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Studies on development of a Leishmanial vaccine

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I wish you could have been with me at this moment to share this with me.

Without your support, I would not have reached this. I miss you.

Rest in Peace

Abstract

Leishmaniasis is a disease caused by infection with the obligate intracellular parasite of the genus *Leishmania*. Leishmaniasis is a public health problem; it is estimated that approximately 12-15 million people worldwide are infected. An estimated 1.5-2 million new cases occur each year, 350 million people are at risk of infection, and it causes 70000 deaths per year. Therefore, 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 live non-pathogenic *L. tarentolae* transfected with gamma glutamylcysteine synthetase (γGCS) from three different species (*L. donovani, L. mexicana* and *L. major*).

Previous studies for expressing recombinant γGCS using *E. coli* as an expression system have shown that it was not possible to produce pure full-length recombinant γGCS protein. Therefore, in this project, using an expression vector that is phylogenically more like *Leishmania. L. tarentolae* has been used as an expression system for eukayotic recombinant proteins. Studies of the expression of *L. donovani, L. mexicana* and *L. major* γGCS recombinant proteins showed that parasites integrated with a green fluorescent protein γGCS-His gene gave stable expression of the fusion protein for six months without the requirement for antibiotics to maintain expression of the gene insert. Supplementing the culture medium with hydrogen peroxide (H_2O_2) increased proliferation of parasites in cell culture. The addition of a 12 μM of H_2O_2 supplement increased the number of the parasites in cell culture, which can conclude that expression of the GFP-γGCS-His recombinant protein has been increased.

Expression of the recombinant GFP-γGCS-His protein produced full-length protein and truncated protein, but after isolation from an affinity column, it was impossible to produce pure full-length protein for all three transfected parasites. Therefore, this purification method failed to remove non-specific protein contamination.

The live non-pathogenic lizard parasite, *L. tarentolae*, expressing elected *Leishmania* antigens has recently provided a promising new approach as a safe

and effective live vaccine candidate to prevent Leishmaniasis. Here, this study evaluated the immunoprotective potential of a live vaccine against *L. major* infection in BALB/c mice, using *L. tarentolae* transfected with the GFP-γGCS-His sequence gene from one of three different species (*L. donovani, L. major* or *L. mexicana*) or a 'triple vaccine' using a 1:1:1mixture of *L. tarentolae* transfected with the GFP-γGCS-His sequence gene of the three pathogenic species. Vaccination with transfected *L. tarentolae* with GFP-γGCS-His gene from *L. donovani, L. major* and *L. mexicana* (triple vaccine) induced significant parasite-specific Th1 immune responses based on antibody titres and cytokine production *in vitro* in stimulated splenocytes and popliteal lymph nodes from immunised mice. Vaccination by subcutaneous injection with the triple vaccine caused the highest percentage reduction in parasite burdens compared to controls ± SE, was 94% ± 0.01 in *L. major* infected mice. Vaccination with *L.t L. maj* GFP-γGCS-His, *L. mex* GFP-γGCS-His and *L. don* GFP-γGCS-His parasites failed to give significant protection against *L. major* infection, but vaccination with *L. t L. maj* GFP-γGCS-His resulted in an 86% ± 0.01 suppression in parasite burden compared to controls.

In conclusion, the results of this study indicate that vaccination with transfected *L. tarentolae* parasites against *L. major* infection enhanced the protective efficacy and that the triple vaccine is a potential vaccine candidate.

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
ddH2O	Doubled distilled water
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
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HN	Hemagglutinine neuraminidase
HPRT	Hypoxanthine-guanine phosphorribosyl transferase
HRP	Horseradish peroxidase
IC_{50}	Half maximal inhibitory concentration
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
KCl	Potassium chloride
МΦ	Macrophages
MALD1	Matrix-assisted laser desorption/ionization mass spectrometry
MHC	Major histocompatibility complex
mRNA	messenger RNA
MCL	Mucocutaneous Leishmaniasis
NaCl	Sodium chloride
NK	Natural killer
NADPH	Nicotinamide Adenine Dinucleotide Phosphate Oxidase
NO	Nitric oxide
LACK	Leishmania activated C kinase
LdonγGCS	*L. donovani γGCS*
LmexγGCS	*L. mexicana γGCS*

LmajγGCS	*L. major γGCS*
L.t LdonγGCS	*L. tarentolae L. don γGCS*
L.t LmajγGCS	*L. tarentolae L. maj γGCS*
L.t LmexγGCS	*L. tarentolae L. mex γGCS*
LV82-luc	Luciferase expressing *L. donovani* (LV82 strain)
$NiCl_2$	Nickel chloride
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
TBS	Tris-buffered saline
Th	T-helper cells
TNF-α	Tumour necrosis factor-α
T reg T	T regulatory
TSH	Trypanothione
VL	Visceral Leishmaniasis
X-gel	5-bromo-4-chloro-3-indoyl-beta-D-galactopyranoside

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CHAPTER 1. GENERAL INTRODUCTION

1.1. Leishmaniasis

Leishmaniasis is a disease caused by infection with the obligate intracellular parasite of the genus *Leishmania*. The disease occurs mainly in the tropical and subtropical region of the world. Alexander Russell was the first person to describe *Leishmania* in 1756 (Sharma and Singh, 2009) but it was not until 1903, when L. H. Donovan and W.B. Leishman independently identified *Leishmania* as the cause of infection in patients in India, by demonstrating the presence of parasites in splenic tissue (Bern and Chowdhury, 2006). Leishmaniasis is a public health problem; it is estimated that approximately 12-15 million people worldwide are infected. An estimated 1.5-2 million new cases occur each year, 350 million people are at risk of infection, and it causes 70000 deaths per year (WHO, 2015). Leishmaniasis is endemic in 89 countries, and is found in Asia, Africa, the Americas and the Mediterranean region (Kashif *et al.*, 2016). *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 around 20 *Leishmania* species have been identified, most of which cause disease in animals and humans (Duthie *et al.*, 2012).

Leishmaniasis can be divided into 3 main clinical forms i.e. visceral Leishmaniasis (VL), cutaneous Leishmaniasis (CL) and mucocutaneous Leishmaniasis (MCL) (Singh *et al.*, 2012), which are caused by different species of *Leishmania* (Figure 1.1).

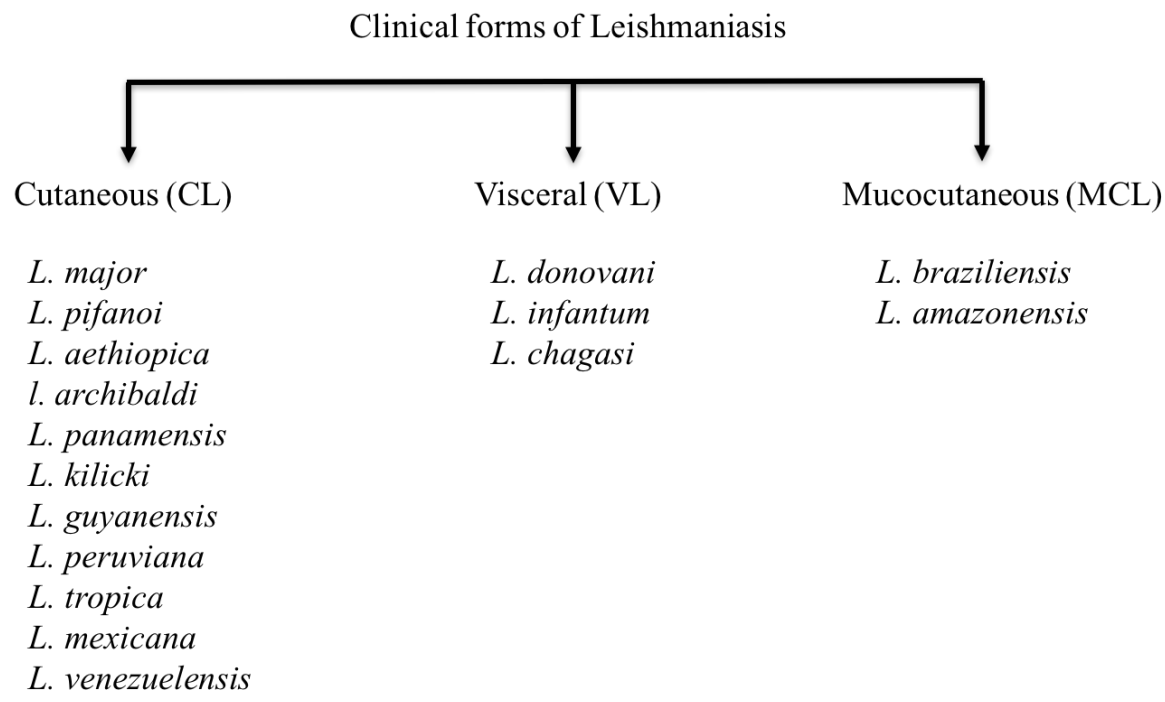


Figure 1.1 List of *Leishmania* species which cause different clinical forms of Leishmaniasis.

Cases of CL have been reported in many countries of the Old and New World. The period of CL incubation in human is from 2 to 8 weeks, though a period of longer than 8 phases has been noticed. An erythematous papule is the first indication of the disease where the body is exposed to the sandfly bite. The size of the papule increases and develops to a nodule. Finally, the nodule starts to ulcerate and coatings over. Usually, the edge is higher and distinct. Multiple lesions could be occurred, mainly when a coat of sandflies faced the patient. The ulcer will be painful when a secondary fungal or bacterial infection, but not if the ulcer caused by CL only. Healing of *L. mexicana* lesions naturally tends in months, while *L. braziliensis* may take years to heal. A depressed scar remains after healing, usually is round, however, can be irregular (Nylen and Eidsmo, 2012).

MCL is potentially life threatening and requires drug treatment to cure (David and Craft, 2009). The mucosal form usually occurs after an initial cutaneous infection. However, MCL mostly appears months or years after the CL. It is characterised by a lesion or a papule that develops into a painless ulcer. The ulcer rarely heals spontaneously, but instead metastasises to various mucocutaneous

regions, including the nasal septum, mouth, nasopharynx, and sometimes the genitalia. Ninety percent of cases of mucosal Leishmaniasis are found in Brazil, Bolivia, and Peru (Goto and Lauletta Lindoso, 2012). They result in scarring and disfigurement and can cause pulmonary aspiration and death.

VL can be classified into two types according to the transmission characteristics. The zoonotic form, where dogs are the main reservoir, occurs in the Mediterranean basin, China, the Middle East, and South America. This form is caused by infection with *L. infantum*. The anthroponotic form, where transmission occurs 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 VL, also known as kala-azar, meaning Black fever (Mcgwire and Satoskar, 2014) has symptoms such as fever, weight loss, cough, diarrhoea, dizziness, vomiting, bleeding of gums, pain in the limbs and a grossly enlarged abdomen because 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 1.2 shows the geographical distribution of Leishmaniasis.

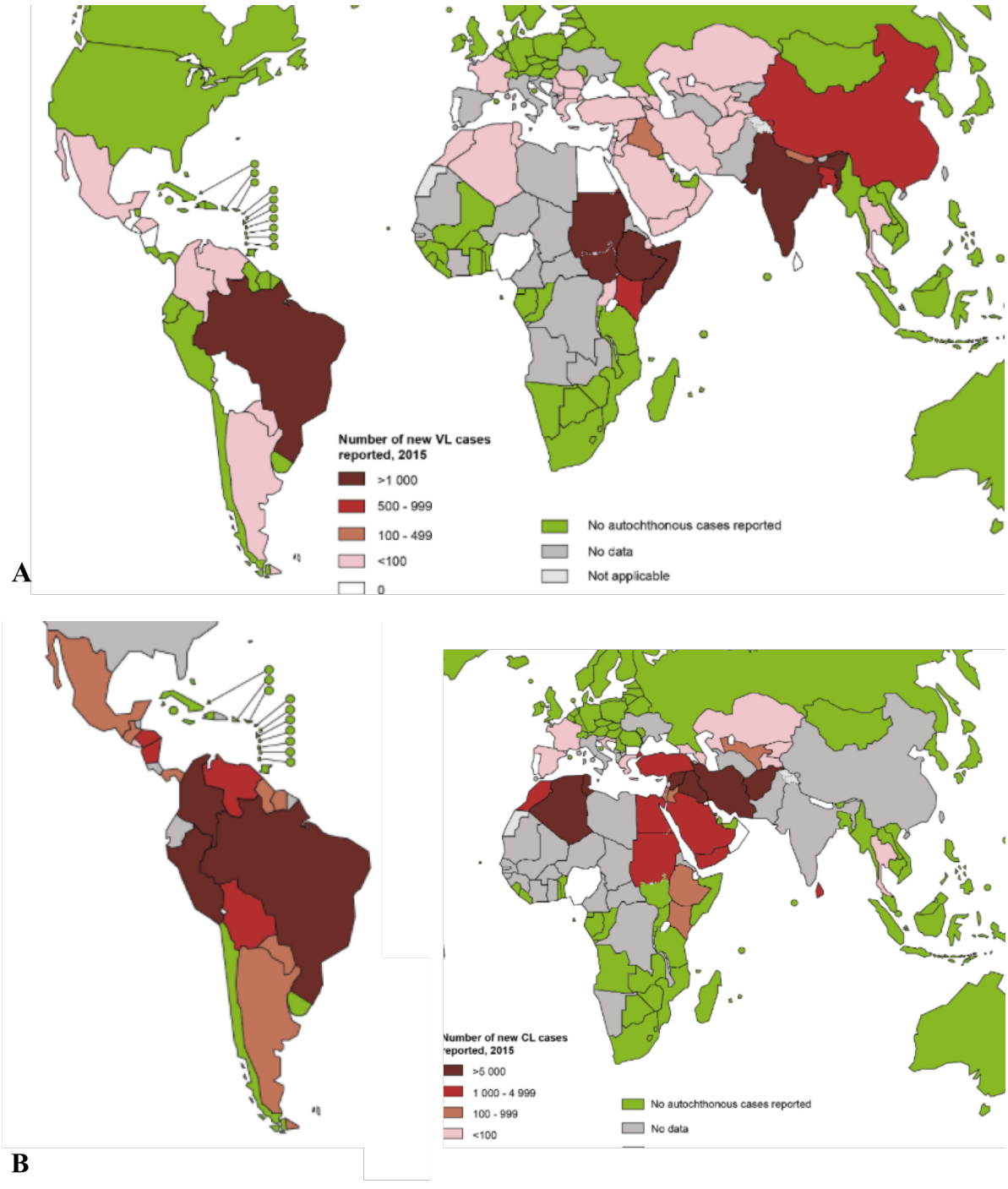


Figure 1.2 Geographical distribution of visceral Leishmaniasis in the Old and New World (A), areas show the distribution of VL. (B) Geographical distribution of cutaneous and mucocutaneous Leishmaniasis in the Old and New World, areas show the distribution of CL (adapted from WHO 2015).

Post kala-azar dermal Leishmaniasis (PKDL) is a dermal development of VL. 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). Although it is not life threating, it is considered a public health problem since patients remain reservoirs of the disease (Ganguly *et al.*, 2010). 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 occurs in people who are otherwise well, although more serious signs of facial ulcers can occur (Adams *et al.*, 2013).

VL 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 have more severe VL symptoms (Hurissa *et al.*, 2010). This is probably due to the dysregulation effects of the virus or parasite on the immune system of the host (Okwor and Uzonna, 2013).

1.2. Life cycle of *Leishmania*

Leishmania parasites are dimorphic organisms (Figure 1.3), which live and multiply as a non-motile form (amastigote) within a parasitophorous vacuole (PV) in mammalian host cells; or as a motile form (promastigote), which lives in the female sandfly (Burchmore and Barrett, 2001). The motile promastigote measures 15-20 μm in length and has a large central nucleus and kinetoplast (Figure 1.4). Parasites live inside the midgut of the female sandfly, where they attach to epithelial cells and multiply as a non-virulent form (procyclic promastigote). The promastigotes then develop into the virulent form (metacyclic promastigote), stop dividing, leaving the midgut and migrate to the insect's mouthparts (Barbieri, 2006). Specific changes occur during the transformation from procyclic to metacyclic promastigote which enable the parasites to evade and survive inside the mammalian host, for example, there is upregulation of glycolipid, lipophosphoglycan (LPG) and the glycoprotein (gp63) on the parasite's surface, and changes in its enzyme content (Dominguez *et al.*, 2003). It is possible to speculate that specific capping of certain adhesiotopes at

the flagellar tip of *Leishmania* parasite, such as LPG, gp63, or other surface molecules, might account for oriented attachment and entry of the parasite into MØ through receptor/ligand interactions (Forestier *et al.*, 2011). Alternatively, the potential role of the flagellum as a sensory organ may point toward a triggered release of parasite proteins during host cell attachment, which may in turn modulate host cell phagocytic activity

When an infected female sandfly is feeding on an uninfected human it deposits metacyclic promastigote into the skin. Then the promastigotes start to differentiate into the non-motile amastigote form (Figure 1.3). Amastigotes are non-motile, either round or oval in shape, with a diameter of 2-5μm with a large kinetoplast and nucleus. They are colourless and have a homogenous cytoplasm. amastigotes exist in acidic PV inside an infected macrophage (MØ). Activation of phagocytic cells triggers a respiratory burst which leads to antimicrobial agents production e.g. reactive oxygen species (ROS) such as superoxide, hydrogen peroxide (H_2O_2) and reactive nitrogen species (RNS) such as nitric oxide, which are toxic to *Leishmania* amastigotes (Birnbaum and Craft, 2011). *Leishmania* escape the degradation in the host cells by evolving numerous mechanisms (Duclos and Desjardins, 2000). Proliferation of the amastigotes inside the hostile causing lysis to the MØs and infection of other MØs by the amastigotes from the surrounding environment. When sandfly feed by taken blood from the human host they take up *Leishmania* infected macrophages and parasites released to the fly's gut. LPG and gp63 on the surface of procyclic promastigotes prevent the parasites from lysis by the sandfly's gut. Moreover, LPG assists the parasites to attached into the epithelial of the insect's gut (Dostalova and Volf, 2012).

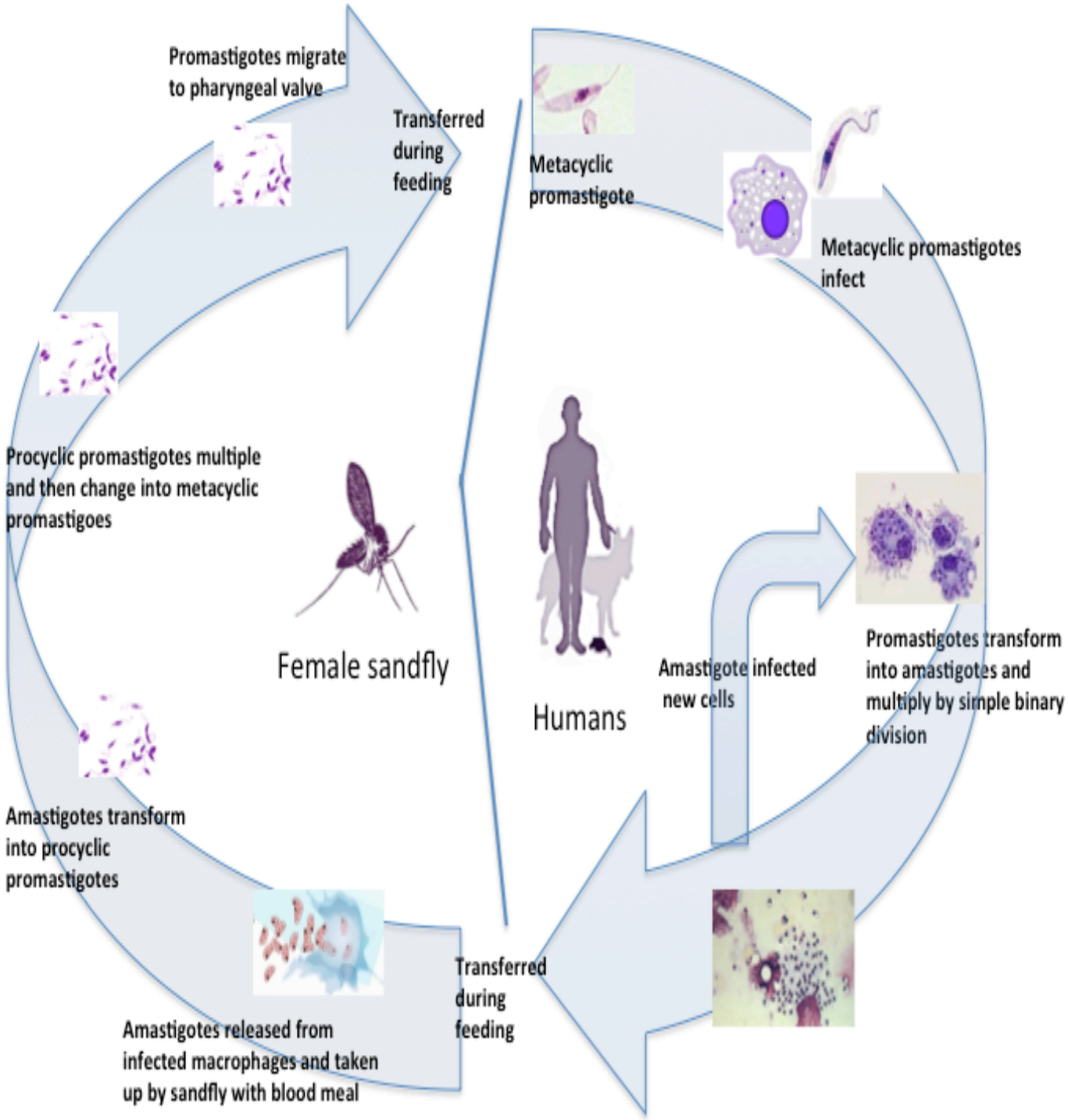


Figure 1.3 The life cycle of *Leishmania,* adapted from (Harhay *et al.*, 2011)

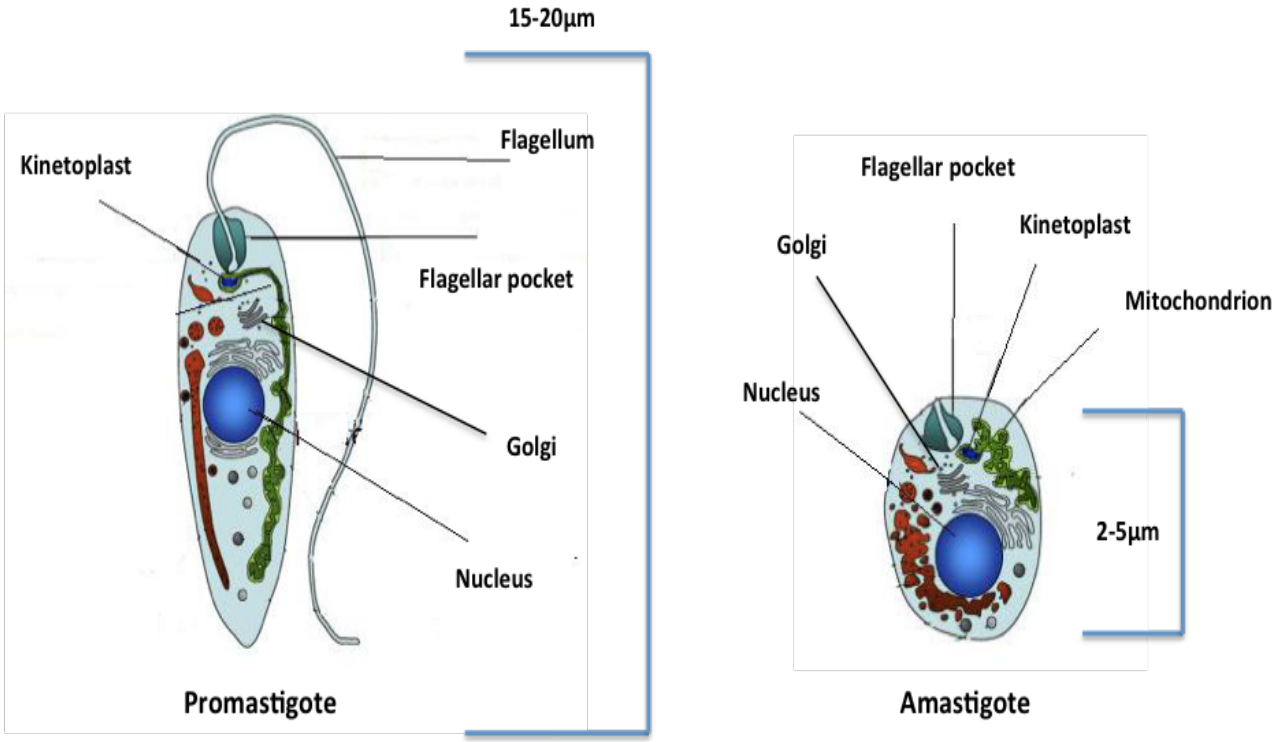


Figure 1.4 Diagram showing the shape of *Leishmania* promastigotes and amastigotes, adapted from (Besteiro *et al.*, 2007).

