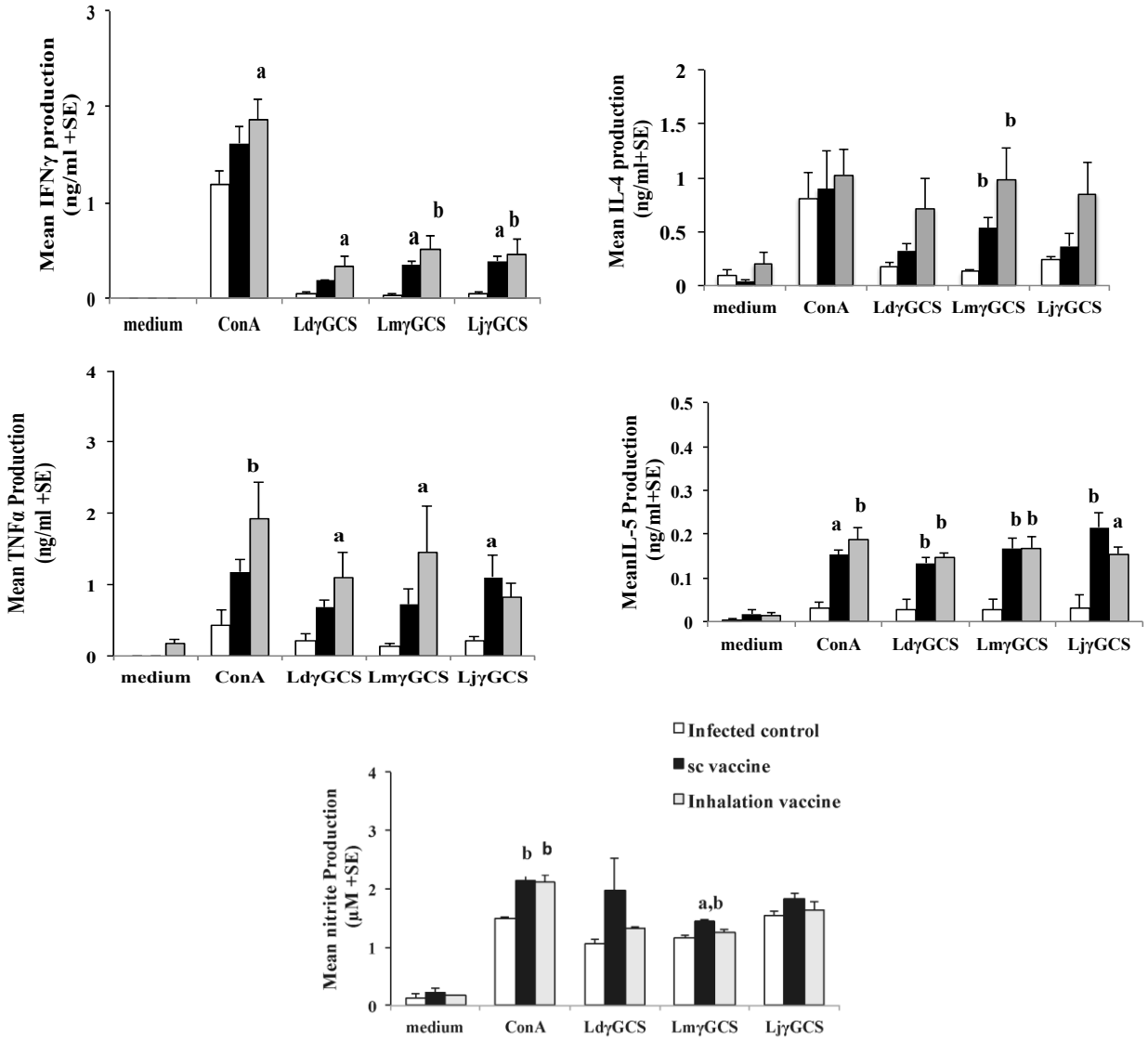


Figure 7.19 The effect of vaccination on mice infected with *L. mexicana*. Mice shown in figure 7.15, were vaccinated on days -28 and day -14 mice by subcutaneous injection with PBS (control), recombinant γGCS protein joint treatment of *L. donovani* (LdγGCS), *L. mexicana* (LmγGCS) and *L. major* (LjγGCS) 20 µg/dose (s.cLdLmLjγGCS) or inhalation (inLdLmLjγGCS) with 50 µg each protein. On day 0 mice were infected with 1 x 10^7 *L. mexicana* promastigotes by subcutaneous injection into footpad. On day 28 mice (n=5/treatment) were sacrificed and IFN-γ, IL-5, IL-4, TNF-α and nitrite, production by splenocytes stimulated with medium alone (unstimulated), γGCS or ConA was determined. $^{a}p < 0.05$, $^{b}p < 0.01$, $^{c}p < 0.001$, n=5/treatment.

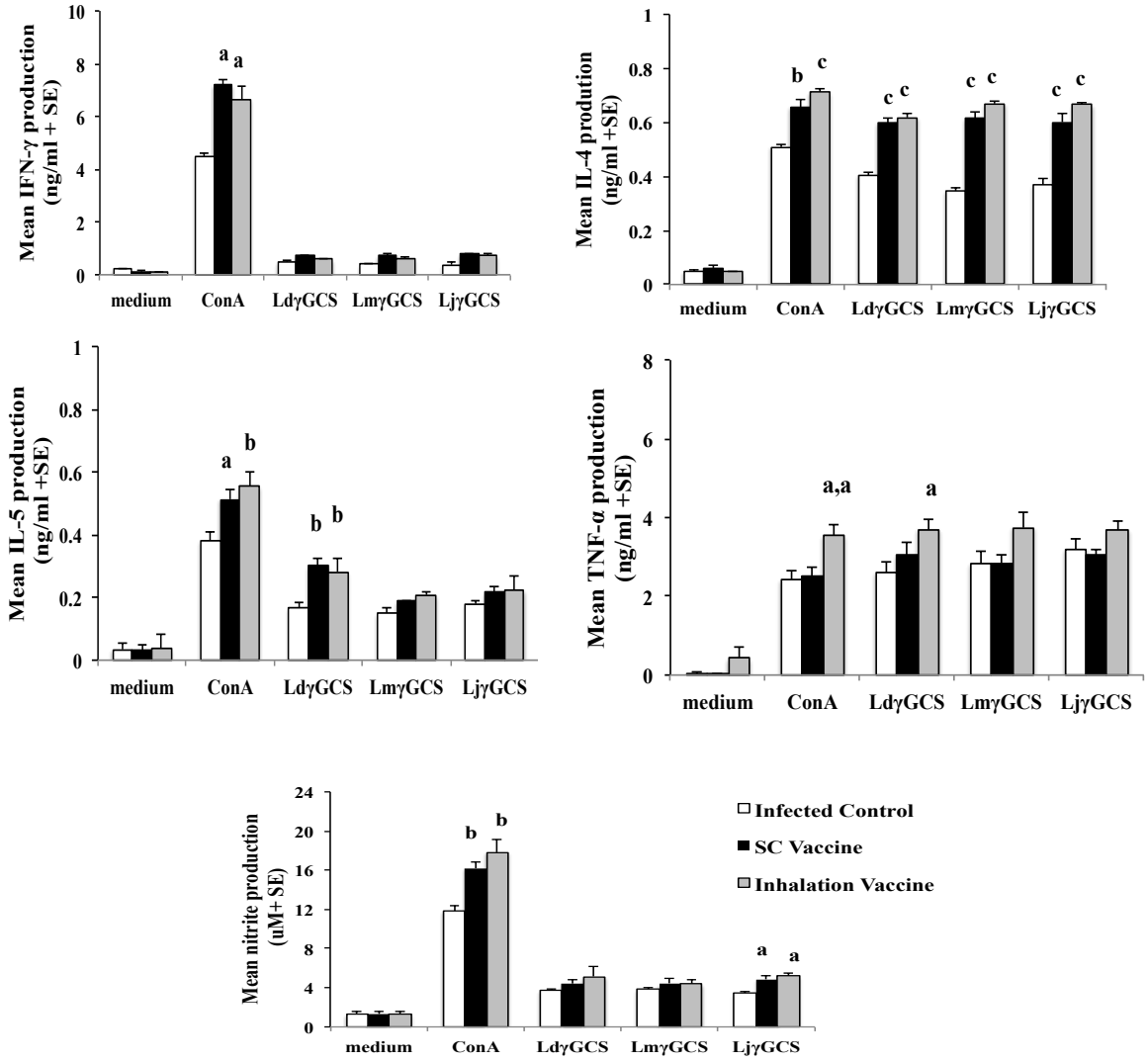


Figure 7.20 The effect of vaccination on mice infected with *L. major*. Mice shown in figure 7.15, were vaccinated on days -28 and day -14 mice by subcutaneous injection with PBS (control), recombinant γGCS protein joint treatment of *L. donovani* (LdγGCS), *L. mexicana* (LmγGCS) and *L. major* (LjγGCS) 20 µg/dose (s.cLdLmLjγGCS) or inhalation (inLdLmLjγGCS) with 50 µg each protein. On day 0 mice (n=5/treatment) were infected with 1 x 10^7 *L. major* promastigotes by subcutaneous injection into footpad. On day 28 mice were sacrificed and IFN-γ, IL-4, IL-5, TNF-α and nitrite, production by splenocytes stimulated with medium alone (un stimulated), γGCS or ConA was determined. $^{a}p < 0.05$, $^{b}p < 0.01$, $^{c}p < 0.001$, n=5/treatment.

7.2.5 Determination of the ability of the triple vaccine given by the subcutaneous route to protect against infection in a murine model of VL

In this study, the ability of subcutaneous injection with the triple vaccine (LdLmLjγGCS) to protect mice against infection with *L. donovani* amastigotes was determined. Immunisation with the triple vaccine did not protect mice against infection as vaccinated mice and infected controls had similar parasite burdens on day 21 post-infection (Table 7. 5).

Table 7.5 The effect of the vaccination with the triple vaccine on the parasite burden of *L. donovani* infected mice. Mice were treated by subcutaneous injection with PBS (control) or the triple recombinant γGCS vaccine (LdγGCS, LmγGCS and LjγGCS, using 20 μg of each protein/dose, vaccine) on days -28 and -14. On day 0 mice were infected with 1 x 10^7 *L. donovani* amastigotes by iv injection into the tail vein

	Mean parasite burdens ± SE		
	Spleen	**Liver**	**Bone marrow**
Control	12 ± 3	157 ± 50	31 ± 22
Vaccine	7 ± 3	321 ± 56	108 ± 55

Vaccination with the triple vaccine induced the production of both IgG1 and IgG2a specific antibodies (Figure 7.21 & 7.22). Specific IgG1 and IgG2a antibodies to the LdγGCS, LmγGCS and LjγGCS proteins were detected in appreciable amounts from day 7 after immunisation and levels were significantly higher than infected controls over the course of infection ($p < 0.05$).

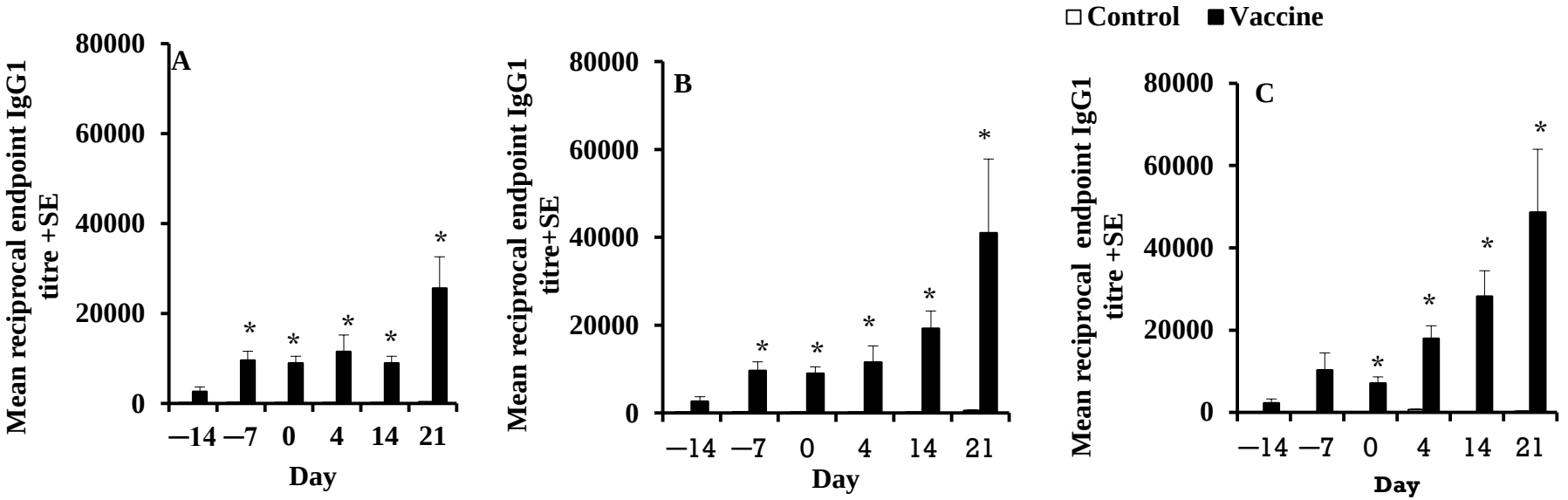


Figure 7.21 The effect of vaccination with γGCS on IgG1 antibody titres of mice shown in Table 7.5 to *L. donovani* γGCS antigen (A), *L. mexicana* γGCS antigen (B), and *L. major* γGCS antigen (C) throughout the study. Mice were treated by subcutaneous injection with PBS (control) or the triple recombinant γGCS vaccine (LdγGCS, LmγGCS and LjγGCS, using 20 μg of each protein/dose, vaccine) on days -28 and -14. On day 0 mice were infected with 1 x 10^7 *L. donovani* amastigotes by iv injection into the tail vein $^*p < 0.05$ comparing control and vaccinated groups with triple recombinant γGCS vaccine (LdγGCS, LmγGCS and LjγGCS, using 20 μg of each protein/dose, vaccine), n=5/treatment.

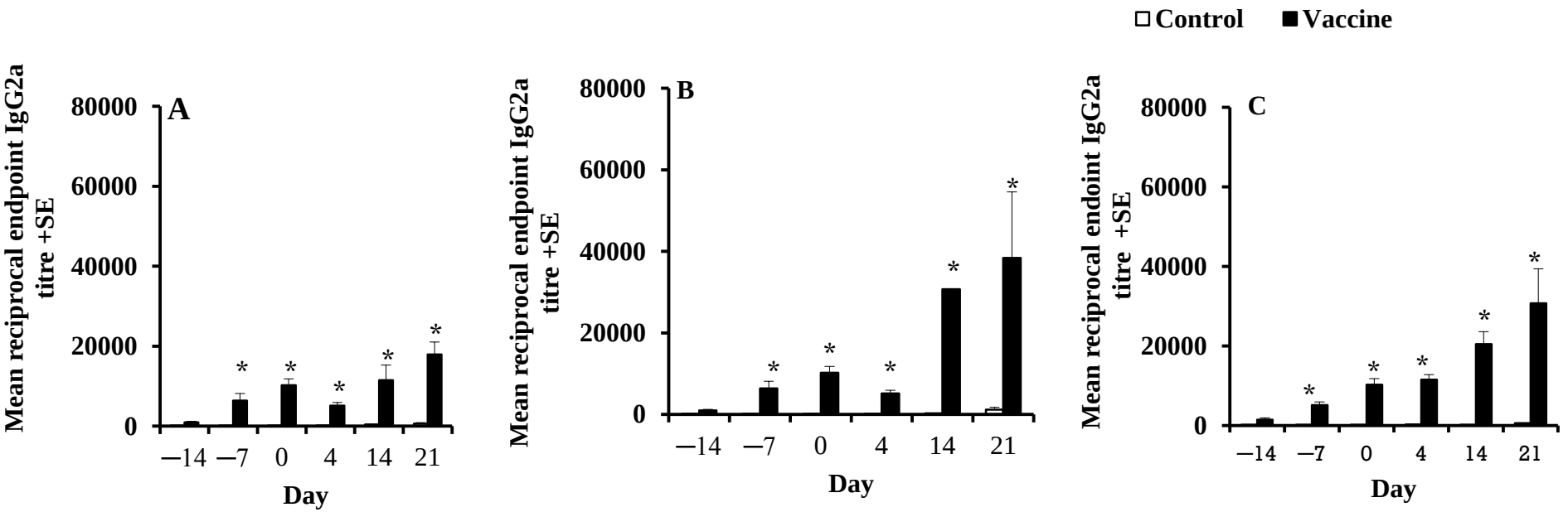


Figure 7.22 The effect of vaccination with γGCS on IgG2a antibody titres of mice shown in Table 7.5 to *L. donovani* γGCS antigen (A), *L. mexicana* γGCS antigen (B), and *L. major* γGCS antigen (C) throughout the study. Mice were treated by subcutaneous injection with PBS (control) or the triple recombinant γGCS vaccine (LdγGCS, LmγGCS and LjγGCS, using 20 µg of each protein/dose, vaccine) on days -28 and -14. On day 0 mice were infected with 1 x 10^7 *L. donovani* amastigotes by iv injection into the tail vein $^*p < 0.05$ comparing control and vaccinated groups with triple recombinant γGCS vaccine (LdγGCS, LmγGCS and LjγGCS, using 20 µg of each protein/dose, vaccine), n=5/treatment.

Cytokine and nitrite responses of spleen cells taken from mice shown in Figure 7.23 were also assessed to determine whether vaccination influenced cellular immune responses. Vaccination enhanced IFN-γ production by spleen cells from vaccinated mice compared to controls and there was higher amount of IFN-γ produced by vaccinated group but no significant difference between both groups for cell stimulated with ConA. Antigen stimulation resulted in significant ($p < 0.05$) levels of IFN-γ being produced by cells from the vaccinated group.

The cells from two groups produced similar levels of nitrite and IL-12. IL-10 and IL-4 was significantly higher ($p < 0.05$) compared to infected control values, perhaps indicating that vaccination preferentially boosted Th2 cellular immune responses in infected mice.

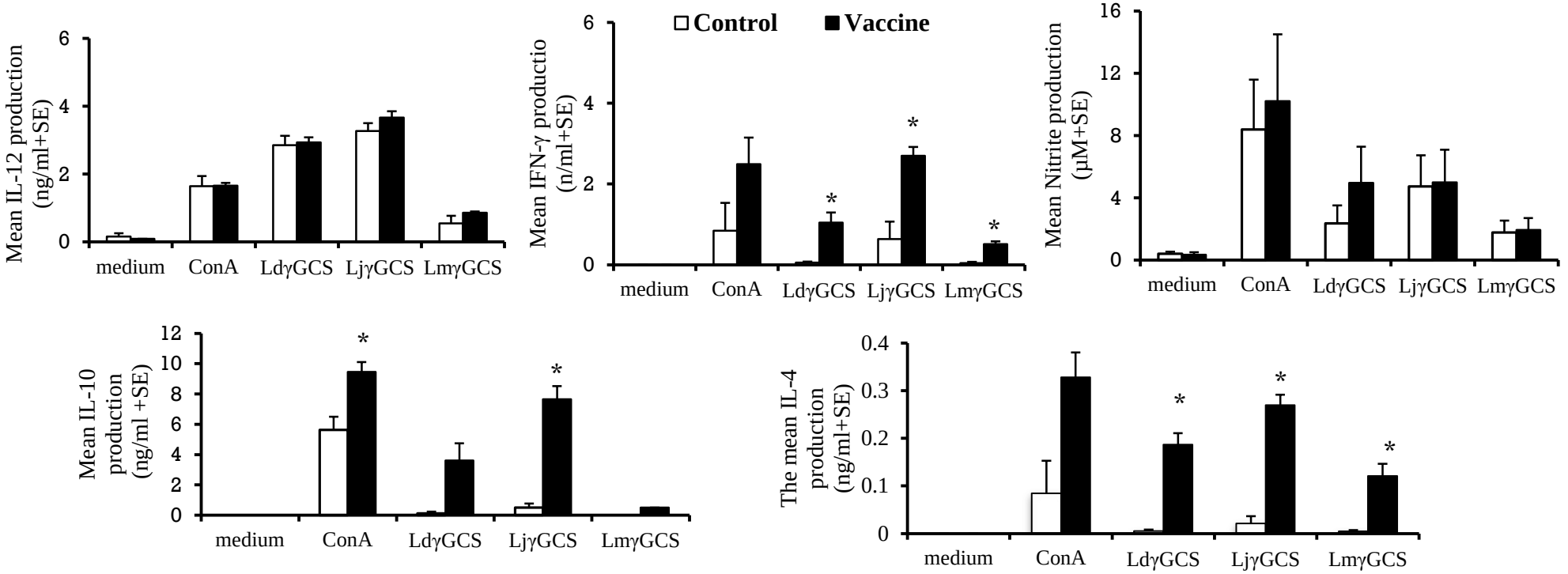


Figure 7.23 The effect of vaccination with triple recombinant γGCS vaccine (LdγGCS, LmγGCS and LjγGCS, using 20 μg of each protein/dose, vaccine) on cytokine and nitrite production of mice were infected with 1 x 10^7 *L. donovani* amastigotes by iv injection into the tail vein. Spleen cells isolated from the mice at sacrifice on day 14 post infection, were cultured with medium alone (unstimulated control, medium), one of the recombinant γGCS antigens or ConA and IFN-γ, IL-4, IL-12, IL-10 and nitrite levels in cell supernatants determined. $^*p < 0.05$, comparing control and vaccinated mice n=5/treatment.

7.2.6 Determination of the triple vaccine given by inhalation to protect against infection in a hamster model of VL

In this study, the ability of the triple vaccine (LdγGCS, LmγGCS and LjγGCS proteins) to protect against *L. donovani* infection was determined using pulmonary route in a hamster model. Using the larger animal species allowed direct treatment of animals with a known dose of the triple vaccine.

Immunisation with the triple vaccine using inhalation protected hamsters against *L. donovani* resulting in a significant reduction in liver ($p < 0.05$) and bone marrow ($P < 0.01$) parasite burdens compared to control values, but splenic parasite burdens were similar in the two groups of hamsters (Figure 7.24).

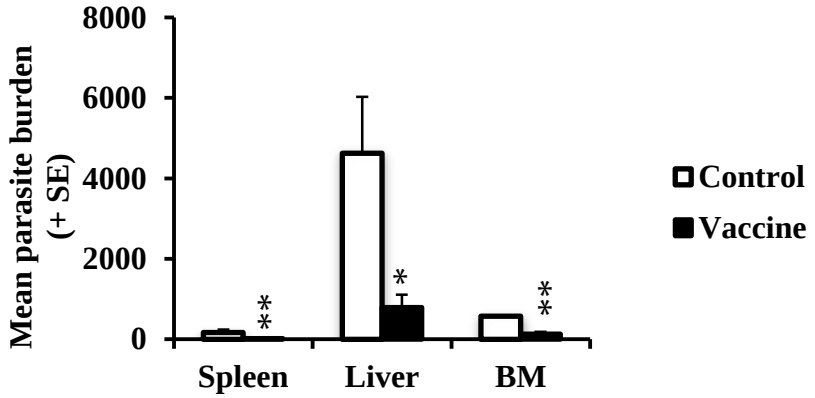


Figure 7.24 The effect of vaccination with the triple vaccine on *L. donovani* spleen, liver, and bone marrow burdens of control and immunised hamsters. Hamsters were immunised by inhalation with PBS (control) or the triple vaccine (50 µg of each of the three recombinant γGCS proteins/dose on days −28 and −14). On day 0, hamsters were infected with 1 x 10^7 *L. donovani* amastigotes by iv injection into the tail vein. On day 14 post-infection parasite burdens in the spleen, liver, and bone marrow determined. $^{**}p < 0.01$, $^{*}p < 0.05$ compared to control values, n=6/treatment.

Immunization via the pulmonary route with triple vaccine resulted in significant production of IgG specific antibodies ($p< 0.05$) to all three recombinant γGCS proteins by day -15 preinfection and day 14 post-infection. In addition, vaccinated hamsters had significantly higher IgG antibody titres to all three of the recombinant γGCS proteins (Figure 7.25).