1.3. Immunity to *Leishmania*

Innate (MØs, dendritic cells [DCs] and neutrophils) and adaptive (T and B cells) components of the immune system are involved in immunity against *Leishmania*. Innate immune responses are important for clearing invading pathogens and influence the ensuing adaptive immune responses. Figure 1.5 shows the cells involved in initial responses to *Leishmania* parasites. Recruitment of neutrophils and monocytes to the site of infection occurs as a result of the local inflammatory response caused by the sandfly bite (Mougneau *et al.*, 2011). It is believed that the complement system is an effective first barrier to help clear *Leishmania* parasites (Gurung and Kanneganti, 2015). The most important pathway involved in *Leishmania* clearance is the alternative pathway. However, studies have shown that anti-*Leishmania* IgM antibodies are involved in the Classical pathway and this pathway is important for *Leishmania* promastigote agglutination and killing (Pearson and Steigbigel, 1980, Navin *et al.*, 1989). The first cells at the site of

infection are neutrophils, which are thought to participate in the containment of *Leishmania* within an hour of infection (Ricardo-Carter *et al.*, 2013). Neutrophils could be involved in the defense against *Leishmania* infection or play a role in disease-exacerbating activity. The main function of neutrophils is phagocytosis of the invading microorganisms and killing via the generation of oxygen metabolites and the release of lytic enzymes stored in their granules (Mollinedo *et al.*, 2010). Neutrophils can release their nuclear DNA associated with granular and cytoplasmic protein to the extracellular milieu, to form neutrophil extracellular traps (NETs), which cause cell death by a process called NETosis (De Menezes *et al.*, 2016). *Leishmania* promastigotes are taken up by macrophages by phagocytosis; and the parasites transform to amastigotes and replicate within the MØs phagolysosomes (Reiner and Locksley, 1995, Soong *et al.*, 2012). MØs can be the cell that is responsible for the parasite killing or the host cells for *Leishmania* replication. Pro-inflammatory cytokines produced by infected MØs causes the recruitment of pro-inflammatory cells e.g. neutrophils, mast cells and eosinophils to the site of infection. Phagocytosis of *Leishmania* promastigotes by MØs activates a respiratory burst and production of ROS (Basu and Ray, 2005). This leads to the generation of nitric oxide (NO) and N-hydroxy-L-arginine (LOHA) (Liew *et al.* 1990). One of the cytokines that is produced from activated MØs that is important in protection against *Leishmania* infection is interleukin 12 (IL-12). This cytokine leads to upregulation of interferon gamma (IFN-γ) by T cells and NK cells. This leads to the generation of Th1-type responses and T cell dependent and independent MØs activation, resulting in inducible nitric oxide synthase (iNOS) and NO production, culminating in parasite killing (Trinchieri, 1998). Production of mast cell-derived mediators, immunoglobulin G mediated mechanisms and cytokines/chemokines released by MØs and neutrophils, lead to the recruitment of DCs. These cells have an important role in linking the innate with the adaptive immune response against *Leishmania* (Reiner and Locksley, 1995). Tables 1.1 summarise the cytokines induced during *Leishmania* infection.

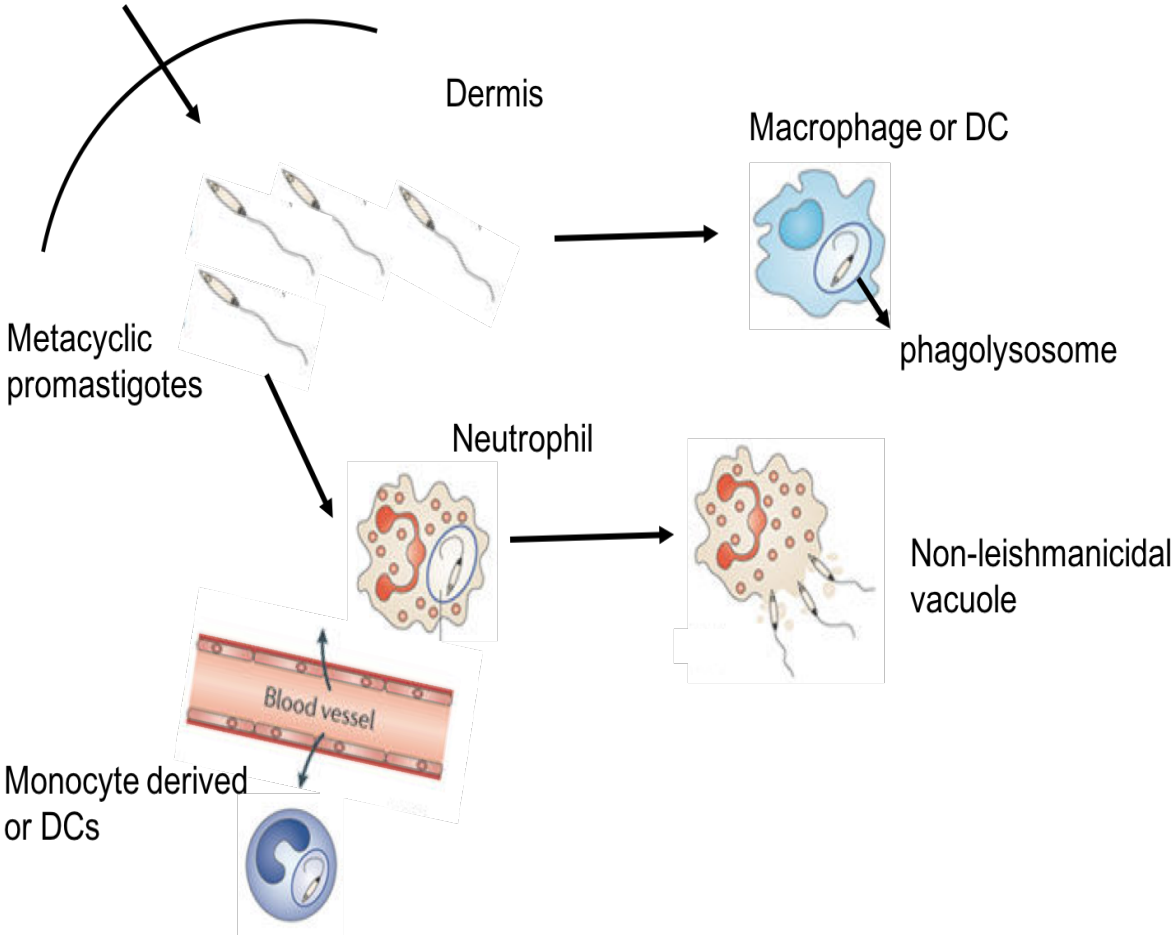


Figure 1.5 The cells involved in the uptake of *Leishmania* parasites. Metacyclic promastigotes are deposited in the dermis in a mixture of immunomodulatory salivary secretions and parasite-derived proteophosphoglycans. Metacyclic promastigotes from the initial inoculum (or those that have been released from infected neutrophils) are phagocytosed by tissue-resident macrophages and dermal DCs. Recruited neutrophils swarm around the extracellular metacyclic promastigotes and engulfing many in non-Leishmanial vacuoles. The death of neutrophils releases metacyclic promastigotes that may be pre-conditioned for survival in other myeloid cells. Alarmins (such as high mobility group protein B1 (HMGB1) and IL-1β), which are released from ruptured neutrophils, possibly help in drawing inflammatory monocyte-derived DCs (moDCs) to the local site. Infected moDCs may enable parasite traffic to the draining lymph node, adapted from (Kaye and Scott, 2011).

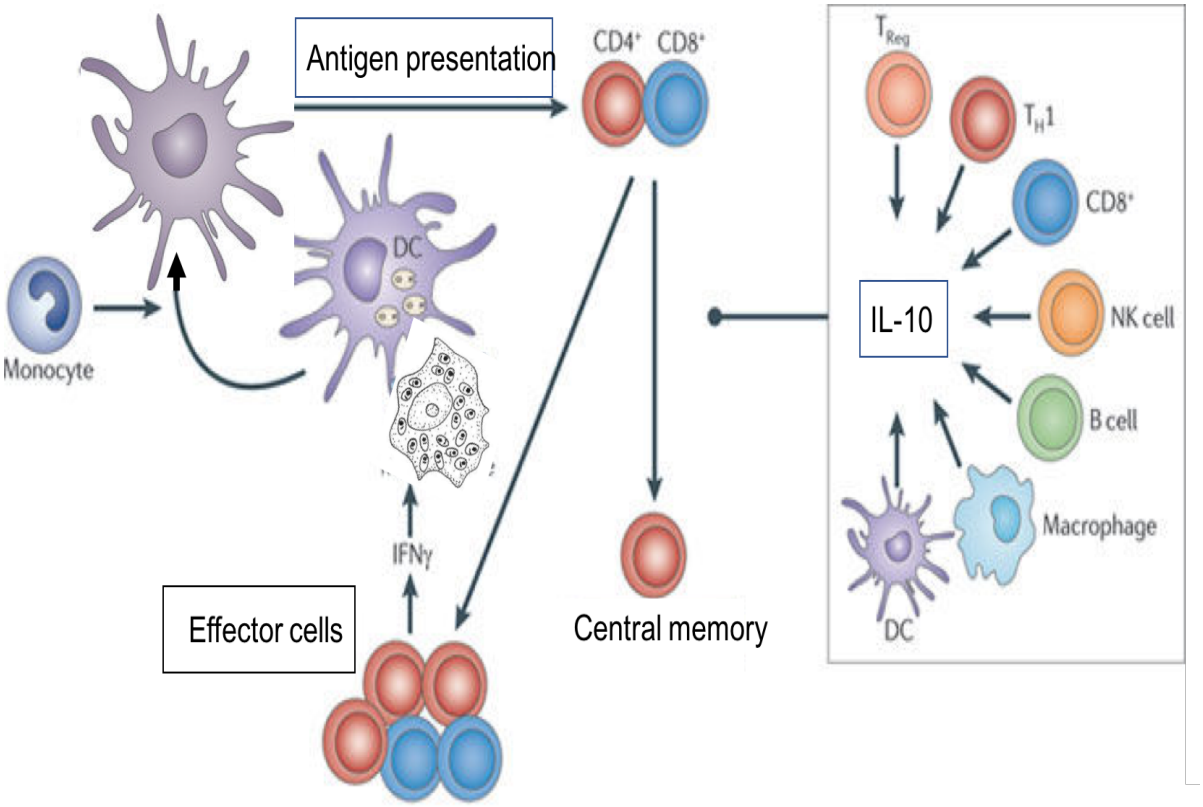


Figure 1.6 Cells involved in immunity to *Leishmania*. Monocytes infiltrate the site of infection and differentiate into DCs. DCs become infected but fail to become activated, whereas local uninfected DCs upregulate major histocompatibility complex class II (MHC class II). MØs are also infected by the 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 T helper (TH1) cells, which produce interferon-γ (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 IFN-γ. Control of the response is largely mediated by the production of interleukin-10 (IL-10), which can come from several different cell types, including regulatory T (TReg) cells, TH1 cells, CD8+ cells, natural killer (NK) cells, B cells, MØs and DCs, adapted from (Kaye and Scott, 2011)

Stimulation of a parasite-specific Th2 response leads to the production of IL-10 by MØs, mast cells, and T regulatory cells. This cytokine inhibits the production of pro-inflammatory mediators such as TNF-α, ROS, IL-12 and RNS by MØs and DCs (Bogdan, 2012), and favours the survival of *Leishmania* within infected MØs. It also inhibits antigen presentation pathways of MØsand DCs. The lack of protective immunity in VL is believed to be related to the production of IL-10 (Nylen and Sacks, 2007). The important of IL-10 in susceptibility to *L. donovani* was shown in studies where treatment with an anti–IL-10 receptor antibody promoted clinical cure of *L. donovani* in mice (reviewed by Kaye and Scott, 2011). Adaptive T regulatory cells (Tregs) which produce IL-10 and may co-express IFN-γ, have been shown to be important in suppression of anti-Leishmanial immunity in human VL. Studies suggest that lack of regulation of IFN-γ secretion by Tregs was associated with development of chronic human dermal Leishmaniasis caused by species of *Leishmania* (*Viannia*) (Rodriguez-Pinto *et al.*, 2012). 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 non-healing strain of *L. major* (Ansari *et al.*, 2011).

DCs present Leishmanial antigens to NK cells, resulting in the production of IFN-γ (Bajenoff *et al.*, 2006). *Leishmania* parasites have evolved a variety of mechanisms to interfere with DC function, which inhibit DC activation during *Leishmania* infection. Infection of DCs with amastigotes leads to a reduction in phosphorylation and degradation of vital molecules involved in Janus Kinase/Signal transduction, and a reduction in Activator of Transcription (JAK/STAT) (Soong, 2008, Gupta *et al.*, 2013). Mechanisms that prevent activation of DCs, and subsequently inhibit T cell priming, impair NK cell activation and suppress IL-12 and IFN-γ production.

The immunological responses associated with healing or non-healing of CL infection are summarised in Figure 1.7. Clinical studies have shown that healing in CL caused by *L. major* infection correlates with development of parasite-specific Th1 response and production of IL-12. In contrast, susceptibility to infection is characterised by the development of a parasite-specific 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 MØ, 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).

Table 1.1 Cytokines involved in immunity to *Leishmania* species

Cytokine	Sources	Function	References
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-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)
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 MØ.	(Ansari *et al.*, 2006, Kima and Soong, 2013)
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)
IL-10	MØ, mast cell, Tregs	Induce non-healing disease, maintaining latent infection and concomitant immunity.	(Alexander and Bryson, 2005, Ansari *et al.*, 2011)
IL-12	DCs and MØs	IL-12 enhances innate NK response to induce differentiation of CD4+ naïve T cells to Th1 cell.	(Torti and Feldman, 2007)
IL-13	Th2 cells	Promotes antibody production by B cells. Inhibits IL-12 function.	(Solbach and Laskay, 2000, Cummings *et al.*, 2010)
IL-17	T helper 17 cells	Stimulates endothelial cells, neutrophils, MØ, and epithelial cells to produce IL-1, IL-6, and TNFα.	(Bacellar *et al.*, 2009)
IL-18	MØ, DCs 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	DCs	Induce production of IL-17	*(Mudigonda et al., 2012)*
IL-33	Th2 cells, MØ, NK cells.	It plays opposing roles. Involved Th1/Th2 response	(Rostan *et al.*, 2013)

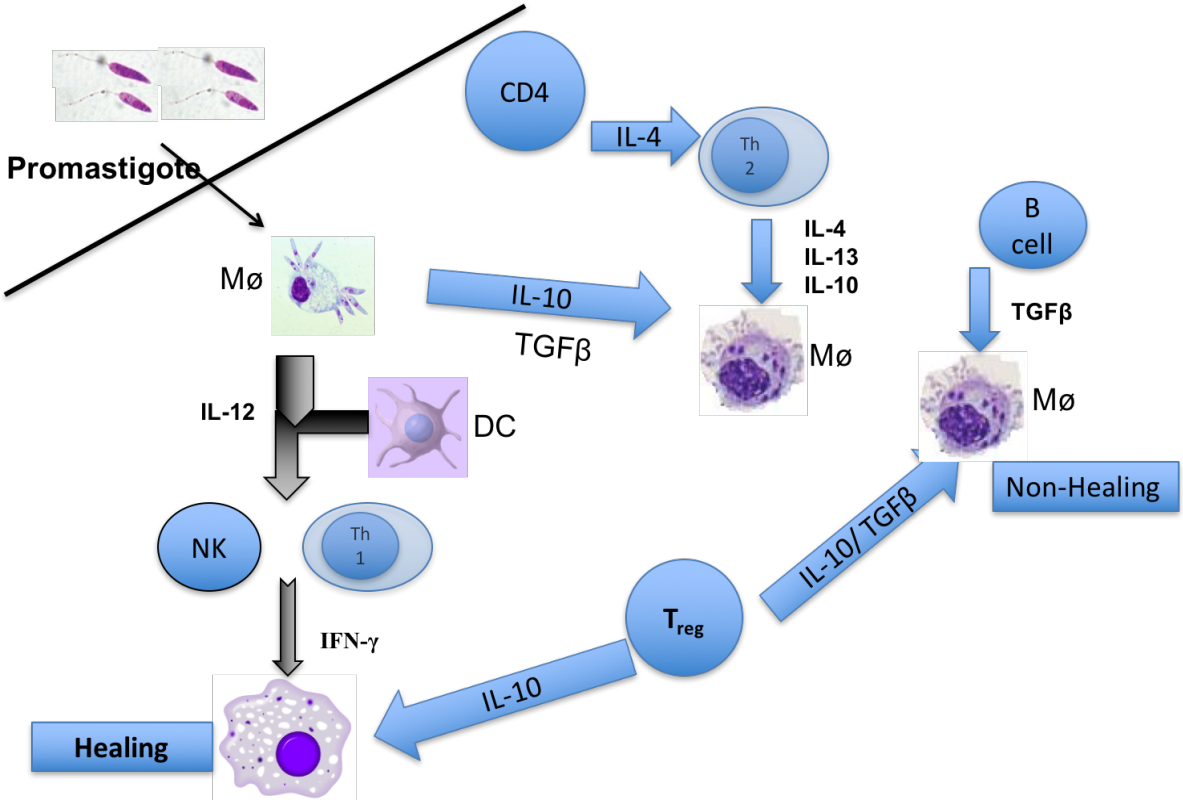


Figure 1.7 The healing or non-healing immune responses associated with infection in *L. major*. In susceptible infection, IL-4 and IL-10 play crucial role for suppressing the healing response by leading the Th2 immune response. IL-12 and IFN-γ are the protective cytokines based on their ability to stimulate Th1 immune response. DCs are the source of IL-12 during early infection as infected MØ shows a decreased ability to produce IL-12 in response to infection, adapted from (Sharma and Singh, 2009)

The immune response to *L. mexicana* is complex and the parasite is highly resistant to neutrophil or MØ killing. There is evidence that infection with *L. mexicana* suppresses IL-12 production by MØs and DCs. 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 DCs to the draining lymph nodes may result in the insufficient priming of a Th1 response (Petritus *et al.*, 2012).

Human VL has a complicated immunology and it is characterised by mixed Th1/Th2 responses (Figure 1.8). A suppressed parasite-specific Th1 response, along with an elevated parasite-specific Th2 occurs during active disease. Protective immunity requires an up-regulation of Th1 responses, and this has been shown to occur after successful chemotherapy treatment (Engwerda *et al.*, 2004). Immunity against VL is organ-specific and in 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 IFN-γ. A hepatic granuloma consists of a core of parasitised 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 iNOS, and thus are unable to make NO. They 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 NO is an important anti-Leishmanial agent (Bunn *et al.*, 2014, 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 using IL-12 gene deficient mice highlighted Th1 cytokines importance in the immune response against *L. donovani*. 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* infection, and in both groups granuloma formulation was accelerated when parasite killing was augmented (Deak *et al.*, 2010).

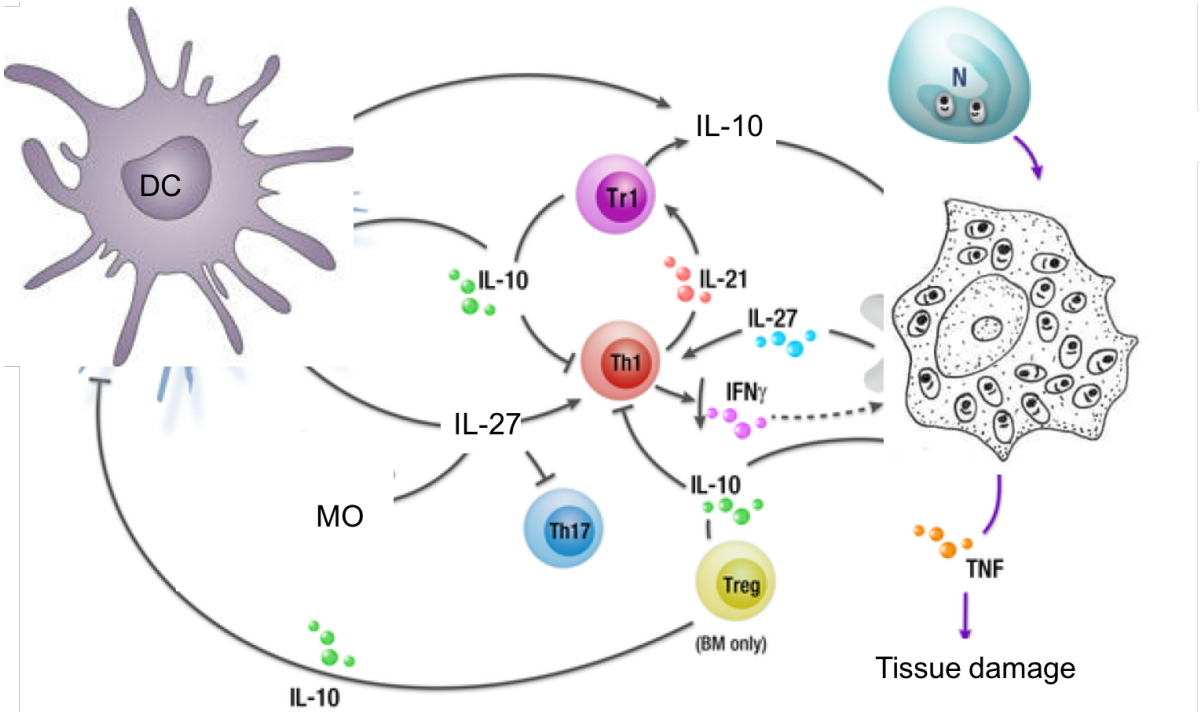


Figure 1.8 An overview of cellular response during *L. donovani* infection. During an established *L. donovani* infection, a subset of regulatory DCs in the spleen can produce IL-10 that promotes the expansion of IL-10-producing Tregs1, as well as inhibiting antimicrobial mechanisms in MØs and other phagocytic cells (including suppression of ROI and RNI generation). IL-27 produced by regulatory DCs and MØs, along with T cell–derived IL-21, can drive the differentiation of Th1 cells into Tregs1, as well as inhibit Th17 development. IL-10 produced by Tregs1 cells can suppress antigen presentation, contributing to T cell dysfunction, as well as down-regulate $CD4^+$ T cell IFN-γ production. There has been a report that IL-10 can also be produced by Treg cells in the BM of VL patients. Although uptake of infected neutrophils undergoing apoptosis by MØs contributes to the establishment of *L. major* infection in mice, no such mechanism has yet been described during *L. donovani* infection, adapted from (Faleiro *et al.*, 2014)

1.4. Treatment of Leishmaniasis

The third most devastating vector-borne disease after malaria and African trypanosomiasis is Leishmaniasis (Sharifi *et al.*, 2015). Access to medicines to treat the disease is challenging in poor countries that have the highest burden of cases (WHO, 2016). Chemotherapy is considered as the major method used to control Leishmaniasis, but there are drawbacks to this method of treatment: as it is slow acting, expensive and most of the drugs have toxic side effects (Table 1.2). In addition, most anti-Leishmanial drugs have been around a long time and there are few new treatments entering the clinic. The therapy is further complicated by the fact that many of the infected individuals are children and drug resistance has limited the clinical utility of pentavalent antimonial compounds, which were the first line treatment for VL. Lipid formulations of amphotericin B are an important advancement in therapy, as they require fewer doses, they are more effective and less toxic than amphotericin B solution. However, their high cost precludes their use. Miltefosine is the first oral medicine for the treatment of VL, but drug resistance in the parasite population is already limiting the use of this drug (Chavez-Fumagalli *et al.*, 2015).

Many factors including not continuous of the medicines by the suppler, which are produced by one manufacturer e.g quality, low production capacity and lack of adequate forecast of needs can be the main issues for Leishmaniasis elimination (WHO, 2015). In 2016 Gilead Sciences signed a contract of 5 years for the donation through WHO of 380000 vials of liposomal amphotericin B including funding for enhancing the diagnosis and treatment for eligible endemic countries for VL.

Table 1.2 Drugs used in treatment of Leishmaniasis

Drug	Route of administration	Dose	Mode of Action	References
Pentavalent antimonials	Intramuscular or intravenous injections	20 mg/ kg/ day for 28 days	Inhibits trypanothione reductase and increases the ROS	(Frezard *et al.*, 2009)
Amphotericin B	Intravenous	0.75-1 mg/ kg for 20 infusions	Impairs permeability of plasma membrane	(Sundar and Chatterjee, 2006)
Liposomal amphotericin B	Intravenous	5 mg/ kg, 4–10 doses over 10–20 days	Impairs permeability of plasma membrane	(Sinha *et al.*, 2011, Sundar and Chakravarty, 2010)
Paromomycin	Parenteral for VL. Topical for CL	11mg/ kg /21 days/im Topical up to 20 days.	Induces respiratory dysfunction	(Sundar and Chatterjee, 2006, Wiwanitkit, 2012)
Pentamidine	Parenteral administration Intravenous	1 mg/ kg daily for 15 days	Interferes with DNA synthesis	(Das *et al.*, 2009)
Miltefosine	Oral treatment for VL	100 mg daily for 28 days	Inhibits membrane signalling pathways	(Dorlo *et al.*, 2012, Bhandari *et al.*, 2012, Sundar and Olliaro, 2007)

1.5. Vaccination

The most efficient strategy for preventing Leishmaniasis would be vaccination (Mutiso *et al.*, 2013) which would give long term protection against a disease and would be effective against drug resistance parasites (Nagill and Kaur, 2011). Vaccines can be defined as a particular class of drugs, which do not have a direct effect on a pathogen. The stimulation of suitable and effective immune responses, are the origin for the defensive efficiency of a vaccine. Development of a vaccine required a long process, which includes costly laboratory studies to define their efficiency and safety (Pappalardo *et al.*, 2010). A vaccine against *Leishmania* should be safe to use, produce a high level of long-lived efficacy, be cost efficient and easy to store and administer. Also, the vaccine should have the ability to stimulate cell-mediated immunity against *Leishmania* (Nagill and Kaur, 2011). Issues such as antigenic complexity, cost, variability of organisms and the mixed type of responses produced in the host are limiting the development of hundreds of potential vaccines for clinical use (Singh and Sundar, 2012). In addition, virulence factor variations between species and the type of immune responses induced e.g. LPG is a virulence factor for *L. major* (Spath *et al.*, 2000), but not for *L. mexicana* (Ilg *et al.*, 2001). Several *Leishmania* vaccine candidates have been tested and these have used different approaches. These include, vaccines consisting of whole parasites as live attenuated or killed vaccines, subunit vaccines, recombinant protein vaccines and DNA vaccines (Nagill and Kaur, 2011). In addition, sandfly salivary components have been suggested as potential vaccine candidates on their own (Collin *et al.*, 2009, Gomes *et al.*, 2012) or in combination with *Leishmania* vaccines (Alvar *et al.*, 2013). Figure 1.9 shows the types of vaccines that have been used against Leishmaniasis.

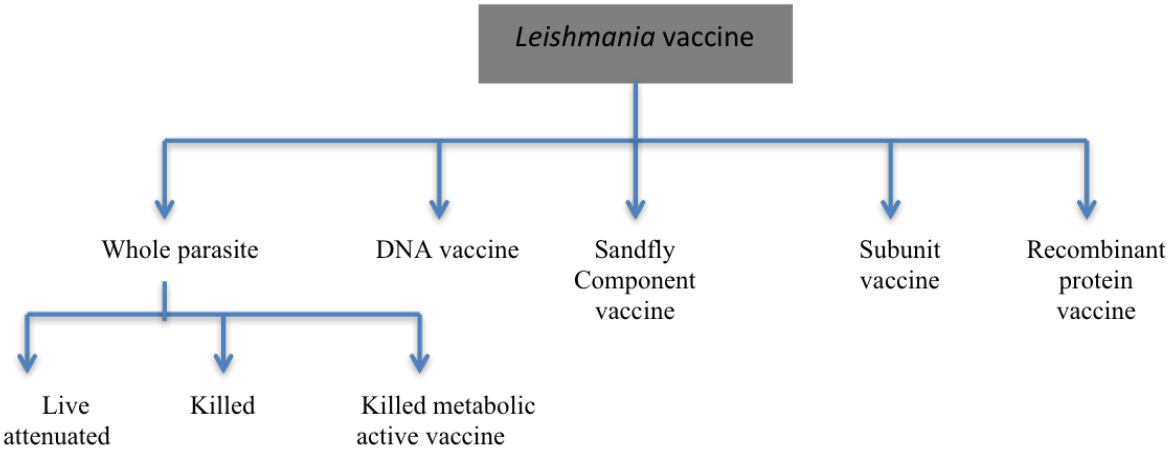


Figure 1.9 Types of vaccines that have been used to protect against Leishmaniasis.

The first type of vaccine used against *Leishmania* was 'Leishmanisation', 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. Live *L. major* promastigotes grown from infected lesion exudates have also been used as a prophylactic vaccine in Iran, where vaccination decreased the incidence of CL. A mixture of live virulent *L. major* parasites and killed parasites was used in Uzbekistan (Khamesipour *et al.*, 2006, Coler and Reed, 2005). In 1937, a live promastigote *L. major* vaccine was used in Russia (Coler and Reed, 2005) and several thousand people were vaccinated with live *L. major* in Turkmenistan (Kellina and Strelkova, 2010). However, this type of vaccine is not suitable because of ethical and safety reasons, as its use is associated with a high risk of parasite latency after cure (Kedzierski, 2011). An alternative approach is to use a non-pathogenic such as *L. tarentolae* as a live vaccine, as it can differentiate to the amastigote form within mammalian macrophages (Breton *et al.*, 2005). *L. tarentolae* can cause DC activation, induction of T cell proliferation and the production of IFN-γ. *L. tarentolae* has been used as a vaccine vector candidate in several studies against *L. donovani* or *L. major* infection. In studies the parasite has been transfected with the gene sequences of cysteine proteinases (type I and

II, CPA/CPB) and sand fly salivary antigen (PpSP15) (Zahedifard *et al.*, 2014), *L. donovani* A2 antigen and cysteine proteinases A (CPA and CPB without its unusual C-terminal extension (CPB^{-CTE}) (Saljoughian *et al.*, 2013), and lipophosphoglycan 3 (Pirdel and Farajnia, 2017).

An alternative approach is to use an attenuated vaccine where the virulence of a pathogen has been reduced so that the vaccine can induce a protective immune response without causing the disease. The parasite can be attenuated by irradiation, mutagenic chemical treatment or through genome alteration (Kobets *et al.*, 2012). Live attenuated parasites can deliver complete antigens to antigen presenting cells (Kedzierski, 2011). Vaccination using attenuated lines of *L. mexicana* or *L. major* protected susceptible BALB/c mice from infection with the wild-type parasite. For example, attenuated *L. mexicana* parasite was prepared by culturing promastigotes in the presence of gentamicin that resulted in the development of promastigotes with a different morphology (i.e. longer) but they had the same growth rate as the original wild-type parasite. 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 but still caused infection. Thus, vaccinated mice had slow developing lesions, and at 18 weeks after infection had the mean lesion size of approximately 500 mm^3, and lesions reduced in size over time (Daneshvar *et al.*, 2003).

A killed vaccine is safe to use and should be low cost. Methods to obtain a killed pathogen included heating e.g. autoclaving (Nagill *et al.*, 2009), formaldehyde treatment (Mutiso *et al.*, 2010), or repeated cycles of freezing and thawing (Okwor *et al.*, 2010). For example, a killed *L. major* vaccine was produced using promastigotes fixed with formalin (KLM). This vaccine was given to BALB/c mice in combination with adjuvants used to enhance the immunogenicity of the vaccine i.e. alum, BCG or montanide ISA 720 (MISA). All three combinations gave significant protection against infection, and immunity was associated with an antigen-specific T helper 1 immune response. However, significant inflammation occurred at the vaccine site in mice immunised with the killed vaccine and BCG (Joshua M Mutiso, 2009). In a study in Ecuador, vaccination of school children

with two doses of *L. amazonensis* and *L. mexicana* promastigotes mixed with BCG gave 73% protection against infection (Armijos *et al.*, 1998). Indicating that BCG can be used safely in a clinical vaccine. An autoclaved *L. major* promastigote vaccine has passed phase I-II clinical trials in Iran. Using BCG as an adjuvant to increase immunogenicity of this vaccine could be incorporated into the national vaccination programme in endemic areas (Dowlati *et al.*, 1996, Modabber, 2010). Another type of vaccine used in studies is a metabolically active (KBMA) vaccine. *L. infantum* and *L. chagasi* promastigotes treated with the psoralen compound amotosalen, and exposed to low doses of UV radiation, were used to immunise mice against infection. The parasites were undetectable within the mice organs and *in vitro* study showed that the vaccine parasites retained the ability to enter macrophages and induce nitric oxide production. This vaccine given by subcutaneous injection protected mice infection virulent parasites (Bruhn *et al.*, 2012).

A nucleic acid or DNA vaccine was shown to protect against *Leishmania* infection in 1990 by Wolff and co-workers. In this type of vaccine, the gene sequence encoding for a target protein is cloned into an expression vector. The purified DNA vaccine is then administered by intradermal or intramuscular injection (Coban *et al.*, 2011). DNA vaccines can have single or multiple gene sequences in the plasmid allowing vaccination against a single antigen or combination of antigens. The plasmid-encoding antigen is translated in the cell, which takes up the plasmid, and subsequently degraded in the cytoplasm of the host cell. The cell can then present these degraded fragments through different pathways to specific T cells and/or B cells, resulting in induction of an immune response which can subsequently protect against a live infection (Coban *et al.*, 2013). These types of vaccines are safe because they do not contain pathogens that could revert to their virulent form (Kobets *et al.*, 2012). DNA vaccines against *Leishmania* have been used in many studies. For example, a plasmid vaccine containing gene sequences for L. major gp63 protein induced a strong type 1 T helper (Th1) response and protected BALB/c mice against *L. major* infection (Xu and Liew, 1995). In a later study *L. mexicana* gp63 cDNA achieved a higher

protection than immunization with a soluble *Leishmania* antigen (SLA). In addition, protection was associated with a higher Th1 immune response (Rezvan *et al.*, 2011).

Subunit vaccines use protein antigens, which are easy to purify and detect (Mutiso *et al.*, 2013). The first purified *Leishmania* sub fraction vaccine was purified from *L. donovani* promastigote glycoprotein fraction. This subunit vaccine named the 'Fructose mannose ligand (FML)' has passed Phase I-III clinical trials and is given with the adjuvant produced from *Quillaja saponaria* (saponin). This vaccine is licensed as Leishmune® and is used to vaccinate against canine VL. The first European canine *Leishmania* vaccine CanLeish®, approved in 2011, contains protein excreted from *L. infantum*, and the dominant antigen is the promastigote surface antigen (Day, 2011). However, there is still no approved vaccine against Leishmaniasis for humans.

Whole sandfly saliva or components of saliva were tested for their ability to vaccinate against *Leishmania* (Kobets *et al.*, 2012). Insect saliva contains immunomodulatory molecules, which induce species-specific humoral and cellular response in the host (Drahota *et al.*, 2009), which can give protection against *Leishmania* after limited to short-term exposure to sandflies immediately before infection (Rohousova *et al.*, 2011).

Recombinant protein is the most common method for vaccination used now. The protein is produced using recombinant DNA technology, which allows the production of the high amounts of the protein. 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). A number of methods and expression systems are used, and *Escherichia coli* is the most common expression system. However, the protein may be expressed in an insoluble form in inclusion bodies. It is possible to refold protein recovered from inclusion bodies, but the method used may be involved and time-consuming. And sometimes refolding to an active protein is simply not possible (Chan *et al.*, 2010). Several *Leishmania* recombinant proteins have been produced for vaccination,

e.g. surface expressed glycoprotein leishmaniolysin (Olobo *et al.*, 1995, Mazumder *et al.*, 2011). Studies have shown that recombinant proteins can protect against Leishmaniasis in animal models (Sachdeva *et al.*, 2009, Kaur *et al.*, 2011, Kobets *et al.*, 2012, Hezarjaribi *et al.*, 2013), where vaccination resulted in a significantly decrease in parasite burdens compared to controls.

1.6. *Leishmania* tarentolae

Several eukaryotic expression systems have been used for expression of eukaryotic proteins. Trypanosomatidae can also be used to produce eukaryotic proteins and they also have unique features which may be important in for protein expression for similar species, including RNA editing, polycistronic transcription followed by trans-splicing reaction, and regulation of gene expression at the post-transcriptional level (Teixeira, 1998). Trypanosomatidae are also rich in glycoproteins that are represent more than 10% of total protein (Ferguson, 1997). Similarity of oligosaccharide structures of their glycoprotein to those of mammalians, which in some cases complex-type oligosaccharides with α-linked galactose, fucose and sialic acid residues can be incorporated. Trypanosomatidae have been successfully used as a heterologous expression system to express recombinant proteins that were biologically active (Zhang *et al.*, 1995). *L. tarentolae*, a parasite of the gecko *Tarentolae anuularis*, is a member of the family Trypanosomatidae that is not pathogenic to humans. They are notorious for finding unique solutions to general process of the eukaryotic cell as RNA editing, arrangement of genes in tandem arrays, polycistronic transcription followed by trans-splicing and regulation of gene expression exclusively at the post-transcriptional level (Basile and Peticca, 2009). It shows rapid growth rates *in vitro* and has simple nutrient requirements (Simpson *et al.*, 1996). *L.tarentolae* has been used to express several heterologous proteins produced as intracellular proteins or secreted proteins, and could be used for with large-scale production (Basile and Peticca, 2009). *L. tarentolae* can be transformed using different expression vectors, where recombinant expression cassettes can be stably integrated into the genomic DNA to produce protein constitutively using

endogenous RNA polymerase I or II, or for inducible expression using co-integrated promoters for RNA polymerase I or heterologous RNA polymerases such as T7 or T3 (Mureev *et al.*, 2007).

1.6.1. Differences between *L. tarentolae* and pathogenic genome species

L. tarentolae lacks several genes coding for proteins implicated in trafficking. Indeed, the β1/β2-adaptins (LmjF11.0990; LmjF36.5595), μ-adaptin (LmjF31.3035) and the epsilon–adaptin (LmjF30.1545) were absent from *L. tarentolae*. Adaptins are involved in the formation of clathrin-associated adaptor protein (AP) complexes, which play a key role in the transport of proteins by regulating the formation of transport vesicles as well as cargo selection between the trans-Golgi network, endosomes, lysosomes and the plasma membrane. The calcium-dependent membrane binding proteins copines (LmjF28.1190) and Ras-like small GTP-binding proteins (LmjF36.1820), both involved in cell signalling and/or membrane trafficking/transport pathways and exocytosis, were also absent from *L. tarentolae*. The endosomal/lysosomal membrane-bound acid phosphatase (LmjF28.2650), potentially involved in intracellular trafficking, is also missing from *L. tarentolae*. *L. tarentolae* also lacks the phosphatidylinositol 3-kinase 2 gene (LmjF14.0020) and the phosphatidylinositol-4-phosphate 5-kinase gene (LmjF26.2495) whose activities were linked to a diverse set of key cellular functions, including intracellular trafficking. The Tubby protein 1, that has been reported to bind phosphatidylinositol 4,5-bisphosphate on the plasma membrane and facilitate macrophage phagocytosis, is not present in *L. tarentolae* (Raymond *et al.,* 2012).

1.7. Role of gamma glutamylcysteine synthetase (γGCS)

Gamma glutamylcysteine synthetase (glutamate cysteine Ligase, γGCS) catalyses the rate limiting step of glutathione (L-gamma-glutamyl-L-cysteinylglycine, GSH) biosynthesis. The GSH non-allosteric feedback controlled the activity of γGCS, the transcription processing of the enzyme due to the availability of cysteine factors effecting the production of the enzyme (Lu, 2009). GSH is a tripeptide thiol in mammals, beside it is a crucial component for cells as it plays several vital functions such as sustaining intracellular redox balance and protecting against possible toxic agents which cause chemical or oxidative stress (Manta *et al.*, 2013). One of the most important antioxidant defense system of *Leishmania* is trypanothione (TSH) and TSH reductase, whereas mammalian cells depend on GSH and GSH reductase to control their intracellular thiol redox state. GSH could efficiently reduce the oxidized cell components, forming oxidized GSH disulphide (Van Assche *et al.*, 2011, Olin-Sandoval *et al.*, 2012). The four-major low molecular mass thiols that are Trypanosomatids contain: GSH, mono-glutathionyl spermidine, TSH and ovothiol A. Species, growth phase of the parasite and life stage factors that the amount of each molecule depends on. Trypanosomes and *Leishmania* have trypanothione reductase instead of glutathione reductase (Krauth-Siegel and Comini, 2008). A study demonstrated that γGCS is essential for the survival of *Leishmania* against oxidative agents using *L. infantum* GSH1 null mutants. It found that parasites copy an additional copy of the γGCS gene every time they tried to delete the gene and (Mukherjee *et al.*, 2009), studying the γGCS as drug target (Frearson *et al.*, 2007). Moreover, Carter and her research group concluded that γGCS is crucial to the persistence 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).

1.8. Luciferase-expressing *Leishmania*

The enzyme Different organisms produce bioluminescence naturally e.g. bacteria, fungi, fish, and insects. The oxidation reaction between the enzyme luciferase and its substrate luciferin generating the bioluminescence, which usually requires energy and oxygen (Kuchimaru *et al.*, 2016). 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). Animal bioluminescent imaging is now 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. The method can be used to continually monitor a single individual, so it allows a reduction in the amount of inter-animal variation (Baker, 2010). There are many different types of luciferase enzymes available e.g. 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 impractical for large animal models (Close *et al.*, 2011). Firefly luciferase generates bioluminescence in a two-step reaction. The first step involves the conversion 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 1.10). 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).

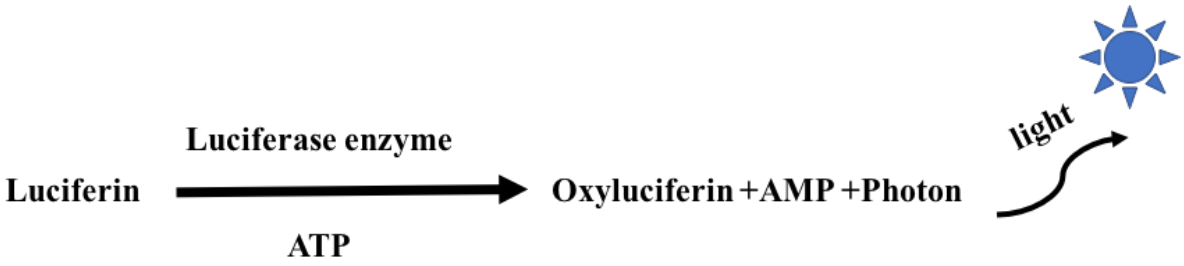


Figure 1.10 The reaction of luciferin with luceriferase enzyme in light production

The typical method for monitoring *Leishmania* parasite burdens 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 using bioluminescent imaging have been developed for *in vitro* drug screening or *in vivo* determination of parasite infection level (Dube *et al.*, 2009). The reporter gene used to produce bioluminescence 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 reporter 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 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 the study

Previous studies have shown that vaccination with the DNA encoding for *L. donovani* γGCS protected BALB/c mice against infection (Carter *et al.*, 2007). Subsequent studies showed that vaccination with recombinant γGCS protein gave significant protection against *L. donovani* (Henriquez *et al.*, 2010), *L. major* or *L. mexicana* infection (Campbell *et al.*, 2012). The recombinant γGCS protein was expressed and purified using an *E. coli* expression system, but there were problems with recombinant protein expression and it was not possible to produce pure full-length protein. One way to overcome would be to use an expression vector that is phylogenically more like *Leishmania. L. tarentolae* has been developed as an eukaryotic expression system for the production of recombinant proteins and can be considered as an alternative expression system to mammalian expression system (Sodoyer, 2004). The main advantage of using these parasites is their high and stable growth rate, where a maximum cell density of 1 x 10^9 parasites/ml can be achieved (Fritsche *et al.*, 2007). In addition, they can be cultivated in low cost medium supplemented with hemin. In this project, the feasibility of using *L. tarentolae* instead of *E. coli* to produce recombinant γGCS protein for vaccine studies was determined.

Therefore, the aims of this project were to:

1. Clone the γGCS gene sequence of *Leishmania* γGCS from *L. major*, *L. mexicana* or *L. donovani* into the expression vector pSSU-INT to produce N terminal EGFP and C terminal His-tag which was suitable to be stably integrated into the ribosomal RNA locus of the *Leishmania* genome.
2. Optimise the expression conditions for *Leishmania* γGCS to increase protein yield from the three different *L. tarentolae* constructs.
3. Determine if treatment with BSO, H_2O_2 or glutathione improved the growth and production of recombinant γGCS by transfected *L. tarentolae* promastigotes.
4. Compare the ability of live vaccine of *L. tarentolae* transfected with different *Leishmania* γGCS gene sequences to protect against *Leishmania* infection.

CHAPTER 2. MATERIALS AND METHODS

2.1. Materials

Acrylamide 40% v/v Bis Solution	Bio-Rad Laboratories GmbH, HeidemannstraBe, München, Germany
Agarose (electrophoresis grade)	Techmate Ltd, Milton Keyes, UK
alkaline phosphatase conjugate	BD Biosciences, Oxford, UK
Ammonium chlorid	Fisons Scientific Equipment, Loughborough, England.
Ammonium Persulfate (APS)	Sigma Aldrich, Irvine, UK
Anti-mouse IgG HRP-linked Antibody	New England Biolabs, Hitchin, UK
Antibiotics (Ampicillin, Kanamycin, Tetracyclines, Chloramphenicol, Hygromycin B and Blasticidin S Hydrochloride)	Sigma Aldrich, Irvine, UK
Bromophenol blue stain	Sigma Aldrich, Irvine, UK
cobalt chloride	Fisher Scientific UK Ltd, Loughborough, UK
cOmplete™	Roche Diagnostics, Burgess Hill, UK
Coomassie Brilliant Blue R Stain	Sigma Aldrich, Irvine, UK
Cytokines standards	BD Biosciences, Oxford, UK
D-(+)-Glucose	Sigma Aldrich, Irvine, UK
D-luciferin potassium salt	Caliper Life Science, Massachusetts, USA
DAPI	Sigma Aldrich, Irvine, UK
Detection anti-cytokines antibodies	BD Biosciences, Oxford, UK
DMSO	Techmate Ltd, Milton Keyes, UK
DNA ladder 1 Kb	New England Biolabs, Hitchin, UK
DTT	Sigma Aldrich, Irvine, UK
EDTA	Sigma Aldrich, Irvine, UK
Escherichia coli strains Rosetta blue, BL21 and DH5α	Novagen, London, UK
Ethanol	Sigma Aldrich, Irvine, UK
Ethidium Bromide	Sigma Aldrich, Irvine, UK
Foetal calf serum	Gibco BRL, Paisley, UK
Formaldehyde (37% v/v)	Carl Roth, Karlsruhe, Germany
GFP (4B10) Mouse mAb	New England Biolabs, Hitchin, UK

Giemsa stain	Sigma Aldrich, Irvine, UK
Glycerol	Sigma Aldrich, Irvine, UK
Glycine	Fisher Scientific UK Ltd, Loughborough, UK
Hemin	Sigma Aldrich, Irvine, UK
Hepes	Sigma Aldrich, Irvine, UK
His-Tag (D3I10) XP® Rabbit mAb (HRP Conjugate)	New England Biolabs, Hitchin, UK
HisGraviTrap columns	GE Healthcare, Amersham, UK
HisTrap® columns	GE Healthcare, Amersham, UK
Horseradish peroxidase (HRP) conjugated goat antimouse IgG1 and IgG2a	Southern Biotechnology Associates Inc, Birmingham, USA
Human T Cell Nucleofector Kit	Amaxa Biosystems, Gaithersburg, USA
Imidazole	Sigma Aldrich, Irvine, UK
Isopropanol	Techmate Ltd, Milton Keyes, UK
L-broth medium	Sigma Aldrich, Irvine, UK
L-glutamine	Gibco BRL, Paisley, UK
LB agar (MILLER)	Merck kGaA, Darmstadt, Germany
Lithium Chloride	Sigma Aldrich, Irvine, UK
Methanol	Sigma Aldrich, Irvine, UK
Mouse anti-Hamster igg HRP	Southern Biotechnology Associates Inc, Birmingham, USA
Novex® ECL	Invitrogen, Paisley, UK
Ortho-Phenanthroline	Sigma Aldrich, Irvine, UK
Penicillin-streptomycin	Gibco BRL, Paisley, UK
pET-24a plasmid	Invitrogen, Paisley, UK
Pharmingen capture	BD Biosciences, Oxford, UK
Platinum® Taq DNA High Fidelity polymerase	Invitrogen, Paisley, UK
Ponceau S solution	Sigma Aldrich, Irvine, UK
Potassium Chloride	Sigma Aldrich, Irvine, UK
Potassium Nitrate	Sigma Aldrich, Irvine, UK
Prestained protein markers (7-175	New England Biolabs, Hitchin, UK

kda)	
Protein assay dye reagent	Bio-Rad Laboratories GmbH, HeidemannstraBe, München, Germany
PureLink™ Quick Plasmid Miniprep Kit	Invitrogen, Paisley, UK
Puromycin dihydrochloride	Calbiochem EMB Chemicals Inc, Darmstadt, Germany
Qiagen gel extraction kit	Qiagen, Crawley, UK
QIAprep Spin Miniprep Kit	Qiagen, Crawley, UK
Restriction endonucleases	New England Biolabs, Hitchin, UK
Reverse Transcriptase kit	Promega, Southampton, UK
RNase A (bovine pancreas)	Roche Diagnostics, Mannheim, Germany
RPMI 1640 medium	Gibco BRL, Paisley, UK
Shrimp alkaline phosphatase	Roche Diagnostics, Mannheim, Germany
Sodium Chloride	Sigma Aldrich, Irvine, UK
Sodium dihydrogen orthophosphate 1-hydrate	AnalaR BDH Laboratory, Poole, England
Sodium Dodecyl Sulfate (SDS)	Sigma Aldrich, Irvine, UK
Sodium Phosphate Dibasic	Sigma Aldrich, Irvine, UK
Southern Biotechnology Associates Inc, Birmingham, USA	Southern Biotechnology Associates Inc, Birmingham, USA
T4 DNA Ligase kit	Qiagen, Crawley, UK
TEMED	Sigma Aldrich, Irvine, UK
TOPO® TA cloning® kit	Invitrogen, Paisley, UK
Triton X-100	Techmate Ltd, Milton Keyes, UK
Trizma	Sigma Aldrich, Irvine, UK
Trizol® reagent	Invitrogen, Paisley, UK
Tween-20	Sigma Aldrich, Irvine, UK
X-ray films	Santa Cruz Biotechnology Inc, Dallas, Texas, USA
Yeast Extract Medium	Sigma Aldrich, Irvine, UK
β-mercaptoethanol	Techmate Ltd, Milton Keyes, UK

2.2. Animals and Parasites

Male and female age matched BALB/c (20-25 g) in-house inbred mice supplied from the University of Strathclyde colony were used in studies. Luciferase-expressing strains of *L. donovani* (*Ldon*luc, derived from MHOM/ET/67: LV82) were supplied by Dr Carter and *L. tarentolae* (strain Parrot-TarII), *L. mexicana* (strain *Lmex*luc, derived from MNYC/BZ/M379) and *L. major* (*Lmaj*luc, derived from WHOM/IR/173) were supplied by Dr Wiese. Studies were carried out in accordance with local ethical approval and had United Kingdom Home Office approval.

2.3. Molecular biology methods

2.3.1. Production of competent cells

Competent *E. coli* cells were prepared using the method of Hanahan (1983). A single *E. coli* colony of the parental line was picked from an LB agar plate and used to inoculate 3 ml of LB broth. The culture was grown overnight in a shaking incubator (Innova 4230/4400, New Brunswick Scientific, Edison, NJ, USA) at 37°C and 500 µl of the culture were added to 100 ml of fresh LB broth. The culture was grown until it reached an optical density of 0.2 using a wavelength of 600 nm (OD600). The culture was maintained on ice for 15 min, then 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 CH3CO2Kpo, 15% v/v aqueous glycerol, adjusted to pH 5.8), pooled and incubated on ice for 90 min. The cells were pelleted as before, then 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 aqueous glycerol, adjusted to pH 6.8) and incubated on ice for 15 min. The resulting competent cells were aliquoted in 200 µl volumes into 1.5 ml sterile microfuge tubes, snap-frozen in liquid nitrogen, and stored at -80°C until required.