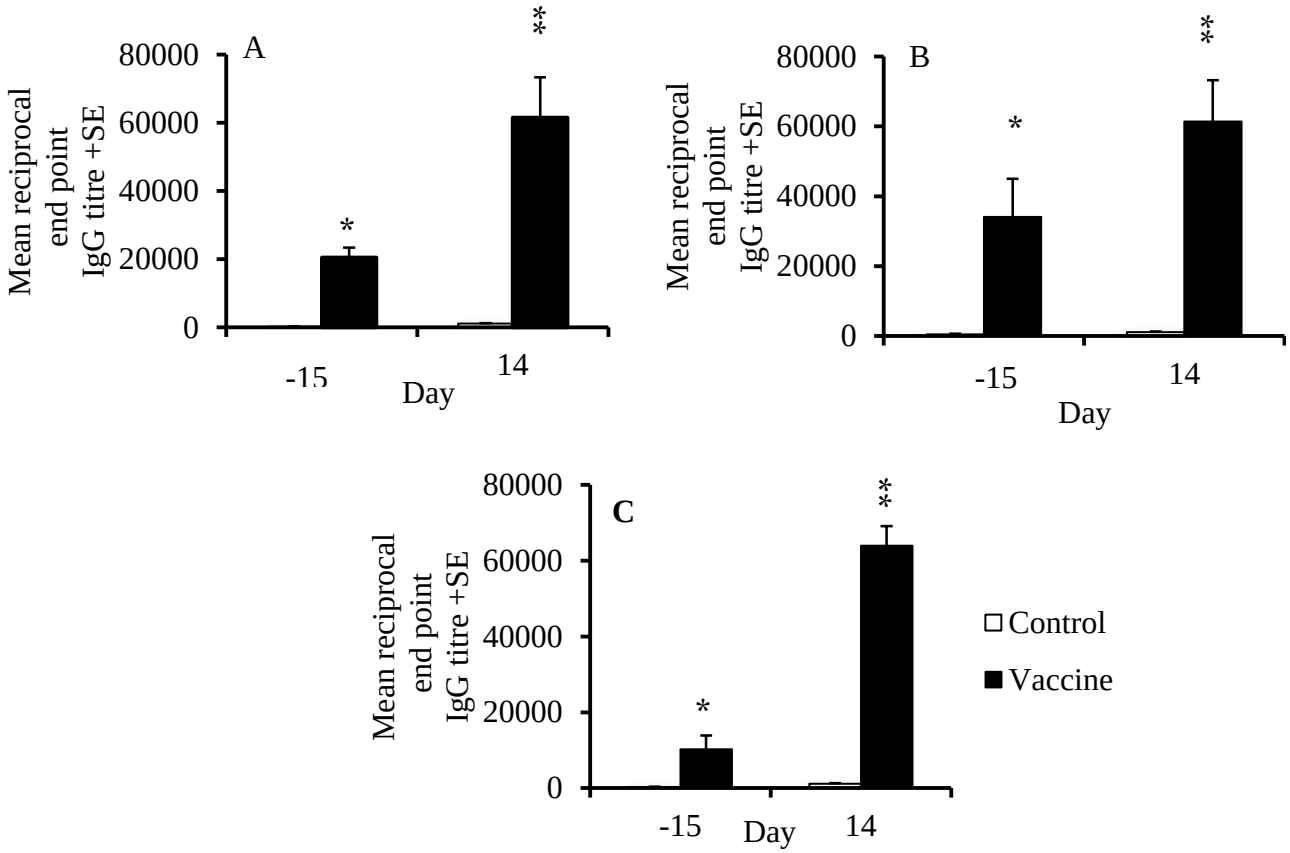


Figure 7.25 The effect of vaccination with triple vaccine on specific IgG antibody responses of hamsters pre-infection and post-infection. Hamsters, shown in figure 7.24, were immunized by inhalation with PBS or the triple vaccine (50µg of each of the three recombinant γGCS proteins on days −28 and −14). Specific IgG antibody titres to LdγGCS (A), LmγGCS (B) and LjγGCS (C) proteins were determined pre-infection (day -15) and on day 14 post-infection. $^{*}p < 0.05$, $^{**}p < 0.01$ comparing control and vaccinated groups, n=6/treatment.

The effect of vaccination on the ability of splenocytes from hamsters to proliferate in response to stimulation with specific antigen or ConA was determined using a resazurin based assay. Using resazurin in proliferation assays is simple to carry out and multiple measurements on the same cell population is possible (Al-Nasiry *et al.*, 2007, Larson *et al.*, 1997). Resazurin (7-hydroxy-3H-phenoxazin-3-one 10-oxide) is a redox dye that can be reduced by metabolically active cells to resorufin and manifested as visual colour change from blue (resazurin) to pink (resorufin) (Anoopkumar-Dukie *et al.*, 2005). The resorufin fluorescence can be measured at 570 nm, where there is a linear correlation between absorbance and cell number (Perrot *et al.*, 2003). Spleen cells from immunised hamsters which were incubated with LmγGCS or LjγGCS antigens have a significantly higher cell proliferative responses compared to infected controls ($p < 0.05$, Figure 7.26). Cells from vaccinated hamsters incubated with ConA or the LdγGCS antigen also had a higher absorbance compared to cells from infected control hamsters but the results were not significantly different.

Spleen cells from vaccinated hamsters produced significantly higher amounts of nitrite level compared to infected control hamsters ($p < 0.05$) in response to all three specific antigens and ConA stimulation (Figure 7.27).

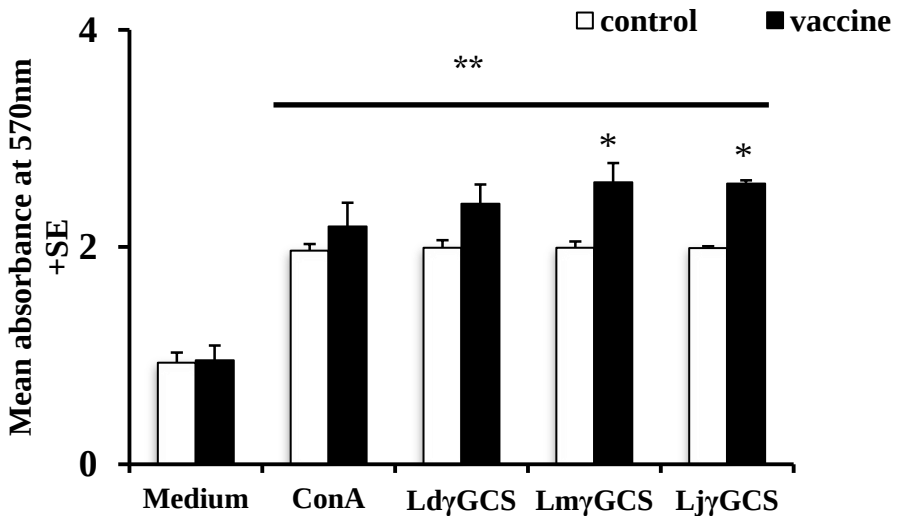


Figure 7.26 The effect of vaccination on the proliferative response of *L. donovani* infected hamster. Spleen cells from hamsters were immunised by inhalation with PBS (control) or the triple vaccine (50 µg of each of the three recombinant γGCS proteins/dose on days −28 and −14). On day 0, hamsters were infected with 1 x 10^7 *L. donovani* amastigotes by iv injection into the tail vein. On day 14 post-infection were ssacrificed and spleen cells were incubated with medium alone (control), LdγGCS protein (25 µg/ml), LmγGCS protein (25 µg/ml), LjγGCS protein (25 µg/ml) or Con A (5 µg/ml), and 72 hr incubated in 37°C. Resazurin was used to measure cell proliferation after 72 hr, by adding, 20µl of resazurin from the stock solution prepared at 0.0125% (w/v) resazurin salt in PBS solution, was added to each well and the plates are further incubated for 18h. Fluorescence reading was detected at 570 nm. $^{*}p < 0.05$, $^{**}p < 0.01$ comparing control and vaccinated groups. n=6/treatment.

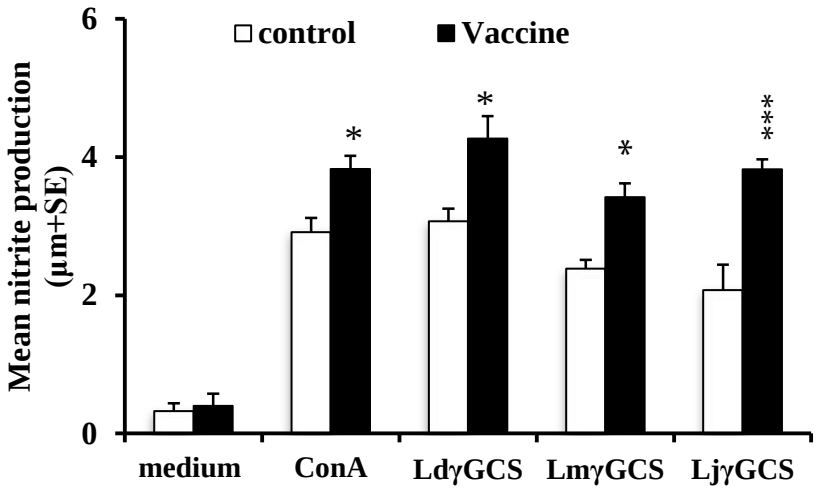


Figure 7.27 Nitrite levels in the cells supernatants of cells from Hamsters. Spleen cells from hamsters were immunised by inhalation with PBS (control) or the triple vaccine (50 µg of each of the three recombinant γGCS proteins/dose on days −28 and −14). On day 0, hamsters were infected with 1 x 10^7 *L. donovani* amastigotes by iv injection into the tail vein. On day 14 post-infection were ssacrificed and spleen cells were incubated with medium alone (control), LdγGCS protein (25 µg/ml), LmγGCS protein (25 µg/ml), LjγGCS protein (25 µg/ml) or Con A (5 µg/ml), and 72 hr incubated in 37°C. $^{*}p < 0.05$, $^{***}p < 0.001$ comparing control and vaccinated groups n=6/treatment.

The type of immune response stimulated in vaccinated hamsters was determined by measuring nitrite levels in the supernatant of stimulated spleen cells and cytokines levels in the spleen of vaccinated and control hamsters using real time PCR, where gene expression was calculated relative to control value using the $2^{-\Delta\Delta C_T}$ method and expression was normalised to the hamster housekeeping gene (HPRT).

Significantly higher levels of mRNA for IL-4, which was present in vaccinated hamsters compared to controls ($p < 0.05$, Figure 7.28). Splenic IL-12 mRNA levels were similar in vaccinated hamsters and infected controls but IL-12 mRNA levels were nearly 12 fold higher in the liver of vaccinated hamsters compared to infected controls ($p < 0.01$). Both liver and spleen mRNA levels for IFN-γ were significantly higher in vaccinated hamsters compared to infected controls ($p < 0.01$, Figure 7.28).

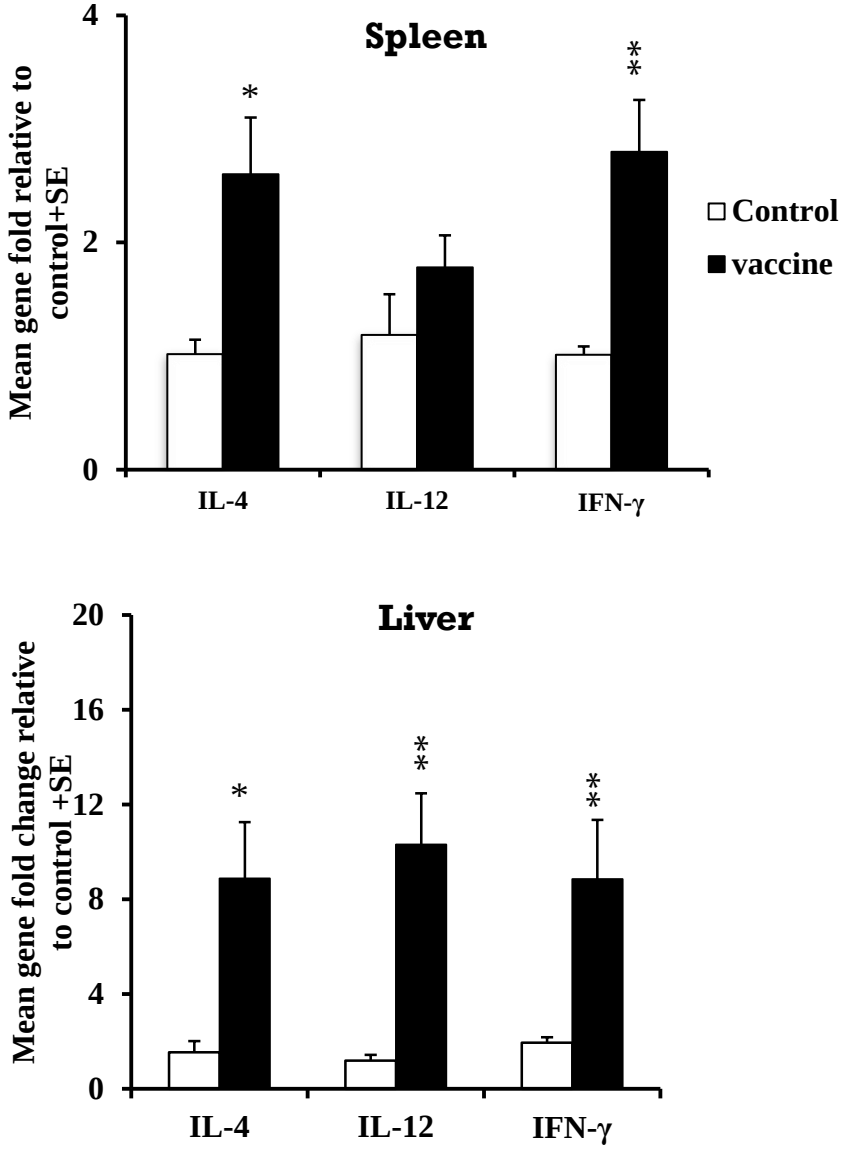


Figure 7.28 The effect of vaccination on IL-4, IL-12 and IFN-γ of hamsters were immunised by inhalation with PBS (control) or the triple vaccine (50 µg of each of the three recombinant γGCS proteins/dose on days −28 and −14). On day 0, hamsters were infected with 1 x 10^7 *L. donovani* amastigotes by iv injection into the tail vein. On day 14 post-infection were ssacrificed and cytokines detected from liver and spleen using real time PCR. Cytokine mRNA levels in the spleen and liver were relative to the hamster housekeeping gene (HPRT) using the $2^{-\Delta\Delta C}{}_T$ method. $^{*}p < 0.05$, $^{**}p < 0.01$, comparing vaccinated and infected control groups, n=6/treatment.

7.3 Discussion

Although preventive vaccines are recognized as the best and most cost effective protection measure against pathogens, there is still no vaccine available to protect people against leishmaniasis. *Leishmania* vaccine development has proven to be a difficult and challenging task and this is probably related to the complexity of immune responses involved in protection against the different *Leishmania spp.* and the cost of vaccine development (Evans and Kedzierski, 2012).

A recombinant protein prepared using *E. coli* could be contaminated with endotoxin, which would be harmful to humans. Therefore endotoxin levels need to be kept as low as possible to prevent adverse side effects (Robertson, 2013). Researchers involved in preclinical vaccine research typically have very little guidance on acceptable endotoxin levels for preclinical evaluation. According to United States Pharmacopeia, United States Food and Drug Administration, and the European Medicines Agency, an acceptable level of endotoxin is less than 10 EU/mL (Brito and Singh, 2011). In this study, endotoxin contamination was variable between different batches of recombinant protein but processing to remove endotoxin gave levels that were <3 Eu/ml. Therefore, in vaccine studies animals were dosed with less than 1.5 Eu/dose even when giving the triple vaccine at a total dose of 150 µg protein. This is higher that the levels reported for a commercial human influenza vaccine where acceptable endotoxin levels were 0.304–0.380 EU/mL (Geier *et al.*, 2003). However, lower than that of reported for a purified recombinant H1N1 influenza vaccine, where the endotoxin level was 8.7 EU/mL (Pushko *et al.*, 2010).

The results of this study indicated that vaccination with γGCS recombinant protein from one *Leishmania* species gave cross-immunity against another species. For example, an antibody response against *L. mexicana* γGCS antigen was obtained for mice vaccinated with the *L. donovani* γGCS antigen and vice versa. Few studies have focused on cross-protection using *Leishmania* but early studies using sequential infections with distinct species did suggest complex cross-protection relationships occur. For example, immunisation of mice with heat-killed *L. donovani* induced protection against a subsequent infection with *L. major* (Bebars *et al.*, 2000). Also, cross-immunity was investigated in monkeys, which had self-cured a *Leishmania* infection or were cured of their *Leishmania* infection using antimony-based chemotherapy. They found that a self-healing CL infection caused by *L. major* induced significant protection against *L. amazonensis* but did not protect against *L. braziliensis* infection. In contrast, monkeys that recovered from *L. braziliensis* or *L. chagasi* following chemotherapeutic intervention were protected against challenge with *L. braziliensis* or *L. amazonensis* (Porrozzi *et al.*, 2004). Cross immunity was investigated using *L. major* promastigote exogenous antigens (*Lm*SEAgs) which have been shown to protect against *L. major* infection in BALB/c mice. These antigens have been used to develop serologic assays that efficiently detected antibodies to *Leishmania* (both IgM and IgG) in VL patients (Ryan *et al.*, 2002).

The results of this study showed that it was possible to immunise mice against *L. mexicana, L. major* and *L. donovani* using a triple vaccine that consisted of recombinant γGCS proteins from *L. mexicana, L. major* and *L. donovani.* My studies showed that the amount of protein used had a fundamental effect on the level of

protection obtained. In addition, vaccination with a double or triple combination γGCS vaccine gave more protection than vaccination with a single recombinant γGCS protein. Other studies have also shown that vaccination with more than one protein can protect against *Leishmania spp.* but they tend to use different types of protein. Coler *et al.* (2002), immunised BALB/c mice with a recombinant polyprotein comprising a tandem fusion of the *Leishmania* antigens thiol-specific antioxidant, *L. major* stress-inducible protein 1 (LmSTI1), and *Leishmania* elongation initiation factor (LeIF). They concluded that it induced a potent Th1-type immune response and protected against CL and *L. infantum* infection (Coler *et al.*, 2007). The three antigens (TSA, LmSTI1, and LeIF) induced protection in the BALB/c mouse model of *L. major* either as a prophylactic (TSA and LmSTI1) or therapeutic (LeIF) vaccine (Skeiky *et al.*, 1995, Webb *et al.*, 1998).

The results of my study showed that it was possible to immunise mice against *L. mexicana* and *L. major* by the pulmonary route using the triple vaccine. Vaccination by inhalation with a total dose of 150 µg of the triple vaccine gave significant protection against a high bolus dose of promastigote parasites but the effect was transient. However increasing the administered dose to 150 µg protein gave a higher level of protection (98% reduction in parasite burden), which was similar to that obtained by subcutaneous injection with a total dose of 60 µg of the same vaccine, (98% reduction in parasite burdens). Disis and his group (2004), study to estimate whether vaccine dose impacts on immunogenicity, a study using intradermal HER-2/neu-overexpressing breast or ovarian cancer and an oncogenic protein, patients were

given a low (25 μg), intermediate (150 μg), or high (900 μg) vaccine dose. The patients receiving the highest dose of recombinant protein against breast cancer developed specific immunity more rapidly than those who received the lowest dose (Disis *et al.*, 2004). In this study, mice were not directly dosed with a fixed amount of protein by inhalation due to technical difficulties caused by their small size and this will partly explain why a larger dose is required. This could either be related to the route or it could be a consequence of the practical constraints associated with using a murine model since drug studies have already shown that only a fraction of the dose applied to the nebuliser actually reaches the lungs (Alsaadi *et al.*, 2012). In addition damage to the protein may also occur during nebulisation due to the shear force applied (Depreter *et al.*, 2013; Lu, 2012) Obviously in a larger animal for administration or clinical treatment, a specific dose can be administered and using the most appropriate nebuliser or altering the formulation of the vaccine e.g. dry powder or inclusion of chemical stabilisers, can prevent protein degradation during nebulisation. In this study a whole body exposure system was used which could result in some of the dose being taken orally when the mice groom their fur. However it is unlikely that this route gave any protection as we have found that immunisation by oral dosing by gavage with *L. donovani* γGCS recombinant protein did not protect against infection (unpublished data), presumably because the protein was degraded in the stomach of the mice. Assessment of antibody levels indicated that exposure to the larger protein dose did have a dose dependent effect on antibody levels and did not induce tolerance to the proteins. An earlier study using intranasal immunization with Leish-111f induced serum Leish-111f-specific IgG2a production, biased IFN-γ

production from spleen cells and protective immunity against *L. major* infection (Sakai *et al.*, 2010). The results showed that inhalation was effective for vaccine delivery to protect against *L. mexicana* and *L. major* infection. Inhalation as a mucosal vaccination route is a non-invasive alternative approach for not only mucosal pathogens but also systemic pathogens, since it induces both mucosal and systemic immune reactions (Sakai *et al.*, 2010).

In this study, vaccination by the pulmonary route induced higher specific Th1 responses in the spleen and lungs based on IFN-γ and IL-5 production. Pulmonary administration was associated with Th1 responses based on the ratio of IgG2a/IgG1 responses. Vaccination with each γGCS protein resulted in similar Th1 and Th2 responses using IgG2a and IgG1 as proxies. However, in *L. mexicana* infected mice, a polarised Th1 response predominated for the first 14 days but reverted to a mixed Th1/Th2 response by day 28. Typically, a predominant Th2 response is a feature of *L. mexicana* infection and is associated with IgG1 and IL-10 production (Buxbaum, 2013). In studies using B6 IgG1 knockout mice, which are unable to produce IgG1, the mice developed earlier and stronger IgG2a responses to *L. mexicana* and were more resistant to the infection (Chu *et al.*, 2010). Therefore, the enhanced Th1 responses observed up to day 14 in this study are not surprising and were associated with significant parasite reduction. By day 28, parasite burdens at the infection site were low and a down regulation of Th1 response may have occurred in a quest to minimise abnormal pathology at the site of infection.

In mice infected with *L. major* differences in the immune response were observed between routes of vaccine administration, a mixed Th1/Th2 response was more apparent. On day 4 post-infection in subcutaneously vaccinated mice whereas pulmonary vaccinated mice had a predominant Th1 response. On days 14 and day 21, both vaccination groups had a slightly higher Th1 than Th2 response present, especially against LjγGCS protein. This mirrors other studies where mice vaccinated using a *L. major* recombinant ribosomal protein protection had an enhanced Th1 response and significantly higher antigen specific IgG2a titres (Ramirez *et al.*, 2013). Furthermore, ineffective vaccination using heat shock proteins from *L. major* promastigotes resulted in a Th2 response and disease exacerbation (Holakuyee *et al.*, 2012).

Protective immunity against *Leishmania* is species-specific but ultimately depends on the ability of an infected macrophage to kill the intracellular parasite (Okwor *et al.*, 2012). Microbicidal macrophage mechanisms are stimulated by cytokines such as IFN-γ and TNF-α, although alternatively activated macrophages can kill *Leishmania* parasites via the production of nitric oxide when they are stimulated with IFN-γ/LPS (Mylonas *et al.*, 2009). Data from the present study shows that protection against *L. mexicana* or *L. major* in vaccinated mice was associated with enhanced nitrite production by splenocytes stimulated with ConA or specific antigen, which is an indirect measurement of nitric oxide production. In previous studies, using a tri-fusion *Leishmania* protein, researchers showed that protection was associated with Th1 type

immunity, i.e specific IgG2a production based on IFN-γ production and protected against *L. major* infection (Sakai *et al.*, 2002; Sakai *et al*, 2010).

Immunization against experimental VL in murine models has been reported to be more difficult to achieve than for the cutaneous forms. This may be due to the more complex situation in regarding the immunopathology of murine infection with VL species. In particular, the outcome of VL infection in mice does not depend on the Th1 versus Th2 subset expansion (Alexander and Bryson, 2005). In mice, clearance of hepatic parasite burden occurs after 2 weeks post infection due to efficient granuloma formation resulting from interaction of *Leishmania*-specific T cells with parasitized dendritic cells and resident macrophages. Parasite killing in mature granulomas leads to resolution of hepatic infection in about 56 days in experimental VL. Hence, vaccine efficacy and disease progression in mice can be predicted from the degree of maturation of hepatic granulomas in challenged animals, which correlate with cell-mediated immunity (Carrion *et al.*, 2006). In my study, subcutaneous immunisation against *L. donovani* infection did not protect BALB/c mice against infection, as vaccinated and control mice had similar liver, spleen and bone marrow parasite burdens. However, the triple vaccine induced high IgG1/IgG2a titre, IFN-γ, IL-10 and IL-4 production. There was no difference between vaccinated and non vaccinated groups in IL-12 and nitrite production. Researchers have suggested that antibodies are not relevant for vaccine-induced protection in the absence of an appropriate cellular response. Overall, both $CD4^+$ and $CD8^+$ T cells are required to mediate parasite control and successful vaccination requires IL-12 driven IFN-γ-dependent Th1/Th2 mixed

response against VL. However, high IL-4 and IL-10 production in immunised animals, despite very high IFN-γ, may lead to vaccine failure and disease exacerbation in VL (reviewed by Das and Ali, 2012). Melby *et al.*, (2001) used LACK (*Leishmania* homolog of receptors for activated C kinase) antigen, and found that it protected against cutaneous *L. major* infection by redirecting the early T-cell response away from a pathogenic interleukin-4 (IL-4) response and toward a protective Th1 response. While it did not protect against *L. donovani* infection it induced a strong parasite-specific Th1 response.

Compared to mice, hamsters seem to be a better model for progressive VL expressing clinical features like hepatosplenomegaly, immunosuppression, anaemia, and death, closely simulating human VL. Impaired macrophage activation in liver and spleen due to lack of NOS2 expression throughout disease progression seems to be the major cause of uncontrolled parasite multiplication and death in hamsters (Melby *et al.*, 2001). High innate susceptibility of hamsters to *L. donovani* makes them a more suitable model for studying immunopathology of VL than evaluation of protective immunity (Nieto *et al.*, 2011). In my study vaccination with the triple γGCS vaccine (50μg/recombinant protein/dose) protected hamsters against *L. donovani* infection, resulting in a significant reduction in liver and bone marrow parasite burdens. Vaccination induced high specific IgG antibody titres, enhanced mRNA levels of IFN-γ and IL-4 in the liver and spleen, stimulated proliferation of spleen cells and their ability to produce nitrite, an indirect measure of nitric oxide production. It is well documented that in *Leishmania* infections, macrophages become activated by IFN-γ

released from parasite-specific T cells. They are then able to destroy intracellular parasites such as *Leishmania* through the production of several mediators, principal among which is NO (Soong *et al.*, 2012). The recovery from *Leishmania* infection depends on the induction of the Th1 response with production of IFN-γ and IL-12 and enhanced expression of NO synthase (Kushawaha *et al.*, 2011). In this study, we have also observed that the triple vaccine given by inhalation vaccine induces IFN-γ and IL-12 production.