2.3.2. Production of transformed *E. coli*

Transformation is a generic process, which involves the direct intake or combination of exogenous DNA from the environment through cell membrane. Pure plasmid was used to transform to competent bacterial cells. Competent

cells, taken from the -80°C freezer, were thawed on ice. A 1 µl aliquot of the relevant plasmid preparation was added to 100µl of competent cells and the bacteria were incubated on ice for 1 hour. The bacteria were heat shocked by incubating in water bath at 42°C for 90 sec. The bacteria were then incubated on ice for 5 mins and then 800 µl of LB broth added. The bacteria were incubated at 37°C using a thermomixer for an hour. Finally, 100µl or 200µl of the transformed *E. coli* cells were evenly distributed on a sterile Petri dish containing 20 ml LB agar, supplemented with 100 µg/ml (ampicillin), 50 µg/ml (kanamycin) or 20 µg/ml (tetracycline), using a sterile spreader. The inoculated agar plates were incubated upside down over night at 37°C and then single colonies were selected using a sterile 100 µl pipette tip. Each colony was transferred to 10 ml LB-broth medium supplemented with the same antibiotic(s) used in selection. The cultures were incubated overnight in a shaking incubator at 37°C and 220 rpm and the used to prepare glycerol stocks or to isolate a plasmid of interest.

2.3.3. Preparation of glycerol stocks

In order to prepare glycerol stocks, 500 µl of an overnight bacterial culture was carefully mixed with 500 µl sterile glycerol in a sterile cryotube. The mixture was incubated for 5 min on ice and subsequently stored at -80°C until required.

2.3.4. Isolation of plasmid DNA from *E. coli*

Plasmid DNA was isolated from bacterial using two methods:

2.3.4.1. Plasmid DNA mini-preparation (TENS method, Zhou *et al*., 1990)

A single colony picked from a plate used to grow transformed *E. coli*, was used to inoculate 3 ml of LB broth containing the appropriate antibiotic(s). The culture was incubated overnight at 37°C, 225 rpm in a shaker incubator. The next day, 1.5 ml of the culture was transferred into a 1.5 ml microfuge tube and centrifuged at 11,000 × g at room temperature for 30 s. The majority of the supernatant was decanted, and the cell pellet was resuspended in the remaining broth (approximately 100 µl) by vortexing vigorously. Three hundred µl TENS solution was added and the resulting mixture was vortexed for 4 s then 150 µl of 3M

sodium acetate (pH 5.2) was added and the solution was vortexed for 3 s (samples kept on ice until all had been processed). The mixture was then centrifuged at 11,000 × g at 4°C for 15 min. The supernatant was transferred to a fresh microfuge tube and any DNA present was precipitated by the addition of 900 µl of ice-cold 100% ethanol. Following centrifugation under the same conditions as before, the pellet was washed with 1 ml of ice-cold 70% v/v aqueous ethanol, and then centrifuged for 5 min at 11,000 × g at 4°C. The supernatant was discarded, and the pellet was air-dried before being resuspended in 40 µl of ddH_2O.

2.3.4.2. Plasmid DNA midi-preparation using Macherey & Nagel Kits

LB medium (100 ml) containing the appropriate antibiotic(s) was inoculated with a single colony from a spread LB plate of transformed *E. coli*. The culture was incubated at 37°C with 220 rpm agitation overnight. (A sterile glycerol culture, if required, was prepared as previously described 2.3.3). The overnight culture was centrifuged for 15 min at 4,000 x g, 4°C. Subsequent steps followed the instructions of the manufacturer's manual "Plasmid DNA Purification" in the chapter "High-copy plasmid purification" until the elution of the plasmid DNA. The elute was distributed between six 1.5 ml microfuge tubes; 833 µl of eluate was mixed with 583 µl of isopropanol, before being centrifuged for 30 min at 15,800 x g and 4°C. The supernatant was decanted and the DNA pellets were washed with 1 ml of 70% ice-cold ethanol then centrifuged for 10 min at 15,800 x g, 4°C. The pellets were air-dried and resuspended in a total volume of 120 µl. The DNA was stored at -20°C until required.

2.3.5. Determination of DNA concentration

The Nanodrop2000c (Thermo Scientific, Wilmington, USA) was used to determine DNA concentrations in plasmid samples, using double distilled water (ddH2O) as the blank. 1 µl of the sample DNA was added directly to the micro-volume pedestal and the concentration determined using the supplied software.

2.3.6. Cleavage of DNA using type II restriction endonucleases

All restriction endonucleases were used according to the manufacturer's instructions and diluted using the manufacturer's supplied buffers. Analytical digests of plasmid DNA were performed in a total volume of 15 µl using 1 µg of DNA and 5-10 U of the appropriate enzyme or in a volume of 100 µl using 10-20 µg DNA and 30-60 U of the appropriate enzyme. Samples were incubated for up to 3 h at the appropriate temperature. The sample was supplemented with 2 µg of RNase A if the plasmid was isolated via the TENS method. The resulting DNA fragments were separated by gel electrophoresis using 0.8-1.2% (w/v) agarose gels prepared using 0.5× TBE, containing 0.3 µg/ml ethidium bromide. The DNA sample were mixed with 1/10 volume of 10× DNA loading buffer before loaded into the gel pockets. Electrophoresis was performed at 120 V in 0.5 × TBE for 60 min. Nucleic acids were visualised by UV illumination low intensity UV light (λ = 365 nm) as it was intercalated with ethidium bromide, and photographed for analysis. The DNA bands of interest were extracted using a clean scalpel and the DNA present isolated using a NucleoSpin Extract II kit, and eluted in 20-40 µl ddH2O. The linearised plasmid DNA was treated with shrimp alkaline phosphatase (SAP) to prevent re-ligation of the digested fragments. Thus 25.5 µl plasmid DNA was incubated with 3 µl of 10 × SAP buffer and 1.5 µl SAP and incubated at 37°C for 2 hours. The enzyme was then inactivated by heating the mixture to 65°C for 20 min.

2.3.7. Ligation of DNA fragments

Ligation was then carried out to incorporate a DNA fragment into a new DNA vector so that a new plasmid could be obtained. Thus, using a reaction volume of 15 µl, 50-100 ng of the DNA vector was and incubated with three times more of the DNA fragment to be inserted in the presence of 1.5 µl 10× T4 ligase buffer and 1 U T4 DNA ligase. The reaction was incubated overnight at 13°C in a thermocycler and the resulting plasmid was used to transform *E. coli* bacteria.

2.3.8. Insertion of γGCS into the pSSU *Leishmania* plasmid

Production of recombinant γGCS from *L. tarentolae* required transfection of the parasite with the pSSU plasmid containing the gene sequence for γGCS. In

addition, the DNA sequence needed to be flanked with two markers, GFP Tag, and His Tag, for downstream applications i.e. protein purification (His tag) and visualisation studies (GFP tag). Figure 2.1 explains the steps required to clone the whole sequence into *L. tarentolae*, which involved three main steps which required the production of different plasmids to acquire the correct gene sequence for transformation of *L. tarentolae.*

First, the plasmid pUC-GCS-GFPB was digested with *NdeI*, and *XbaI* to give three fragments (241 bp, 125 bp, and 2882 bp). The 241 bp fragment was isolated by agarose gel electrophoresis. The band, which correlated to the correct fragment size, was excised from the gel used to separate the DNA fragments and purified from the gel extracted using the NucleoSpin Extract II Kit. The pSSU-INT-lmmkk plasmid was then digested with *NdeI*, and *XbaI* to give two fragments (1836 bp, and 6687 bp). The 6687 bp fragment was isolated using gel electrophoresis and then treated with shrimp alkaline phosphatase (as described above). The 241 bp fragment was ligated with the 6687 bp using the T4 DNA ligase (as mentioned in 2.3.7). The resulting construct (plasmid pSSU4GCS), containing both gene sequences, was then used to transform *E. coli* using Amp (100 μg/ml) as the selection pressure to identify positive colonies which contained the correct plasmid (pSSU4GCS plasmid). Single cell colonies containing the pSSU4GCS plasmid were then grown up in transfected *E. coli* and stored as glycerol stocks or isolated from the transformed bacteria using the NucleoSpin Extract II Kit.

The pSSU4GCS plasmid was then digested with *NdeI* and *XhoI* to give 2 fragments (230 bp and 6792 bp). The 6792 bp fragment was isolated by gel electrophoresis and treated as above with shrimp alkaline phosphatase (SAP). The plasmid pSSU-INT-lmmkk was digested with *XhoI* and *NdeI* to give 3 fragments (671 bp, 948 bp, and 6904 bp) and the 671 bp fragment was isolated by gel electrophoresis. The isolated 671 bp and 6792 bp fragment were ligated together to form the plasmid pSSU1-6mcsHIS as above and used to transform *E. coli*, using Amp (100 μg/ml) as the selection pressure to obtain positive clones. Single cell colonies containing the pSSU1-6mcsHIS plasmid were then grown up in transfected *E. coli* and stored

as glycerol stocks or isolated from single colonies of the transformed bacteria using a NucleoSpin Extract II Kit.

Secondly, plasmid pUC-GCS-GFPB was digested with with *BamHI* and *NotI* to give three fragments (17 bp, 40 bp, and 3191 bp). The 3191 bp fragment was isolated by gel electrophoresis, and dephosphorylated using the NucleoSpin Extract II Kit. The expression plasmid pET24aGCS was digested with *BamHI* and *NotI* to give 2 fragments (2068 bp and 7540 bp) and the 2068 bp fragment was isolated using gel electrophoresis. The 3191 bp fragment from pUC-GCS-GFPB was ligated with the 2068 bp fragment of plasmid pET24aGCS and the resulting plasmid (pUC-GCS-His) was used to transform into *E. coli*, using Amp (100μg/ml) as selection pressure for positive colonies.
Then, plasmid pSSUMCSHis was digested with *NruI* to give two fragments 75 bp, and 7388 bp (the small fragment might be difficult to see), and the 7388 bp fragment was isolated by gel electrophoresis and then the 7388 bp allowed to re-ligate and form new plasmid (pSSUMCS).

Next, plasmids pUC-GCS-His and pTH6cGFPn were digested with *MfeI* to give fragments 2139 bp/3121 and a linear DNA 7697 bp respectively. The 2139 bp fragment was ligated to the linear DNA form of pTH6cGFPn and the resulting plasmid (pTHGFP-GCS-His) was used to transform into *E. coli*, using Amp (100μg/ml) as selection pressure for positive colonies.

Finally, the pTH6cGFPn plasmid was cleaved with *PmeI*/*AvrII* and the fragment containing the GFP-GCS-His was ligated with the bone pSSUMCS plasmid that cleaved with *NruI*/*XbaI* to give the plasmid pSSU-GFP-GCS-His. This plasmid containing the γGCS sequence with a His tag at the C terminal, and a GFP gene sequence at the N terminal. This plasmid was used to transfect *L. tarentolae*.

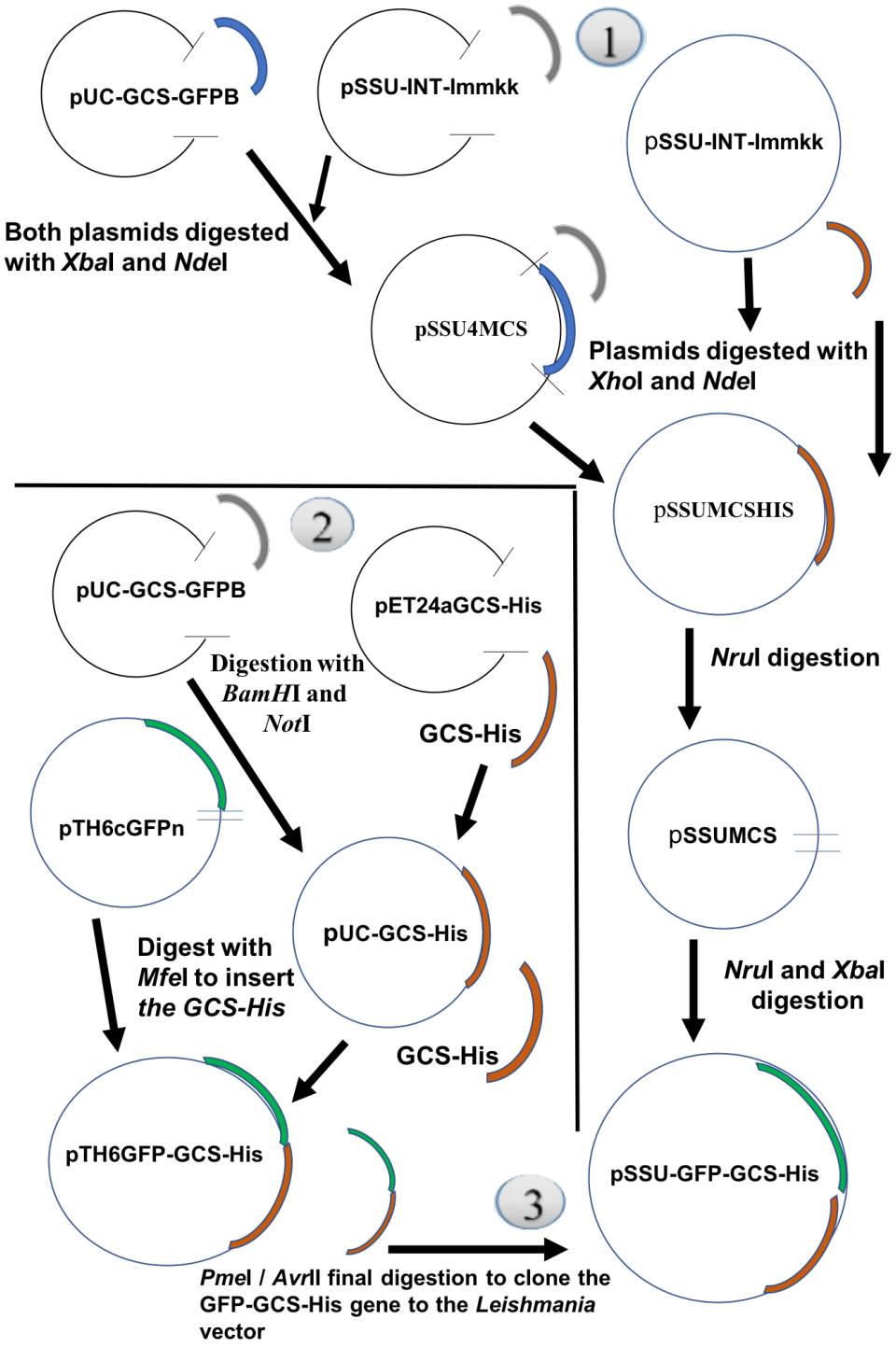


Figure 2.1 Cloning diagram of plasmids using to produce the pSSU-GFP-GCS-His plasmid. (1) Preparation of a *Leishmania* expression plasmid. (2) Combining sequence encoding γGCS-His with GFP. (3) Insertion of the GFP-γGCS-His construct into the *L. tarentolae* plasmid.

2.3.9. Transfection of *L. tarentolae* with pSSU-GFP-GCS-His gene sequence

The Amaxa Human T Cell Nucleofector Kit was used for all transfections. Late log-phase *L. tarentolae* promastigotes (3×10^7) were sedimented by centrifuging at 5,600 x g for 20 s and resuspended in 100 µl of Human T Cell Nucleofector solution, containing the supplement. provided in the kit. The cell suspension was transferred to the provided cuvette and 1-5 µg of linearised DNA or 5 µg of plasmid DNA were added. After gentle mixing the cells were electroporated in the Amaxa Nucleofector II using program V-033 and then incubated on ice for 10 min before being transferred into 10 ml of complete HOMEM medium (HOMEM medium supplemented with 10% v/v foetal calf serum). The cells were incubated at 27°C for 24 hours after which, antibiotics were added according to the resistance gene of the transfected DNA, and the cultures were distributed to two 96 well plates (200 µl/well) in a 1:4 and 1:40 dilution. The plates were sealed with parafilm and incubated at 27°C until resistant cells grew (10-14 days).

2.3.10. 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, supplemented with 62.6 mM EDTA, 2.5 M LiCl and 4% v/v Triton X- 00). After incubation at room temperature for 5 min, 400 µl of ice cold phenol were added to the suspension, and the mixture was end-over-end rotated at 4°C for 5 min. The mixture was then centrifuged at 15,800 g at 4°C for 10 min and the resulting aqueous upper layer transferred to a fresh microfuge 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 min 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 resuspended in 100 µl T10 E0.1 buffer (10 mM Tris-HCL pH 8.0

supplemented with 0.1 mM EDTA) 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).

2.3.11. Primer sequences used in studies

The primers shown in Table 2.1 were used in studies.

Table 2.1 Primers used in studies

Primer name	**Primer sequence**	**Use**
LeishSSU.for	Forward 5'-GATCTGGTTGATTCTGCCAGTAG-3'	Sequencing of plasmids used in producing *L. tarentolae* expression vector
GCSR1	Reverse 5'-TCGATCATCTTCTCGTACAT-3'	Sequencing of plasmids used in producing *L. tarentolae* expression vector
LtWTGCS-R-5	Reverse 5'-CTCCTCGCCCCAAAGAAATG-3'	*L. tarentolae* native γGCS specific primers to determine deletion of native γGCS from LtaP18
Hygint.rev	Reverse 5'- GCAATAGGTCAGGCTCTCGC-3'	*L. tarentolae* native γGCS specific primers to determine deletion of native γGCS from LtaP18
Blasticidin-int.rev	Reverse 5- GCAATAGGTCAGGCTCTCGC-3'	*L. tarentolae* native γGCS specific primers to determine deletion of native γGCS from LtaP18

2.3.12. Polymerase Chain Reaction (PCR)

Genomic DNA (gDNA) isolated from transfected *L. tarentolae* was used to assess the integration of γGCS into the ribosomal DNA gene locus (Bolhassani *et al.*, 2011). For this purpose, a pair of primers including one primer which would hybridise to upstream sequence not present on the plasmid used (LeishSSufor as forward primer) and one which hybridised within the expression cassette (GCSR1 as reverse primer). A final volume of 25 µl reactions were set up in 200 µl PCR tubes which contained 30 ng template DNA (prepared as described in 2.3.10), 10 µM of each primer, 5µl of the supplied buffer that included $MgCl_2$ and dNTPs and 1 unit My Taq™ DNA Polymerase. The PCR conditions consisted of an initial denaturation at 94ºC for 3 min, followed by 30 cycles of denaturation at 94ºC for 45 seconds and annealing at 54°C for 30 seconds. A final extension was carried out at 72ºC for 90 seconds (1min per 1Kb product) using a gradient PCR machine (Eppendorf Mastercycle Gradient, Hamburg, Germany). The amplification products were analysed by separating DNA products using agarose gel electrophoresis. In addition, diagnostic PCR reactions to confirm that the correct gene sequenced were cloned into the plasmids and including native γGCS-specific primers (LtUPStr-F-4, Blasticidin-int.rev, Hygint.rev and LtWTGCS-R-5 as one forward and three reverse primers, respectively) were carried out to determine the deletion of native *L. tarentolae* γGCS from LtaP18. 1640 gene and integration of hygromycin and blasticidin into the same gene of transfected *L. tarentolae* parasites.

2.4. Protein expression and purification

2.4.1. The effect of using different culture medium on the growth *L. tarentolae*

The effect of culturing *L. tarentolae* in complete HOMEM or yeast extract medium (Fritsche *et al.*, 2007) on parasite growth was determined. *L. tarentolae* promastigotes were initially acclimatised to the yeast extract medium by passaging them for 2-3 week in this medium. *L. tarentolae* promastigotes ($1x10^6$) from complete HOMEM medium or yeast extract medium were added to a sterile tissue culture flask containing 5 ml of the appropriate medium (n = 3) and

cultured until they reached the log phase (normally 5 days). A 10 µL sample was removed from each flask and used to determine the parasite conc/ml. This data was used to track the growth of the parasites until they reached the late log phase, where the number of cells stopped increasing.

2.4.2. Recombinant protein production studies using *L. tarentolae*

Production of recombinant protein was carried out using modifications of the method described by (Fritsche *et al.*, 2007). An initial culture was prepared by adding 1 x 10^6 parasites of 10 ml of yeast extract in a 25 cm^2 tissue culture flask and the culture was incubated at 26ºC with agitation at 100 rpm using a shaking incubator until the parasites reached late log phase (2 x 10^7 cells). The culture was then transferred to a 150 cm^2 tissue culture flasks and 100 ml of yeast extract medium added. The culture was incubated as before as until a cell density of 2x10^7 cells/ml was obtained. The parasites were then pelleted by centrifugation (2000 x g, 20ºC, 10 min) and the resulting parasites were resuspended in 1 L fresh yeast extract medium. The parasites were then transferred to a 2 L shaker flask with four baffles, then cultured as before. The parasite density was monitored daily and the effect of cell density on recombinant protein expression determined. At the end of the incubation period the parasites present were pelleted by centrifugation at 4000 g for 15 min at 4ºC. The resulting pellet was washed with buffer (21 mM HEPES pH 7.5 supplemented with 137 mM NaCl, 5 mM KCl) and centrifuged as before. The pellet was then resuspended in 10 ml buffer (50 mM sodium phosphate, 500 mM NaCl, pH 7.4) containing 1mM DTT, 20 mM immidazole and complete protease inhibitors cocktail tablet (1 tablet/ 10 ml). The suspension was kept on ice where possible throughout processing to minimise protein degradation. Soluble protein was released from the parasite cells by sonication using a Branson sonifier S250 (G Heinemann Ultraschall Laborte Chnik, Schwabisch, Germany) fitted with a 10mm tip. The suspension was sonicated using 2 pulses of 30 seconds at intensity 5, with 2-min breaks between pulses to allow the probe to cool down. The resulting suspension was centrifuged at 20000 x g, 4ºC for 30 min and soluble fractions collected. Immobilized metal ion affinity chromatography (IMAC) or gravity (HisGraviTrap) columns charged with cobalt or nickel ions was used to isolate

the histidine tagged recombinant protein form the soluble parasite fraction. The following buffers were used in soluble protein purification studies: binding buffer (50 mM sodium phosphate supplemented with 500 mM NaCl, 1mM DTT, 20 mM imidazole, and 1 tablet of Roche protease inhibitor EDTA-free pH 7.4), washing buffer (50mM sodium phosphate supplemented with 500 mM NaCl, 1 mM DTT, and 50 mM imidazole, pH X) and elution buffer (50 mM sodium phosphate, supplemented with 500 mM NaCl, 1 mM DTT, 500mM imidazole, pH X). The soluble parasite protein was loaded on to a 1 ml HisTrap column using a 10-ml syringe or decanted on to the HisGraviTrap column. The column was washed extensively with 10 column volumes of binding buffer, followed by 10 column volume of washing buffer. Recombinant histidine tag protein was eluted twice using elution buffer. Eluted samples were aliquoted into microfuge tubes and kept at -20ºC or -80ºC until required. The amount of protein present in each fraction was determined using the Bio-Rad protein assay reagent (see section 2.4.5) and the purity of the eluted protein was analysed using SDS polyacrylamide gel electrophoresis (see section 2.4.6). The presence of full length recombinant protein in samples was assessed by identification of a protein band at the correct molecular weight for γGCS (109 kDa) and that western blot studies showed the presence of a histidine tag and GFP tag on this full-length protein (see section 2.4.7). It was the intention to use MALDI as an additional method if these two criteria were fulfilled.

2.4.3. Supernatant Harvesting

Ten ml of supplemented HOMEM medium (10% FCS, 1% P/S) supernatant was harvested from cell culture at late logarithmic stage by centrifugation at 2500 × g, 4°C, 15 min. One ml of supernatant was harvested at 13000 × g, 4°C for 15 min and stored at 5 ± 3°C. Cells were resuspended in 10ml non-supplemented HOMEM medium incubated at 26°C for 24 h, 48 h, 72 h and supernatant harvested by centrifugation as before with the supplemented medium. Supernatant harvested from wild type *L. tarentolae* was used as a negative control and from *L. mexicana* was used as a positive control for secreted acid phosphatase activity.

2.4.4. Secreted acid phosphatase assay

Protein expression detection was performed by 96 well plate colorimetric assay for secreted phosphatase acid activity in harvested supernatant samples with *p*-nitrophenylphosphate as enzyme substrate. Hundred µl total sample volume in each well, 20 µl supernatant/media sample, 70 µl 50mM *p*-nitrophenylphosphate in 100mM sodium acetate buffer pH 5.0. Supernatant volumes were assayed in triplicate of 10 µl, and 20 µl with corresponding volumes of media made to 20 µl sample volume. After substrate addition to the 96 well plate the assay was incubated at 37°C for 30 min to 1 h. On completion of the incubation period the enzyme activity was neutralised by addition of 10 µl 2M sodium hydroxide solution. A visible colour change can be immediately detected on addition of 2M sodium hydroxide. Yellow positive and pink for negative for secreted acid phosphatase activity. Absorbance readings for the assay were measured at wavelength 405nm using a spectrophotometer (Spectramax M5, Molecular Devices, USA).

2.4.5. Determination of protein levels in samples

The protein concentration was determined using the Bio-Rad protein assay reagent. Briefly, 10 µl of protein standards (BSA 0.1-1 mg/ml) or 10 µl of recombinant protein sample were added to appropriate wells of a 96 well ELISA plate. Two hundred µl of Bio-Rad protein assay reagent, pre-diluted 1:5 with distilled water, were added to each sample. The absorbance of the samples was measured at O.D. 595 nm using a 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.

2.4.6. SDS polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE gels were used to separate proteins from affinity purified soluble protein extracts or from total *Leishmania* cell lysate preparations. *L. tarentolae* lysate was prepared from 5 x 10^8 parasites grown as described in (2.4.2). The parasites were concentrated by centrifugation at 2500 x g at 4°C for 15 min and

resuspended in 5 ml washing buffer (HEPES pH 7.5, 21 mM, NaCl 137 mM, and KCl 5mM). The suspension was pelleted again and the parasites were resuspended in buffer (HEPES pH 7.5, 21 mM, NaCl 137 mM, and KCl 5mM, o-phenanthroline 10mM and 1 tablet of Roche protease inhibitors EDTA-free) to give 1 x 10^8 parasites/ml. The cells were pelleted by centrifugation at 5600 g for 2 min at 4°C, the supernatant removed, and the pellet was snap frozen in liquid nitrogen and stored at -80°C until required. SDS-PAGE gels were prepared at room temperature and consisted of 10 % v/v acrylamide for stacking gels and resolving gels. SDS sample buffer (TBS buffer, SDS 0.1%, o-phenanthroline 10mM, DTT 50mM, 1 tablet of Roche protease inhibitors EDTA-free and SDS loading buffer) was added to the sample and the sample was heated at 95°C for 10 min to denature the proteins present, then 25-30 µl was loaded into the gel pocket. The samples were separated at 30 mA until the dye front reached the end of the gel. A pre-stained NEB protein molecular weight marker (7-175 KDa) was used to estimate the molecular weight of separated proteins. The proteins present were visualised using Coomassie R250 staining solution by placing the gel in 25 ml Coomassie R250 staining solution (0.1% Coomassie Brilliant Blue R-250, 50% methanol and 10%, glacial acetic acid) for approximately 60 min, with gentle agitation. The gel was then washed with destaining solution (40% methanol and 10% glacial acetic acid), which was changed several times, until the separated protein bands were easily distinguished and the blue background minimised. The gels were photographed for analysis and either dried or stored in ddH2O.

2.4.7. Western blot studies

The production of γGCS by transfected *L. tarentolae* promastigotes was determined using Western blot studies. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) then transferred to an Immobilon-P PolyvinylideneDifluoride (PVDF) or nitrocellulose membrane by electroblotting using a XCell SureLock™ E10001 (Invitrogen™, Carlsbad, CA92008 USA) system at a current of 4 mA/cm^2 of gel for 90 min. Prior to setting up the electro blot six Whatman papers, cut to the size of the resolving gel, were soaked in transfer buffer (12 mM Trizma, 96 mM glycine (v/v) methanol) then 3

layered on top of each other followed by resolving gel. The nitrocellulose membrane (soaked in transfer buffer for 1 min) or PVDF membrane (soaked in 100% methanol for 1 min) was subsequently washed in transfer buffer and placed on top of the filter layers and then an additional three more papers layered on top. Following blotting, the membrane was incubated for one h at 37°C in an appropriate blocking solution (5% w/v milk powder, 0.2 %, v/v, Tween-20 in PBS pH 7.4) at 110 rpm in a shaking incubator. The membrane was then washed three time for 5 min using wash buffer (PBS pH 7.4 supplemented with 2%, v/v, Tween-20). The membrane was then incubated with mouse anti-GFP antibody (1/1000-1/10,000 in blocking solution) or rabbit anti-His HRP conjugate (1/1000-1/10,000 in blocking solution) and incubated overnight rotating at 4°C or for one hour with gentle agitation at 37°C. The membrane was then washed 4 times as before at room temperature. If the membrane was incubated with mouse anti-GFP antibody then it was incubated with anti-mouse IgM horseradish peroxidase (HRP) linked antibody (1/2000 dilution in in blocking solution) for 1 h at room temperature or 37°C. The membrane was then washed three times as before then washed twice for 5 min in PBS pH 7.4. The membrane was then incubated with a 1:1 mix of Super Signal Peroxide solution and Signal Enhancer (1 ml v/v mixture), then placed between two sheets of plastic in a radiographic cassette and exposed to X-ray film (CEA medical X ray film screen, AgFa healthcare NV, Mortsel, Belgium) for 1 sec to 1 hour. The resulting X ray film was then photographed and the results analysed.

2.5. Vaccine studies

BALB/c mice (n = 5/treatment) were injected subcutaneously in the shaven rump on days 0 and 14 with 100 µl PBS pH 7.4 alone (control), or PBS pH 7.4 containing $1x10^7$/ml live wild-type (WT) L. *tarentolae* parasites, *L. tarentolae* transfected with different γGCS genes (*L.don* γGCS, *L.maj* γGCS or *L.mex* γGCS) or a mixture of all three transfected *L. tarentolae* parasites (1:1:1 ratio). Blood was collected from the tail vein of each mouse over the course of the experiment and samples were incubated overnight or for a minimum of 12 hours at 4ºC so that the blood clotted. The resulting serum was collected after centrifugation of samples at (13,000 x g) on a bench top centrifuge (Centrifuge 5415R, Eppendorf, Hamburg,

Germany for 10 min at 4ºC. The serum samples were stored at -20 ºC until required. After two weeks after the second immunisation (day 28), mice were infected by subcutaneous injection in the footpad with 10 µl incomplete RPMI-1640 medium (RPMI 1640 supplemented with 100 µg/ml penicillin/streptomycin and L-glutamine) containing $1x10^7$ *L. major* luciferase expressing promastigotes (*Lmaj*Luc). 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 (Alsaadi *et al.*, 2012). Mice were imaging 5 min after intraperitoneal injection of luciferin solution (150 mg/Kg). The amount of bioluminescence (BLI) emitted in each region of interest (ROI) was determined using the Living Image software, and the results were recorded as photons/sec emitted. At the end of the experiment (day 56) the mice were sacrificed and blood was collected from each mouse under aseptic conditions. The blood was treated as above and the serum collected stored at -20°C until specific antibody levels were determined. Spleen and lymph nodes were removed and used in lymphocyte proliferation assays. The infected footpad of each mouse was removed and disrupted in 5 ml incomplete RPMI-1640 medium using the end of a sterile 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 BLI emitted by the homogenate, (diluted 1:1 with luciferin solution, 300 µg/ml) in incomplete RPMI-1640 medium was determined.

2.5.1. Preparation of soluble *L. major* antigen

A crude promastigote parasite extract was prepared using a freeze-thaw protocol. Cells ($1x10^8$/tube) from promastigote parasite culture(s) were harvested by centrifugation at 3000 rpm for 15 min. Parasite pellets were washed in PBS pH 7.4 and centrifugation at 3000 rpm for 15 min. Parasite pellets were resuspended in PBS pH 7.4 and freeze-thawed in liquid nitrogen 3 times. 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.

2.5.2. Specific antibody responses

Serum IgG1 and IgG2a antibody end point titres against soluble promastigote antigen prepared from WT *L. major* parasites 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 *L. major* soluble antigen solution (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 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 anti-mouse IgG1 or IgG2a 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 min 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.

2.5.3. Lymphocyte proliferation studies

Spleens and lymph nodes were removed at sacrifice under aseptic condition and single cell suspensions prepared in incomplete RPMI-1640 medium (RPMI-1640, 100 µg/mL penicillin/streptomycin and 200 mM L-glutamine). 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 x g (BioFuge Fresco, Heraeus instruments, supplied by Thermo Scientific, Hemel Hempstead, UK) for 5 min at 4ºC. The lymph node cell

pellet from each animal was resuspended in 1 ml RPMI-1640 incomplete medium (RPMI 1640 medium supplemented with 100 µ g/mL penicillin– streptomycin and 200mML-glutamine) and the cell concentration determined by mixing 15 µl of the cell suspension 1:1 with Trypan blue solution before loading into a haemocytometer and viewed at x400 magnification on a (Nikon Eclipse E400 Microscope, Nikon UK limited, London, UK. The spleen cell pellet was resuspended in 3 ml Boyle's solution (0.007 M NH4Cl, 0.0085 M Tris, pH 7.2) and the resulting suspension was incubated for 5 min at room temperature. The spleen cell suspension was centrifuged as before for 5 min and the cells resuspended in 5 ml RPMI-1640 medium. This process was repeated to ensure that all the Boyle's solution had been removed. Cells were then resuspended in 1 ml RPMI-1640 complete medium (incomplete RPMI 1640 medium supplemented with 10% v/v foetal calf serum) and the cell concentration determined. Dead cells stain blue and the number of viable cells/ml was determined. In all cases cell viability was > 97%. Spleen or lymph node 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), *L. major* soluble antigen (50 µg/ml, PBS pH 7.4) or concanavalin A (10 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.

2.5.4. 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-5 or IFN-γ, 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 suitable 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 proper 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 chloride, pH 10.4]) were added to the appropriate wells of the plate, before incubating at room temperature in the dark for 20-60 min. The absorbance of the samples at 405 nm 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. The mean cytokine production (ng/ml ± SE) for each treatment was determined.

2.5.5. 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 15 min. 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. The mean nitrite concentration (µM ± SE) for each treatment was determined.

2.6. Statistical Analysis

Data was analysed using GraphPad Prism Version 6.00 for Mac, GraphPad Software, La Jolla California USA, www.graphpad.com. Normally distributed data was analysed using a student's t-test to compare two treatments or one-way

analysis of variance (ANOVA) for 3 or more treatments, combined with Fisher's LSD test post-hoc. Non-parametric data from *in vitro* or *in vivo* studies were analysed using a Mann Whitney U test to compare two treatments, or a Kruskal Wallis test for 3 or more treatments followed by a Dunn's ad hoc test. A p value of < 0.05 was considered significant.

CHAPTER 3. Transfection of *L. tarentolae* and production of recombinant γGCS

3.1. Introduction

The most effective technique for producing good quality protein on a large scale and with high purity is using recombinant DNA technology. This technology facilitates protein production by using the gene of interest cloned into an expression vector (Li, 2011). Restriction endonucleases and DNA ligases are the crucial for the isolation and preparation of gene sequences and insertion of the appropriate DNA segments into plasmids (Chen *et al.*, 2013). Type II restriction endonucleases are the most common enzymes used for gene mapping and reconstruction of DNA sequences, because they recognise specific nucleotide sites and cleave just at these sites (Pingoud and Jeltsch, 2001). The ability of the restriction enzyme to produce the correct DNA sequence can be assessed by determining the size of fragments purified using agarose gel electrophoresis. This shows that a specific DNA sequence has been inserted/isolated and that it was in the correct orientation. The DNA fragments are inserted into the plasmid using DNA ligase, which forms phosphodiester bonds (Lee *et al.*, 2012). In most cases *E. coli* is used as an expression system as it is easy to growth, has well developed methods of protein expression and is relatively cheap to cultivate. However, *E. coli* does not produce the post translational modifications e.g. correct folding, required for functional eukaryotic proteins (Niculae *et al.*, 2006). A variety of eukaryotic and prokaryotic heterologous expression systems have been developed to produce recombinant proteins, but none is universal applicable for production of proteins for commercial use e.g. therapeutic proteins or vaccines. *Trypanosoma* and *Leishmania* spp. human parasites have been used as heterologous expression system as they have unique features in their transcription and translation machinery e.g. trimming of RNA, organisation of genes in tandem arrays, polycistronic transcription followed by trans-splicing, and regulation of gene expression almost exclusively at the post-transcriptional level (Teixeira, 1998). In addition, these parasites are rich in glycoproteins, and up to 10% of their total proteins have oligosaccharide structures, which makes their expression systems more similar to mammalian systems compared to prokaryotes such as *E. coli*. (Ferguson, 1997, Parodi, 1993). *L. tarentolae,* a parasite of the gecko *Tarentolae annularis,* has been developed as heterologous protein expression system. It is a member of the Trypanosomatidae family and

has the advantage that it has a rapid growth rates and simple nutrient requirements. This parasite is non-pathogenic to humans so it does not require the stringent safety precautions required for pathogenic *Leishmania spp*., making it a safer system for expressing *Leishmania* proteins (Breitling *et al.*, 2002). *L. tarentolae* has been used to express several heterologous proteins produced as intracellular proteins or secreted proteins, and could be used for with large-scale production (Basile and Peticca, 2009). *L. tarentolae* can be transformed using different expression vectors, where recombinant expression cassettes can be stably integrated into the genomic DNA to produce protein constitutively using endogenous RNA polymerase I or II, or for inducible expression using co-integrated promoters for RNA polymerase I or heterologous RNA polymerases such as T7 or T3 (Mureev *et al.*, 2007). Inducible expression requires the presence of the selection factor to maintain production of the protein, which may make the system more expensive compared to a constitutive system. Low levels of transcription of genes can occur if they are integrated into genomic regions driven by RNA polymerase II (pol II), as there are no strong pol II promoters known in trypanosomatids (Clayton, 1999). Therefore, it is better to use a system where polymerase I (pol I) drives expression as it drives high levels of transgene expression in trypanosomatids. In order to ensure production of the correct protein reporter genes e.g. antibiotic resistance, is integrated into the gene construct of interest by homologous recombination into a ribosomal RNA site. As the gene sequence is flanked by the required gene sequences to allow transcription of the gene, then it is transcribed by RNA polymerase I when it is translating the ribosomal RNA in both stages of *Leishmania* life cycle (Misslitz *et al.*, 2000). In this study, the gene sequence for γGCS of *L. donovani*, *L. major* or *L. mexicana* was cloned into the pSSU-INT expression vector (vector map shown in Appendix 1) by introducing *BamHI* and *NotI* restriction sites into the gene sequence. Figure 3.1 shows the different steps undertaken to clone each gene sequence of γGCS into the expression vector. This involved a multiple stage process and used more than one plasmid. This was to allow (i) preparation of an expression vector with the proper multiple cloning sites (MCS) needed for insertion of the gene sequence, (ii) to combine the GFP reporter gene sequence

along with the γGCS and (iii) to insert the his-tag gene sequence as one fragment into the MCS of the expression vector.

Post-transcriptional modulation of proteins in *Leishmania* depends on regions found in the 3' coding sequences known as intergenic regions (IRs) e.g. DST, 1.7K or CPB 2.8K (Taheri *et al.*, 2016). *Leishmania* genes are transcribed as polycistronic RNA and functional mRNAs are produced by trans-splicing at the 5'end and cleavage and polyadeneylation at the 3' end. The EGFP-γGCS-His gene sequence produced in this project is flanked with the *L. mexicana* IR of cysteine proteinase B, a 2.8 Kb gene (CPB2.8Kb), which should ensure high expression of the downstream integrated gene into an 18S rRNA gene (Misslitz *et al.*, 2000). Figure 3.2 shows how the EGFP-γGCS-His gene should be integrated into the genomic small sub-unit rRNA locus of *L. tarentolae*. The GFP tag allows a way to track the parasites within the host after administration, although the GFP signal may not be strong enough for *in vivo* imaging studies (Misslitz *et al.*, 2000). Fluorescent or bioluminescent imaging technologies have been used to study of host-*Leishmania* interactions to define their molecular mechanisms in a cellular context, and investigate disease progression. For example, GFP derived from the jellyfish *Aequorea victoria* and been cloned and used in different applications as a reporter gene. It has been used to monitor gene expression *in vivo* (Cirillo *et al.*, 1998, Halford *et al.*, 2004) and to study localization of intracellular protein patterns (Boes *et al.*, 2002, Elliott and O'hare, 1999). GFP gene has been used as reporter for recombinant gene expression in *Leishmania* spp. (Beattie and Kaye, 2011, Fumarola *et al.*, 2004), *Plasmodium* species (Jongco *et al.*, 2006) *Trypanosoma* (Darocha *et al.*, 2004) and *Toxoplasma* (Striepen *et al.*, 1998). Other reporter genes used in studies include ß-galactosidase, chloramphenicol acetyltransferase (CAT), firefly luciferase and bacterial luciferase (D'aiuto *et al.*, 2008, Dube *et al.*, 2009). GFP fluorescence is easy to use for imaging and quantification, it has low toxicity and can be detected using a fluorescence microscope, a fluorimeter, or a fluorescent activated cell sorter (FACS) (Bolhassani *et al.*, 2011). GFP has been used for the detection of Ag-specific T cells response of tumour identification (Zhang *et al.*, 2004). Also, expression of GFP

has been shown to be stable for up to 72 h by monitoring amastigote numbers in an *in vivo* study (Bolhassani *et al.*, 2011).

The recombinant protein must be purified from the transfected parasites and identifying a method that can be used in large scale manufacture is important. Affinity tags are used to allow purification of virtually any protein and this process does not require any knowledge of the biochemical properties of the induced protein (Arnau *et al.*, 2006). Affinity tags are exogenous amino acids sequences with a high affinity for a specific biological or chemical ligand (Fassina *et al.*, 2001). The pSSUE-GFP-TEV-γGCS-His expression plasmid was designed to ensure high expression of the recombinant protein and it will be expressed as an intracellular fusion protein within the *L. tarentolae* promastigote with an EGFP tag (N-terminal GFP-tag) and histidine tag (c-terminal his-tag). The GFP-tag is engineered into the protein to allow cellular localisation of expressed protein to be studied. The His-tag allows purification using metal affinity chromatography as the histidine will bind to a metal ion such as Co^{+2}, Ni^{+2}, Cu^{+2} or Zn^{+2} (Bornhorst and Falke, 2000). The His-tag consists of six polyhistidine residues and six histidine residues are sufficient to allow high-affinity interactions with an immobilized metal matrices (IMAC) such as nickel-nitrilotriacetic acid (Ni^{+2}-NTA), or Co^{+2}-carboxylmethylaspartate (Co^{2+}-CMA), coupled to a solid resin (Chaga *et al.*, 1999, Hochuli *et al.*, 1987). Differences in these resins control the binding affinity of a Hi-tagged protein and its purity after isolation. Ni^{2+}-NTA resin has higher binding affinity for the His-tag compared to Co^{+2}-CMA resins but they exhibit less non-specific protein binding compared to Ni^{+2}-NTA resins (Bornhorst and Falke, 2000). Thus, when a mixture of soluble proteins is passed through the affinity column, the His-tagged recombinant protein is held in the column while other proteins pass through in the overflow and this separates the target protein from the mixture. The column is then washed with a high concentration of imidazole to elute the bound recombinant protein from the column as it has a higher binding affinity for nickel than histidine, so it replaces histidine on the column`s binding sites (Tropea *et al.*, 2009). However, FDA guidelines for clinical vaccines have stated that any recombinant vaccine protein can only contain amino acids residues from the protein of interest. Therefore, it

must be possible to remove any tags from the recombinant vaccine product (Parenky *et al.*, 2014). Therefore, the gene construct was engineered so that an enzyme cleavage site i.e. TEV protease site, was present between the GFP and γGCS proteins. Thus the His-tag portion of the fusion protein can be removed by treating the protein with bovine carboxypeptidase B (Waugh, 2011) after isolation from the affinity column. In previous studies *L. donovani* γGCS recombinant protein was expressed and purified as a His-tagged protein in *E. coli* (Campbell *et al.*, 2012, Henriquez *et al.*, 2010). However, in these studies γGCS was mainly expressed in inclusion bodies, which are cytoplasmic aggregates of proteins (Bernaudat *et al.*, 2011). Truncated version of γGCS was produced during isolation from *E. coli*, probably due to exposure to urea during extraction. It then proved difficult to purify the truncated proteins from the full-length protein. It is hoped that production using *L. tarentolae* will not have the same problem.

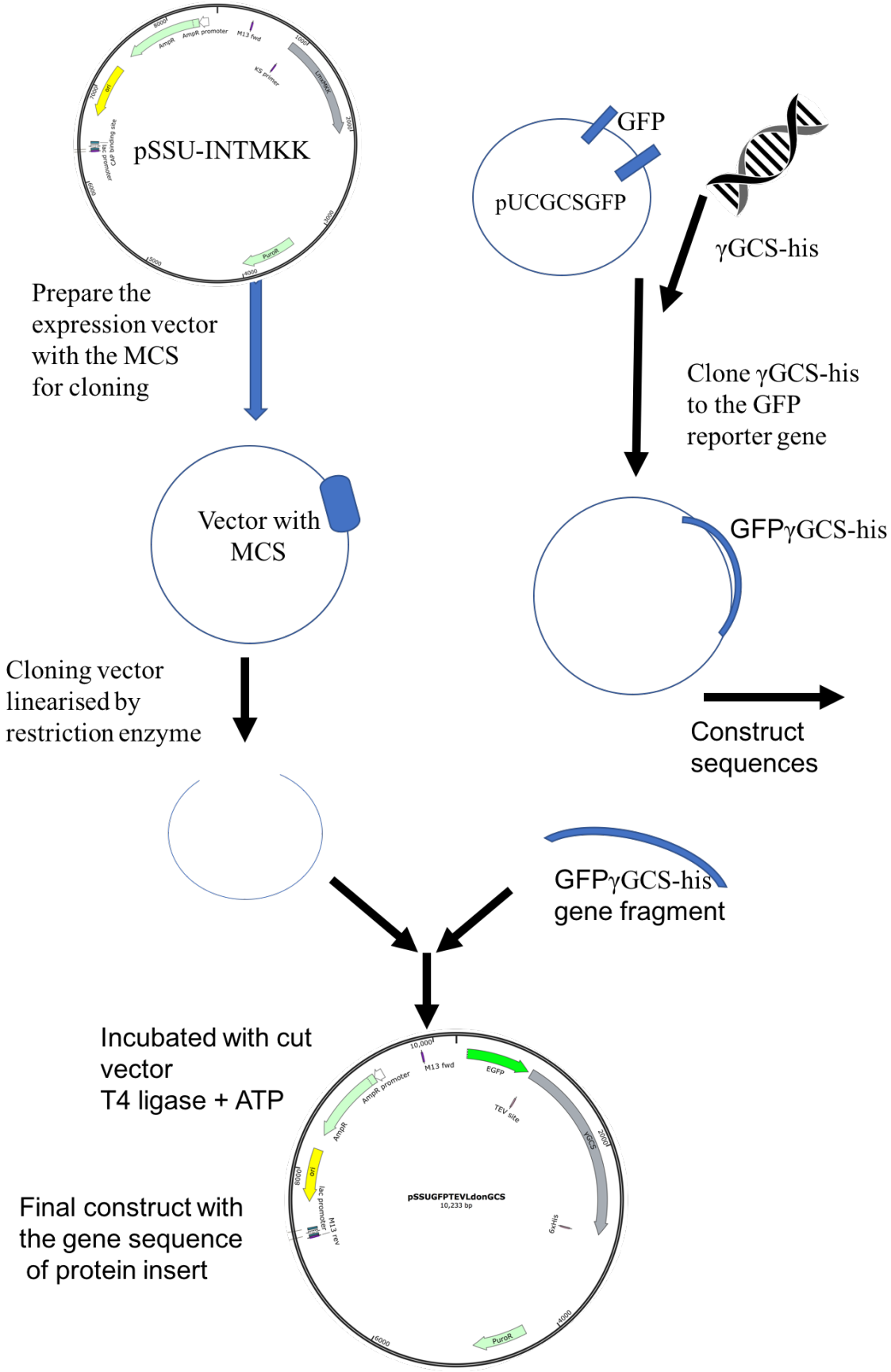


Figure 3.1 The procedure for engineering the γGCS gene sequence into the expression vector. Full plasmid maps are shown in Appendix 1.

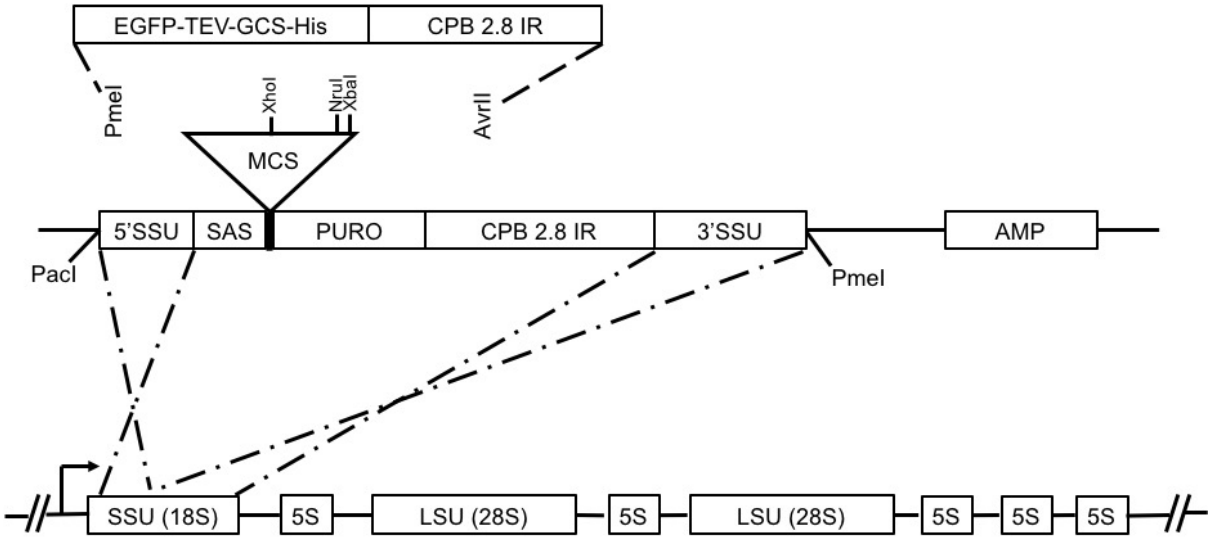


Figure 3.2 Constructs used for the integration of γGCS and its tags into the genomic small sub-unit rRNA locus. The top map shows the gene sequence required to be integrated into *L. tarentolae* followed by the CPB 2.8 IR. pSSU-INT/EGFP-TEV-GCS-His obtained by integration of the top fragment into the MCS of pSSU-INT. Linearised pSSU-INT structure is presented by the middle map. 5'SSU and 3'SSU identical to the required sequence needed for homologous recombination into an 18S rRNA; SAS refers to a sequence bearing a splice acceptor site, MCS to the multiple cloning site, PURO to the puromycin gene and CPB 2.8 IR to the IR of the CPB 2.8 IR derived from *L. mexicana.* Bottom map shows putative integration into a rRNA locus. The arrow reveals transcription direction on the rRNA genes.