Adjuvants enhance the immune response. However, some studies have shown vaccine protection against infection without use of adjuvants. Early studies showed that *L. major* promastigotes exogenous antigens (*Lm*SEAgs) administered in the absence of adjuvants are highly immunogenic molecules that protect BALB/c mice against challenge infection with *L. major* (Tonui *et al.*, 2004). In this study the triple protein induced protection without any adjuvants. However, preliminary vaccine studies using the triple vaccine and the subcutaneous route of administration studies, where the vaccine was incorporated into non ionic surfactant and the lower dose 20 μg of the three recombinant γGCS proteins was used, protection against *L. mexicana* infection was obtained (unpublished data).

In summary, the results of this study indicate that a triple *Leishmania* γGCS vaccine offered significantly better protection against *L. mexicana* infection than using a single γGCS protein vaccine. A triple vaccine given by inhalation protected against *L.*

mexicana and *L. major* infection in murine model and *L. donovani* infection in hamster model, and studies using *L. mexicana* showed that the level of protection was similar to that obtained using the injectable vaccine. Vaccination using inhalation or subcutaneous routes did not protect completely against leishmaniasis, therefore further studies to improve the vaccine (e.g. using adjuvants) should be explored. In addition ways reduce the dose of protein given using the triple *Leishmania* γGCS vaccine should be explored.

Chapter 8: General conclusion and Future work

Leishmaniasis is an infectious disease caused by the protozoan parasite *Leishmania*. At present, there are a number of drugs available for the treatment of leishmaniasis but ideally a vaccine is required to prevent infection. Previous studies in our laboratory have concluded that intramuscular immunization with a plasmid containing the *L. donovani* gene sequence for gamma glutamyl cysteine synthetase protected against *L. donovani* (Carter *et al.,* 2007) and immunisation with recombinant γGCS protein gave significant protection against *L. donovani* infection (Henriquez *et al.,* 2010) and *L. major* and *L. mexicana* (Campbell *et al.,* 2012*).* However, they did not give sterile immunity against infection. Therefore, this study was to determine if a combination vaccine using recombinant γGCS from three *Leishmania* species gave better protection and if vaccination by inhalation were possible, as this would have many advantages over injectable vaccine. In this study the following aims were achieved.

First, the γGCS sequences of all three *Leishmania* species were successfully cloned into the pET-24a expression vector. The sequencing studies showed that the γGCS gene sequences for all three *Leishmania* species were in the correct orientation for expression and the sequences were 100% homologous to the published γGCS sequences for the three *Leishmania* species. The recombinant *Leishmania* γGCS proteins for *L. donovani, L. mexicana* and *L. major* were expressed in *E. coli* and a method which gives a large amount of protein from just 100 ml broth was identified. However, most of the induced recombinant protein was located in the insoluble fraction of *E. coli* cells. Purification of recombinant *Leishmania* γGCS protein under

denaturing conditions resulted is the production of a truncated protein as well as a full length protein. The recombinant protein was identified as *Leishmania* γGCS using MALDI studies. Improving the purification technique could allow isolation of full-length protein only.

Multiple sequence alignment of γGCS from *L. donovani, L. major, L. mexicana, T. brucei* and human shows that the 80% similarity between human γGCS and *Leishmania* was different, especially in binding cysteine and sulfoxime inhibitors (Appendix 5). *E. coli* γGCS was not included in the above alignment because the low level of homology in the cysteine binding region prevented an accurate alignment using clustalw. The amino acid identity between *Leishmania* species were *L. donovani* /*L. major* 94.8%, *L. donovani*/*L. mexicana* 91%, *L. major/ L. mexicana* 88.9% (Appendix 4).

Secondly, the specific activity of recombinant *Leishmania* γGCS for all three species was determined and found to be lower than that obtained for *Trypanosoma brucei* (Lueder and Phillips, 1996), *Ascaris suum* (Hussein and Walter, 1995) or rat catalytic subunit (Huang *et al*., 1993). My study showed that BSO is cytotoxic to both the promastigotes and intracellular amastigote stages of all three *Leishmania* species and there are species-specific differences in BSO susceptibility, with *L. donovani* being more sensitive to BSO treatment.

Thirdly, this study showing that luciferase expressing *Leishmania* can be used in *in vitro* and *in vivo* studies and there was an excellent linear correlation between parasite number and luciferase activity. In this study (chapter 5) luciferase expressing *L. mexicana* had the highest infectivity rate compared to *L. major* or *L. donovani*, where 99% of bone marrow macrophages were infected using *L. mexicana* at a parasite: host cell ratio of 10:1, whereas higher parasite: host cell ratios were required for the other two *Leishmania spp*. (*L. major*, 20:1; *L. donovani* 40:1). *In vivo*, (chapter 6) the luciferase expressing *L. mexicana* and *L. major* had the ability to infect rump and footpad, Also the parasite burden was high after weeks of infection and lesion developed quickly. Furthermore, the role of phagocytic cells was investigated in this study. There was a significant difference between rump and footpad infected with *L. mexicana* promastigotes in the chemiluminescence emitted, which was used as a marker of phagocytic cells as neutrophils and macrophages producing an oxidative environment as light expression occurs in response to the luminol given to mice. Rump lesions of mice infected with *L. mexicana* emitted more chemiluminescence than footpad infected mice. However, in mice infected with *L. major* promastigote, there were no any significant different between both sites.

Vaccination with the combination vaccine using recombinant *Leishmania* γGCS from *L. donovani*, *L. mexicana* and *L. major* gave significantly better protection against *L. mexicana* infection than vaccination with single or double γGCS recombinant protein. Treatment by inhalation vaccine with triple combination recombinant *Leishmania* γGCS was also as effective as subcutaneous administration vaccine for protection

against *L. mexicana* or *L. major* infection in a murine model and *L. donovani* in hamster model. This correlates with published studies where multicomponent vaccines are thought be a better option for vaccination against *Leishmania* (Das and Ali, 2012).

There is currently great interest in developing mucosal vaccines against a variety of microbial pathogens. The primary reason for using a mucosal route of vaccination is that most infections affect or start from a mucosal surface, and that in these infections, topical application of a vaccine is often required to induce a protective immune response, For examples include gastrointestinal infections caused by *Helicobacte pylori*, *Vibrio cholerae*, enterotoxigenic *E. coli*, *Clostridium difficile*, rotaviruses and calici viruses; respiratory infections caused by *Mycoplasma pneumoniae*, influenza virus and respiratory syncytial virus; and sexually transmitted genital infections caused by HIV, *Chlamydia trachomatis*, *Neisseria gonorrhoeae* and herpes simplex virus (Holmgren and Czerkinsky, 2005).

The development of mucosal vaccines, whether for prevention of infectious diseases or for oral-tolerance immunotherapy, requires efficient antigen delivery and adjuvant systems. Ideally, such systems should be protect the vaccine from physical elimination and enzymatic digestion, target mucosal inductive sites including membrane, or M cells, and at least for vaccines against infections, appropriately stimulate the innate immune system to generate effective adaptive immunity (Fujkuyama *et al.*, 2012). Mucosal immune responses in the humoral-secretory arm of the immune system develop earlier than systemic immune responsiveness, conferring a logistical advantage for mucosal vaccination in infants. On the other hand, it seems that mucosal tolerance develops much later, explaining, in part, the frequency of food allergies in

young children. There is yet no precise knowledge regarding the ontogeny of the different mucosal regulatory cells for which selective targeting and activation by appropriate delivery systems and immunomodulating agents could be advantageous for preventing allergies and tissue-damaging inflammatory reactions (Kristensen and Chen, 2013). In this study, vaccination by the inhalation or subcutaneous route did not give complete protection against infection but this could possibly be improved by changing the vaccination protocol. For example, addition of adjuvants to boost immune recognition, as Leish-111f antigen when used with MPL-SE adjuvant the protection was increased against canine leishmaniasis (Sachdeva *et al.*, 2009).

Future studies using other methods could be tested to improve the expression of full length soluble *Leishmania* γGCS e.g. with ion exchange studies or gel filtration after nickel column purification. This would allow crystallisation of γGCS for the three species so that 3-D modelling studies could be carried out to identify the active site in enzyme and the mechanism of inhibitor binding to the enzyme. Understanding the crystal structure of the enzyme would help in the discovery of new specific drugs against leishmaniasis. Improving the expression of recombinant proteins in an expression host is the best way to improve protein yield and perhaps changing the affinity tag e.g. using glutathione S-transferase (GST) would give better protein recovery (Young *et al.*, 2012). Also using a monoclonal antibody could help purify full length protein (Liu *et al.*, 2010). Perhaps changing the expression host to prevent the formation of inclusion bodies could help in the production of the full length protein e.g. using yeast or a *Leishmania* expression system (Sevastsyanovich *et al.*, 2009). For

example, recombinant human c-reactive protein was expressed as a soluble protein in *L. tarentolae* but expressed as an inclusion bodies when using *E. coli* expression system (Dortay *et al*., 2011).

Future studies investigating differences in susceptibility to BSO inhibition between *Leishmania* species could be useful to do. A purified antibody against *Leishmania* γGCS that would help to recognized the expression of γGCS inside *Leishmania* amastigotes and promastigotes using western blotting and investigates the mechanism that resistance and susceptibility to BSO. Also using HPLC to detect the amount of thiol inside *Leishmania* amastigotes and promastigotes would allow comparison between species after addition of BSO (Mukherjee e*t al*., 2009). Another protocol that could be useful is to add a protein marker e.g. fluorescent tag to γGCS *Leishmania* and follow its function in each species using fluorescence microscopy studies.

Pulmonary vaccines offer a new approach for vaccine delivery. However, studies to obtain a proper understanding of how antigens are processed after pulmonary administration and which aerosol and formulation characteristics are the most important for inducing protection are required (Tonnis *et al.*, 2012). The role of DCs in inhalation *Leishmania* γGCS vaccination against leishmaniasis was important. The DCs are found throughout the body and are especially prominent at mucosal surfaces. Immature type DCs are enriched underneath the epithelium of mucosal inductive sites and are poised to capture antigens. When antigen uptake occurs, these DCs change

their phenotype by expressing higher levels of MHC class II and costimulatory molecules and move to T-cell areas of inductive sites for Ag presentation. Future studies could determine the role of pulmonary dendritic cells in antigen uptake and transport to the draining lymph node, and flow cytometer studies could investigate the role of $CD8^+$ T and $CD4^+$ T cells in immunity to the triple vaccine (Nembrini *et al.*, 2011). This could help to improve the vaccine formulation to increase vaccine immunogenicity and reduce the protein dose. Secretory (S)-IgA antibody is a major player in the mucosal immune system and is locally produced in effector tissues (Kristensen and Chen, 2013). The future study to detect the presence of Ag-specific S-IgA antibody at mucosal effector sites other than the inductive sites where initial Ag sampling occurred is definitive evidence for the common mucosal immune system. Previous studies, fund that M cell-targeting delivery system may be of play role in developing effective mucosal vaccines. Furthermore, it is likely that M cells are also involved in the induction of oral tolerance (Fujkuyama *et al*, 2012). Therefore, the future study to investigate the role of M cells in delivery of inhalation *Leishmania* γGCS vaccine could be important in vaccine formulation.

References

ABDELHAK, S., LOUZIR, H., TIMM, J., BLEL, L., BENLASFAR, Z., LAGRANDERIE, M., GHEORGHIU, M., DELLAGI, K. & GICQUEL, B. 1995. Recombinant BCG expressing the *Leishmania* surface antigen Gp63 induces protective immunity against *Leishmania major* infection in BALB/c mice. *Microbiology,* 141 (Pt 7)**,** 1585-92.

ADAMS, E. R., VERSTEEG, I. & LEEFLANG, M. M. 2013. Systematic review into diagnostics for post-Kala-Azar dermal leishmaniasis (PKDL). *J Trop Med,* 2013**,** 150746.

AL-NASIRY, S., GEUSENS, N., HANSSENS, M., LUYTEN, C. & PIJNENBORG, R. 2007. The use of Alamar Blue assay for quantitative analysis of viability, migration and invasion of choriocarcinoma cells. *Hum Reprod,* 22**,** 1304-9.

ALEKSIC, M., DUSHEK, O., ZHANG, H., SHENDEROV, E., CHEN, J. L., CERUNDOLO, V., COOMBS, D. & VAN DER MERWE, P. A. 2010. Dependence of T cell antigen recognition on T cell receptor-peptide MHC confinement time. *Immunity,* 32**,** 163-74.

ALEXANDER, J. & BROMBACHER, F. 2012. T helper1/t helper2 cells and resistance/susceptibility to *leishmania* infection: is this paradigm still relevant? *Front Immunol,* 3**,** 80.

ALEXANDER, J. & BRYSON, K. 2005. T helper (h)1/Th2 and *Leishmania*: paradox rather than paradigm. *Immunol Lett,* 99**,** 17-23.

ALEXANDER, J., COOMBS, G. H. & MOTTRAM, J. C. 1998. *Leishmania mexicana* cysteine proteinase-deficient mutants have attenuated virulence for mice and potentiate a Th1 response. *J Immunol,* 161**,** 6794-801.

ALLAHVERDIYEV, A. M., BAGIROVA, M., ELCICEK, S., KOC, R. C. & OZTEL, O. N. 2011. Effect of human urine on cell cycle and infectivity of *Leismania* species promastigotes in vitro. *Am J Trop Med Hyg,* 85**,** 639-43.

ALSAADI, M., ITALIA, J. L., MULLEN, A. B., RAVI KUMAR, M. N., CANDLISH, A. A., WILLIAMS, R. A., SHAW, C. D., AL GAWHARI, F., COOMBS, G. H., WIESE, M., THOMSON, A. H., PUIG-SELLART, M., WALLACE, J., SHARP, A., WHEELER, L., WARN, P. & CARTER, K. C. 2012. The efficacy of aerosol treatment with non-ionic surfactant vesicles containing amphotericin B in rodent models of leishmaniasis and pulmonary aspergillosis infection. J Control Release, 160, 685-91.

ANSAR, NASIM AKHTAR, KUMAR, RAJIV, GAUTAM,SHALINI, NYLEN, SUSANNE, SINGH ,OM PRAKASH, SUNDAR SHYAM and SACKS DAVID. 2011. IL-27 and IL-21 are associated with T Cell IL-10 responses in human Visceral Leishmaniasis. *The Journal of Immunology*.186.7 3977-3985

ALVAR, J., CROFT, S. L., KAYE, P., KHAMESIPOUR, A., SUNDAR, S. & REED, S. G. 2013. Case study for a vaccine against leishmaniasis. *Vaccine,* 31 Suppl 2**,** B244-9.

AMEEN, M. 2010. Cutaneous leishmaniasis: advances in disease pathogenesis, diagnostics and therapeutics. *Clin Exp Dermatol,* 35**,** 699-705.

ANDREU, N., ZELMER, A. & WILES, S. 2011. Noninvasive biophotonic imaging for studies of infectious disease. *FEMS Microbiol Rev,* 35**,** 360-94.

ANOOPKUMAR-DUKIE, S., CAREY, J. B., CONERE, T., O'SULLIVAN, E., VAN PELT, F. N. & ALLSHIRE, A. 2005. Resazurin assay of radiation response in cultured cells. *Br J Radiol,* 78**,** 945-7.

ANSARI, N. A., RAMESH, V. & SALOTRA, P. 2006. Interferon (IFN)-gamma, tumor necrosis factor-alpha , interleukin-6, and IFN-gamma receptor 1 are the major immunological determinants associated with post-kala azar dermal leishmaniasis. *J Infect Dis,* 194**,** 958-65.

ANTINORI, S., CALATTINI, S., PIOLINI, R., LONGHI, E., BESTETTI, G., CASCIO, A., PARRAVICINI, C. & CORBELLINO, M. 2009. Is real-time polymerase chain reaction (PCR) more useful than a conventional PCR for the clinical management of leishmaniasis? *Am J Trop Med Hyg,* 81**,** 46-51.

ARIYANAYAGAM, M. R. & FAIRLAMB, A. H. 2001. Ovothiol and trypanothione as antioxidants in trypanosomatids. *Mol Biochem Parasitol,* 115**,** 189-98.

ARMIJOS, R. X., WEIGEL, M. M., AVILES, H., MALDONADO, R. & RACINES, J. 1998. Field trial of a vaccine against New World cutaneous leishmaniasis in an at-risk child population: safety, immunogenicity, and efficacy during the first 12 months of follow-up. *J Infect Dis,* 177**,** 1352-7.

ARNAU, J., LAURITZEN, C., PETERSEN, G. E. & PEDERSEN, J. 2006. Current strategies for the use of affinity tags and tag removal for the purification of recombinant proteins. *Protein Expr Purif,* 48**,** 1-13.

ARRICK, B. A., GRIFFITH, O. W. & CERAMI, A. 1981. Inhibition of glutathione synthesis as a chemotherapeutic strategy for trypanosomiasis. *J Exp Med,* 153**,** 720-5.

ASHUTOSH, GUPTA, S., RAMESH, SUNDAR, S. & GOYAL, N. 2005. Use of *Leishmania donovani* field isolates expressing the luciferase reporter gene in in vitro drug screening. *Antimicrob Agents Chemother,* 49**,** 3776-83.

ATOSUO, J. T. & LILIUS, E. M. 2011. The real-time-based assessment of the microbial killing by the antimicrobial compounds of neutrophils. *ScientificWorldJournal,* 11**,** 2382-90.

BACELLAR, O., FARIA, D., NASCIMENTO, M., CARDOSO, T. M., GOLLOB, K. J., DUTRA, W. O., SCOTT, P. & CARVALHO, E. M. 2009. Interleukin 17 production among patients with American cutaneous leishmaniasis. *J Infect Dis,* 200**,** 75-8.

BAKER, M. 2010. Whole-animal imaging: The whole picture. *Nature,* 463**,** 977-80.

BANULS, A. L., HIDE, M. & PRUGNOLLE, F. 2007. *Leishmania* and the leishmaniases: a parasite genetic update and advances in taxonomy, epidemiology and pathogenicity in humans. *Adv Parasitol,* 64**,** 1-109.

BARBIERI, C. L. 2006. Immunology of canine leishmaniasis. *Parasite Immunol,* 28**,** 329-37.

BEBARS, M. A., EL SEROUGI, A. O., MAKLED, K. M., MIKHAEL, E. M., ABOU GAMRA, M. M., EL SHERBINY, M., MOHAREB, A. W. & MOHAMMED,

E. A. 2000. An experimental vaccine providing heterologous protection for *Leishmania* species in murine model. *J Egypt Soc Parasitol,* 30**,** 137-56.

BERNAUDAT, F., FRELET-BARRAND, A., POCHON, N., DEMENTIN, S., HIVIN, P., BOUTIGNY, S., RIOUX, J. B., SALVI, D., SEIGNEURIN-BERNY, D., RICHAUD, P., JOYARD, J., PIGNOL, D., SABATY, M., DESNOS, T., PEBAY-PEYROULA, E., DARROUZET, E., VERNET, T. & ROLLAND, N. 2011. Heterologous expression of membrane proteins: choosing the appropriate host. *PLoS One,* 6**,** e29191.

BESSETTE, P. H., ASLUND, F., BECKWITH, J. & GEORGIOU, G. 1999. Efficient folding of proteins with multiple disulfide bonds in the *Escherichia coli* cytoplasm. *Proc Natl Acad Sci U S A,* 96**,** 13703-8.

BESTEIRO, S., WILLIAMS, R. A., COOMBS, G. H. & MOTTRAM, J. C. 2007. Protein turnover and differentiation in *Leishmania*. *Int J Parasitol,* 37**,** 1063-75.

BEVERLEY, S. M. 1991. Gene amplification in *Leishmania*. *Annu Rev Microbiol,* 45**,** 417-44.

BINS, A. D., VAN DEN BERG, J. H., OOSTERHUIS, K. & HAANEN, J. B. 2013. Recent advances towards the clinical application of DNA vaccines. *Neth J Med,* 71**,** 109-17.

BIRNBAUM, R. & CRAFT, N. 2011. Innate immunity and *Leishmania* vaccination strategies. *Dermatol Clin,* 29**,** 89-102.

BIVAS-BENITA, M., OTTENHOFF, T. H., JUNGINGER, H. E. & BORCHARD, G. 2005. Pulmonary DNA vaccination: concepts, possibilities and perspectives. *J Control Release,* 107**,** 1-29.

BOGDAN, C. 2012. Natural killer cells in experimental and human leishmaniasis. *Front Cell Infect Microbiol,* 2**,** 69.

BRANDTZAEG, P. 2010. The mucosal immune system and its integration with the mammary glands. *J Pediatr,* 156**,** S8-15.

BRITO, L. A. & SINGH, M. 2011. Acceptable levels of endotoxin in vaccine formulations during preclinical research. *J Pharm Sci,* 100**,** 34-7.

BRITTO, C., RAVEL, C., BASTIEN, P., BLAINEAU, C., PAGES, M., DEDET, J. P. & WINCKER, P. 1998. Conserved linkage groups associated with large-scale chromosomal rearrangements between Old World and New World *Leishmania* genomes. *Gene,* 222**,** 107-17.

BROGAN, J., LI, F., LI, W., HE, Z., HUANG, Q. & LI, C. Y. 2012. Imaging molecular pathways: reporter genes. *Radiat Res,* 177**,** 508-13.

BRUHN, K. W., BIRNBAUM, R., HASKELL, J., VANCHINATHAN, V., GREGER, S., NARAYAN, R., CHANG, P. L., TRAN, T. A., HICKERSON, S. M., BEVERLEY, S. M., WILSON, M. E. & CRAFT, N. 2012. Killed but metabolically active *Leishmania infantum* as a novel whole-cell vaccine for visceral leishmaniasis. *Clin Vaccine Immunol,* 19**,** 490-8.

BURZA, S., NABI, E., MAHAJAN, R., MITRA, G. & LIMA, M. A. 2013. One-Year Follow-up of Immunocompetent Male Patients Treated With Miltefosine For Primary Visceral Leishmaniasis in Bihar, India. *Clin Infect Dis*.

BUXBAUM, L. U. 2013. *Leishmania mexicana* Infection Induces IgG to Parasite Surface Glycoinositol Phospholipids that Can Induce IL-10 in Mice and Humans. *PLoS Negl Trop Dis,* 7**,** e2224.

CAMPBELL, S. A., ALAWA, J., DORO, B., HENRIQUEZ, F. L., ROBERTS, C. W., NOK, A., ALAWA, C. B., ALSAADI, M., MULLEN, A. B. & CARTER, K. C. 2011. Comparative assessment of a DNA and protein *Leishmania donovani* gamma glutamyl cysteine synthetase vaccine to cross-protect against murine cutaneous leishmaniasis caused by *L. major* or *L. mexicana* infection. *Vaccine,* 30**,** 1357-63.

CARLSEN, E. D., HAY, C., HENARD, C. A., POPOV, V., GARG, N. J. & SOONG, L. 2013. *Leishmania amazonensis* amastigotes trigger neutrophil activation, but resist neutrophil microbicidal mechanisms. *Infect Immun*.

CARRION, J., NIETO, A., IBORRA, S., INIESTA, V., SOTO, M., FOLGUEIRA, C., ABANADES, D. R., REQUENA, J. M. & ALONSO, C. 2006. Immunohistological features of visceral leishmaniasis in BALB/c mice. *Parasite Immunol,* 28**,** 173-83.

CARTER, K. C., HENRIQUEZ, F. L., CAMPBELL, S. A., ROBERTS, C. W., NOK, A., MULLEN, A. B. & MCFARLANE, E. 2007. DNA vaccination against the parasite enzyme gamma-glutamylcysteine synthetase confers protection against *Leishmania donovani* infection. *Vaccine,* 25**,** 4502-9.

CARTER, K. C., HUTCHISON, S., BOITELLE, A., MURRAY, H. W., SUNDAR, S. & MULLEN, A. B. 2005. Sodium stibogluconate resistance in *Leishmania donovani* correlates with greater tolerance to macrophage antileishmanial responses and trivalent antimony therapy. *Parasitology,* 131**,** 747-57.

CARTER, K. C., HUTCHISON, S., HENRIQUEZ, F. L., LEGARE, D., OUELLETTE, M., ROBERTS, C. W. & MULLEN, A. B. 2006. Resistance of *Leishmania donovani* to sodium stibogluconate is related to the expression of host and parasite gamma-glutamylcysteine synthetase. *Antimicrob Agents Chemother,* 50**,** 88-95.

CARTER, K. C., SUNDAR, S., SPICKETT, C., PEREIRA, O. C. & MULLEN, A. B. 2003. The *in vivo* susceptibility of *Leishmania donovani* to sodium stibogluconate is drug specific and can be reversed by inhibiting glutathione biosynthesis. *Antimicrob Agents Chemother,* 47**,** 1529-35.