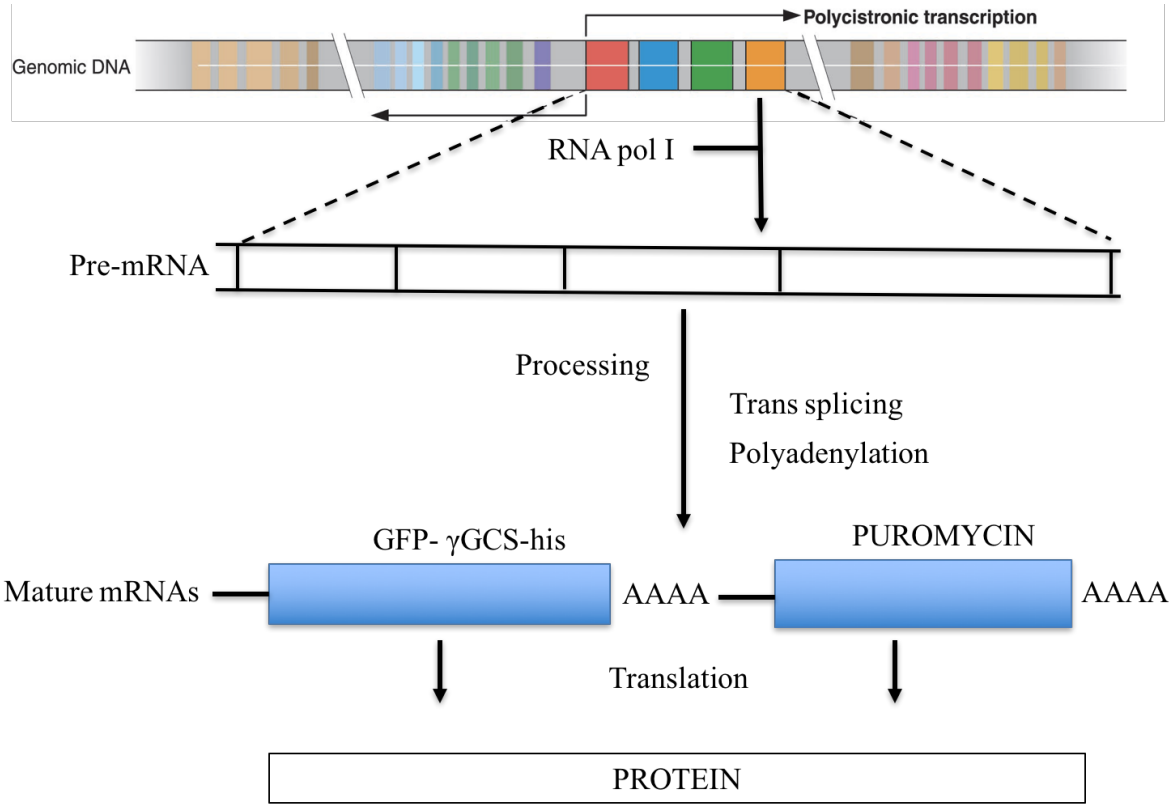


Figure 3.3 Processing of pre-mRNAs in *Leishmania*. Gene regulation in *Leishmania* and related trypanosomatids shares unique features that include polycistronic transcription of large RNA units by an -amanitin sensitive RNA polymerase II, probably in the absence of promoter elements, and pre-mRNA processing into monocistronic mRNAs through a posttranscriptional control mediated by trans-splicing and polyadenylation. Trans-splicing and polyadenylation are mechanistically coupled in these parasites.

It is important to be able to grow transfected parasites in high quantities so that sufficient recombinant protein can be extracted from transfected parasites. Therefore, it is important to use a media that gives high parasite growth, ideally at a low cost. Media used to grow *Leishmania* promastigotes are mainly liquid and of animal origin, supplemented with animal serum or blood (Fritsche *et al.*, 2007). Brain Heart Infusion (BHI) is commonly used but it can be contaminated with heterologous contaminants from viruses or prions (Robb 1975, Yamamoto and Akama 1969). This may be a particular problem as the health worries caused by cases of Bovine spongiform encephalopathy (BSE; Mad Cow's disease) have shown. These contaminants can also stimulate immune reactions in immunized hosts (Fang *et al.*, 2006). Ideally a defined medium with the minimum number of

constituents is required, which would allow a high rate of growth for *L. tarentolae* and favour production of the recombinant protein as quick as possible. This could be achieved using batch production or ideally using a continuous production method. A variety of media have been developed to cultivate *Leishmania* promastigotes but a review of the vast number of publications show there is no universal media for all *Leishmania* species, perhaps because there are species differences in the nutrition requirement for growth (Merlen *et al.*, 1999, O'daly and Rodriguez, 1988). Most studies on parasite growth have focused on *in vitro* cultivation of parasites from a clinical sample for diagnosis purposes (Merlen *et al.*, 1999) but the medium used is often supplemented with 10-30% v/v FCS but this is very expensive e.g. a 500-ml bottle of FCS can cost between £104.00-£358.00 based on current prices charged by Sigma-Aldrich Company Ltd, Dorset, UK. Although this price would be lower for bulk purchases. One constituent that is essential for parasite growth in serum-free medium is hemin (Fritsche *et al.*, 2007). Heme compounds are essential growth factors for the growth of *Leishmania* parasite as it lacks the complete biosynthesis pathway for heme (Pal and Joshi-Purandare, 2001). Studies have shown that *Leishmania* lacks two essential enzymes for biosynthesis of heme*;* aminolevulinate dehydratase and prophobilinogen deaminase. Therefore, addition of exogenous heme as hemin chloride is an obligatory supplement for the growth of promastigotes and differentiation to amastigotes *in vitro* (Chang *et al.*, 1975, Chang and Chang, 1985). However, the role of hemin in the metabolism of *Leishmania* is currently unknown, but it is important as a prosthetic group for numerous proteins, a source of energy and is crucial as an intracellular regulator for metabolic pathways involved in respiration and protein synthesis (Pal and Joshi-Purandare, 2001). Yeast extract medium (YE), supplemented with hemin used for large scale fermentation took less time (6.7 h) and gave a higher cell density (0.65-1 x 10^9 cell/ml) than parasites grown under similar conditions in Brain Heart Infusion media supplemented with hemin (Meehan *et al.*, 2000). Various heterologous proteins have been successfully expressed using *L. tarentolae* such as proprotein convertase 4, a member of Ca^{2+}-dependent mammalian subtilases, human laminin-332, and a tissue type plasminogen activator (Basak *et al.*, 2008, Phan *et*

al., 2009, Hemayatkar *et al.*, 2010). These proteins were expressed at levels of up to 300 µg/ml (Mureev *et al.*, 2009).

Glutathine (GSH) is responsible for the maintenance of the intracellular redox environment in nearly all living organisms. *Leishmania* lacking glutathione reductase and instead they have trypanothione (TSH), a conjugate of GSH and spermidine which consider to be the main anti-oxidant thiol of Kinetoplastida parasites (Romao *et al.*, 2006). One of the major biological functions of the TSH pathway is to regulate oxidative and probably, nitrosative stress by shuttling reducing equivalents from NADPH to hydroperoxides and peroxynitrites (Fiorillo *et al.*, 2012). It is possible that transfected *L. tarentolae* parasites may be more resistant to oxidative stress as they have an additional copy of the γGCS gene. Therefore, addition of a compound that causes oxidative stress/inhibits γGCS, without killing the parasites, to the culture medium used to grow the parasites recombinant protein production. Buthionine sulfoximine (BSO) is an inhibitor of γGCS, which causes a complete and irreversible depletion in GSH levels. Previous studies have shown that BSO can inhibit the survival of *L. donovani* both *in vivo* and *in vitro* (Carter *et al.*, 2003). Therefore, the effect of treatment with BSO or hydrogen peroxide, a known oxidant, on the GFP-γGCS-His fusion protein expression was determined. In addition, production of γGCS is regulated by intracellular GSH levels (Moskaug *et al.*, 2005). Therefore, internal GSH levels may have important influence on expression of the fusion protein.

The main objectives of this study were to:

1. produce a γGCS gene sequence of *L. donovani, L. major* or *L. mexicana* construct which is flanked with a EGFP-tag (N terminal) and his-tag (C terminal) and introduce a TEV protease cleavage site into the EGFP-γGCS-His construct, between GFP and γGCS sequence, so that the EGFP peptide can be cleaved from the recombinant fusion protein.
2. Clone the constructed fragment EGFP-γGCS-His into the expression vector pSSU-INT so that it can be used to transfect *L. tarentolae* and give stable integration of the gene sequence into a ribosomal RNA locus. This would

allow constitutive production of recombinant *L. donovani, L. major* or *L. mexicana* γGCS protein from transfected *L. tarentolae.*

3. Determine where GFP-γGCS-His fusion protein is expressed within transfected *L. tarentolae* promastigotes and identify the optimal medium for growth and recombinant protein production by transfected *L. tarentolae* promastigotes.
4. Determine if treatment with buthionine sulfoximine (BSO), hydrogen peroxide (H_2O_2) or glutathione (GSH) improved the growth and recombinant protein production by transfected *L. tarentolae* promastigotes.
5. Optimise the expression and purification of recombinant γGCS of the three *Leishmania* species i.e. *L. donovani, L. major* or *L. mexicana* and obtain enough recombinant γGCS from the three *Leishmania* species for enzyme and vaccine studies.

3.2. Results

3.2.1. Cloning of *Leishmania* γGCS gene

The γGCS gene sequence of *L. major, L. mexicana* or *L. donovani* was successfully cloned into the pET24a expression vector in previous studies (Doro, 2014). This plasmid was grown up as described in section 2.3.2 and isolated from transfected *E. coli* using the Midi prep kit as described in section 2.3.4.2. The isolated plasmid was digested with the restriction enzymes *BamHI*/*NotI*. The isolated pET24aGCS were of high purity as their A260/A280 ratio was > 1.80. The resulting fragments were separated by gel electrophoresis (Fig 3.4) and the γGCS gene sequence had a predicted size of 2068 bp and it was isolated from agarose gel as described in section 2.3.6. The pUC-GCS-GFPB vector was quantified and linearised using *BamHI*/*NotI*. It had a fragment with a predicted size of 17, 40 and 3191 bp and the largest fragment was isolated from the gel as described in section 2.3.9 (Fig 3.4). The γGCS gene sequence of each of the three species was then integrated into the linearised pUC-GCS-GFPB vector and the resulting plasmid used to transfect competent *E. coli.* The plasmid present in the bacteria was isolated using a minprep as described in section 2.3.4.1. The resulting plasmid (pUC-GCS-His) was

digested with *EcoRV*, *NcoI*, *XbaI*, *EcoRI*, *KpnI*, *ScaI*, *ClaI*, *NdeI* and *NcoI* restriction enzymes. The resulting products were separated by agarose gel electrophoresis. The size of the fragments showed that the pUC-GCS-GFPB vector contained the relevant *Leishmania* γGCS-his gene insert (Figure 3.5) for all the selected colonies.

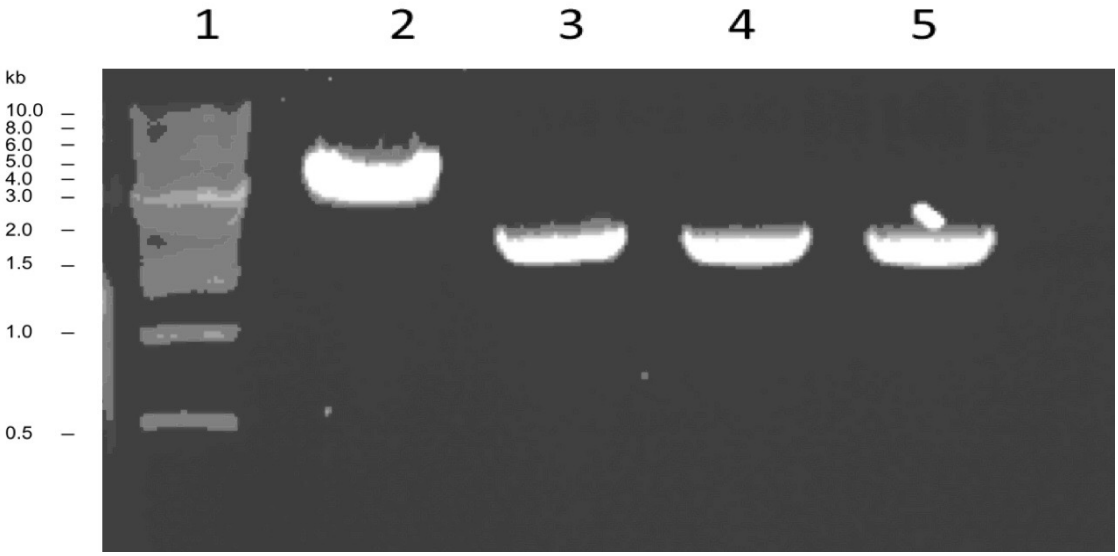


Figure 3.4 Results from enzymatic digestion of pUC-GCS-GFPB, showing fragments present (A), pET24a *L. donovani* γGCS (B), pET24a *L. major* γGCS (C), and pET24a *L. mexicana* γGCS (D) with the restriction enzymes BamHI/NotI. M indicates the molecular weight marker. Ethidium bromide was used to stain the agarose gel to allow visualization of the DNA containing bands under UV light.

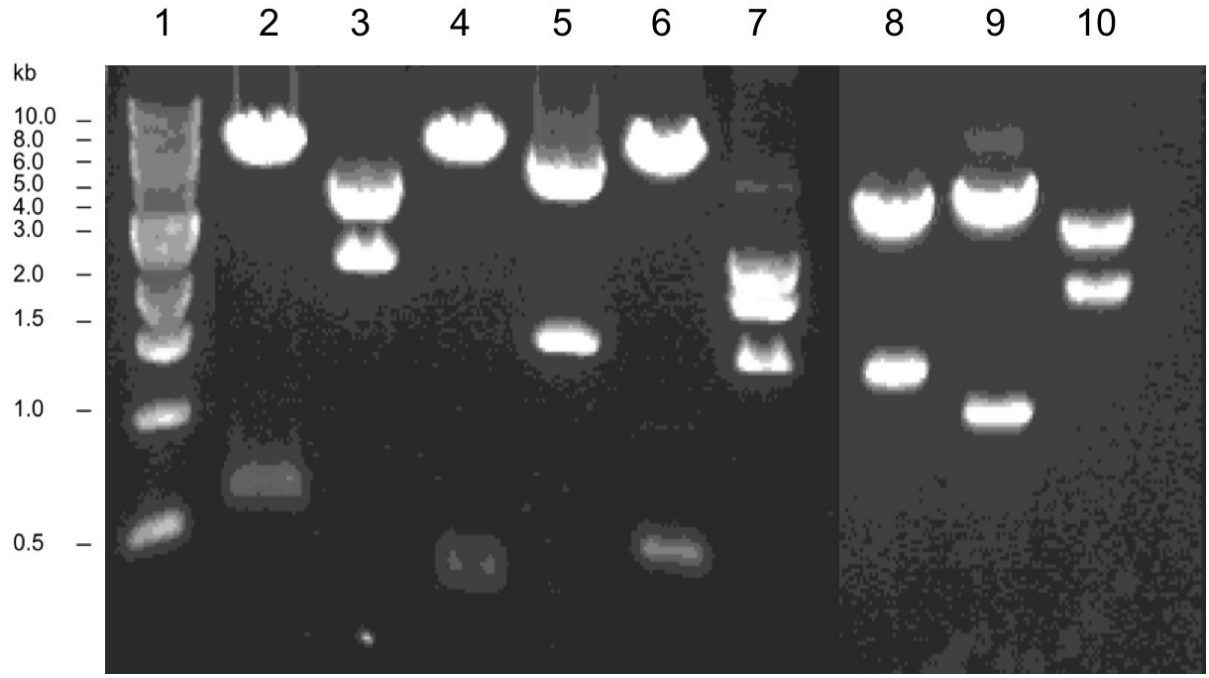


Figure 3.5 Results of the DNA fragments present after digestion of the pUC-GCS plasmid, containing inserts of the γGCS gene from different *Leishmania* spp., with EcoRV, NcoI, XbaI, EcoRI, KpnI, ScaI, ClaI, NdeI, NcoI respectively. The agarose gel was stained with ethidium bromide so that the DNA bands were visualised when the gel was viewed under UV light. The pUC-GCS plasmid of *L. donovani* (2, 3, and 4), *L. major* (5, 6, and 7), *L. mexicana* (8 ,9, and 10).

3.2.2. Cloning of *Leishmania* γGCS into pSSU-INT expression vector.

The GFP-γGCS gene sequence then had to be cloned into the plasmid pSSU-INT-lmmkk so that the GFP-γGCS-His was flanked with the sequence for SSU. This allow the integration of the gene construct into the *Leishmania* genomic DNA. Digestion of the pUC-GCS-GFPB plasmid with *XbaI*/*NdeI*, results in three fragments of the following sizes: 125 bp, 241 bp, and 2882 bp. The pSSU-INT-lmm vector was digested with *XbaI*/*NdeI* restriction enzymes to give fragments with a size of 1836 bp and 6687 bp. The pSSU-INT-lmm vector was then digested with *XhoI*/*NdeI* to give three fragments, with size of 671, 948, and 6904 bp (Figure 3.6). The 241 bp fragment from pUC-GCS-GFPB plasmid and the 6687 bp pSSU-INT-lmm vector were isolated from the agarose gel after gel electrophoresis as described on section 2.3.6 and ligated together using DNA ligase enzyme (Lee *et al.*, 2012). The resulting plasmid was called pSSU4MCS. This plasmid was then digested with *ScaI* to ensure that the plasmid had the correct gene sequence inserted in the right orientation. Figure 3.7 shows the results for plasmids purified from 9 individual colonies of transfected *E. coli* XL1 blue competent cells. Only sample 6 and 9 were positive for the plasmid based on the predicted size of the DNA fragments. Colony 6 was used to produce large quantities of the plasmid.

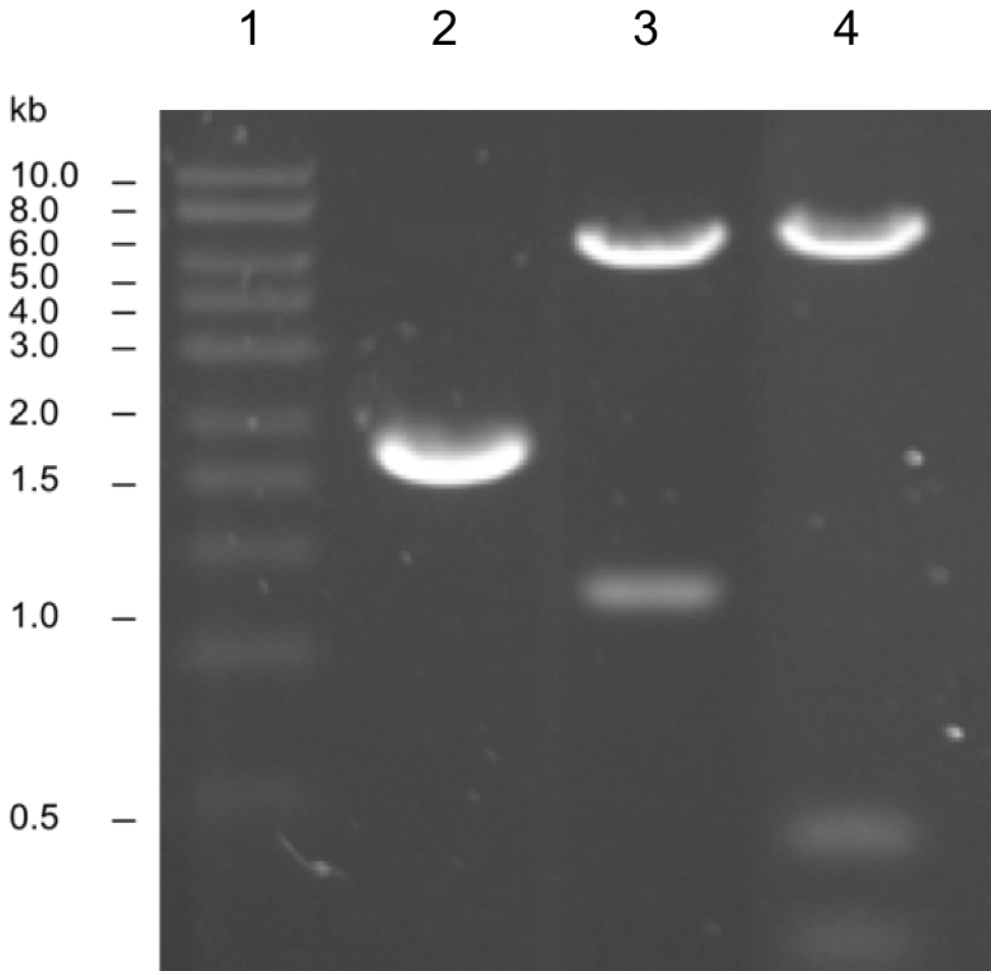


Figure 3.6 Results of pUC-GCS-GFPB and pSSU-int-lmm vector DNA products after digestion with the restriction enzymes XbaI/NdeI or XhoI/NdeI. pUC-GCS-GFPB digested with XbaI/NdeI (A), pSSU-int-lmm digested with XbaI/NdeI (B), pSSU-int-lmm digested with XhoI/NdeI (C) were subjected to gel electrophoresis. The size of the DNA fragments was obtained using the molecular weight marker (M) run on the same gel. DNA fragments of the size 241, 6687, and 671 bp respectively were present. The agarose gel was stained with ethidium bromide so that the DNA bands were visualised when the gel was viewed under UV light.

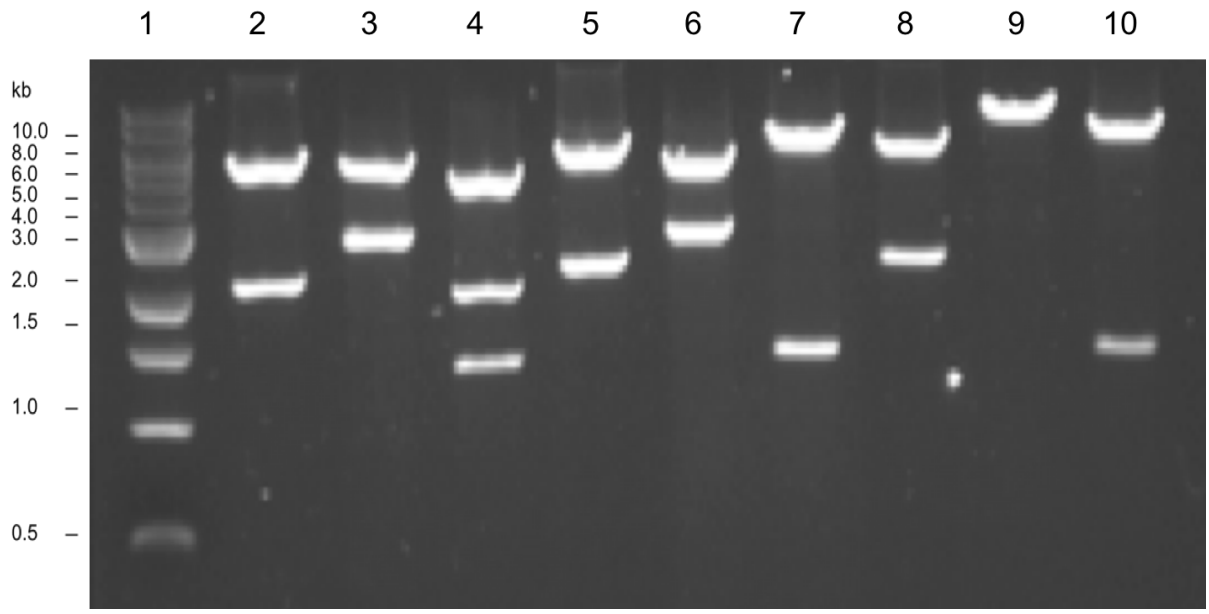


Figure 3.7 Results of the pSSU4GCS plasmid digestion with the ScaI restricted enzyme. The agarose gel was stained with ethidium bromide so that the DNA bands were visualised when the gel was viewed under UV light. The correct plasmid would give 2 fragments with a size of 5672 and 1350 bp.

Next, the SSUmcs sequence had to be engineered into a plasmid. To achieve this the plasmid pSSU4GCS was digested with *XhoI*/*NdeI* to give fragments with a size of 230 bp 6792 bp. The 671 bp fragment was isolated after digestion of pSSU-int-lmm. It was then ligated with the 6792 bp fragment from pSSU4GCS digested plasmid, to form a new plasmid called pSSUmcsHIS. *E. coli* XL1 blue competent cells were transfected with the pSSUmcsHIS plasmid and ten individual colonies were selected. Plasmid was prepared from each of the 10 clones and digested with the restriction enzyme *PvuI* to confirm that the selected clone contained the pSSUmcsHIS plasmid based on the fragments produced (Figure 3.8). Sample 8 was negative while the other 9 colonies were all positive. One of positive colonies was grown and used to produce large quantities of the pSSUmcsHIS plasmid. This plasmid was then digested with *NruI* to get rid of the histidine residue from the plasmid and the resulting product was re-ligated to form the vector pSSUmcs.