CHAKRAVARTY, J., KUMAR, S., TRIVEDI, S., RAI, V. K., SINGH, A., ASHMAN, J. A., LAUGHLIN, E. M., COLER, R. N., KAHN, S. J., BECKMANN, A. M., COWGILL, K. D., REED, S. G., SUNDAR, S. & PIAZZA, F. M. 2011. A clinical trial to evaluate the safety and immunogenicity of the LEISH-F1+MPL-SE vaccine for use in the prevention of visceral leishmaniasis. *Vaccine,* 29**,** 3531-7.

CHAN, W. C., LIANG, P. H., SHIH, Y. P., YANG, U. C., LIN, W. C. & HSU, C. N. 2010. Learning to predict expression efficacy of vectors in recombinant protein production. *BMC Bioinformatics,* 11 Suppl 1**,** S21.

CHAVEZ-FUMAGALLI, M. A., COSTA, M. A., OLIVEIRA, D. M., RAMIREZ, L., COSTA, L. E., DUARTE, M. C., MARTINS, V. T., OLIVEIRA, J. S., OLORTEGI, C. C., BONAY, P., ALONSO, C., TAVARES, C. A., SOTO, M. & COELHO, E. A. 2010. Vaccination with the *Leishmania infantum* ribosomal

proteins induces protection in BALB/c mice against *Leishmania chagasi* and *Leishmania amazonensis* challenge. *Microbes Infect,* 12**,** 967-77.

CHEN, Z., HE, Y., SHI, B. & YANG, D. 2013. Human serum albumin from recombinant DNA technology: Challenges and strategies. *Biochim Biophys Acta,* 1830**,** 5515-5525.

CHU, N., THOMAS, B. N., PATEL, S. R. & BUXBAUM, L. U. 2010. IgG1 is pathogenic in *Leishmania mexicana* infection. *J Immunol,* 185**,** 6939-46.

CLAES, F., VODNALA, S. K., VAN REET, N., BOUCHER, N., LUNDEN-MIGUEL, H., BALTZ, T., GODDEERIS, B. M., BUSCHER, P. & ROTTENBERG, M. E. 2009. Bioluminescent imaging of *Trypanosoma brucei* shows preferential testis dissemination which may hamper drug efficacy in sleeping sickness. *PLoS Negl Trop Dis,* 3**,** e486.

CLAEYS BOUUAERT, C., LIPKOW, K., ANDREWS, S. S., LIU, D. & CHALMERS, R. 2013. The autoregulation of a eukaryotic DNA transposon. *Elife,* 2**,** e00668.

CLOSE, D. M., XU, T., SAYLER, G. S. & RIPP, S. 2011. In vivo bioluminescent imaging (BLI): noninvasive visualization and interrogation of biological processes in living animals. *Sensors (Basel),* 11**,** 180-206.

COBAN, C., KOBIYAMA, K., AOSHI, T., TAKESHITA, F., HORII, T., AKIRA, S. & ISHII, K. J. 2011. Novel strategies to improve DNA vaccine immunogenicity. *Curr Gene Ther,* 11**,** 479-84.

COBAN, C., KOBIYAMA, K., JOUNAI, N., TOZUKA, M. & ISHII, K. J. 2013. DNA vaccines: A simple DNA sensing matter? *Hum Vaccin Immunother,* 9.

COLER, R. N., GOTO, Y., BOGATZKI, L., RAMAN, V. & REED, S. G. 2007. Leish-111f, a recombinant polyprotein vaccine that protects against visceral Leishmaniasis by elicitation of CD4+ T cells. *Infect Immun,* 75**,** 4648-54.

COLER, R. N. & REED, S. G. 2005. Second-generation vaccines against leishmaniasis. *Trends Parasitol,* 21**,** 244-9.

COLER, R. N., SKEIKY, Y. A., BERNARDS, K., GREESON, K., CARTER, D., CORNELLISON, C. D., MODABBER, F., CAMPOS-NETO, A. & REED, S. G. 2002. Immunization with a polyprotein vaccine consisting of the T-Cell antigens thiol-specific antioxidant, *Leishmania major* stress-inducible protein 1, and *Leishmania* elongation initiation factor protects against leishmaniasis. *Infect Immun,* 70**,** 4215-25.

COLLIN, N., GOMES, R., TEIXEIRA, C., CHENG, L., LAUGHINGHOUSE, A., WARD, J. M., ELNAIEM, D. E., FISCHER, L., VALENZUELA, J. G. & KAMHAWI, S. 2009. Sand fly salivary proteins induce strong cellular immunity in a natural reservoir of visceral leishmaniasis with adverse consequences for *Leishmania*. *PLoS Pathog,* 5**,** e1000441.

COLOTTI, G. & ILARI, A. 2011. Polyamine metabolism in *Leishmania*: from arginine to trypanothione. *Amino Acids,* 40**,** 269-85.

CONNELL, N. D., MEDINA-ACOSTA, E., MCMASTER, W. R., BLOOM, B. R. & RUSSELL, D. G. 1993. Effective immunization against cutaneous leishmaniasis with recombinant bacille Calmette-Guerin expressing the *Leishmania* surface proteinase gp63. *Proc Natl Acad Sci U S A,* 90**,** 11473-7.

COURRET, N., FREHEL, C., GOUHIER, N., POUCHELET, M., PRINA, E., ROUX, P. & ANTOINE, J. C. 2002. Biogenesis of *Leishmania*-harbouring parasitophorous vacuoles following phagocytosis of the metacyclic promastigote or amastigote stages of the parasites. *J Cell Sci,* 115**,** 2303-16.

CUMMINGS, H. E., TULADHAR, R. & SATOSKAR, A. R. 2010. Cytokines and their STATs in cutaneous and visceral leishmaniasis. *J Biomed Biotechnol,* 2010**,** 294389.

DALTON, T. P., CHEN, Y., SCHNEIDER, S. N., NEBERT, D. W. & SHERTZER, H. G. 2004. Genetically altered mice to evaluate glutathione homeostasis in health and disease. *Free Radic Biol Med,* 37**,** 1511-26.

DALTON, T. P., DIETER, M. Z., YANG, Y., SHERTZER, H. G. & NEBERT, D. W. 2000. Knockout of the mouse glutamate cysteine ligase catalytic subunit (Gclc) gene: embryonic lethal when homozygous, and proposed model for moderate glutathione deficiency when heterozygous. *Biochem Biophys Res Commun,* 279**,** 324-9.

DANESHVAR, H., COOMBS, G. H., HAGAN, P. & PHILLIPS, R. S. 2003. *Leishmania mexicana* and *Leishmania major*: attenuation of wild-type parasites and vaccination with the attenuated lines. *J Infect Dis,* 187**,** 1662-8.

DANTAS-TORRES, F., SOLANO-GALLEGO, L., BANETH, G., RIBEIRO, V. M., DE PAIVA-CAVALCANTI, M. & OTRANTO, D. 2012. Canine leishmaniosis in the Old and New Worlds: unveiled similarities and differences. *Trends Parasitol,* 28**,** 531-8.

DAS, A. & ALI, N. 2012. Vaccine Development Against *Leishmania donovani*. *Front Immunol,* 3**,** 99.

DAVID, C. V. & CRAFT, N. 2009. Cutaneous and mucocutaneous leishmaniasis. *Dermatol Ther,* 22**,** 491-502.

DAVIES, D. R. & COHEN, G. H. 1996. Interactions of protein antigens with antibodies. *Proc Natl Acad Sci U S A,* 93**,** 7-12.

DAY, M. J. 2011. The immunopathology of canine vector-borne diseases. *Parasit Vectors,* 4**,** 48.

DE OLIVEIRA GOMES, D. C., SCHWEDERSKY, R. P., BARBOSA DE-MELO, L. D., DA SILVA COSTA SOUZA, B. L., DE MATOS GUEDES, H. L., LOPES, U. G. & ROSSI-BERGMANN, B. 2012. Peripheral expression of LACK-mRNA induced by intranasal vaccination with PCI-NEO-LACK defines the protection duration against murine visceral leishmaniasis. *Parasitology,* 139**,** 1562-9.

DEAK, E., JAYAKUMAR, A., CHO, K. W., GOLDSMITH-PESTANA, K., DONDJI, B., LAMBRIS, J. D. & MCMAHON-PRATT, D. 2010. Murine visceral leishmaniasis: IgM and polyclonal B-cell activation lead to disease exacerbation. *Eur J Immunol,* 40**,** 1355-68.

DECUYPERE, S., RIJAL, S., YARDLEY, V., DE DONCKER, S., LAURENT, T., KHANAL, B., CHAPPUIS, F. & DUJARDIN, J. C. 2005. Gene expression analysis of the mechanism of natural Sb(V) resistance in *Leishmania donovani* isolates from Nepal. *Antimicrob Agents Chemother,* 49**,** 4616-21.

DECUYPERE, S., VANAERSCHOT, M., RIJAL, S., YARDLEY, V., MAES, L., DE DONCKER, S., CHAPPUIS, F. & DUJARDIN, J. C. 2008. Gene expression

profiling of *Leishmania (Leishmania) donovani*: overcoming technical variation and exploiting biological variation. *Parasitology,* 135**,** 183-94.
DEPRETER, F., PILCER, G. & AMIGHI, K. 2013. Inhaled proteins: challenges and perspectives. *Int J Pharm,* 447**,** 251-80.
DERMINE, J. F., GOYETTE, G., HOUDE, M., TURCO, S. J. & DESJARDINS, M. 2005. *Leishmania donovani* lipophosphoglycan disrupts phagosome microdomains in J774 macrophages. *Cell Microbiol,* 7**,** 1263-70.
DISIS, M. L., SCHIFFMAN, K., GUTHRIE, K., SALAZAR, L. G., KNUTSON, K. L., GOODELL, V., DELA ROSA, C. & CHEEVER, M. A. 2004. Effect of dose on immune response in patients vaccinated with an her-2/neu intracellular domain protein--based vaccine. *J Clin Oncol,* 22**,** 1916-25.
DO MONTE-NETO, R. L., COELHO, A. C., RAYMOND, F., LEGARE, D., CORBEIL, J., MELO, M. N., FREZARD, F. & OUELLETTE, M. 2011. Gene expression profiling and molecular characterization of antimony resistance in *Leishmania amazonensis. PLoS Negl Trop Dis,* 5**,** e1167.
DOMINGUEZ, M., MORENO, I., AIZPURUA, C. & TORANO, A. 2003. Early mechanisms of *Leishmania* infection in human blood. *Microbes Infect,* 5**,** 507-13.
DORTAY, H., SCHMOCKEL, S. M., FETTKE, J. & MUELLER-ROEBER, B. 2011. Expression of human c-reactive protein in different systems and its purification from *Leishmania tarentolae*. *Protein Expr Purif,* 78**,** 55-60.
DOSTALOVA, A. & VOLF, P. 2012. *Leishmania* development in sand flies: parasite-vector interactions overview. *Parasit Vectors,* 5**,** 276.
DOWLATI, Y., EHSASI, S., SHIDANI, B. & BAHAR, K. 1996. Stepwise safety trial of a killed *Leishmania* vaccine in Iran. *Clin Dermatol,* 14**,** 497-502.
DOWNING, T., IMAMURA, H., DECUYPERE, S., CLARK, T. G., COOMBS, G. H., COTTON, J. A., HILLEY, J. D., DE DONCKER, S., MAES, I., MOTTRAM, J. C., QUAIL, M. A., RIJAL, S., SANDERS, M., SCHONIAN, G., STARK, O., SUNDAR, S., VANAERSCHOT, M., HERTZ-FOWLER, C., DUJARDIN, J. C. & BERRIMAN, M. 2011. Whole genome sequencing of multiple *Leishmania donovani* clinical isolates provides insights into population structure and mechanisms of drug resistance. *Genome Res,* 21**,** 2143-56.
DRAHOTA, J., LIPOLDOVA, M., VOLF, P. & ROHOUSOVA, I. 2009. Specificity of anti-saliva immune response in mice repeatedly bitten by Phlebotomus sergenti. *Parasite Immunol,* 31**,** 766-70.
DUBE, A., GUPTA, R. & SINGH, N. 2009. Reporter genes facilitating discovery of drugs targeting protozoan parasites. *Trends Parasitol,* 25**,** 432-9.
DUCLOS, S. & DESJARDINS, M. 2000. Subversion of a young phagosome: the survival strategies of intracellular pathogens. *Cellular Microbiology,* 2**,** 365-377.
DUTHIE, M. S., RAMAN, V. S., PIAZZA, F. M. & REED, S. G. 2012. The development and clinical evaluation of second-generation leishmaniasis vaccines. *Vaccine,* 30**,** 134-41.
EVANS, K. J. & KEDZIERSKI, L. 2012a. Development of Vaccines against Visceral Leishmaniasis. *J Trop Med,* 2012**,** 892817.

FASSINA, G., RUVO, M., PALOMBO, G., VERDOLIVA, A. & MARINO, M. 2001. Novel ligands for the affinity-chromatographic purification of antibodies. *J Biochem Biophys Methods,* 49**,** 481-90.

FAUNDEZ, M., PINO, L., LETELIER, P., ORTIZ, C., LOPEZ, R., SEGUEL, C., FERREIRA, J., PAVANI, M., MORELLO, A. & MAYA, J. D. 2005. Buthionine sulfoximine increases the toxicity of nifurtimox and benznidazole to *Trypanosoma cruzi*. *Antimicrob Agents Chemother,* 49**,** 126-30.

FERNANDEZ-ROBLEDO, J. A. & VASTA, G. R. 2010. Production of recombinant proteins from protozoan parasites. *Trends Parasitol,* 26**,** 244-54.

FERRO, V. A. & CARTER, K. C. 2006. Mucosal immunisation: Successful approaches to targeting different tissues. *Methods,* 38**,** 61-4.

FILARDY, A. A., PIRES, D. R. & DOSREIS, G. A. 2011. Macrophages and neutrophils cooperate in immune responses to *Leishmania* infection. *Cell Mol Life Sci,* 68**,** 1863-70.

FIORILLO, A., COLOTTI, G., BOFFI, A., BAIOCCO, P. & ILARI, A. 2012. The crystal structures of the tryparedoxin-tryparedoxin peroxidase couple unveil the structural determinants of *Leishmania* detoxification pathway. *PLoS Negl Trop Dis,* 6**,** e1781.

FRAGA, H. 2008. Firefly luminescence: a historical perspective and recent developments. *Photochem Photobiol Sci,* 7**,** 146-58.

FREARSON, J. A., WYATT, P. G., GILBERT, I. H. & FAIRLAMB, A. H. 2007. Target assessment for antiparasitic drug discovery. *Trends Parasitol,* 23**,** 589-95.

FUJKUYAMA Y, TOKUHARA D, KATAOKA K, GILBERT RS, MCGHEE JR, YUKI Y, KIYONO H, FUJIHASHI K. 2012. Novel vaccine development strategies for inducing mucosal immunity. *Expert Rev Vaccines*. 11(3):367-79

GANGULY, S., DAS, N. K., BARBHUIYA, J. N. & CHATTERJEE, M. 2010. Post-kala-azar dermal leishmaniasis--an overview. *Int J Dermatol,* 49**,** 921-31.

GARCIA-HERNANDEZ, R., MANZANO, J. I., CASTANYS, S. & GAMARRO, F. 2012. *Leishmania donovani* develops resistance to drug combinations. *PLoS Negl Trop Dis,* 6**,** e1974.

GEIER, M. R., GEIER, D. A. & ZAHALSKY, A. C. 2003. Influenza vaccination and Guillain Barre syndrome small star, filled. *Clin Immunol,* 107**,** 116-21.

GENG, L., XIN, W., HUANG, D.-W. & FENG, G. 2006. A universal cloning vector using vaccinia topoisomerase I. *Molecular Biotechnology,* 33**,** 23-28.

GHAFFARIFAR, F., JORJANI, O., SHARIFI, Z., DALIMI, A., HASSAN, Z. M., TABATABAIE, F., KHOSHZABAN, F. & HEZARJARIBI, H. Z. 2013. Enhancement of immune response induced by DNA vaccine cocktail expressing complete LACK and TSA genes against *Leishmania major*. *APMIS,* 121**,** 290-8.

GIPP, J. J., CHANG, C. & MULCAHY, R. T. 1992. Cloning and nucleotide sequence of a full-length cDNA for human liver gamma-glutamylcysteine synthetase. *Biochem Biophys Res Commun,* 185**,** 29-35.

GIUDICE, E. L. & CAMPBELL, J. D. 2006. Needle-free vaccine delivery. *Adv Drug Deliv Rev,* 58**,** 68-89.

GNOTH, S., SIMUTIS, R. & LUBBERT, A. 2010. Selective expression of the soluble product fraction in Escherichia coli cultures employed in recombinant protein production processes. *Appl Microbiol Biotechnol,* 87**,** 2047-58.

GOKCE, G., OZSARLAK-SOZER, G., OKTAY, G., KIRKALI, G., JARUGA, P., DIZDAROGLU, M. & KERRY, Z. 2009. Glutathione depletion by buthionine sulfoximine induces oxidative damage to DNA in organs of rabbits *in vivo. Biochemistry,* 48**,** 4980-7.

GOMES, R., OLIVEIRA, F., TEIXEIRA, C., MENESES, C., GILMORE, D. C., ELNAIEM, D. E., KAMHAWI, S. & VALENZUELA, J. G. 2012. Immunity to sand fly salivary protein LJM11 modulates host response to vector-transmitted *leishmania* conferring ulcer-free protection. *J Invest Dermatol,* 132**,** 2735-43.

GONG, P., CEDERBAUM, A. I. & NIETO, N. 2004. Heme oxygenase-1 protects HepG2 cells against cytochrome P450 2E1-dependent toxicity. *Free Radic Biol Med,* 36**,** 307-18.

GOODMAN, W. A., LEVINE, A. D., MASSARI, J. V., SUGIYAMA, H., MCCORMICK, T. S. & COOPER, K. D. 2009. IL-6 signaling in psoriasis prevents immune suppression by regulatory T cells. *J Immunol,* 183**,** 3170-6.

GOONEWARDENE, R., DAILY, J., KASLOW, D., SULLIVAN, T. J., DUFFY, P., CARTER, R., MENDIS, K. & WIRTH, D. 1993. Transfection of the malaria parasite and expression of firefly luciferase. *Proc Natl Acad Sci U S A,* 90**,** 5234-6.

GOTO, H. & LINDOSO, J. A. 2010. Current diagnosis and treatment of cutaneous and mucocutaneous leishmaniasis. *Expert Rev Anti Infect Ther,* 8**,** 419-33.

GRIFFITH, O. W. 1982. Mechanism of action, metabolism, and toxicity of buthionine sulfoximine and its higher homologs, potent inhibitors of glutathione synthesis. *J Biol Chem,* 257**,** 13704-12.

GROSS, S., GAMMON, S. T., MOSS, B. L., RAUCH, D., HARDING, J., HEINECKE, J. W., RATNER, L. & PIWNICA-WORMS, D. 2009. Bioluminescence imaging of myeloperoxidase activity in vivo. *Nat Med,* 15**,** 455-61.

GUPTA, R., KUSHAWAHA, P. K., SAMANT, M., JAISWAL, A. K., BAHARIA, R. K. & DUBE, A. 2012. Treatment of *Leishmania donovani*-infected hamsters with miltefosine: analysis of cytokine mRNA expression by real-time PCR, lymphoproliferation, nitrite production and antibody responses. *J Antimicrob Chemother,* 67**,** 440-3.

HALSTEAD, S. B., MAHALINGAM, S., MAROVICH, M. A., UBOL, S. & MOSSER, D. M. 2010. Intrinsic antibody-dependent enhancement of microbial infection in macrophages: disease regulation by immune complexes. *Lancet Infect Dis,* 10**,** 712-22.

HAMILTON, D., WU, J. H., ALAOUI-JAMALI, M. & BATIST, G. 2003. A novel missense mutation in the gamma-glutamylcysteine synthetase catalytic subunit gene causes both decreased enzymatic activity and glutathione production. *Blood,* 102**,** 725-30.

HANAHAN, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol,* 166**,** 557-80.

HARRIS, P. T., RAGHUNATHAN, K., SPURBECK, R. R., ARVIDSON, C. G. & ARVIDSON, D. N. 2010. Expression, purification, crystallization and preliminary X-ray studies of Lactobacillus jensenii enolase. *Acta Crystallogr Sect F Struct Biol Cryst Commun,* 66**,** 938-40.

HARTINGER, D., HEINL, S., SCHWARTZ, H. E., GRABHERR, R., SCHATZMAYR, G., HALTRICH, D. & MOLL, W. D. 2010. Enhancement of solubility in *Escherichia coli* and purification of an aminotransferase from Sphingopyxis sp. MTA144 for deamination of hydrolyzed fumonisin B(1). *Microb Cell Fact,* 9**,** 62.

HENRIQUEZ, F. L., CAMPBELL, S. A., ROBERTS, C. W., MULLEN, A. B., BURCHMORE, R. & CARTER, K. C. 2010. Vaccination with recombinant *Leishmania donovani* gamma-glutamylcysteine synthetase fusion protein protects against *L. donovani* infection. *J Parasitol,* 96**,** 929-36.

HEZARJARIBI, H. Z., GHAFFARIFAR, F., DALIMI, A., SHARIFI, Z. & JORJANI, O. 2013. Effect of IL-22 on DNA vaccine encoding LACK gene of *Leishmania major* in BALB/c mice. *Exp Parasitol,* 134**,** 341-8.

HIBI, T., NII, H., NAKATSU, T., KIMURA, A., KATO, H., HIRATAKE, J. & ODA, J. 2004. Crystal structure of gamma-glutamylcysteine synthetase: insights into the mechanism of catalysis by a key enzyme for glutathione homeostasis. *Proc Natl Acad Sci U S A,* 101**,** 15052-7.

HOLAKUYEE, M., MAHDAVI, M., MOHAMMAD HASSAN, Z. & ABOLHASSANI, M. 2012. Heat shock proteins enriched-promastigotes of *Leishmania major* inducing Th2 immune response in BALB/c mice. *Iran Biomed J,* 16**,** 209-17.

HOLMGREN, J. & CZERKINSKY, C. 2005. Mucosal immunity and vaccines. *Nat Med,* 11**,** S45-53.

HUANG, C. S., MOORE, W. R. & MEISTER, A. 1988. On the active site thiol of gamma-glutamylcysteine synthetase: relationships to catalysis, inhibition, and regulation. *Proc Natl Acad Sci U S A,* 85**,** 2464-8.

HUGENTOBLER, F., YAM, K. K., GILLARD, J., MAHBUBA, R., OLIVIER, M. & COUSINEAU, B. 2012. Immunization against *Leishmania major* infection using LACK- and IL-12-expressing *Lactococcus lactis* induces delay in footpad swelling. *PLoS One,* 7**,** e30945.

HURISSA, Z., GEBRE-SILASSIE, S., HAILU, W., TEFERA, T., LALLOO, D. G., CUEVAS, L. E. & HAILU, A. 2010. Clinical characteristics and treatment outcome of patients with visceral leishmaniasis and HIV co-infection in northwest Ethiopia. *Trop Med Int Health,* 15**,** 848-55.

HUSSEIN, A. S. & WALTER, R. D. 1995. Purification and characterization of gamma-glutamylcysteine synthetase from Ascaris suum. *Mol Biochem Parasitol,* 72**,** 57-64.

HUYNH, T. T., HUYNH, V. T., HARMON, M. A. & PHILLIPS, M. A. 2003. Gene knockdown of gamma-glutamylcysteine synthetase by RNAi in the parasitic protozoa *Trypanosoma brucei* demonstrates that it is an essential enzyme. *J Biol Chem,* 278**,** 39794-800.

ILG, T., DEMAR, M. & HARBECKE, D. 2001. Phosphoglycan repeat-deficient *Leishmania mexicana* parasites remain infectious to macrophages and mice. *J Biol Chem,* 276**,** 4988-97.

JAAFARI, M. R., GHAFARIAN, A., FARROKH-GISOUR, A., SAMIEI, A., KHEIRI, M. T., MAHBOUDI, F., BARKHORDARI, F., KHAMESIPOUR, A. & MCMASTER, W. R. 2006. Immune response and protection assay of recombinant major surface glycoprotein of *Leishmania* (rgp63) reconstituted with liposomes in BALB/c mice. *Vaccine,* 24**,** 5708-17.

JING, L., CHEN, L., DU,L., and MINGONG,L., 2013. Cage the firefly lucerferin a strategey of developing bioluminescence probe.*Chem. Soc. Rev*.42, 662-676.

KAPOOR, P., SACHDEV, M. & MADHUBALA, R. 2000. Inhibition of glutathione synthesis as a chemotherapeutic strategy for leishmaniasis. *Trop Med Int Health,* 5**,** 438-42.

KAUR, T., SOBTI, R. C. & KAUR, S. 2011. Cocktail of gp63 and Hsp70 induces protection against *Leishmania donovani* in BALB/c mice. *Parasite Immunol,* 33**,** 95-103.

KAZI, K. M., MANDAL, A. S., BISWAS, N., GUHA, A., CHATTERJEE, S., BEHERA, M. & KUOTSU, K. 2010. Niosome: A future of targeted drug delivery systems. *J Adv Pharm Technol Res,* 1**,** 374-80.

KEDZIERSKI, L. 2010. Leishmaniasis Vaccine: Where are We Today? *J Glob Infect Dis,* 2**,** 177-85.

KEDZIERSKI, L. 2011. Leishmaniasis. *Hum Vaccin,* 7**,** 1204-14.

KELLINA, O. I. & STRELKOVA, M. V. 2010. [Investigations on leishmaniases at the E.I. Martsinovsky Institute of Medical Parasitology and Tropical Medicine]. *Med Parazitol (Mosk)***,** 19-22.

KELLY, B. S., ANTHOLINE, W. E. & GRIFFITH, O. W. 2002. *Escherichia coli* gamma-glutamylcysteine synthetase. Two active site metal ions affect substrate and inhibitor binding. *J Biol Chem,* 277**,** 50-8.

KIMURA, A. & KISHIMOTO, T. 2010. IL-6: regulator of Treg/Th17 balance. *Eur J Immunol,* 40**,** 1830-5.

KIRKPATRICK, C. E., NOLAN, T. J. & FARRELL, J. P. 1987. Rate of *Leishmania*-induced skin-lesion development in rodents depends on the site of inoculation. *Parasitology,* 94 (Pt 3)**,** 451-65.

KOBETS, T., GREKOV, I. & LIPOLDOVA, M. 2012. Leishmaniasis: prevention, parasite detection and treatment. *Curr Med Chem,* 19**,** 1443-74.

KORF, U., KOHL, T., VAN DER ZANDT, H., ZAHN, R., SCHLEEGER, S., UEBERLE, B., WANDSCHNEIDER, S., BECHTEL, S., SCHNOLZER, M., OTTLEBEN, H., WIEMANN, S. & POUSTKA, A. 2005. Large-scale protein expression for proteome research. *Proteomics,* 5**,** 3571-80.

KRAUTH-SIEGEL, R. L. & COMINI, M. A. 2008. Redox control in trypanosomatids, parasitic protozoa with trypanothione-based thiol metabolism. *Biochim Biophys Acta,* 1780**,** 1236-48.

KRISTENSEN, D. & CHEN, D. 2013. Strategies to advance vaccine technologies for resource-poor settings. *Vaccine,* 31 Suppl 2**,** B157-62.

KUMAR, D., SINGH, R., BHANDARI, V., KULSHRESTHA, A., NEGI, N. S. & SALOTRA, P. 2012. Biomarkers of antimony resistance: need for expression

analysis of multiple genes to distinguish resistance phenotype in clinical isolates of *Leishmania donovani*. *Parasitol Res,* 111**,** 223-30.

KUMAR, G. P., RAJESHWARRAO, POGAKU 2011. Nonionic surfactant vesicular systems for effective drug delivery—an overview. *Acta Pharmaceutica Sinica B,* 1**,** 208-219.

KUSHAWAHA, P. K., GUPTA, R., SUNDAR, S., SAHASRABUDDHE, A. A. & DUBE, A. 2011. Elongation factor-2, a Th1 stimulatory protein of *Leishmania donovani*, generates strong IFN-gamma and IL-12 response in cured *Leishmania*-infected patients/hamsters and protects hamsters against *Leishmania* challenge. *J Immunol,* 187**,** 6417-27.

LAMBERT, P. H. & LAURENT, P. E. 2008. Intradermal vaccine delivery: will new delivery systems transform vaccine administration? *Vaccine,* 26**,** 3197-208.

LANG, T., GOYARD, S., LEBASTARD, M. & MILON, G. 2005. Bioluminescent *Leishmania* expressing luciferase for rapid and high throughput screening of drugs acting on amastigote-harbouring macrophages and for quantitative real-time monitoring of parasitism features in living mice. *Cell Microbiol,* 7**,** 383-92.

LARSON, E. M., DOUGHMAN, D. J., GREGERSON, D. S. & OBRITSCH, W. F. 1997. A new, simple, nonradioactive, nontoxic in vitro assay to monitor corneal endothelial cell viability. *Invest Ophthalmol Vis Sci,* 38**,** 1929-33.

LEBOWITZ, J. H., SMITH, H. Q., RUSCHE, L. & BEVERLEY, S. M. 1993. Coupling of poly(A) site selection and trans-splicing in *Leishmania*. *Genes Dev,* 7**,** 996-1007.

LEE, H. C., BUTLER, M. & WU, S. C. 2012. Using recombinant DNA technology for the development of live-attenuated dengue vaccines. *Enzyme Microb Technol,* 51**,** 67-72.

LEW, J. 2008. Bioluminescence the first 300 years. *Journal of Siberian Federal University, Biology* 3,149-205.

LEWIS-WAMBI, J. S., SWABY, R., KIM, H. & JORDAN, V. C. 2009. Potential of l-buthionine sulfoximine to enhance the apoptotic action of estradiol to reverse acquired antihormonal resistance in metastatic breast cancer. *J Steroid Biochem Mol Biol,* 114**,** 33-9.

LIAVE, E. LECOEUR, H. BESSE, A. PRINA, G. LANG, T. 2011. A combined luciferase imaging and reverse transcription polymerase chain reaction assay for the study of *Leishmania* amastigote burden and correlated mouse tissue transcript fluctuations. *Cellular Microbiology,* 13(1) 81-91

LI, Y. 2011. Recombinant production of antimicrobial peptides in *Escherichia coli*: a review. *Protein Expr Purif,* 80**,** 260-7.

LIU, H. F., MA, J., WINTER, C. & BAYER, R. 2010. Recovery and purification process development for monoclonal antibody production. *MAbs,* 2**,** 480-99.

LIU, Y., DORING, J. & HUREK, T. 2012. Bias in topoisomerase (TOPO)-cloning of multitemplate PCR products using locked nucleic acid (LNA)-substituted primers. *J Microbiol Methods,* 91**,** 483-6.

LLAMBRICH, A., ZABALLOS, P., TERRASA, F., TORNE, I., PUIG, S. & MALVEHY, J. 2009. Dermoscopy of cutaneous leishmaniasis. *Br J Dermatol,* 160**,** 756-61.

LU, D. 2012. Recent development in aerosol devices for pulmonary vaccine delivery. *Beijing Da Xue Xue Bao,* 44**,** 683-7.

LU, S. C. 2009. Regulation of glutathione synthesis. *Mol Aspects Med,* 30**,** 42-59.

LUEDER, D. V. & PHILLIPS, M. A. 1996. Characterization of *Trypanosoma brucei* gamma-glutamylcysteine synthetase, an essential enzyme in the biosynthesis of trypanothione (diglutathionylspermidine). *J Biol Chem,* 271**,** 17485-90.

LUERSEN, K., WALTER, R. D. & MULLER, S. 2000. *Plasmodium falciparum*-infected red blood cells depend on a functional glutathione de novo synthesis attributable to an enhanced loss of glutathione. *Biochem J,* 346 Pt 2**,** 545-52.

MAKINO, T., SKRETAS, G. & GEORGIOU, G. 2011. Strain engineering for improved expression of recombinant proteins in bacteria. *Microb Cell Fact,* 10**,** 32.

MANNING, M. C., CHOU, D. K., MURPHY, B. M., PAYNE, R. W. & KATAYAMA, D. S. 2010. Stability of protein pharmaceuticals: an update. *Pharm Res,* 27**,** 544-75.

MANTA, B., COMINI, M., MEDEIROS, A., HUGO, M., TRUJILLO, M. & RADI, R. 2013. Trypanothione: a unique bis-glutathionyl derivative in trypanosomatids. *Biochim Biophys Acta,* 1830**,** 3199-216.

MAZUMDER, S., MAJI, M. & ALI, N. 2011. Potentiating effects of MPL on DSPC bearing cationic liposomes promote recombinant GP63 vaccine efficacy: high immunogenicity and protection. *PLoS Negl Trop Dis,* 5**,** e1429.

MCFARLANE, E., CARTER, K. C., MCKENZIE, A. N., KAYE, P. M., BROMBACHER, F. & ALEXANDER, J. 2011. Endogenous IL-13 plays a crucial role in liver granuloma maturation during *Leishmania donovani* infection, independent of IL-4Ralpha-responsive macrophages and neutrophils. *J Infect Dis,* 204**,** 36-43.

MCGWIRE, B. S. & SATOSKAR, A. R. 2013. Leishmaniasis: clinical syndromes and treatment. *QJM*.

MCMAHON-PRATT, D. & ALEXANDER, J. 2004. Does the *Leishmania major* paradigm of pathogenesis and protection hold for New World cutaneous leishmaniases or the visceral disease? *Immunol Rev,* 201**,** 206-24.

MEITIN, C. A., BENDER, B. S. & SMALL, P. A., JR. 1994. Enteric immunization of mice against influenza with recombinant vaccinia. *Proc Natl Acad Sci U S A,* 91**,** 11187-91.

MELBY, P. C., YANG, J., ZHAO, W., PEREZ, L. E. & CHENG, J. 2001. *Leishmania donovani* p36(LACK) DNA vaccine is highly immunogenic but not protective against experimental visceral leishmaniasis. *Infect Immun,* 69**,** 4719-25.

MEYER, S., LORENZ, C., BASER, B., WORDEHOFF, M., JAGER, V. & VAN DEN HEUVEL, J. 2013. Multi-host expression system for recombinant production of challenging proteins. *plos one,* 8**,** e68674.

MICHEL, G., FERRUA, B., LANG, T., MADDUGODA, M. P., MUNRO, P., POMARES, C., LEMICHEZ, E. & MARTY, P. 2011. Luciferase-expressing *Leishmania infantum* allows the monitoring of amastigote population size, in vivo, ex vivo and in vitro. *PLoS Negl Trop Dis,* 5**,** e1323.

MILLINGTON, O. R., MYBURGH, E., MOTTRAM, J. C. & ALEXANDER, J. 2010. Imaging of the host/parasite interplay in cutaneous leishmaniasis. *Exp Parasitol,* 126**,** 310-7.

MINNE, A., LOUAHED, J., MEHAUDEN, S., BARAS, B., RENAULD, J. C. & VANBEVER, R. 2007. The delivery site of a monovalent influenza vaccine within the respiratory tract impacts on the immune response. *Immunology,* 122**,** 316-25.

MISRA, A., GANGA, S. & UPADHYAY, P. 1999. Needle-free, non-adjuvanted skin immunization by electroporation-enhanced transdermal delivery of diphtheria toxoid and a candidate peptide vaccine against hepatitis B virus. *Vaccine,* 18**,** 517-23.

MISRA, I. & GRIFFITH, O. W. 1998. Expression and purification of human gamma-glutamylcysteine synthetase. *Protein Expr Purif,* 13**,** 268-76.

MISSLITZ, A., J.C. MOTTRA, P. OVERATH, T. AEBISCHER, TARGETED integration into a rRNA locus results in uniform and high level expression of transgenes in *Leishmania* amastigotes, *Mol. Biochem. Parasitol.* 107 (2000) 251–261

MODABBER, F. 2010. Leishmaniasis vaccines: past, present and future. *Int J Antimicrob Agents,* 36 Suppl 1**,** S58-61.

MONDAL, D., HUDA, M. M., KARMOKER, M. K., GHOSH, D., MATLASHEWSKI, G., NABI, S. G. & KROEGER, A. 2013. Reducing visceral leishmaniasis by insecticide impregnation of bed-nets, Bangladesh. *Emerg Infect Dis,* 19**,** 1131-4.

MONDAL, S., BHATTACHARYA, P. & ALI, N. 2010. Current diagnosis and treatment of visceral leishmaniasis. *Expert Rev Anti Infect Ther,* 8**,** 919-44.

MOORE, J. W., MOYO, D., BEATTIE, L., ANDREWS, P. S., TIMMIS, J. & KAYE, P. M. 2013. Functional complexity of the *Leishmania* granuloma and the potential of in silico modeling. *Front Immunol,* 4**,** 35.

MORADIN, N. & DESCOTEAUX, A. 2012. *Leishmania* promastigotes: building a safe niche within macrophages. *Front Cell Infect Microbiol,* 2**,** 121.

MOREIRA, D., SANTAREM, N., LOUREIRO, I., TAVARES, J., SILVA, A. M., AMORIM, A. M., OUAISSI, A., CORDEIRO-DA-SILVA, A. & SILVESTRE, R. 2012. Impact of continuous axenic cultivation in *Leishmania infantum* virulence. *PLoS Negl Trop Dis,* 6**,** e1469.

MORRIS, D., KHURASANY, M., NGUYEN, T., KIM, J., GUILFORD, F., MEHTA, R., GRAY, D., SAVIOLA, B. & VENKETARAMAN, V. 2013. Glutathione and infection. *Biochim Biophys Acta,* 1830**,** 3329-49.

MOUGNEAU, E., BIHL, F. & GLAICHENHAUS, N. 2011. Cell biology and immunology of *Leishmania*. *Immunol Rev,* 240**,** 286-96.

MUDIGONDA, P., MUDIGONDA, T., FENERAN, A. N., ALAMDARI, H. S., SANDOVAL, L. & FELDMAN, S. R. 2012. Interleukin-23 and interleukin-17: importance in pathogenesis and therapy of psoriasis. *Dermatol Online J,* 18**,** 1.

MUKHERJEE, A., PADMANABHAN, P. K., SINGH, S., ROY, G., GIRARD, I., CHATTERJEE, M., OUELLETTE, M. & MADHUBALA, R. 2007. Role of ABC transporter MRPA, gamma-glutamylcysteine synthetase and ornithine

decarboxylase in natural antimony-resistant isolates of *Leishmania donovani*. *J Antimicrob Chemother,* 59**,** 204-11.

MUKHERJEE, A., ROY, G., GUIMOND, C. & OUELLETTE, M. 2009. The gamma-glutamylcysteine synthetase gene of *Leishmania* is essential and involved in response to oxidants. *Mol Microbiol,* 74**,** 914-27.

MUTISO, J. M., MACHARIA, J. C. & GICHERU, M. M. 2010. A review of adjuvants for *Leishmania* vaccine candidates. *J Biomed Res,* 24**,** 16-25.

MUTISO, J. M., MACHARIA, J. C., KIIO, M. N., ICHAGICHU, J. M., RIKOI, H. & GICHERU, M. M. 2013. Development of *Leishmania* vaccines: predicting the future from past and present experience. *J Biomed Res,* 27**,** 85-102..

MUTISO M. J., KARIUKI TM, GICHERU MM 2009. Montanide ISA 720 is more effective than BCG as an adjuvant for *Leishmania* killed vaccine in BALB/c mice. *IJIB,* 7**,** 107–116.

MYLER, P. J. & STUART, K. D. 2000. Recent developments from the *Leishmania* genome project. *Curr Opin Microbiol,* 3**,** 412-6.

MYLONAS, K. J., NAIR, M. G., PRIETO-LAFUENTE, L., PAAPE, D. & ALLEN, J. E. 2009. Alternatively activated macrophages elicited by helminth infection can be reprogrammed to enable microbial killing. *J Immunol,* 182**,** 3084-94.

NABORS, G. S. & FARRELL, J. P. 1994. Site-specific immunity to *Leishmania major* in SWR mice: the site of infection influences susceptibility and expression of the antileishmanial immune response. *Infect Immun,* 62**,** 3655-62.

NAGILL, R. & KAUR, S. 2011. Vaccine candidates for leishmaniasis: a review. *Int Immunopharmacol,* 11**,** 1464-88.

NAGILL, R., MAHAJAN, R., SHARMA, M. & KAUR, S. 2009. Induction of cellular and humoral responses by autoclaved and heat-killed antigen of *Leishmania donovani* in experimental visceral leishmaniasis. *Parasitol Int,* 58**,** 359-66.

NASCIMENTO, I. P. & LEITE, L. C. 2012. Recombinant vaccines and the development of new vaccine strategies. *Braz J Med Biol Res,* 45**,** 1102-11.

NEMBRINI, C., STANO, A., DANE, K. Y., BALLESTER, M., VAN DER VLIES, A. J., MARSLAND, B. J., SWARTZ, M. A. & HUBBELL, J. A. 2011. Nanoparticle conjugation of antigen enhances cytotoxic T-cell responses in pulmonary vaccination. *Proc Natl Acad Sci U S A,* 108**,** E989-97.

NEUBER, H. 2008. Leishmaniasis. *J Dtsch Dermatol Ges,* 6**,** 754-65.

NIETO, A., DOMINGUEZ-BERNAL, G., ORDEN, J. A., DE LA FUENTE, R., MADRID-ELENA, N. & CARRION, J. 2011. Mechanisms of resistance and susceptibility to experimental visceral leishmaniasis: BALB/c mouse versus syrian hamster model. *Vet Res,* 42**,** 39.

NOVAGEN 2003. pET System Manual. Novagen, uk.

NYLEN, S., D. SACKS. 2007. Interleukin-10 and the pathogenesis of human visceral leishmaniasis. *Trends Immunol.* **28**: 378–3

NYLEN, S. & EIDSMO, L. 2012. Tissue damage and immunity in cutaneous leishmaniasis. *Parasite Immunol,* 34**,** 551-61.

NYLEN, S. & GAUTAM, S. 2010. Immunological perspectives of leishmaniasis. *J Glob Infect Dis,* 2**,** 135-46.

O'DWYER, P. J., HAMILTON, T. C., LACRETA, F. P., GALLO, J. M., KILPATRICK, D., HALBHERR, T., BRENNAN, J., BOOKMAN, M. A., HOFFMAN, J., YOUNG, R. C., COMIS, R. L. & OZOLS, R. F. 1996. Phase I trial of buthionine sulfoximine in combination with melphalan in patients with cancer. *J Clin Oncol,* 14**,** 249-56.

OHTAKE, Y. & YABUUCHI, S. 1991. Molecular cloning of the gamma-glutamylcysteine synthetase gene of Saccharomyces cerevisiae. *Yeast,* 7**,** 953-61.

OKWOR, I., KURIAKOSE, S. & UZONNA, J. 2010. Repeated inoculation of killed *Leishmania major* induces durable immune response that protects mice against virulent challenge. *Vaccine,* 28**,** 5451-7.

OKWOR, I., MOU, Z., LIU, D. & UZONNA, J. 2012. Protective immunity and vaccination against cutaneous leishmaniasis. *Front Immunol,* 3**,** 128.

OKWOR, I. & UZONNA, J. E. 2013. The immunology of *Leishmania*/HIV co-infection. *Immunol Res,* 56**,** 163-71.

OLIN-SANDOVAL, V., GONZALEZ-CHAVEZ, Z., BERZUNZA-CRUZ, M., MARTINEZ, I., JASSO-CHAVEZ, R., BECKER, I., ESPINOZA, B., MORENO-SANCHEZ, R. & SAAVEDRA, E. 2012. Drug target validation of the trypanothione pathway enzymes through metabolic modelling. *FEBS J,* 279**,** 1811-33.

OLIVIER, M., ATAYDE, V. D., ISNARD, A., HASSANI, K. & SHIO, M. T. 2012. *Leishmania* virulence factors: focus on the metalloprotease GP63. *Microbes Infect,* 14**,** 1377-89.

OLOBO, J. O., ANJILI, C. O., GICHERU, M. M., MBATI, P. A., KARIUKI, T. M., GITHURE, J. I., KOECH, D. K. & MCMASTER, W. R. 1995. Vaccination of vervet monkeys against cutaneous leishmaniosis using recombinant *Leishmania 'major* surface glycoprotein' (gp63). *Vet Parasitol,* 60**,** 199-212.

OLSEN, S. 2006. Applications of isothermal titration calorimetry to measure enzyme kinetics and activity in complex solutions. *Thermochimica Acta,* 448**,,** 12–18.

OPPENHEIMER, L., WELLNER, V. P., GRIFFITH, O. W. & MEISTER, A. 1979. Glutathione synthetase. Purification from rat kidney and mapping of the substrate binding sites. *Journal of Biological Chemistry,* 254**,** 5184-90.

OSORIO, Y., MELBY, P. C., PIRMEZ, C., CHANDRASEKAR, B., GUARIN, N. & TRAVI, B. L. 2003. The site of cutaneous infection influences the immunological response and clinical outcome of hamsters infected with *Leishmania panamensis*. *Parasite Immunol,* 25**,** 139-48.

OTRANTO, D. & DANTAS-TORRES, F. 2013. The prevention of canine leishmaniasis and its impact on public health. *Trends Parasitol,* 29**,** 339-45.

PAPPALARDO, F., PENNISI, M., CASTIGLIONE, F. & MOTTA, S. 2010. Vaccine protocols optimization: in silico experiences. *Biotechnol Adv,* 28**,** 82-93.

PATTON, J. S. & BYRON, P. R. 2007. Inhaling medicines: delivering drugs to the body through the lungs. *Nat Rev Drug Discov,* 6**,** 67-74.

PAVLI, A. & MALTEZOU, H. C. 2010. Leishmaniasis, an emerging infection in travelers. *Int J Infect Dis,* 14**,** e1032-9.

PEDELACQ, J. D., NGUYEN, H. B., CABANTOUS, S., MARK, B. L., LISTWAN, P., BELL, C., FRIEDLAND, N., LOCKARD, M., FAILLE, A., MOUREY, L., TERWILLIGER, T. C. & WALDO, G. S. 2011. Experimental mapping of soluble protein domains using a hierarchical approach. *Nucleic Acids Res,* 39**,** e125.

PENNINCKX, M. J. 2002. An overview on glutathione in Saccharomyces versus non-conventional yeasts. *FEMS Yeast Res,* 2**,** 295-305.

PERROT, S., DUTERTRE-CATELLA, H., MARTIN, C., RAT, P. & WARNET, J. M. 2003. Resazurin metabolism assay is a new sensitive alternative test in isolated pig cornea. *Toxicol Sci,* 72**,** 122-9.

PERRY, K. & AGABIAN, N. 1991. mRNA processing in the Trypanosomatidae. *Experientia,* 47**,** 118-28.

PETERNEL, S., GRDADOLNIK, J., GABERC-POREKAR, V. & KOMEL, R. 2008. Engineering inclusion bodies for non denaturing extraction of functional proteins. *Microb Cell Fact,* 7**,** 34.

PETERSEN, C. A. 2009. Leishmaniasis, an emerging disease found in companion animals in the United States. *Top Companion Anim Med,* 24**,** 182-8.

PETRITUS, P. M., MANZONI-DE-ALMEIDA, D., GIMBLET, C., GONZALEZ LOMBANA, C. & SCOTT, P. 2012. *Leishmania mexicana* induces limited recruitment and activation of monocytes and monocyte-derived dendritic cells early during infection. *PLoS Negl Trop Dis,* 6**,** e1858.

PHAM, N. K., MOURIZ, J. & KIMA, P. E. 2005. *Leishmania pifanoi* amastigotes avoid macrophage production of superoxide by inducing heme degradation. *Infect Immun,* 73**,** 8322-33.

PICADO, A., DASH, A. P., BHATTACHARYA, S. & BOELAERT, M. 2012. Vector control interventions for visceral leishmaniasis elimination initiative in South Asia, 2005-2010. *Indian J Med Res,* 136**,** 22-31.

PICADO, A., SINGH, S. P., RIJAL, S., SUNDAR, S., OSTYN, B., CHAPPUIS, F., URANW, S., GIDWANI, K., KHANAL, B., RAI, M., PAUDEL, I. S., DAS, M. L., KUMAR, R., SRIVASTAVA, P., DUJARDIN, J. C., VANLERBERGHE, V., ANDERSEN, E. W., DAVIES, C. R. & BOELAERT, M. 2010. Longlasting insecticidal nets for prevention of *Leishmania donovani* infection in India and Nepal: paired cluster randomised trial. *BMJ,* 341**,** c6760.

PIGNON, J. P., TRIBODET, H., SCAGLIOTTI, G. V., DOUILLARD, J. Y., SHEPHERD, F. A., STEPHENS, R. J., DUNANT, A., TORRI, V., ROSELL, R., SEYMOUR, L., SPIRO, S. G., ROLLAND, E., FOSSATI, R., AUBERT, D., DING, K., WALLER, D., LE CHEVALIER, T. & GROUP, L. C. 2008. Lung adjuvant cisplatin evaluation: a pooled analysis by the LACE Collaborative Group. *J Clin Oncol,* 26**,** 3552-9.

PINTO, E. F., PINHEIRO, R. O., RAYOL, A., LARRAGA, V. & ROSSI-BERGMANN, B. 2004. Intranasal vaccination against cutaneous leishmaniasis with a particulated leishmanial antigen or DNA encoding LACK. *Infect Immun,* 72**,** 4521-7.

PORROZZI, R., TEVA, A., AMARAL, V. F., SANTOS DA COSTA, M. V. & GRIMALDI, G., JR. 2004. Cross-immunity experiments between different

species or strains of *Leishmania* in rhesus macaques (Macaca mulatta). *Am J Trop Med Hyg,* 71**,** 297-305.

PRAJEETH, C. K., HAEBERLEIN, S., SEBALD, H., SCHLEICHER, U. & BOGDAN, C. 2011. *Leishmania*-infected macrophages are targets of NK cell-derived cytokines but not of NK cell cytotoxicity. *Infect Immun,* 79**,** 2699-708.

PRINZ, B., SCHULTCHEN, J., RYDZEWSKI, R., HOLZ, C., BOETTNER, M., STAHL, U. & LANG, C. 2004. Establishing a versatile fermentation and purification procedure for human proteins expressed in the yeasts Saccharomyces cerevisiae and Pichia pastoris for structural genomics. *J Struct Funct Genomics,* 5**,** 29-44.

PRUSS-USTUN, A., RAPITI, E. & HUTIN, Y. 2005. Estimation of the global burden of disease attributable to contaminated sharps injuries among health-care workers. *Am J Ind Med,* 48**,** 482-90.