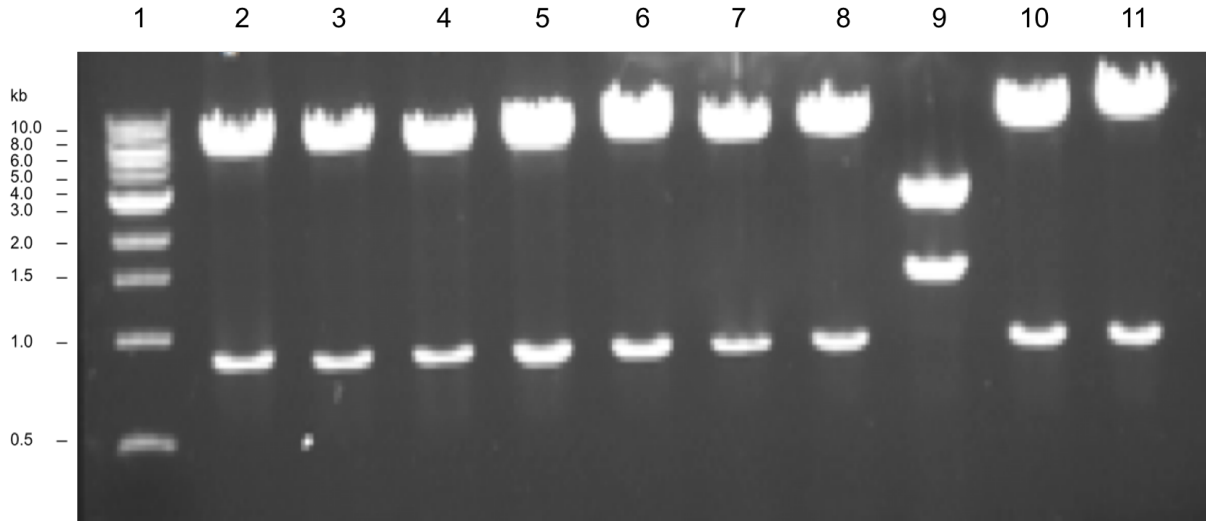


Figure 3.8 Results of digestion with *PvuI* on the fragments produced using plasmids obtained from 10 separate colonies of bacteria transformed with the pSSUmcsHIS plasmid. The agarose gel was stained with ethidium bromide so that the DNA bands were visualised when the gel was viewed under UV light. All the colonies except colony 8 was positive for the pSSUmcsHIS plasmid.

3.2.3. Cloning of *Leishmania* γGCS gene from pUC-GCS into pSSUmcs expression vector

Next the *Leishmania* GFP-γGCS-his gene sequence was cloned into the pSSUmcs vector. The GFP-γGCS-his sequence of the relevant *Leishmania* species was isolated from the pUC-GCS plasmid after digestion with *MfeI* restriction enzyme. This produced two DNA fragments and the smaller fragment, which has the GFP-γGCS and hexa-histidine tag residue gene sequence, was isolated from the agarose gel. The plasmid pTH6cGFPn was also digested with *MfeI* and the larger fragment was ligated with the 2.134 bp fragment from the pUCGFP-GCS-his plasmid (Figure 3.9). This plasmid, called pTHGFPLGCS, was then used to transfect bacteria. This plasmid was digested with *BamHI* then to ensure that the plasmid contained the correct gene sequences in the right orientation (Figure 3.10). Positive clones from 18 individual colonies were identified by digesting purified plasmid with *BamHI*. Three positive clones were obtained for pTHGFP*L.donovani*-GCS, two for pTHGFP*L.mexicana*-GCS and one for pTHGFP*L.major*-GCS. Samples from one positive clone/specie was grown to produce large quantities of the plasmid and this plasmid was digested with five different restricted enzymes to confirm it had the corrected gene sequence engineered into it (Figure 3.11).

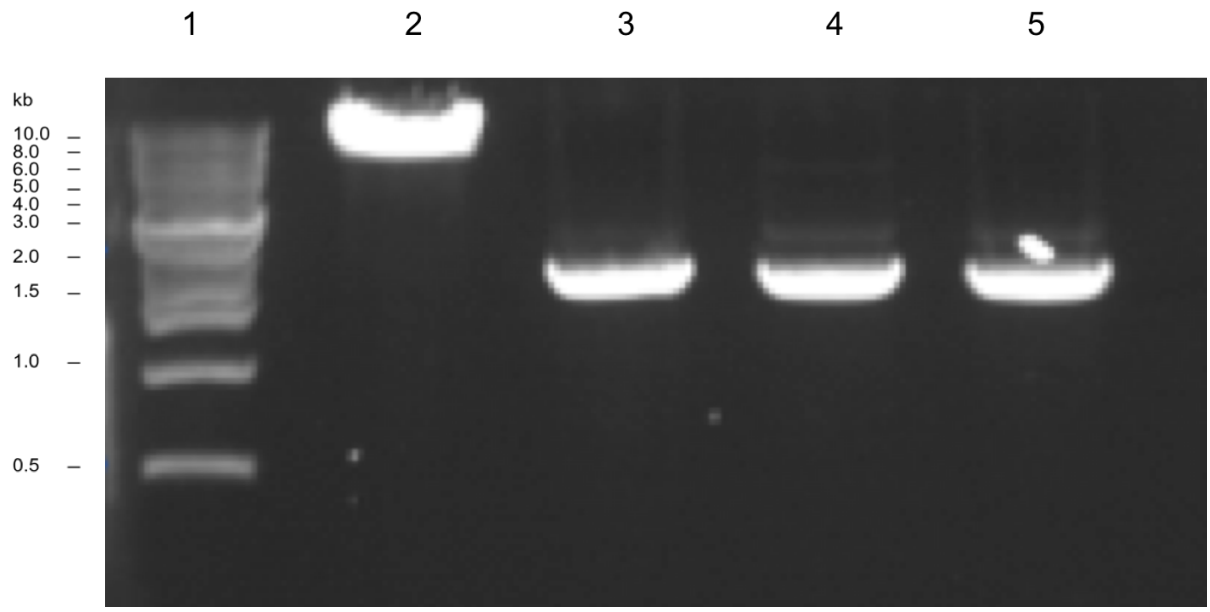


Figure 3.9 Results shows fragments of pTH6cGFPn, and pUCGFP-γGCS respectively. The agarose gel was stained with ethidium bromide so that the DNA bands were visualised when the gel was viewed under UV light. Marker (1). pTH6cGFPn big fragment (2), pUCGFP-*L. donovani* γGCS (3), pUCGFP-*L. major* γGCS (4), pUCGFP-*L.mexicana* γGCS (5)

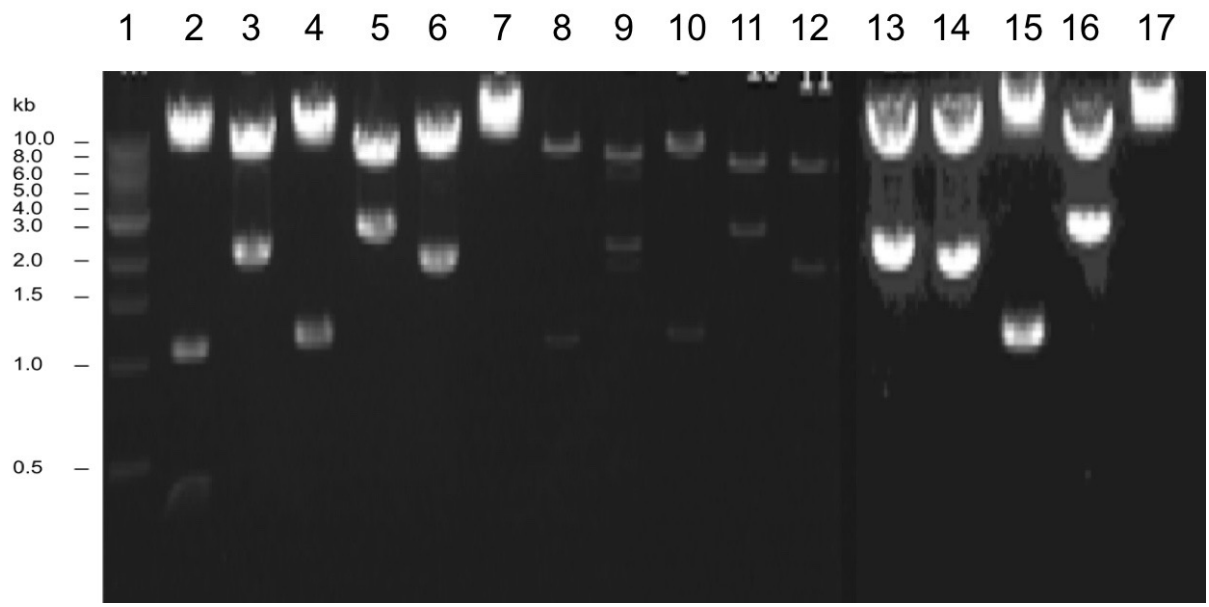


Figure 3.10 Results of the fragments produced after enzymatic digestion of plasmid obtained from different colonies of transfected bacteria. The pTHGFPL.GCS plasmid was digested with XbaI (sample 2, 8 and 13), SpeI (sample 3, 9 and 14), NotI (sample 4, 10 and 15), NcoI (sample 5, 11 and 16) or MfeI (sample 6, 12 and 17) restricted enzyme respectively. The agarose gel was stained with ethidium bromide so that the DNA bands were visualised when the gel was viewed under UV light. The pTHGFP *L.donovani*-GCS (2-6), pTHGFP*L.major* GCS (7-12), pTHGFP *L.mexicana* GCS (13-17), and molecular weight marker (1). All digests were positive according to the predicted molecular weight of fragments size.

The plasmid pTHGFPLGCS had the GFP-γGCS-His tag sequence, but it had to be inserted into an expression vector so that it contained the required gene sequences for integration into *L. tarentolae* i.e. SSU locus. Therefore, the pTHGFPL.GCS plasmid for the relevant *Leishmania* species was digested with *PmeI*/*AvrII* restriction enzymes to give fragments with a size of 2831 bp and 7005 bp (Figure 3.12). The smaller 2831 bp fragment, which contain the GFP and the *Leshmania* γGCS gene sequence, was purified from the gel and ligated into the linearised pSSUMCS plasmid vector. This resulting vector, called pSSUGFP-γGCS-His, was used to transform competent *E. coli* cells and the bacterial were grown to produce large quantity of the plasmid.

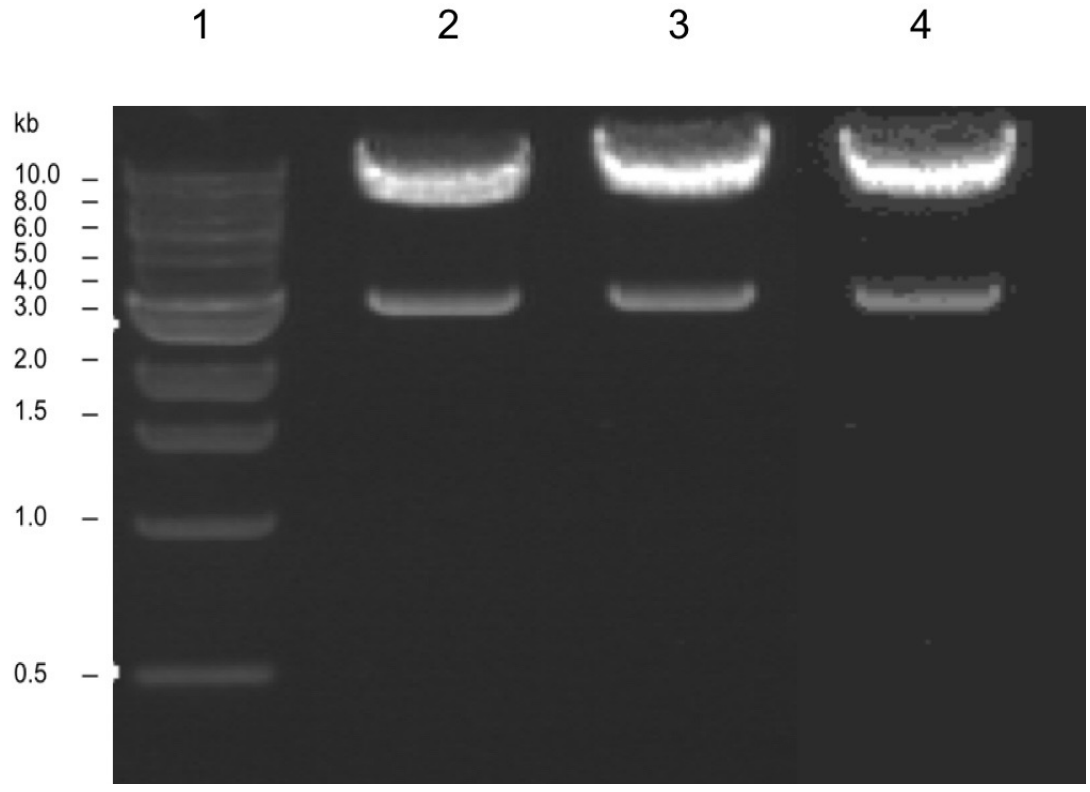


Figure 3.11 Results of pTHGFPL.GCS plasmid products after digestion with PmeI/AvrII restriction enzymes. The agarose gel used in gel electrophoresis was stained with ethidium bromide to allow the DNA bands to be visualised under UV light. Molecular weight marker (1), pTHGFP*L.donovani*γGCS (2), pTHGFP*L.major*γGCS (3), pTHGFP*L.mexicana*γGCS (4). The picture shows that the correct fragment size was obtained for all three species, indicating that the plasmid had the correct gene insert.

3.3. Introducing TEV-protease cleavage site (ENLYFQG)

The γGCS recombinant protein had to have a cleavage site introduced so that it would be possible to remove the GFP tag from the expressed γGCS fusion protein. Therefore, a TEV-protease cleavage site was introduced into the expression vector region between the GFP and γGCS sequence. The respective pSSUGFPGCSHis was linearised by incubating with the restriction enzymes *BamHI* and *AscI* to produce two fragments of 9189 bp and 1020 bp. This DNA sequence was then used as a template for PCR reactions to amplify a fragment containing the TEV-protease sequence. The GFP-TEV-GCS PCR products were analysed by gel electrophoresis (Figure 3.13) and gave the predicted size of 1163 bp for each *Leishmania* spp. The 9189 bp fragment was isolated from the agarose gel used to separate the DNA fragments as described in section 2.3.9. The resulting product was treated with SAP and ligated to the 9189 bp fragment to produce the final construct pSSUGFPTEVγGCSHis. These plasmids sent for sequencing to make sure that the gene sequence was correct and in the right orientation.

These 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 these species (Appendix 2).

Bacteria containing pSSUGFP-TEV-γGCS-His plasmid were grown and large quantities of the relevant plasmid obtained. The plasmids were digested with *PacI*/*PmeI* to prepare the linearised vector 5'SSU-GFPTEV γGCSHis-SSU3' for the transfection into the *L. tarentolae.* Fragments were prepared under sterile conditions using NucleoSpin Extract II kit.

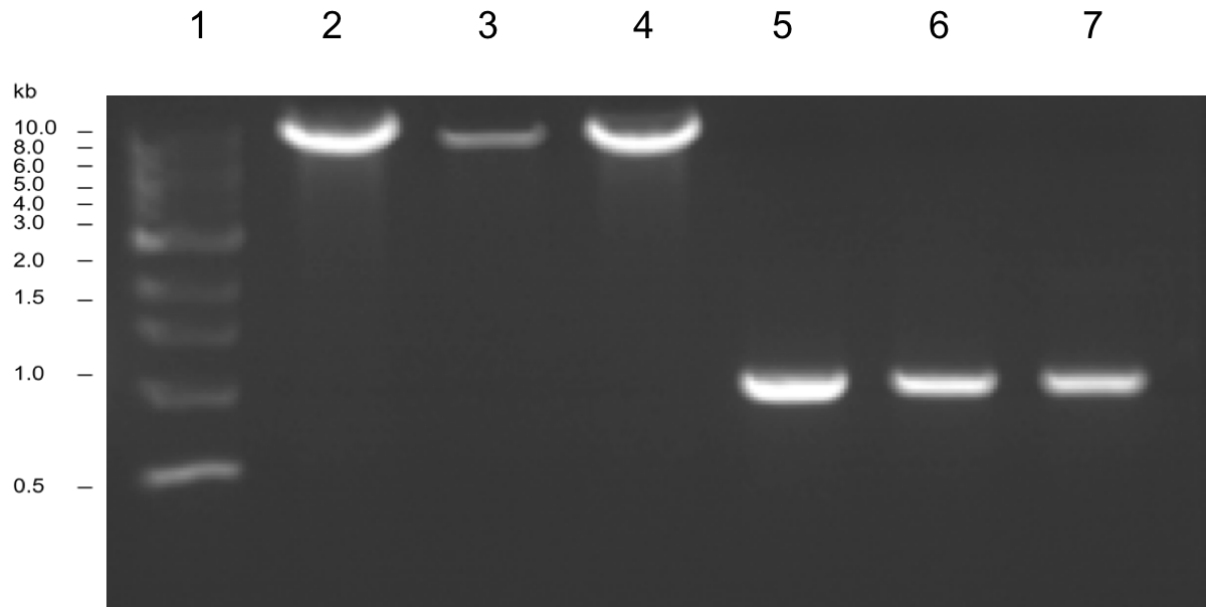


Figure 3.12 Results showing linear fragments of pSSUGFPLγGCS plasmid products after digestion with BamHI and AscI restriction enzymes and the PCR products of GFP-TEV-γGCS. The agarose gel used in gel electrophoresis was stained with ethidium bromide to allow the DNA bands to be visualised under UV light. Molecular weight marker (1), pSSUGFP*L. don*-γGCS (2), pSSUGFP*L. maj*-γGCS (3), pSSUGFP*L. mexi*-γGCS (4), GFPTEV *L. don*-γGCS (5), GFPTEV *L. major*-γGCS (6) and GFPTEV *L. mexi*-γGCS (7). The picture shows that the correct fragment size was obtained for all three species, indicating that the plasmid had the correct gene insert

3.3.1. Localisation of recombinant γGCS expression in transfected *L. tarentolae* cells.

Promastigotes of *L. tarentolae* transfected with the gene sequence for the GFP-γGCS-His fusion protein was examined for the expression of EGFP by epi-fluorescent microscopy. EGFP expression was indicated from the intense green fluorescence of the parasites (Figure 3.14). Fluorescent microscopic images indicate that promastigotes transfected with the integrated GFP-γGCS-His gene sequence were successfully expressing the fusion protein (*L.t L.don*γGCS, Figure 3.14A, *L.t L.maj*γGCS, Figure 3.14B and *L.t L.mex*γGCS Figure 3.14C). Wild-type promastigotes did not express any green fluorescence (Figure 3.14D). Remaining promastigote samples were used for the confirmation of genomic integration by diagnostic PCR. The stability of EGFP expression was monitored over a period of three months post transfection and no decrease in fluorescence intensity was observed over during this period.

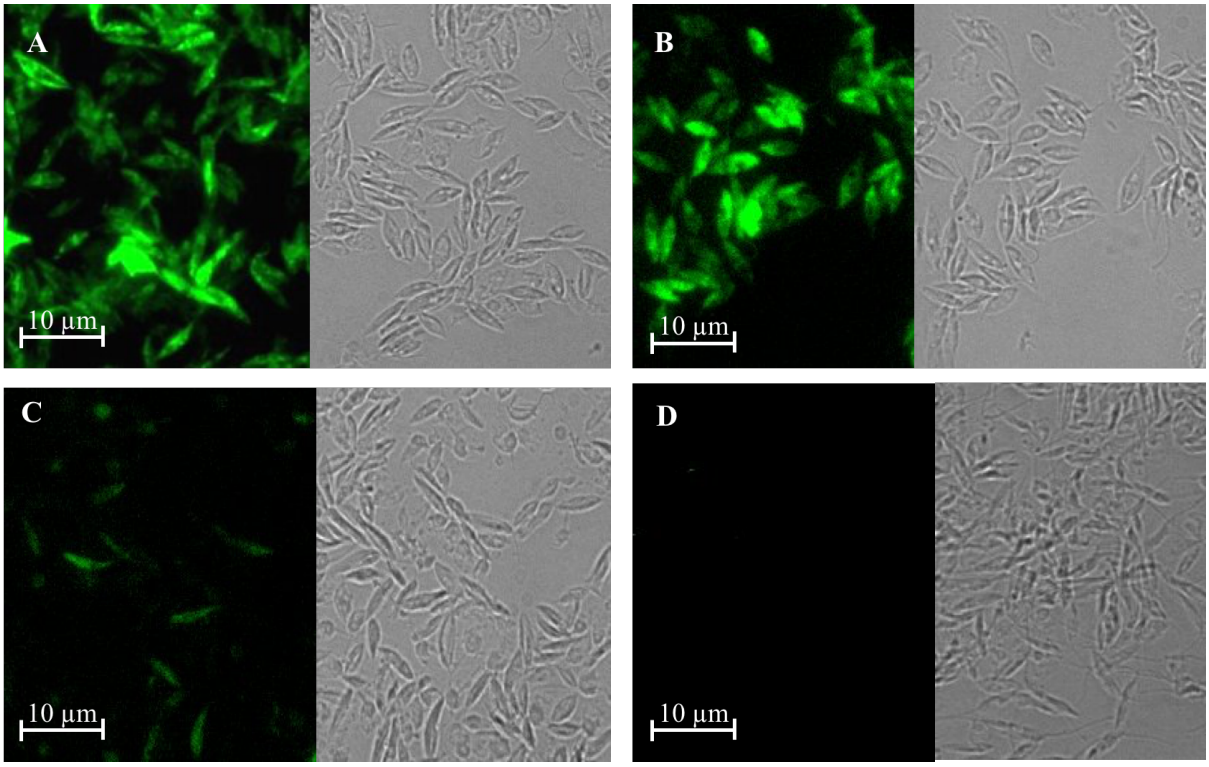


Figure 3.13 Detection of EGFP expression by epi-fluorescent microscopy in transfected *L. tarentolae* promastigotes. Images show expression of GFP-γGCS-His in transfected *L. tarentolae L.t L.don*γGCS (A), *L.t L.maj*γGCS (B), *L.t L.mex*γGCS (C) and *L. tarentolae* WT (D) promastigote (before and after flashing to detect fluorescence). High amount of EGFP were observed in all three transfected *L. tarentolae* promastigotes.

3.3.2. Conformation of the γGCS expression by immunoblotting and PCR

Cell extracts of transgenic parasites were used to detect the expression of GFP-γGCS-His protein using antibodies against GFP and histidine. Expression studies showed that the three γGCS *Leishmania* recombinant proteins were expressed as full-length proteins (MW 109 KDa (Figure 3.15). This study was repeated several times using different protocols to extract purify the recombinant protein from the parasites. Additionally, to confirm EGFP expression cDNA was produced from transfected parasites EGFP-specific primers were used to amplify this sequence, using WT parasites as the control. All transfected parasites showed a dominant band of EGFP (~2000 bp) whereas WT parasites did not have a band (Figure 3.16).

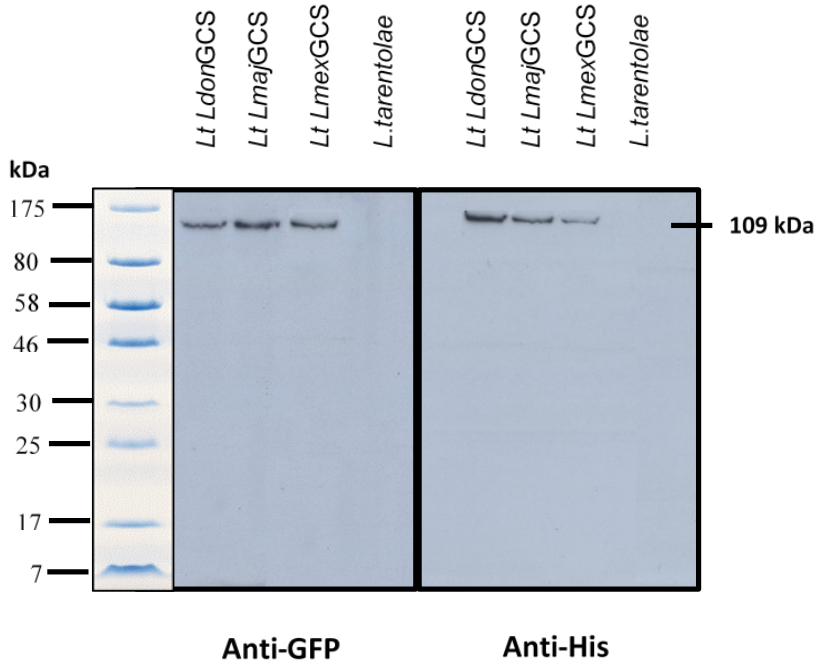


Figure 3.14 Immunoblotting of 2 × 107 *L. tarentolae* (L.t.) promastigotes to detect the presence of γGCS (GCS) recombinant protein. Proteins present in *L. tarentolae* (WT) and transfected parasites (*L.t L.don* γGCS, *L.t L.maj* γGCS, *L.t L.mex* γGCS). A dominant band of 109 KDa that was immunoreactive with anti-GFP/ anti-His antibody detected in all three transgenic *L. tarentolae* parasites. *L. tarentolae* (WT) showed no presence of the relevant band.