PUSHKO, P., KORT, T., NATHAN, M., PEARCE, M. B., SMITH, G. & TUMPEY, T. M. 2010. Recombinant H1N1 virus-like particle vaccine elicits protective immunity in ferrets against the 2009 pandemic H1N1 influenza virus. *Vaccine,* 28**,** 4771-6.

RAGHAVA, S., BARUA, B., SINGH, P. K., DAS, M., MADAN, L., BHATTACHARYYA, S., BAJAJ, K., GOPAL, B., VARADARAJAN, R. & GUPTA, M. N. 2008. Refolding and simultaneous purification by three-phase partitioning of recombinant proteins from inclusion bodies. *Protein Sci,* 17**,** 1987-97.

RAMAMOORTHY, R., DONELSON, J. E. & WILSON, M. E. 1996. 5' sequences essential for trans-splicing of msp (gp63) RNAs in *Leishmania chagasi*. *Mol Biochem Parasitol,* 77**,** 65-76.

RAMIREZ, L., SANTOS, D. M., SOUZA, A. P., COELHO, E. A., BARRAL, A., ALONSO, C., ESCUTIA, M. R., BONAY, P., DE OLIVEIRA, C. I. & SOTO, M. 2013. Evaluation of immune responses and analysis of the effect of vaccination of the *Leishmania major* recombinant ribosomal proteins L3 or L5 in two different murine models of cutaneous leishmaniasis. *Vaccine,* 31**,** 1312-9.

RATHBUN, W. B., SCHMIDT, A. J. & HOLLESCHAU, A. M. 1993. Activity loss of glutathione synthesis enzymes associated with human subcapsular cataract. *Invest Ophthalmol Vis Sci,* 34**,** 2049-54.

RAVINDER, BHASKAR, GANGWAR, S. & GOYAL, N. 2012. Development of luciferase expressing *Leishmania donovani* axenic amastigotes as primary model for in vitro screening of antileishmanial compounds. *Curr Microbiol,* 65**,** 696-700.

REED, S. G., BERTHOLET, S., COLER, R. N. & FRIEDE, M. 2009. New horizons in adjuvants for vaccine development. *Trends Immunol,* 30**,** 23-32.

REIMAO, J. Q., TRINCONI, C. T., YOKOYAMA-YASUNAKA, J. K., MIGUEL, D. C., KALIL, S. P. & ULIANA, S. R. 2013. Parasite burden in *Leishmania* (*Leishmania) amazonensis*-infected mice: validation of luciferase as a quantitative tool. *J Microbiol Methods,* 93**,** 95-101.

REZVAN, H., REES, R. & ALI, S. 2011. *Leishmania mexicana* Gp63 cDNA Using Gene Gun Induced Higher Immunity to *L. mexicana* Infection Compared to Soluble *Leishmania* Antigen in BALB/C. *Iran J Parasitol,* 6**,** 60-75.

RIBEIRO-GOMES, F. L. & SACKS, D. 2012. The influence of early neutrophil-*Leishmania* interactions on the host immune response to infection. *Front Cell Infect Microbiol,* 2**,** 59.

ROBERTSON, C. A. 2013. The science of vaccination: establishing safety and efficacy. *S D Med,* Spec no**,** 38-45.

ROHOUSOVA, I., HOSTOMSKA, J., VLKOVA, M., KOBETS, T., LIPOLDOVA, M. & VOLF, P. 2011. The protective effect against *Leishmania* infection conferred by sand fly bites is limited to short-term exposure. *Int J Parasitol,* 41**,** 481-5.

ROMAO, P. R., FONSECA, S. G., HOTHERSALL, J. S., NORONHA-DUTRA, A. A., FERREIRA, S. H. & CUNHA, F. Q. 1999. Glutathione protects macrophages and *Leishmania major* against nitric oxide-mediated cytotoxicity. *Parasitology,* 118 (Pt 6)**,** 559-66.

ROMAO, P. R., TOVAR, J., FONSECA, S. G., MORAES, R. H., CRUZ, A. K., HOTHERSALL, J. S., NORONHA-DUTRA, A. A., FERREIRA, S. H. & CUNHA, F. Q. 2006a. Glutathione and the redox control system trypanothione/trypanothione reductase are involved in the protection of *Leishmania spp*. against nitrosothiol-induced cytotoxicity. *Braz J Med Biol Res,* 39**,** 355-63.

ROMAO, P. R., TOVAR, J., FONSECA, S. G., MORAES, R. H., CRUZ, A. K., HOTHERSALL, J. S., NORONHA-DUTRA, A. A., FERREIRA, S. H. & CUNHA, F. Q. 2006b. Glutathione and the redox control system trypanothione/trypanothione reductase are involved in the protection of *Leishmania spp*. against nitrosothiol-induced cytotoxicity. *Braz J Med Biol Res,* 39**,** 355-63.

ROSAS, L. E., KEISER, T., BARBI, J., SATOSKAR, A. A., SEPTER, A., KACZMAREK, J., LEZAMA-DAVILA, C. M. & SATOSKAR, A. R. 2005. Genetic background influences immune responses and disease outcome of cutaneous *L. mexicana* infection in mice. *Int Immunol,* 17**,** 1347-57

ROSTAN, O., GANGNEUX, J. P., PIQUET-PELLORCE, C., MANUEL, C., MCKENZIE, A. N., GUIGUEN, C., SAMSON, M. & ROBERT-GANGNEUX, F. 2013. The IL-33/ST2 Axis Is Associated with Human Visceral Leishmaniasis and Suppresses Th1 Responses in the Livers of BALB/c Mice Infected with *Leishmania donovani*. *MBio,* 4.

ROY, G., DUMAS, C., SERENO, D., WU, Y., SINGH, A. K., TREMBLAY, M. J., OUELLETTE, M., OLIVIER, M. & PAPADOPOULOU, B. 2000. Episomal and stable expression of the luciferase reporter gene for quantifying *Leishmania spp.* infections in macrophages and in animal models. *Mol Biochem Parasitol,* 110**,** 195-206.

RYAN, J. R., SMITHYMAN, A. M., RAJASEKARIAH, G. H., HOCHBERG, L., STITELER, J. M. & MARTIN, S. K. 2002. Enzyme-linked immunosorbent assay based on soluble promastigote antigen detects immunoglobulin M (IgM)

and IgG antibodies in sera from cases of visceral and cutaneous leishmaniasis. *J Clin Microbiol,* 40**,** 1037-43.

SACHDEVA, R., BANERJEA, A. C., MALLA, N. & DUBEY, M. L. 2009. Immunogenicity and efficacy of single antigen Gp63, polytope and polytopeHSP70 DNA vaccines against visceral Leishmaniasis in experimental mouse model. *PLoS One,* 4**,** e7880.

SADIKOT, R. T. & BLACKWELL, T. S. 2005. Bioluminescence imaging. *Proc Am Thorac Soc,* 2**,** 537-40, 511-2.

SAFAIYAN, S., BOLHASSANI, A., NYLEN, S., AKUFFO, H. & RAFATI, S. 2011. Contribution of human neutrophils in the development of protective immune response during *in vitro Leishmania major* infection. *Parasite Immunol,* 33**,** 609-20.

SAKAI, S., TAKASHIMA, Y., MATSUMOTO, Y., REED, S. G. & HAYASHI, Y. 2010a. Intranasal immunization with Leish-111f induces IFN-gamma production and protects mice from *Leishmania major* infection. *Vaccine,* 28**,** 2207-13.

SAKAI, S., TAKASHIMA, Y., MATSUMOTO, Y., REED, S. G. & HAYASHI, Y. 2010b. Intranasal immunization with Leish-111f induces IFN-gamma production and protects mice from *Leishmania major* infection. *Vaccine,* 28**,** 2207-13.

SANTOS, D. M., CARNEIRO, M. W., DE MOURA, T. R., FUKUTANI, K., CLARENCIO, J., SOTO, M., ESPUELAS, S., BRODSKYN, C., BARRAL, A., BARRAL-NETTO, M. & DE OLIVEIRA, C. I. 2012. Towards development of novel immunization strategies against leishmaniasis using PLGA nanoparticles loaded with kinetoplastid membrane protein-11. *Int J Nanomedicine,* 7**,** 2115-27.

SARKAR, A., AGA, E., BUSSMEYER, U., BHATTACHARYYA, A., MOLLER, S., HELLBERG, L., BEHNEN, M., SOLBACH, W. & LASKAY, T. 2013. Infection of neutrophil granulocytes with *Leishmania major* activates ERK 1/2 and modulates multiple apoptotic pathways to inhibit apoptosis. *Med Microbiol Immunol,* 202**,** 25-35.

SAUERBORN, M., BRINKS, V., JISKOOT, W. & SCHELLEKENS, H. 2010. Immunological mechanism underlying the immune response to recombinant human protein therapeutics. *Trends Pharmacol Sci,* 31**,** 53-9.

SCHEIN, C. H. 1989. Production of Soluble Recombinant Proteins in Bacteria. *Nat Biotech,* 7**,** 1141 - 1149.

SELVAPANDIYAN, A., DEY, R., GANNAVARAM, S., LAKHAL-NAOUAR, I., DUNCAN, R., SALOTRA, P. & NAKHASI, H. L. 2012. Immunity to visceral leishmaniasis using genetically defined live-attenuated parasites. *J Trop Med,* 2012**,** 631460.

SELVAPANDIYAN, A., DEY, R., NYLEN, S., DUNCAN, R., SACKS, D. & NAKHASI, H. L. 2009. Intracellular replication-deficient *Leishmania donovani* induces long lasting protective immunity against visceral leishmaniasis. *J Immunol,* 183**,** 1813-20.

SERENO, D., ROY, G., LEMESRE, J. L., PAPADOPOULOU, B. & OUELLETTE, M. 2001. DNA transformation of *Leishmania infantum* axenic amastigotes and their use in drug screening. *Antimicrob Agents Chemother,* 45**,** 1168-73.

SEVASTSYANOVICH, Y., ALFASI, S. & COLE, J. 2009. Recombinant protein production: a comparative view on host physiology. *N Biotechnol,* 25**,** 175-80.

SHARMA, S. K. & BANYAL, H. S. 2010. Characterization of Plasmodium berghei glutathione synthetase. *Parasitol Int,* 60**,** 321-3.

SHUMAN, S. 1994. Novel approach to molecular cloning and polynucleotide synthesis using vaccinia DNA topoisomerase. *J Biol Chem,* 269**,** 32678-84.

SIBLEY, L. D. 2011. Invasion and intracellular survival by protozoan parasites. *Immunol Rev,* 240**,** 72-91.

SINGH, B. & SUNDAR, S. 2012. Leishmaniasis: vaccine candidates and perspectives. *Vaccine,* 30**,** 3834-42.

SINGH, S., SHARMA, U. & MISHRA, J. 2011. Post-kala-azar dermal leishmaniasis: recent developments. *Int J Dermatol,* 50**,** 1099-108.

SKEIKY, Y. A., COLER, R. N., BRANNON, M., STROMBERG, E., GREESON, K., CRANE, R. T., WEBB, J. R., CAMPOS-NETO, A. & REED, S. G. 2002. Protective efficacy of a tandemly linked, multi-subunit recombinant leishmanial vaccine (Leish-111f) formulated in MPL adjuvant. *Vaccine,* 20**,** 3292-303.

SKEIKY, Y. A., GUDERIAN, J. A., BENSON, D. R., BACELAR, O., CARVALHO, E. M., KUBIN, M., BADARO, R., TRINCHIERI, G. & REED, S. G. 1995. A recombinant *Leishmania* antigen that stimulates human peripheral blood mononuclear cells to express a Th1-type cytokine profile and to produce interleukin 12. *J Exp Med,* 181**,** 1527-37.

SLABAUGH, M. B., DAVIS, R. E., ROSEMAN, N. A. & MATHEWS, C. K. 1993. Vaccinia virus ribonucleotide reductase expression and isolation of the recombinant large subunit. *J Biol Chem,* 268**,** 17803-10.

SOLOMON, M., PAVLOTZKY, F., BARZILAI, A. & SCHWARTZ, E. 2013. Liposomal amphotericin B in comparison to sodium stibogluconate for *Leishmania braziliensis* cutaneous leishmaniasis in travelers. *J Am Acad Dermatol,* 68**,** 284-9.

SOMMER, J. M., CHENG, Q. L., KELLER, G. A. & WANG, C. C. 1992. In vivo import of firefly luciferase into the glycosomes of *Trypanosoma brucei* and mutational analysis of the C-terminal targeting signal. *Mol Biol Cell,* 3**,** 749-59.

SOONG, L., HENARD, C. A. & MELBY, P. C. 2012. Immunopathogenesis of non-healing American cutaneous leishmaniasis and progressive visceral leishmaniasis. *Semin Immunopathol,* 34**,** 735-51.

SOU, T., MEEUSEN, E. N., DE VEER, M., MORTON, D. A., KAMINSKAS, L. M. & MCINTOSH, M. P. 2011. New developments in dry powder pulmonary vaccine delivery. *Trends Biotechnol,* 29**,** 191-8.

SPATH, G. F., EPSTEIN, L., LEADER, B., SINGER, S. M., AVILA, H. A., TURCO, S. J. & BEVERLEY, S. M. 2000. Lipophosphoglycan is a virulence factor distinct from related glycoconjugates in the protozoan parasite *Leishmania major*. *Proc Natl Acad Sci U S A,* 97**,** 9258-63.

SPELLBERG, B. & EDWARDS, J. E. 2001. Type 1/Type 2 Immunity in Infectious Diseases. *Clinical Infectious Diseases,* 32**,** 76-102.

Stockdale, L. and R. Newton (2013). "A review of preventative methods against human leishmaniasis infection." *PLoS Negl Trop Dis* **7**(6): e2278.

SU, L., XU, C., WOODARD, R. W., CHEN, J. & WU, J. 2013. A novel strategy for enhancing extracellular secretion of recombinant proteins in *Escherichia coli*. *Appl Microbiol Biotechnol,* 97**,** 6705-13.

SULLIVAN, V. J., MIKSZTA, J. A., LAURENT, P., HUANG, J. & FORD, B. 2006. Noninvasive delivery technologies: respiratory delivery of vaccines. *Expert Opin Drug Deliv,* 3**,** 87-95.

TEGEL, H., TOURLE, S., OTTOSSON, J. & PERSSON, A. 2010. Increased levels of recombinant human proteins with the *Escherichia coli* strain Rosetta(DE3). *Protein Expr Purif,* 69**,** 159-67.

THOMAS, C., RAWAT, A., BAI, S. & AHSAN, F. 2008. Feasibility study of inhaled hepatitis B vaccine formulated with tetradecylmaltoside. *J Pharm Sci,* 97**,** 1213-23.

TIUMAN, T. S., SANTOS, A. O., UEDA-NAKAMURA, T., FILHO, B. P. & NAKAMURA, C. V. 2011. Recent advances in leishmaniasis treatment. *Int J Infect Dis,* 15**,** e525-32.

TONNIS, W. F., KERSTEN, G. F., FRIJLINK, H. W., HINRICHS, W. L., DE BOER, A. H. & AMORIJ, J. P. 2012. Pulmonary vaccine delivery: a realistic approach? *J Aerosol Med Pulm Drug Deliv,* 25**,** 249-60.

TONUI, W. K., MEJIA, J. S., HOCHBERG, L., MBOW, M. L., RYAN, J. R., CHAN, A. S., MARTIN, S. K. & TITUS, R. G. 2004. Immunization with *Leishmania major* exogenous antigens protects susceptible BALB/c mice against challenge infection with L. major. *Infect Immun,* 72**,** 5654-61.

TRIGO, J., ABBEHUSEN, M., NETTO, E. M., NAKATANI, M., PEDRAL-SAMPAIO, G., DE JESUS, R. S., GOTO, Y., GUDERIAN, J., HOWARD, R. F. & REED, S. G. 2010. Treatment of canine visceral leishmaniasis by the vaccine Leish-111f+MPL-SE. *Vaccine,* 28**,** 3333-40.

TROPEA, J. E., CHERRY, S. & WAUGH, D. S. 2009. Expression and purification of soluble His(6)-tagged TEV protease. *Methods Mol Biol,* 498**,** 297-307.

TSENG,J and KUNG,A.2012 Invivo imaging of inflammatory phagocytes. *Chemistry and Biology*.19.1199-1209.

UPADHYAY, A. K., MURMU, A., SINGH, A. & PANDA, A. K. 2012. Kinetics of inclusion body formation and its correlation with the characteristics of protein aggregates in Escherichia coli. *PLoS One,* 7**,** e33951.

VAN ASSCHE, T., DESCHACHT, M., DA LUZ, R. A., MAES, L. & COS, P. 2011. *Leishmania*-macrophage interactions: insights into the redox biology. *Free Radic Biol Med,* 51**,** 337-51.

VAN GRIENSVEN, J. & DIRO, E. 2012. Visceral leishmaniasis. *Infect Dis Clin North Am,* 26**,** 309-22.

VAN ROSSUM, T., KENGEN, S. W. & VAN DER OOST, J. 2013. Reporter-based screening and selection of enzymes. *FEBS J,* 280**,** 2979-96.

VARMUS, H., KLAUSNER, R., ZERHOUNI, E., ACHARYA, T., DAAR, A. S. & SINGER, P. A. 2003. Public health. Grand Challenges in Global Health. *Science,* 302**,** 398-9.

VERA, A., GONZALEZ-MONTALBAN, N., ARIS, A. & VILLAVERDE, A. 2007. The conformational quality of insoluble recombinant proteins is enhanced at low growth temperatures. *Biotechnol Bioeng,* 96**,** 1101-6.

VERMEERSCH, M., DA LUZ, R. I., TOTE, K., TIMMERMANS, J. P., COS, P. & MAES, L. 2009. *In vitro* susceptibilities of *Leishmania donovani* promastigote and amastigote stages to antileishmanial reference drugs: practical relevance of stage-specific differences. *Antimicrob Agents Chemother,* 53**,** 3855-9.

VUJANIC, A., SUTTON, P., SNIBSON, K. J., YEN, H. H. & SCHEERLINCK, J. P. 2012. Mucosal vaccination: lung versus nose. *Vet Immunol Immunopathol,* 148**,** 172-7.

VYNOHRADOVA, R. P. 1999. [Units of enzyme activity]. *Ukr Biokhim Zh,* 71**,** 96-9.

WANG, L. 2013. Determining the transport mechanism of an enzyme-catalytic complex metabolic network based on biological robustness. *Bioprocess Biosyst Eng,* 36**,** 433-41.

WANG, S. H., THOMPSON, A. L., HICKEY, A. J. & STAATS, H. F. 2012. Dry powder vaccines for mucosal administration: critical factors in manufacture and delivery. *Curr Top Microbiol Immunol,* 354**,** 121-56.

WEBB, J. R., CAMPOS-NETO, A., OVENDALE, P. J., MARTIN, T. I., STROMBERG, E. J., BADARO, R. & REED, S. G. 1998. Human and murine immune responses to a novel *Leishmania major* recombinant protein encoded by members of a multicopy gene family. *Infect Immun,* 66**,** 3279-89.

WELDRICK, D. P., CHODACKA, B., VOGT, R. & STEENKAMP, D. J. 1999. The effect of buthionine sulfoximine on the growth of *Leishmania donovani* in culture. *FEMS Microbiol Lett,* 173**,** 139-46.

WHO. 2010a. *Control of the leishmaniases* [Online]. Geneva: WHO.

WHO 2010b. Essential leishmaniasis maps. 2010 ed. http://www.who.int/leishmaniasis/leishmaniasis_maps/en/index.html: WHO.

WHO. 2012. *Leishmaniasis: epidemiology and access to medicines* [Online]. Available: http://www.who.int/leishmaniasis/resources/leishmaniasis_epidemiology_access_to_medicine/en/index.html.

WILSON-WELDER, J. H., TORRES, M. P., KIPPER, M. J., MALLAPRAGADA, S. K., WANNEMUEHLER, M. J. & NARASIMHAN, B. 2009. Vaccine adjuvants: current challenges and future approaches. *J Pharm Sci,* 98**,** 1278-316.

WINCKER, P., RAVEL, C., BLAINEAU, C., PAGES, M., JAUFFRET, Y., DEDET, J. P. & BASTIEN, P. 1996. The *Leishmania* genome comprises 36 chromosomes conserved across widely divergent human pathogenic species. *Nucleic Acids Res,* 24**,** 1688-94.

WORD, A. P., PERESE, F., TSENG, L. C., ADAMS-HUET, B., OLSEN, N. J. & CHONG, B. F. 2012. 25-Hydroxyvitamin D levels in African-American and Caucasian/Hispanic subjects with cutaneous lupus erythematosus. *Br J Dermatol,* 166**,** 372-9.

WRIGHT, H. L., MOOTS, R. J., BUCKNALL, R. C. & EDWARDS, S. W. 2010. Neutrophil function in inflammation and inflammatory diseases. *Rheumatology (Oxford),* 49**,** 1618-31.

XU, D. & LIEW, F. Y. 1995. Protection against leishmaniasis by injection of DNA encoding a major surface glycoprotein, gp63, of *L. major*. *Immunology,* 84**,** 173-6.

XU, X., OLIVEIRA, F., CHANG, B. W., COLLIN, N., GOMES, R., TEIXEIRA, C., REYNOSO, D., MY PHAM, V., ELNAIEM, D. E., KAMHAWI, S., RIBEIRO, J. M., VALENZUELA, J. G. & ANDERSEN, J. F. 2011. Structure and function of a "yellow" protein from saliva of the sand fly *Lutzomyia longipalpis* that confers protective immunity against *Leishmania major* infection. *J Biol Chem,* 286**,** 32383-93.

YANG, X., GUO, Y. & WANG, A. 2010. Luminol/antibody labeled gold nanoparticles for chemiluminescence immunoassay of carcinoembryonic antigen. *Anal Chim Acta,* 666**,** 91-6.

YANG, Y. C., CHOU, H. Y., SHEN, T. L., CHANG, W. J., TAI, P. H. & LI, T. K. 2013. Topoisomerase II-mediated DNA cleavage and mutagenesis activated by nitric oxide underlie the inflammation-associated tumorigenesis. *Antioxid Redox Signal,* 18**,** 1129-40.

YOUNG, C. L., BRITTON, Z. T. & ROBINSON, A. S. 2012. Recombinant protein expression and purification: a comprehensive review of affinity tags and microbial applications. *Biotechnol J,* 7**,** 620-34.

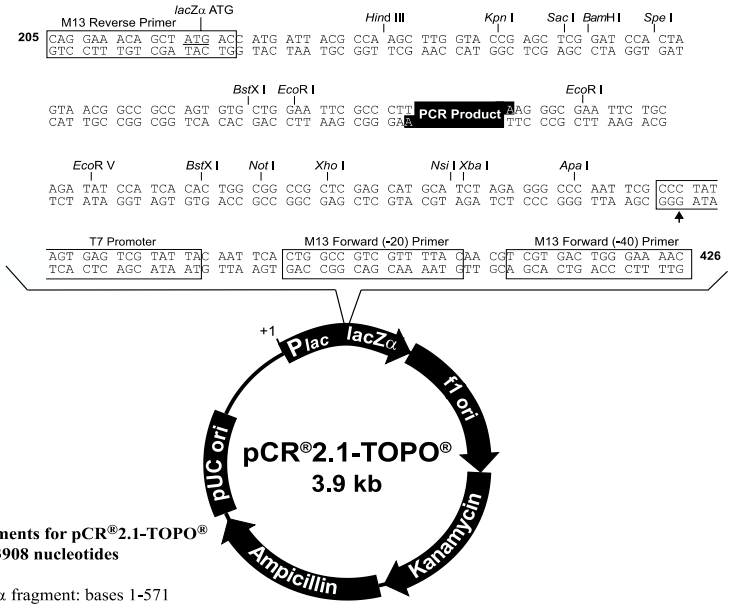
ZHENG, C., ZHAO, Z., LI, Y., WANG, L. & SU, Z. 2011. Effect of IPTG amount on apo- and holo- forms of glycerophosphate oxidase expressed in *Escherichia coli*. *Protein Expr Purif,* 75**,** 133-7.

Appendices

Appendix 1 TOPO® Cloning vector (from Invitrogen Lab manual).

pCR®2.1-TOPO® Map

The map below shows the features of pCR®2.1-TOPO® and the sequence surrounding the TOPO® Cloning site. Restriction sites are labeled to indicate the actual cleavage site. The arrow indicates the start of transcription for T7 polymerase. For the full sequence of the vector, you may download it from our web site or call Technical Service (page 19).



Comments for pCR®2.1-TOPO®
3908 nucleotides

LacZα fragment: bases 1-571
M13 reverse priming site: bases 205-221
Multiple cloning site: bases 234-357
T7 promoter/priming site: bases 364-383
M13 Forward (-20) priming site: bases 391-406
M13 Forward (-40) priming site: bases 411-426
f1 origin: bases 548-962
Kanamycin resistance ORF: bases 1296-2090
Ampicillin resistance ORF: bases 2108-2968
pUC origin: bases 3113-3786

Appendix 2. The gene sequences for *Leishmania* γGCS.

L. donovani γGCS.

GGATCCGGGCTCTTGACGACTGGCGGCGCCCCGATACAGTGGGGCACCGATGCA
AATAGAAAGGCCATTCCGCACGTCAGAGAGCACGGCATTCAGCAGTTCCTCAAC
GTTTTCAAGAACAAAAAGGACCTCCATGGTATGCCGTTTCTCTGGGGAGAGGAG
CTGGAGCACCAGCTAATCCAGATCCACGATAACACGGTTACCCTCAGCACGGAA
AGTGCGATGGTAATGAACAAGCTGAGGGCGCGTCCTGACAACTGCGCCGTGTGG
AACCCCGAATATGGAAGCTTCATGATCGAAAGCACGCCAGACCACCCGTACAGT
CTGTCGGTGGAGAGCCTCGACTCGGTGCAGGACAACATCGAGCGGCGGTACGAC
ATGCTCAACAAGGAGGCACCACCCGGCGTGGTCGGCACCACCTTTGTGACTTTC
CCACTCATGGGCCAGGGCAACTTTGTCCACTGCAGTGATAAGAGTTCTCCGTAC
TCGCAGTCGCTTTTTGTTCCTGATGCGTGCATCAACCAAACGCATCCGCGCTTC
GCGAACCTGACGGCAAACATTCGCCTGCGCCGCGGTCAAAAGGTTTGCGTCCTG
GTGCCTCTGTACATGGACTCCCGTACAATGCAGGACACGGTGGACCCCCAACTA
AACATTGACCTGACTCCACACAACAAGGACATTTTTTACTCCATGAGAGAAAAC
GGCAGGAACATGACCGACGAACTCTACGCGGAGACGGACGCGTCTGCCGCTCTG
CTAGTGCCCAGCAGCTCTCTCGATCCACGCGAGGACTACCCTGTCACCGAGACG
CTGAAGCAGCTCTTCACCCCTGCTACGCTCTACTACTACGCACAGTACTTCACG
GGACAGCGCCGCGAGCATATGCAGGAACGCTACAACGCGTGTAACTGCCCCGTA
ACCTTGGTCAGCCACCCGTGCATCTACATGGACTGCATGGCCTTTGGCATGGGT
AACAGCGCTCTGCAAGTGACGATGCAGCTGGACAACATTCACGAGGCGCGCCAC
GTGTACGACCAGCTCGCCATCTTGTGCCCGGCATTTCTGGCTCTCAGCTCAGCC
ACGCCGTTCCAAAAGGGTCTTCTTTGCGACACCGATGTGCGCTGGCTGACTATC
GCCGGCGCTGTCGACGACCGCCGCGTGGAGGAGGTGCCGCGTATTCTCAAGTCT
CGCTACGACTCCATCTCCGTCTTCATCAGCGACAGAACCGAAAACCTCGAGGAA
TTCAACGATTCACAAATAGCGATAAACCGCTCGTACTGTGAACTTCTGAAGGAC
TCCGGTGTGGACGTGCGGTTGGCGAACCACATTGCACATTTGTTCATTCGAGAT
CCGCTTGTGATGTACGACAAGATGATCGACATCGATGACACGACGCACACGGAG
CACTTTGACAACATCCAGTCCACTAACTGGCAGACAGTGCGCTTCAAGCCTCCG
CCGATAGGAAACGACATTGGCTGGCGCGTTGAGTTCCGCGTGATGGATATTCAG
CCAACACCGTTCGAAAACGCCGCCTTCGCCGTCTTCATTCCGCTTCTCACCAAG
GCCATCATCACCTACAAGCCCTGCTTTTACACCAAGATCTCCATCGTCGACGAG
AATATGGGCCGCGCACATCGCATCAACCCGTGTGGAGAACAATACATTATGCGC
AAGGACATTTTCGCCGACAAGTGCACCGCCAGCGACGAGGAGACGGCGAGGATG
AGCATTGACGAGATCTTCAACGGCAAGGAGGGCGGCTTCTATGGACTCATCCCC
CTCGTGTGCCGCTATCTAGACGACGAGGGGAAGCGAAGTCCCCTCGTAAACTCC
TACCTGAAGTTCCTGTCAATGCGCGCCTCTGGCCGCATTCCCACACCTGCGCAG
TACATGCGCAAGTTTGTCACGACACACCCCGACTACAAGCACGACTCACGCCTC
ACCGACAGCATCGCGCGTGACCTTGTGCAGCGCATGCACGGCCTGGCTGCGAAT
CAGATCCACGACGATGACTACCTTCCCATGAGCTTTTTCACGGCCGATACAGTA
GAGAGCACCAAG**GCGGCCG**CACTCGAGCACCACCACCACCACCACTGA

L. mexicana γGCS.

GGATCCATGGTATTCTTGACGGATGGCGGCGCCGCGATTCAGTGGGGCACCGA
CGCACATAGCAAGGCCATTCCGCACGTCAGAGAGCATGGCATTCAGCAGTTCC
TCAACGTTTTCAAGAACAAGAAGGACCTACATGGCATGCCGTTTCTCTGGGGC
GAGGAGGTGGAACACCAGCTGATCCAGATCCACGATAACACGGTTACCCTCAG
CACAGAAAGTGAGATGGTAATTAACAAGCTGAGAGCGCGTCCTGACAGCTGCG
CCGTGTGGAACTTCGAATATGGCAGCTTCATGGTAGAAAGCACGCCAGACCAC
CCGTACAATCTGTCAGTGGAGAGCCTCGACTCAGTGCAGGACAACATCGCGCG
ACGGTACGACATGCTCAACAAGGAGGCGCCACCTGGCGTGGTCGGCACCACCT
TTGTGACTTTCCCACTCATGGGCCAGGGTAACTTTGTCCACTGCAGTAGCAAG
AGCTCTCCGTACTCTCAGTCGCTTTTTGTTCCCGATGCGTGCATCAACCAAAC
GCATCCGCGCTTCGCGAACCTGACGGCAAACATTCGCTTGCGCCGCGGCCAAA
AGGTTTGCATCCTGGTGCCTCTGTACATGGACACACGTACAATGGAGAACACG
GTGGACCCTCGACTGAACATTGACCTGACTCCACGCAACAATGACATTTTTTA
CTCCATGAGAGAAAACGGCAGGAATACCACCGACGAGCTCTACGCGGAGACGG
ACGCGTTTGCCGCTCCTCTAGTGCCCAGGAGCTCTATCGATCCACGCGAGGAC
TACCCGGCCACCGAGACGCTGAGGCAACTCTTCACCCCTGCCACACTCCGCTA
CTACGCACAGTACTTCACGGAAGAGCACCGCGAGCATATGCAGGAACTCTACA
ACGCGTGTCCCTGCCCTGTACCCTTGGTGAGCCACCCGTGCATCTACATGGAC
TGCATGGCCTTTGGCATGGGTAGCAGCGCTCTGCAAGTGACGATGCAGCTGGA
CAACATTCACGAGGCGCGCCACGTGTATGACCAGCTCGCCATCTTGTGCCCGG
CATTTCTGGCTCTCAGCTCAGCCACGCCGTTTCAGAAGGGTCTTCTTTGCGAC
ACCGATGTGCGCTGGCTGACTATCGCCGGCGCTGTGGACGACCGCCGCGCCGA
GGAGGTGCCGCGTATTCTCAAGTCGCGCTACGACTCCATCTCCGTCTTCATCA
GTGACAGAACCGAAAACCTCGAGGAGTTCAACGATTCACACATAGAGGTGAAC
CGCTCGTACTGTGAACTTCTGAAGGACTCCGGTGTGGACGTGCGGTTGGCGAA
CCACATTGCACATCTGTTTATTCGCGATCCCCTTGTGATGTACGACAAGATGA
TCGACATCGATGACACGACGCACACGGAGCACTTTGATAACATCCAGTCCACT
AATTGGCAGACAATGCGCTTCAAGCCTCCGCCGATAGGCAGCGACATTGGCTG
GCGCGTTGAGTTTCGAGTGATGGATATTCAGCCAACACCATTCGAGAACGCCG
CCTTCGCTGTCTTCATTCCGCTTCTCACCAAGGCCATCGTCAACTACAAGCCC
TGCTTTTACACCAAAATCTCCATCGTCGAGGAGAATATGAGTCGCGCACATCG
CATCAACCCCTGTGGAGAACAATACGTTATGCGTAAGGACATTTTTGCCAACA
AGTGCACCGCCAGCGACGAGGAGACAGCGAGGATGAGCATTGACGAGATCTTC
AACGGCAAGGAGGACGGCTTCTATGGACTCATCCCCCTCGTGTGCCGCTATCT
CGACAGCGAGGGAAAGCGAAGTCCCCTCATAAACTCCTACTTGAAGCTCCTGT
CAATGCGCGCCTCTGGCAGCATTCCCACACCTGCGCAGTACATGCGCAGGTTT
GTCACAACGCACCCCGACTACAAGCACGACTCACGCCTCACCGACAGTATCGC
ACGTGACCTTGTCCAGCACATGCACAGCCTGGCCTCGAATCAGATCCACGACG
ATGACTATCTTCCGATGAGCATCTTCAGGGTCGATTCAGTAGAAAGCACCAAG
GCGGCCGCACTCGAGCACCACCACCACCACTGA

L. major γGCS.

GGATCCGGGCTCTTGACGACTGGCGGCGCCCCAATACAATGGGGCACCGATGC
AAATAGCAAAGCCATTCCGCACGTCAGAGAGCACGGCATTCAACAGTTCCTCA
ACGTTTTCAAAAGCAAAAAGGACCTCCATGGTATGCCGTTTTTCTGGGGAGAG
GAGCTGGAGCACCAGCTGATCCAGCTCCACGATGACACGGTTACCCTCAGCAC
AGAAGGTGCGGAGGTAATGAACAAGCTGAGGGCGCGTCCTGACAACTGTGCCG
TGTGGAATCCCGAATATGGAAGCTTCATGGTCGAAAGCACGCCAGACCACCCT
TACACTCTGTCGGTGGAGAGCCTCGACTCGGTGCAGGACAACATCGCGCGGCG
GTACCACATGCTCAACGAGGAGGCGCCACCCGGCGTGGTCGGCACCACCTTTG
TGACTTTCCCACTCATGGGCCAGGGTAACTTTGTACACTGCAGTGATAAGAGC
TCTCCGTACTCGCAGTCGCTGTTTGTTCCTGATGCGTGCATCAACCAAACGCA
TCCGCGCTTTGCGAACCTGACGGCAAACATTCGCCTGCGCCGCGGTCAAAAGG
TTTGCATCCTGGTGCCTCTGTACGTGGACTCCCGAACAATGCAGGACACGGTG
GACCCCCGACTAAACATTGACCTGACTCCACACAACAAGGACATTTTTCACTC
CAGGAGAGAAAACGGCAGGAGCATGACCGACGAACTCTACGCGCACACGGACG
CGTCTGCCGCTCTGCTAGTGCCGAGTAGCTCTCTCGACCCACGCGAGGACTAC
CCTGTCACCGAGACTCTGAAGCAGCTCTTCACCCCTGCTGCGCTCTACTACTA
CGCACAGTACTTCACGGGACAGCACCGCGAGCATATGCAGGAGCGCTACAACG
CGTGTAACTACCCCGTAACCTTGGTCAGCCACCCGTGCATCTACATGGACTGC
ATGGCCTTTGGCATGGGTAACAGCGCTCTGCAAGTGACGATGCAGCTGGACAA
CATTCACGAGGCGCGCCACGTGTACGACCAGCTCGCCATCTTGTGCCCGGCGC
TTCTGGCTCTCAGCTCAGCCACGCCGTTCCAGAAGGGTCTTCTTTGCGACACC
GATGTGCGCTGGCTGACTATTGCCGGCGCCGTCGACGACCGCCGCGTGGAGGA
GGTGCCGCATATCCTCAAGTCTCGCTACGACTCCATCTCCGTCTTCATCAGCG
ACAGAACCGAAAACCTCGAGGAATTCAACGATTCACAGATAGCGATAAACCGC
TCGTACTATGAACTCCTGAAGGACTCCGGTGTCGACGTGCGGTTGGCGAACCA
CATTGCACATCTGTTCATTCGAGATCCGCTTGTGATGTACGACAAGATGATCG
ACATCGATGACACGACGCACACAGAGCACTTTGACAACATCCAGTCCACTAAC
TGGCAGACAGTGCGCTTCAAGCCTCCGCCGCTAGGCAACGACATTGGCTGGCG
CGTTGAGTTCCGCGTGATGGATATTCAGCCAACGCCGTTCGAGAACGCCGCCT
TCGCCGTCTTCATTCCGCTTCTCACCAAGGCCATCGTCAACTACAAGCCCTGC
TTTTACACCAAGATCTCCATCGTCGACGAGAATATGGGCCGCGCACATCGCAT
CAACCCATGTGGAGAACAATACATTATGCGCAAGGACATTTTCGCCCACAAGT
GCACCGCCAGTGACGAGGAGACGGCGAGGATGAGCATTGACGAGATCTTCAAC
GGCAAGGAGGGCGGATTCTATGGACTCATCCCCCTCGTGTGCCGCTATCTCGA
CGACGAGGGGAAGCGAAGTCCCCTCGTAAACTCCTACTTGAAGTTCCTGTCAA
TGCGCGCCTCTGGCCGCATTCCCACACCTGCGCAGTACATGCGAAAGTTTGTC
ACGACACATCCCGACTACAAACACGACTCACGCCTCACCGACAGCATCGCACG

TGACCTTGTGCAGCGCATGCACGGTCTGGCTTCGAATCAGATCCACGACGATG
ACTACCTTCCCATAAGCGTCTTCAAGGCCACCACAAGAGAGAGTGTCAAG**GCG**
GCCGCACTCGAGCACCACCACCACCACCACTGA

BamH1 **= GGATCC**

NOT1 **= GCGGCCG**

6 HIS = **CACTCGAGCACCACCACCACCACCACTGA**

Appendix 3. Some of MALDI results .

L. mexicana γGCS.

Select Summary Report

Format As | Select Summary (protein hits) | Help

Significance threshold p< 0.05 | Max. number of hits AUTO

Standard scoring ○ MudPIT scoring ◉ Ions score or expect cut-off 0 | Show sub-sets 0

Show pop-ups ◉ Suppress pop-ups ○ | Require bold red ☐

Preferred taxonomy All entries

Re-Search | ◉ All queries ○ Unassigned ○ Below homology threshold ○ Below identity threshold

1. LmxM.18.1660 **Mass:** 79380 **Score:** 3476 **Matches:** 230(169) **Sequences:** 43(40) **emPAI:** 37.53
| organism=Leishmania_mexicana | product=gamma-glutamylcysteine synthetase, putative | location=LmxM.18:682498-684561(+) | leng

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
21	406.1951	810.3757	810.3759	-0.28	0	10	0.2	1	U	R.YLDSEGK.R
151	428.7353	855.4560	855.4562	-0.32	1	44	0.00011	1	U	R.RAEEVPR.I 152
296	456.7282	911.4418	911.4422	-0.46	0	37	0.00048	1	U	R.SYCELLK.D 295
380	470.2395	938.4645	938.4644	0.10	1	(29)	0.0039	1	U	R.RYDMLNK.E 379
383	471.2740	940.5335	940.5342	-0.66	0	46	5.2e-05	1	U	

Select Summary Report

Format As | Select Summary (protein hits) | Help

Significance threshold p< 0.05 | Max. number of hits AUTO

Standard scoring ○ MudPIT scoring ◉ Ions score or expect cut-off 0 | Show sub-sets 0

Show pop-ups ◉ Suppress pop-ups ○ | Require bold red ☐

Preferred taxonomy All entries

Re-Search | ◉ All queries ○ Unassigned ○ Below homology threshold ○ Below identity threshold

1. LmxM.18.1660 **Mass:** 79380 **Score:** 2823 **Matches:** 170(121) **Sequences:** 39(38) **emPAI:** 16.73
| organism=Leishmania_mexicana | product=gamma-glutamylcysteine synthetase, putative | location=LmxM.18:682498-684561(+) | leng

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
152	428.7354	855.4563	855.4562	0.04	1	44	0.0001	1	U	R.RAEEVPR.I 151
298	456.7281	911.4417	911.4422	-0.60	0	36	0.00064	1	U	R.SYCELLK.D 299
378	470.2394	938.4643	938.4644	-0.03	1	27	0.0059	1	U	R.RYDMLNK.E 379 380
385	471.2748	940.5351	940.5342	0.96	0	46	4.4e-05	1	U	R.LNIDLTPR.N 384
433	478.2368	954.4590	954.4593	-0.33	1	(25)	0.0073	1	U	R.RYDMLNK.E 434
500	490.7413	979.4681	979.4685	-0.42	0	(48)	4.9e-05	1	U	R.DPLVMYDK.M 501
546	498.7388	995.4630	995.4634	-0.39	0	52	1.2e-05	1	U	R.DPLVMYDK.M 547
610	510.2852	1018.5559	1018.5560	-0.01	0	63	1.6e-06	1	U	R.FANLTANIR.L 606 607 608 609 611 612
690	523.8029	1045.5913	1045.5920	-0.71	0	34	0.00074	1	U	R.QLFTPATLR.Y 689
695	524.7584	1047.5023	1047.5019	0.37	0	54	1.7e-05	1	U	K.GLLCDTDVR.W 694
786	539.7453	1077.4760	1077.4761	-0.02	0	28	0.0021	1	U	R.TMENTVDPR.L 785

Select Summary Report

Format As | Select Summary (protein hits) | Help

Significance threshold p< 0.05 | Max. number of hits AUTO

Standard scoring ○ MudPIT scoring ◉ Ions score or expect cut-off 0 | Show sub-sets 0

Show pop-ups ◉ Suppress pop-ups ○ | Require bold red ☐

Preferred taxonomy All entries

Re-Search | ◉ All queries ○ Unassigned ○ Below homology threshold ○ Below identity threshold

1. LmxM.18.1660 **Mass:** 79380 **Score:** 1204 **Matches:** 63(54) **Sequences:** 22(21) **emPAI:** 2.43
| organism=Leishmania_mexicana | product=gamma-glutamylcysteine synthetase, putative | location=LmxM.18:682498-684561(+) | leng

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
2	400.1862	798.3578	798.3582	-0.43	0	29	0.0016	1	U	R.YDMLNK.E 1
21	406.1947	810.3749	810.3759	-1.25	0	24	0.0085	1	U	R.YLDSEGK.R 20
104	418.2372	834.4599	834.4593	0.01	1	32	0.0018	1	U	R.KDIFANK.G 103
169	428.7354	855.4563	855.4562	0.11	1	46	6.3e-05	1	U	R.RAEEVPR.I 168
380	470.2394	938.4643	938.4644	-0.03	1	(29)	0.0042	1	U	R.RYDMLNK.E 379
517	471.2746	940.5346	940.5342	0.51	0	24	0.0082	1	U	R.LNIDLTPR.N 516 518
578	478.2361	954.4577	954.4593	-1.67	1	35	0.00077	1	U	R.RYDMLNK.E 579
628	484.2459	966.4773	966.4770	0.30	1	42	0.00021	1	U	R.YLDSEGKR.S 627
838	510.2848	1018.5550	1018.5560	-0.91	0	56	7e-06	1	U	R.FANLTANIR.L 831
945	523.8031	1045.5917	1045.5920	-0.36	0	31	0.0017	1	U	R.QLFTPATLR.Y 944 944
950	524.7581	1047.5016	1047.5019	-0.33	0	55	1.2e-05	1	U	R.GLLCDTDVR.W 949 950 951
1025	531.7481	1061.4817	1061.4812	0.48	0	(18)	0.025	1	U	R.TMENTVDPR.L
1089	539.7456	1077.4767	1077.4761	0.54	0	62	1.2e-06	1	U	R.TMENTVDPR.L 1086 1087 1088 1090 1091
1338	580.2634	1159.5122	1159.5128	-0.51	0	44	5.5e-05	1	U	R.MNDLYSMM.R 1339
1524	589.2972	1176.5799	1176.5808	-0.77	0	42	0.00029	1	U	K.ISIVEENMSR.A
1567	597.7855	1193.5564	1193.5564	-0.04	0	68	4.3e-07	1	U	R.EDVPATETLR.Q 1566 1568
1875	421.8878	1262.6415	1262.6408	0.60	1	19	0.046	1	U	R.RFVTTHPDYK.H 1876
1880	632.3281	1262.6417	1262.6408	0.73	1	(7)	0.61	1	U	R.RFVTTHPDYK.H 1881
2346	683.8154	1365.6162	1365.6169	-0.54	0	(37)	0.00058	1	U	R.INPCGKQVVMR.K 2346

Select Summary Report

Format As | Select Summary (protein hits) | Help

Significance threshold p< 0.05 | Max. number of hits AUTO

Standard scoring ○ MudPIT scoring ◉ Ions score or expect cut-off 0 | Show sub-sets 0

Show pop-ups ◉ Suppress pop-ups ○ | Require bold red ☐

Preferred taxonomy All entries

Re-Search | ◉ All queries ○ Unassigned ○ Below homology threshold ○ Below identity threshold

1. LmxM.18.1660 **Mass:** 79380 **Score:** 2056 **Matches:** 104(82) **Sequences:** 30(29) **emPAI:** 6.45
| organism=Leishmania_mexicana | product=gamma-glutamylcysteine synthetase, putative | location=LmxM.18:682498-684561(+) | leng

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
1	400.1862	798.3578	798.3582	-0.43	0	22	0.0087	1	U	R.YDMLNK.E 2
182	428.7354	855.4562	855.4562	-0.03	1	48	4.2e-05	1	U	R.RAEEVPR.I
464	470.2391	938.4637	938.4644	-0.68	1	29	0.0037	1	U	R.RYDMLNK.E 463 465 466
469	471.2742	940.5339	940.5342	-0.27	0	36	0.00045	1	U	R.LNIDLTPR.N 470

Appendix 4. Alignment for γGCS protein from *L. mexicana, L. major* and *L. donovani.*

using version 3.21 of BOX SHADE software .The amino acid identity was L. don /L. major 94.8% , Ldon/Lmex 91%, Lmajor/Lmex 88.9%

```
Lmex     1 IHDNTVTLSTESEMVTNKLRARPDSCAVWNFEYGSFMVESTPDHPYNLSVESLDSVQDNI
Lmaj     1 LHDDTVTLSTEGAEVMNKLRARPDNCAVWNPEYGSFMVESTPDHPYTLSVESLDSVQDNI
Ldon     1 IHDNTVTLSTESAMVMNKLRARPDNCAVWNPEYGSFMIESTPDHPYSLSVESLDSVQDNI

Lmex    61 ARRYDMLNKEAPPGVVGTTFVTFPLMGQGNFVHCSSKSSPYSQSLFVPDACINQTHPRFA
Lmaj    61 ARRYHMLNEEAPPGVVGTTFVTFPLMGQGNFVHCSDKSSPYSQSLFVPDACINQTHPRFA
Ldon    61 ERRYDMLNKEAPPGVVGTTFVTFPLMGQGNFVHCSDKSSPYSQSLFVPDACINQTHPRFA

Lmex   121 NLTANIRLRRGQKVCILVPLYMDIRTMENTVDPRLNIDLTPRNNDIFYSMRENGRNTTDE
Lmaj   121 NLTANIRLRRGQKVCILVPLYVDSRTMQDTVDPRLNIDLTPHNKDIFHSRRENGRSMTDE
Ldon   121 NLTANIRLRRGQKVCVLVPLYMDSRTMQDTVDPQLNIDLTPHNKDIFYSMRENGRNMTDE

Lmex   181 LYAETDAFAAPLVPRSSIDPREDYPATETLRQLFTPATLRYYAQYFTEEHREHMQELYNA
Lmaj   181 LYAHTDASAALLVPSSSLDPREDYPVTETLKQLFTPAALYYYAQYFTGQHREHMQERYNA
Ldon   181 LYAETDASAALLVPSSSLDPREDYPVTETLKQLFTPATLYYYAQYFTGQRREHMQERYNA

Lmex   241 CPCPVPLVSHPCIYMDCMAFGMGSSALQVTMQLDNIHEARHVYDQLAILCPAFLALSSAT
Lmaj   241 CNYPVTLVSHPCIYMDCMAFGMGNSALQVTMQLDNIHEARHVYDQLAILCPAILALSSAT
Ldon   241 CNCPVTLVSHPCIYMDCMAFGMGNSALQVTMQLDNIHEARHVYDQLAILCPAFLALSSAT

Lmex   301 PFQKGLLCDTDVRWLTIAGAVDDRRAEEVPRILKSRYDSISVFISDRTENLEEFNDSHIE
Lmaj   301 PFQKGLLCDTDVRWLTIAGAVDDRRVEEVPHILKSRYDSISVFISDRTENLEEFNDSQIA
Ldon   301 PFQKGLLCDTDVRWLTIAGAVDDRRVEEVPRILKSRYDSISVFISDRTENLEEFNDSQIA

Lmex   361 VNRSYCELLKDSGVDVRLANHIAHLFIRDPLVMYDKMIDIDDTTHTEHFDNIQSTNWQTM
Lmaj   361 INRSYYELLKDSGVDVRLANHIAHLFIRDPLVMYDKMIDIDDTTHTEHFDNIQSTNWQTV
Ldon   361 INRSYCELLKDSGVDVRLANHIAHLFIRDPLVMYDKMIDIDDTTHTEHFDNIQSTNWQTV

Lmex   421 RFKPPPIGSDIGWRVEFRVMDIQPTPFENAAFAVFIPLLTKAIVNYKPCFYTKISIVPEN
Lmaj   421 RFKPPPLGNDIGWRVEFRVMDIQPTPFENAAFAVFIPLLTKAIVNYKPCFYTKISIVDEN
Ldon   421 RFKPPPIGNDIGWRVEFRVMDIQPTPFENAAFAVFIPLLTKAITTYKPCFYTKISIVDEN

Lmex   481 MSRAHRINPCGEQYVMRKDIFANKCTASDEETARMSIDEIFNGKEDGFYGLIPLVCRYLD
Lmaj   481 MGRAHRINPCGEQYIMRKDIFAHKCTASDEETARMSIDEIFNGKEGGFYGLIPLVCRYLD
Ldon   481 MGRAHRINPCGEQYIMRKDIFADKCTASDEETARMSIDEIFNGKEGGFYGLIPLVCRYLD

Lmex   541 SEGKRSPLINSYLKLLSMRASGSIPTPAQYMRRFVTTHPDYKHDSRLTDSIARDLVQHMH
Lmaj   541 DEGKRSPLVNSYLKFLSMRASGRIPTPAQYMRKFVTTHPDYKHDSRLTDSIARDLVQRMH
Ldon   541 DEGKRSPLVNSYLKFLSMRASGRIPTPAQYMRKFVTTHPDYKHDSRLTDSIARDLVQRMH

Lmex   601 SLASNQIHDDDYLPMSIFFVDSVESTK
Lmaj   601 GLASNQIHDDDYLPISVFKATTRESVK
Ldon   601 GLAANQIHDDDYLPMSFFTADTVESTK
```

Appendix 5. Alignment for γGCS protein from *L. mexicana, L. major L. donovani*, Human γGCS protein, and *T. brucei*

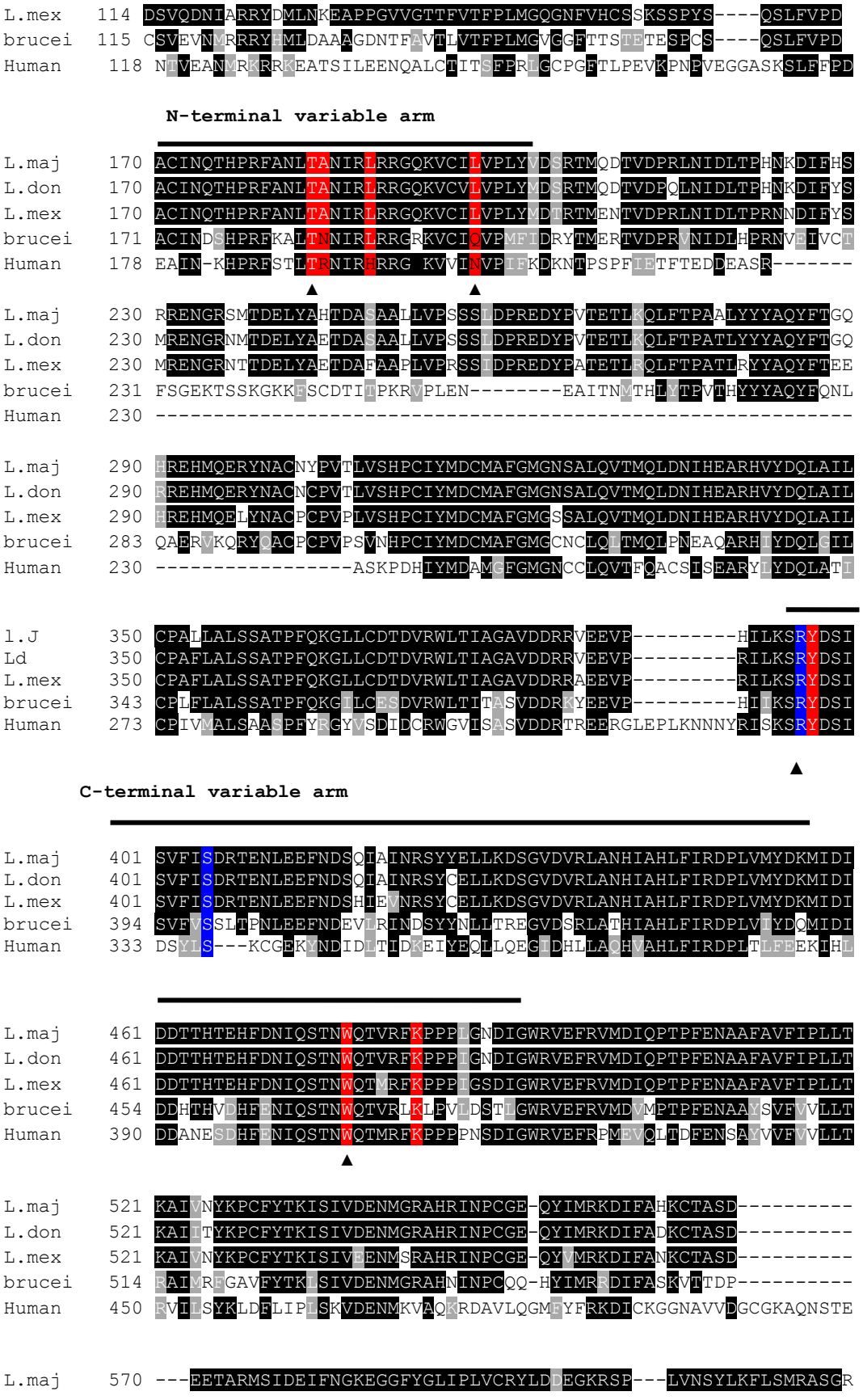
Using version 3.21 of BOXSHADE software. The red area is substrate binding site as described by Hibi *et al*., 2004. Multiple sequence alignment of γGCS from *L. donovani, L. major, L. mexicana, T. brucei* and Human. The structure of the *E. coli* γGCS has been determined as an un-liganded enzyme and as a complex with the sulfoxime inhibitor (2S-2-amino-4 [(2S)-2-carboxybutyl-(R)-sulfonimidoyl]butanoic acid. *E. coli* γGCS was not included in the above alignment because the low level of homology in the cysteine binding region prevented an accurate alignment using clustalw. Instead, a published alignment including *T. brucei,* human and *E. coli* was used to identify residues predicted to be important in binding cysteine and sulfoxime inhibitors.

Red: Position of residues in *E. coli* GCS involved in binding a sulfoxime inhibitor, identified by sequence alignment (Hibi *et al*., 2004).

Blue: Hinge residues in *E. coli* γGCS.

▲ Position of residues in the *E. coli* γGCS involved in binding the carboxylate group of cysteine and the sulfoxime inhibitor.

Only one of the residues involved in carboxylate binding in *E. coli* is conserved in the eukaryote enzymes. The *E. coli* γGCS enzyme has a high specificity for cysteine and a low affinity for BSO, which does not contain a carboxylate.


N-terminal variable arm
C-terminal variable arm


L.don 570 ---EETARMSIDEIFNGKEGGFYGLIPLVCRYLDDEGKRSP---LVNSYLKFLSMRASGR
L.mex 570 ---EETARMSIDEIFNGKEDGFYGLIPLVCRYLDSEGKRSP---LINSYLKLLSMRASGS
brucei 563 ---SENCELTVGEVINGKPGEYYGLIPLVRRYLEEENIQSD---VVEGYLNFISKRACGE
Human 510 LAAEEYTLMSIDTIINGKEGVFPGLIPILNSYLENMEVDVDTRCSILNYLKLIKKRASGE
L.maj 624 IPTPAQYMRKFVTTHPDYKHDSRLTDSIARDLVQRMHGLASNQIHDDDYLPISVFKATTR
L.don 624 IPTPAQYMRKFVTTHPDYKHDSRLTDSIARDLVQRMHGLAANQIHDDDYLPMSFFTADTV
L.mex 624 IPTPAQYMRRFVTTHPDYKHDSRLTDSIARDLVQHMHSLASNQIHDDDYLPMSIFRVDSV
brucei 617 IPTAAQYIRNFVKKHPDYFEDSRLTEQIAHDVVNHVHQLACGGNASESMIGAYTLGSKRQ
Human 570 LMTVARWMREFTANHPDYKQDSVTTDEMNYSLILKCNQIANELCECPELLGSAFRKVKYS
L.maj 684 ESVK----
L.don 684 ESTK----
L.mex 684 ESTK----
brucei 677 REG-----
Human 630 GSKTDSSN

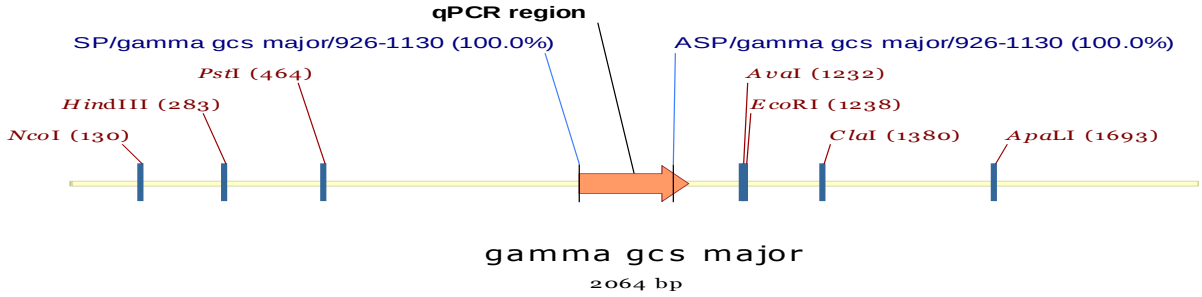
Appendix 6. Primer that used in real time PCR for *Leishmania* γGCS .

The primers contains region of the Gamma-GSC molecule from 931 to 1130

Sense Primer: SP/gamma gcs major/926-1130
CCGTGCATCTACATGGACTGCATGGCCTTTGGCAT
Length: 32 Tm: 74.2 C GC: 56.3
dH: -250.7 kcal/mol dS: -632.8 cal/mol dG: -60.3 kcal/mol

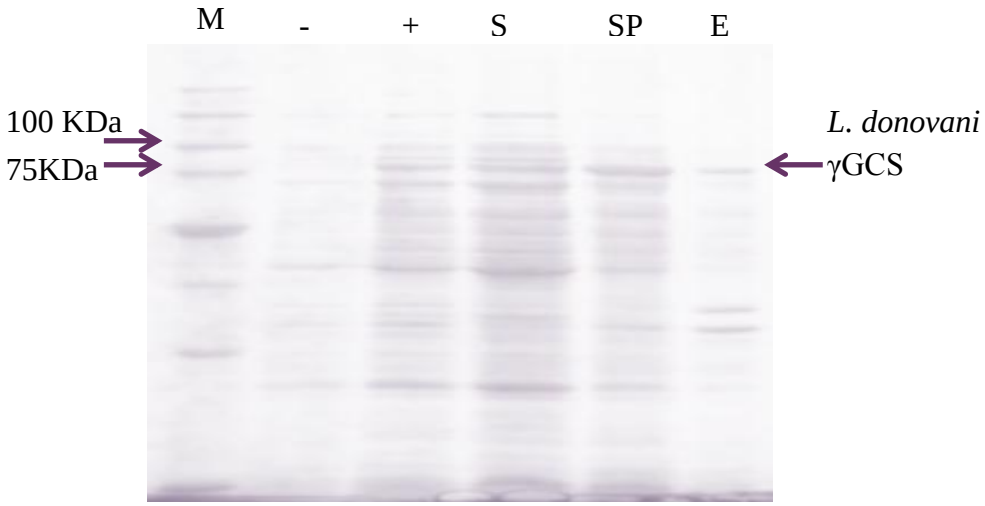
Antisense Primer: ASP/gamma gcs major/926-1130
ATAGTCAGCCAGCGCACATCGGTGTCGC
Length: 28 Tm: 71.4 C GC: 60.7
dH: -220.5 kcal/mol dS: -555.5 cal/mol dG: -53.1 kcal/mol

Tm Difference: 2.8
GC Difference: 4.5

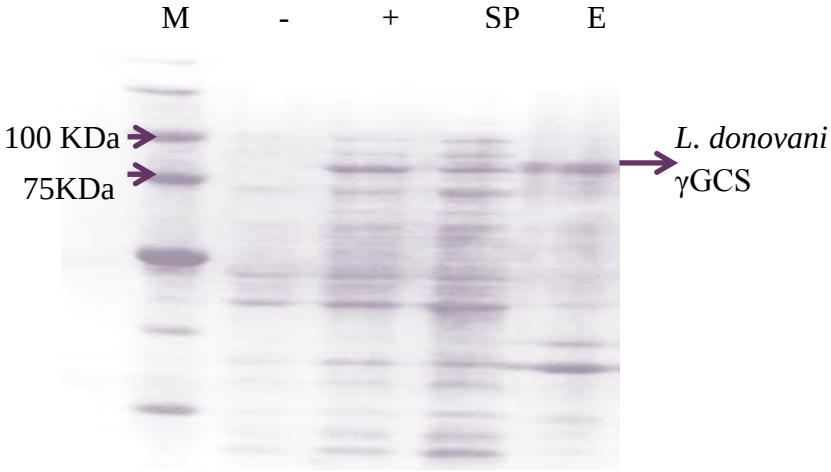



```
 L mexicana    (919) TTGGTGAGCCACCCGTGCATCTACATGGACTGCATGGCCTTTGGCATGGG
L. donovani    (919) TTGGTCAGCCACCCGTGCATCTACATGGACTGCATGGCCTTTGGCATGGG
     Lmajor    (919) TTGGTCAGCCACCCGTGCATCTACATGGACTGCATGGCCTTTGGCATGGG
 _clipseq#2      (1) ------------CCGTGCATCTACATGGACTGCATGGCCTTTGGCATGGG

 L mexicana    (969) TAGCAGCGCTCTGCAAGTGACGATGCAGCTGGACAACATTCACGAGGCGC
L. donovani    (969) TAACAGCGCTCTGCAAGTGACGATGCAGCTGGACAACATTCACGAGGCGC
     Lmajor    (969) TAACAGCGCTCTGCAAGTGACGATGCAGCTGGACAACATTCACGAGGCGC
 _clipseq#2     (39) TAACAGCGCTCTGCAAGTGACGATGCAGCTGGACAACATTCACGAGGCGC

 L mexicana   (1019) GCCACGTGTATGACCAGCTCGCCATCTTGTGCCCGGCATTTCTGGCTCTC
L. donovani   (1019) GCCACGTGTACGACCAGCTCGCCATCTTGTGCCCGGCATTTCTGGCTCTC
     Lmajor   (1019) GCCACGTGTACGACCAGCTCGCCATCTTGTGCCCGGCGCTTCTGGCTCTC
 _clipseq#2     (89) GCCACGTGTACGACCAGCTCGCCATCTTGTGCCCGGCGCTTCTGGCTCTC

 L mexicana   (1069) AGCTCAGCCACGCCGTTTCAGAAGGGTCTTCTTTGCGACACCGATGTGCG
L. donovani   (1069) AGCTCAGCCACGCCGTTCCAAAAGGGTCTTCTTTGCGACACCGATGTGCG
     Lmajor   (1069) AGCTCAGCCACGCCGTTCCAGAAGGGTCTTCTTTGCGACACCGATGTGCG
 _clipseq#2    (139) AGCTCAGCCACGCCGTTCCAGAAGGGTCTTCTTTGCGACACCGATGTGCG

 L mexicana   (1119) CTGGCTGACTATCGCCGGCGC
L. donovani   (1119) CTGGCTGACTATCGCCGGCGC
     Lmajor   (1119) CTGGCTGACTATTGCCGGCGC
 _clipseq#2    (189) CTGGCTGACTAT---------
```

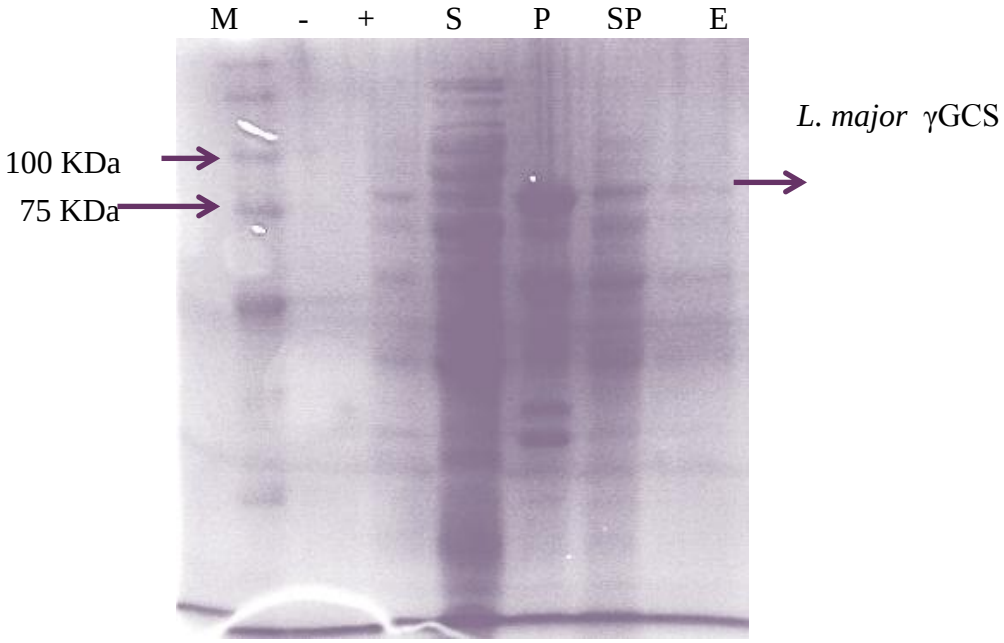
Appendix 7. Proteins picture



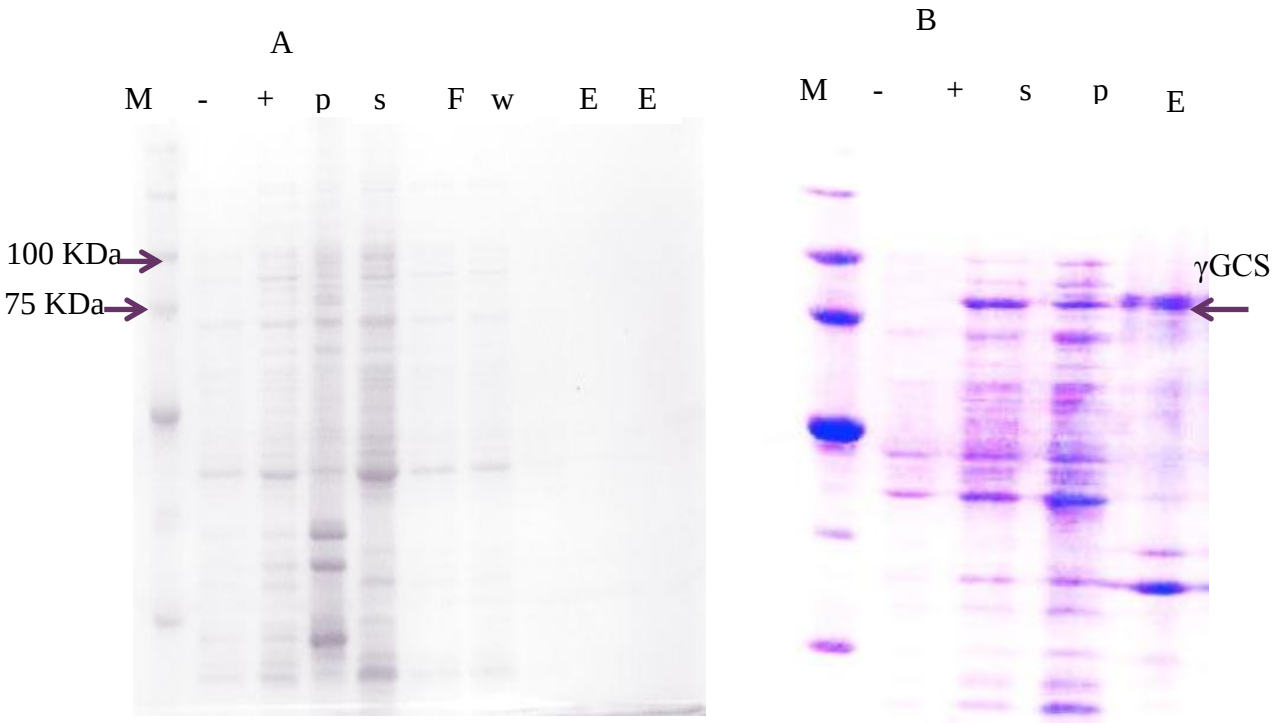
Coomassie stained SDS-PAGE showing *L. donovani* γGCS protein purified under native condition. Protein expression in *E. coli* was induced by adding 0.1mM IPTG and bacteria were incubated at 18ºC overnight, M (molecular mass), uninduced(-), induced(+), supernatant(S), supernatant after add 6M urea (SP). Eluted protein fraction E.



Coomassie stained SDS-PAGE showing *L. donovani* γGCS protein purified under denaturation condition. Protein expression in *E. coli* was induced by adding 0.1mM IPTG and bacteria were incubated at 18ºC overnight, M (molecular mass), uninduced(-), induced(+), supernatant after add 6M urea (SP). Eluted protein fraction E.



Coomassie stained SDS-PAGE showing *L.major* γGCS protein purified under denaturation condition. Protein expression in *E. coli* was induced by adding 0.1mM IPTG and bacteria were incubated at 18ºC overnight, M (molecular mass), uninduced(-), induced(+),supernatant (S), pellet (P), supernatant after add 6M urea (SP). Eluted protein fraction E.



Coomassie stained SDS-PAGE showing the expression of Pet-24a in Rosta Blue without γGCS gene insert on (A), and with *L. donovani* γGCS gene insert (B). Protein expression in *E. coli* was induced by adding 0.1mM IPTG and bacteria were incubated at 18ºC overnight, M (molecular mass), uninduced(-), induced(+),supernatant (S), pellet (P), F (flow thought), W(washing), Eluted protein fraction E.

Appendix 8 Calculations that used in this study.

- **The γGCS enzyme specific activity.**

(Activity of sample- activity of control) /6.22 = X mM/min/ml of sample

Specific activity=X/mg of protein = mM/min/mg

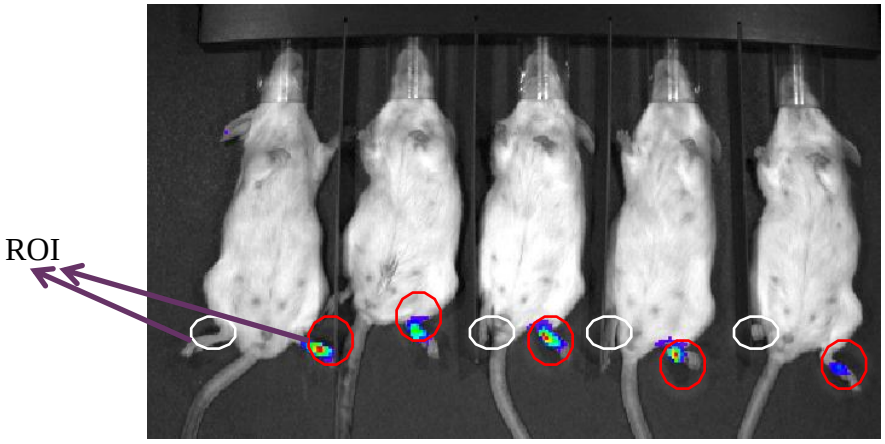
The activity was got from the main table kinetics report.

The extinction coefficient of NADPH is 6.22 mM-1 cm-1. An enzyme oxidizes substrate to product and as such reduces NAPD+ to NAPDH in the reaction. As NADP+ converts to NADPH, an increase at 340 nm is observed.

- **The formal used to calculate percentage of suppression**

% Suppression = [(Mean control- sample value) /Mean control] x100

Appendix 9. Calculations using IVIS .



The amount of bioluminescence emitted in each region of interest (ROI) was determined using the Living Image software, and the results were recorded as photons/sec emitted. The white circle is background; red circle is the BLI imaging from infected site with lucirferase expression *Leishmania.*

Appendix 10. Papers for publication.

- Campbell, S., Alawa, J., Doro, B., Henriquez, F., Roberts, C., Nok, A., Alawa, C., Alsaadi, M., Mullen, A., Carter, C. (2012). Comparative assessment of a DNA and protein *Leishmania* donovani gamma glutamyl cysteine synthetase vaccine to cross-protect against murine cutaneous leishmaniasis caused by *L. major* or *L. mexicana* infection.

Vaccine, 30 (7), 1357-1363

- Development of a recombinant *Leishmania* gamma glutamyl cysteine synthetase vaccine to protect against murine cutaneous leishmaniasis caused by *L. major* or *L. mexicana* infections using the pulmonary route. B. Doro, M. Wiese, R. Birchmore, Suzanne Eadie, G.Westrop, R.A.M. Williams, A. B. Mullen and K. C. Carter. **In preparation**
- The effect of buthionine sulphoximine treatment on the survival of different *Leishmania* species, B. Doro, G. Westrop, M. Wiese, C. Shaw, R.A.M. Williams, R. Burchmore, A.B. Mullen and K.C. Carter**. In preparation**
- Modified ivy saponins have significant acytivity against different *Leishmania* species. Nema Ali Ahmad, Basma Doro, David Preskett, Stephen Strawson, Juma'a R Al Dulayymi, Craig D. Shaw, K.Christine Carter, and Mark S. Baird**. In preparation.**