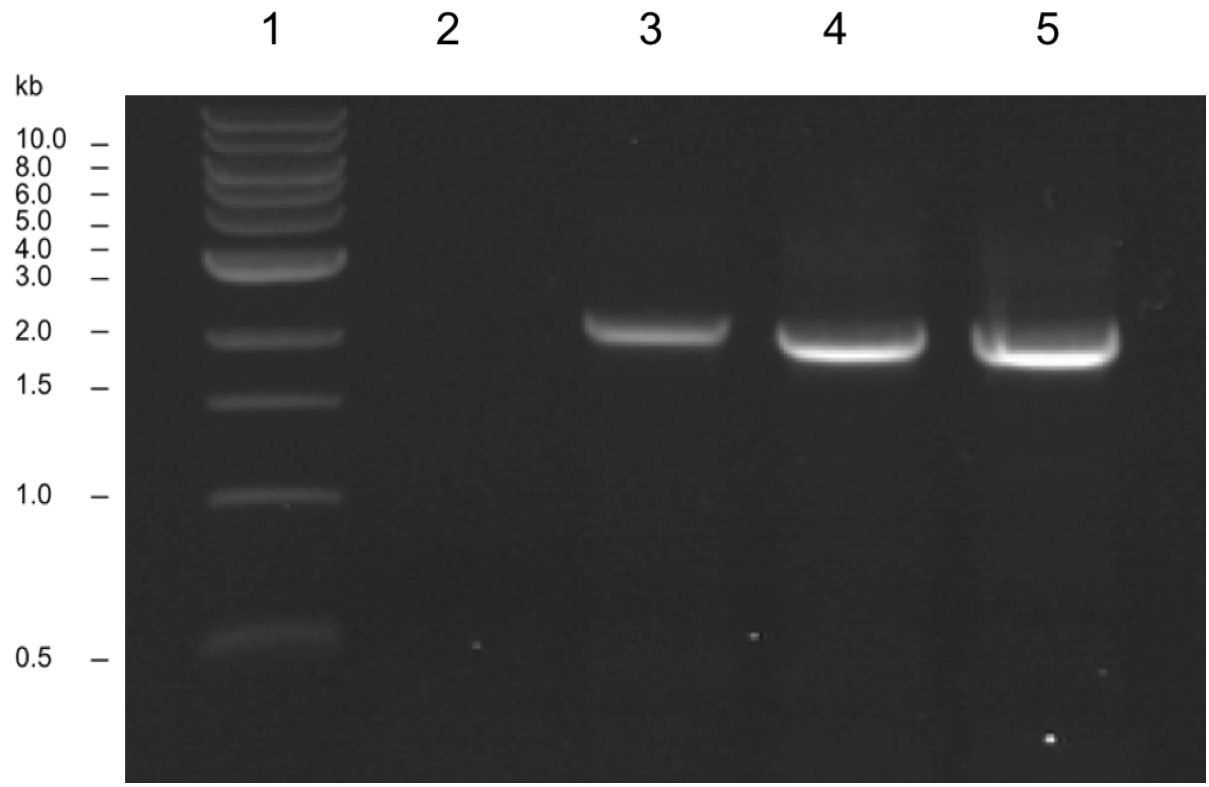


Figure 3.15 Results of the gel used for diagnostic PCR of genomic DNA samples extracted from three transfected *L. tarentolae* (*L.t L.don* γGCS lane 3, *L.t L.maj* γGCS lane 4, *L.t L.mex* γGCS lane 5) and the wild type of *L. tarentolae* (lane 2) for the detection of integration of the GFP-γGCS-His gene into the rRNA locus of the parasites. All transfected parasites showed a dominant band of (~2000bp) whereas the wild type did not produce an amplicon.

3.3.3. Effect of using different media on growth of *L. tarentolae* promastigotes

Previous studies by other researchers have shown that a high yield of recombinant protein from transfected *L. tarentolae* requires a high number of promastigotes. Therefore, the effect of altering culture conditions on cell numbers was determined. In this study, the growth curves of *L. tarentolae* (WT) were obtained by culturing in complete HOMEM medium or yeast extract medium. There was significant better parasite growth in yeast extract medium compared to the HOMEM medium (Figure 3.17, $p<0.0001$). On day 8 yeast extract medium had about three times more promastigotes present compared to HOMEM medium (Yeast medium, mean: 7.4×10^7 cells/ml; HOMEM, mean 2.6×10^7 cells/ml). It is possible that transfection with the GFP-γGCS-His gene sequence from the three-different species of pathogenic *Leishmania* could affect promastigote growth. Therefore, the same study was repeated using the three strains of transfected *L. tarentolae* i.e. *Lt L.don* γGCS, Lt *L.maj* γGCS and *Lt L.mex* γGCS. All three strains grew better in yeast extract medium compared to HOMEM medium (Fig. 3.18). Differences in promastigote growth was not apparent until after day 4 of culture and the promastigotes had not reached stationary phase by day 7. The percentage of promastigotes growth was increased in all three transfected *L. tarentolae* i.e. *Lt L.don* γGCS, Lt *L.maj* γGCS and *Lt L.mex* γGCS (89%, 85% and 76% respectively) compared to WT *L. tarentolae*, which also grew better in yeast medium compared to HOMEM medium (65%, $p<0.0001$). *Lt L.don* γGCS parasites had the highest growth rate increase among the three transfected *L. tarentolae* parasites. Furthermore *Lt L.mex* γGCS had the lowest growth rate increase. This experiment was repeated three times and similar results were obtained.

L. tarentolae promastigotes from cultures showed that the transgenic and WT promastigotes are usually not active when observed under microscope and tend to be immobile. Large scale rotating cultures of parasites in flasks, using a rotating incubator set at 140 rpm, was prepared using the method of Fritsche *et al.*, 2007. Promastigotes often had a circular shape and some were dead, so the

rotation speed was reduced to 90 rpm. This solved this problem and promastigotes now had the normal shape.

The effect of using PBS pH 7.4 to prepare the yeast extract medium instead of phosphate buffer (K_2HPO_4 and KH_2PO_4) on promastigote growth was also determined. This change gave no change in the growth of the parasites in yeast extract medium. In the all the above studies 1 x 10^6 promastigotes were used to start cultures. Reducing this concentration resulted in poor growth so this concentration was maintained in all studies.

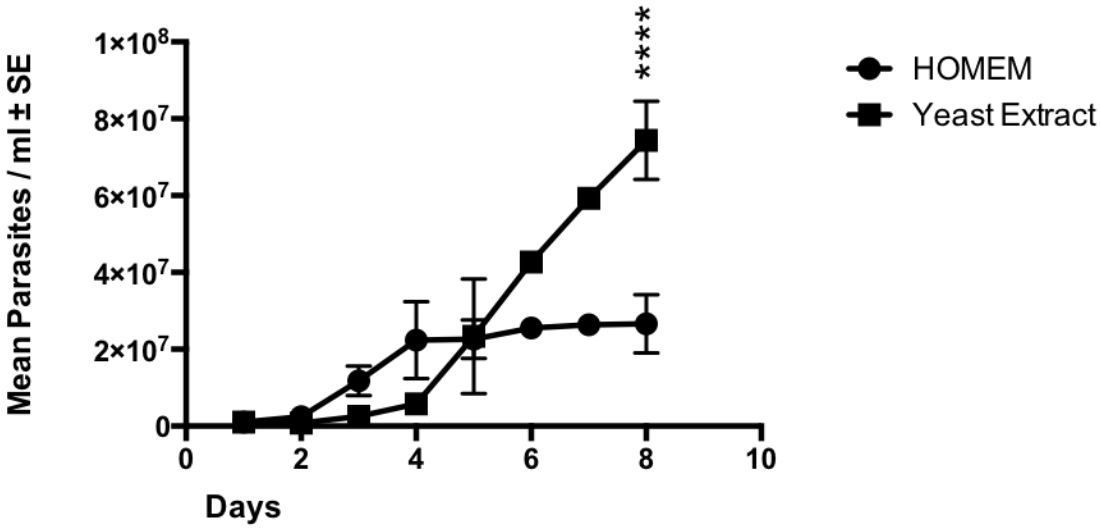


Figure 3.16 The effect of using different medium on the growth of wild- type *L. tarentolae* promastigotes. Promastigotes (1x10^6 cell/ml) were grown at 26°C for up to 8 days in 5 ml HOMEM medium or yeast extract medium (n = 3). Promastigotes numbers were determined by removing 15µL from the culture at the time point shown. ****p<0.0001 promastigotes grew in HOMEM medium compared to promastigotes grew in yeast extract medium day 8

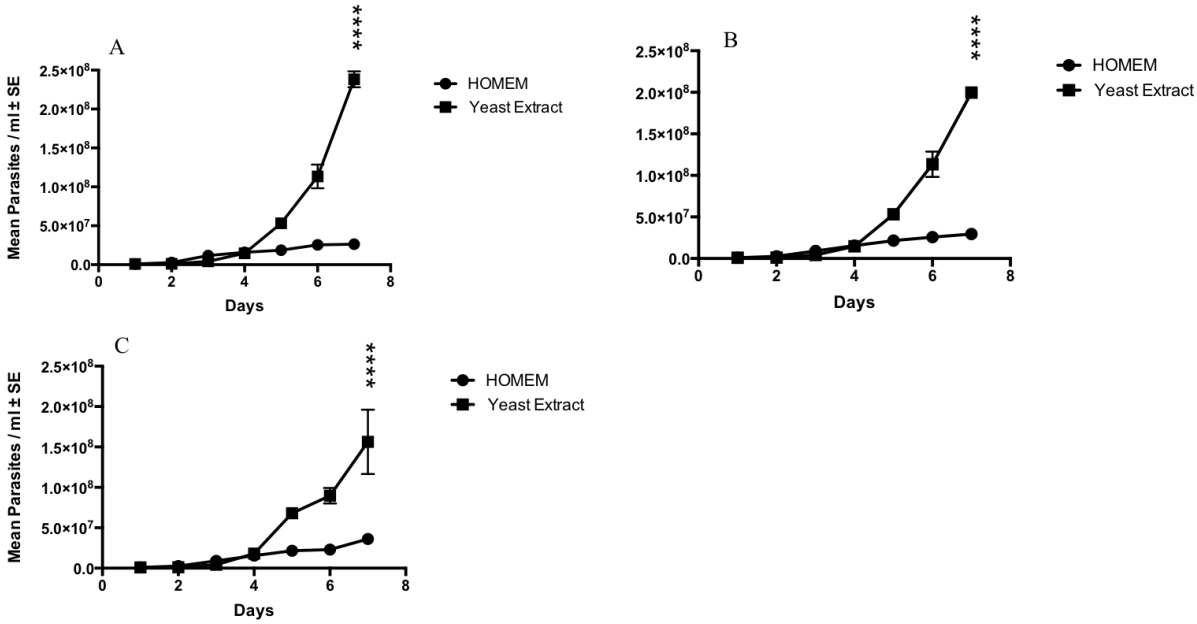


Figure 3.17 The effect of using different medium on the growth of transfected *L. tarentolae* promastigotes (1x10^6 cell/ml) of Lt *L.don* γGCS (A), Lt. *L. maj* γGCS (B) or Lt. *L.mex* γGCS (C) *L. tarentolae* were grown at 26°C for up to 8 days in 5 ml HOMEM medium or yeast extract medium (n = 3). Promastigotes numbers were determined by removing 15µL from the culture at the time point shown. ****p<0.0001 promastigotes grew in HOMEM medium compared to promastigotes grew in yeast extract medium at day 8

3.3.4. The effect of adding different supplements to the yeast extract medium on the growth of transfected *L. tarentolae* promastigotes

3.3.4.1. Hydrogen peroxide (H_2O_2)

Promastigote cultures were supplemented with H_2O_2 (4, 8 or 12 μM) to determine whether using an oxidant improved parasite growth and/or recombinant protein expression. Therefore, the growth of transfected *L. tarentolae* parasites in yeast extract medium with or without H_2O_2 (4, 8, 12 μM) was determined (Figure 3.19). Addition of H_2O_2 significantly increased the growth of the parasites, so that four times more parasites were obtained compared to controls (Table 3.1). The results indicated that addition of H_2O_2 at 12 μM produced the highest cell density compared to controls on day 6 of culture ($p<0.0001$). Parasites transfected with *Lt L.don* γGCS or *Lt. L. maj* γGCS showed approximately the same percentage increase in cell growth, whereas *Lt. L.mex* γGCS had the lowest growth rate increase.

The effect of H_2O_2 on recombinant protein expression in the above studies was determined using immunoblotting. An extract prepared from whole promastigotes ($1x10^8$ cell/lane) was used for the detection of GFP-γGCS-His expression probed using anti-GFP antibody. A band of the correct molecular size for the whole GFP-γGCS-His protein was present in all transfected parasites but not wild type parasites (Figure 3.19). The EGFP expression of the transgenic parasites have a stronger band than the one observed in previous studies where H_2O_2 was not added to the medium for *L.t L.don*γGCS and *L.t L maj*γGCS. However, a similar increase in expression was not observed for *L.t L.mex* γGCS promastigotes. However, further comparative studies using parasites grown with or without H_2O_2 in their culture medium are required.

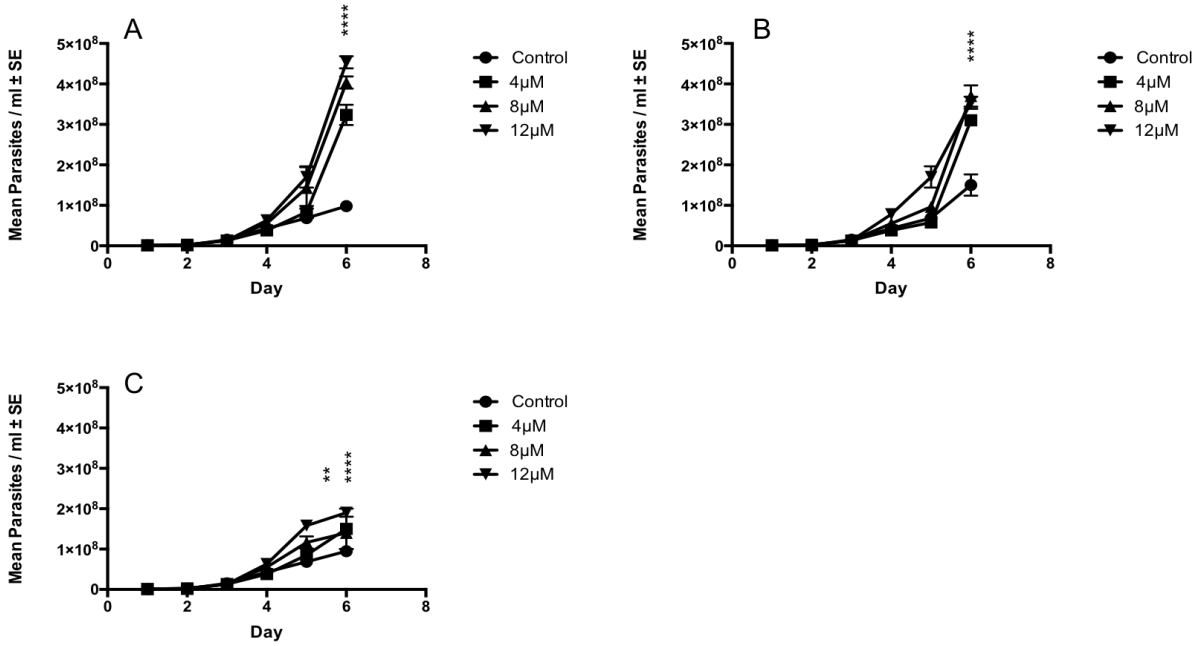


Figure 3.18 The effect of adding H_2O_2 to the culture media on the growth curve for L. tarentolae transfected with γGCS from three different species. Promastigotes (1 x 10^6 cells/ml) of *Lt. L.don* γGCS (A), *Lt.* maj γGCS (B) or *Lt. Lt. mex* γGCS (C) were grown at 26°C for up to 8 days in 5 ml yeast extract medium supplemented with or without H_2O_2 (4, 8, 12 μM, n = 3). ****p< 0.0001 control compared to *Lt. L.don* γGCS and *Lt.maj* γGCS supplement with H_2O_2 (4, 8 and 12 μM) day 6; **** p<0.0001 control compared to *Lt. Lt. mex* γGCS supplemented with H_2O_2 (4 and 8 μM) and **p < 0.001 control compared to H_2O_2 4 μM (*Lt. Lt. mex* γGCS) day 6

Table 3.1 The effect of H_2O_2 treatment on total promastigote number culture on day 6 of culture, n=3. Data obtained from samples shown in Fig. 3.18

Sample name	**Max. Cell number day 6 (cell/ml± SE)**			
	Control	**4μM H_2O_2**	**8μM H_2O_2**	**12μM H_2O_2**
***L.tarentolae* L.don γGCS (Lt. don γGCS)**	9.9 x 10^7 ± 1.5	3.2 x 10^8 ± 1.6	4 x 10^8 ± 8.8	4.5 x 10^8 ± 8.8
***L.tarentolae* L.maj γGCS (Lt. maj γGCS)**	9.7 x 10^7 ± 1.5	1.5 x 10^8 ± 5.7	1.7 x 10^8 ± 1.5	1.9 x 10^8 ± 2.5
***L.tarentolae* L.mex γGCS (Lt. mex γGCS)**	9.9 x 10^7 ± 1.5	3 x 10^8 ± 2.6	3.4 x 10^8 ± 2.3	3.4 x 10^8 ± 3.3

3.3.4.2. L-buthionine sulfoximine (BSO) or Glutathione (GSH)

BSO is a specific irreversible inhibitor of γGCS and GSH acts as a negative regulator of GSH production. Therefore, it is possible that these compounds could influence γGCS expression and either decrease/increase the production of recombinant γGCS by transfected *L. tarentolae* promastigotes. Transfected *L. tarentolae* (*Lt L.don* γGCS, *Lt L.maj* γGCS or *Lt L.mex* γGCS) were cultured in media supplemented with BSO (0.1, 0.5 and 1mM) or GSH (0.5, 1 and 5 mM) to determine the increasing effect of increasing concentration on growth of the transgenic promastigotes (Figures 3.20 and 3.21). Treatment with GSH significantly increased parasite growth, with *Lt L.maj* γGCS and *Lt L.mex* γGCS promastigotes grown in media supplemented with 5 mM GSH having the highest cell density count (cell/ml) on day 6 ($p < 0.0001$). Promastigotes of *Lt L.don* γGCS also showed a significant increase on growth in media supplemented with 5 mM GSH on day 6 ($p< 0.05$) but a concentration of 0.5 mM significantly inhibited parasite growth compared to controls. Culturing the promastigotes with medium supplemented with BSO also increased parasite growth compared to controls ($p <0.0001$), but it had less of an effect than GSH treatment. Cultures supplemented with 1 mM BSO had the highest cell density ($p <0.0001$) and a similar result was obtained for all three transfected parasites.

The parasites from the GSH/BSO studies were also used for the detection of GFP-γGCS-His expression by immunoblotting. A whole promastigote extract (1×10^8 cell/lane) was used for the detection of GFP-γGCS-His expression using anti-GFP antibody. The band corresponding with the molecular size of the relevant gene were observed in all transfected parasites in compare with wild type (data not shown). The EGFP expression of the transgenic parasites apparently showed a dominant stronger band for transgenic parasites cultured with media supplemented with GSH compared to samples incubated with BSO. Culturing with medium supplemented with 5 mM GSH gave the highest protein expression for promastigotes of *L.t L.don* γGCS and *L.t Lmex* γGCS. Treatment with BSO at different concentrations gave the same intensity of GFP-γGCS-His expression for all three transgenic parasites. These results indicated that supplementing the

growth media with GSH had a more favorable effect on the expression of GFP-γGCS-His protein compared to adding BSO.

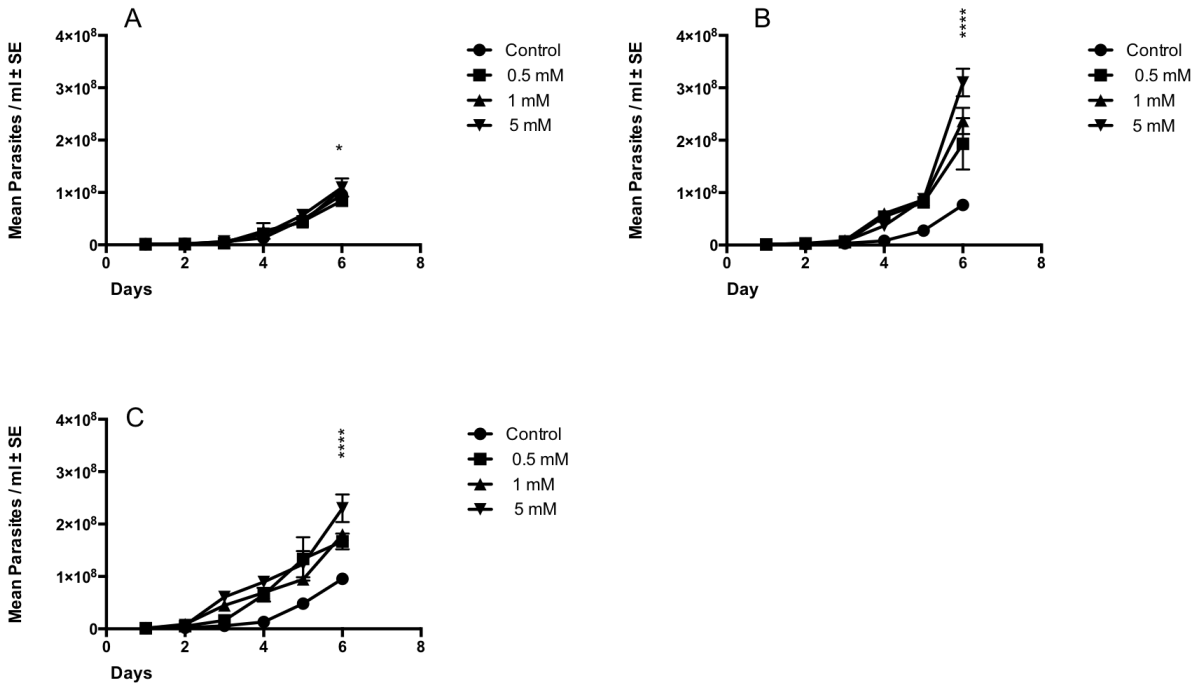


Figure 3.19 The effect of adding increasing concentration of GSH in culture media on the growth of *L. tarentolae* transfected with γGCS from three different *Leishmania* species. Promastigotes (1 x 10^6 cells/ml) of *Lt. L.don* γGCS (A), *Lt.* maj γGCS (B) or *Lt. Lt. mex* γGCS (C) were grown at 26°C for up to 8 days in 5 ml yeast extract medium supplemented with or without GSH (0.5, 1 and 5 mM, n = 3). ****p< 0.0001 control compared to *Lt. L.maj* γGCS and *Lt. mex* γGCS supplement with GSH (0.5, 1 and 5 mM) day 6; **** p<0.0001 control compared to *Lt. Lt. mex* γGCS supplemented with GSH (0.5, 1 and 5 mM) and *p < 0.05 control compared to GSH 5 mM (*Lt. Lt. don* γGCS) day 6

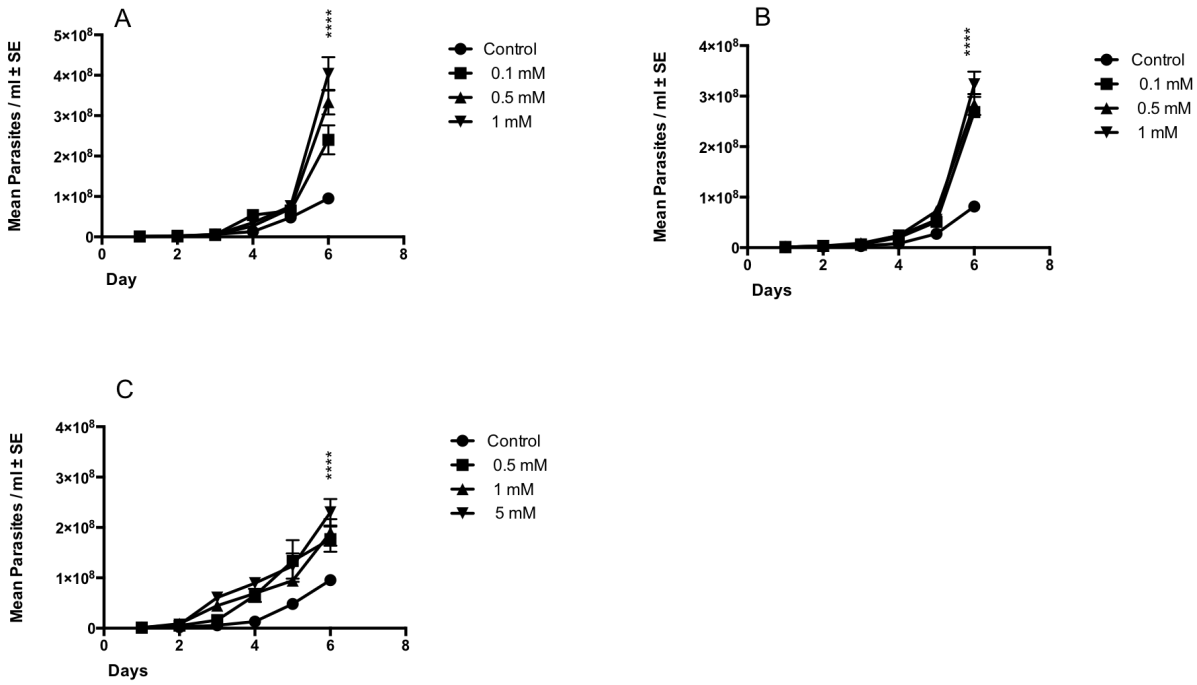


Figure 3.20 The effect of adding increasing concentration of BSO in culture media on the growth curve for *L. tarentolae* transfected with γGCS from three different species. Promastigotes (1 x 10^6 cells/ml) of *Lt. L.don* γGCS (A), *Lt. maj* γGCS (B) or *Lt. mex* γGCS (C) were grown at 26°C for up to 8 days in 5 ml yeast extract medium supplemented with or without BSO (0.1, 0.5 and 1 mM, n = 3). ****p< 0.0001 control compared to BSO 1 mM day 6

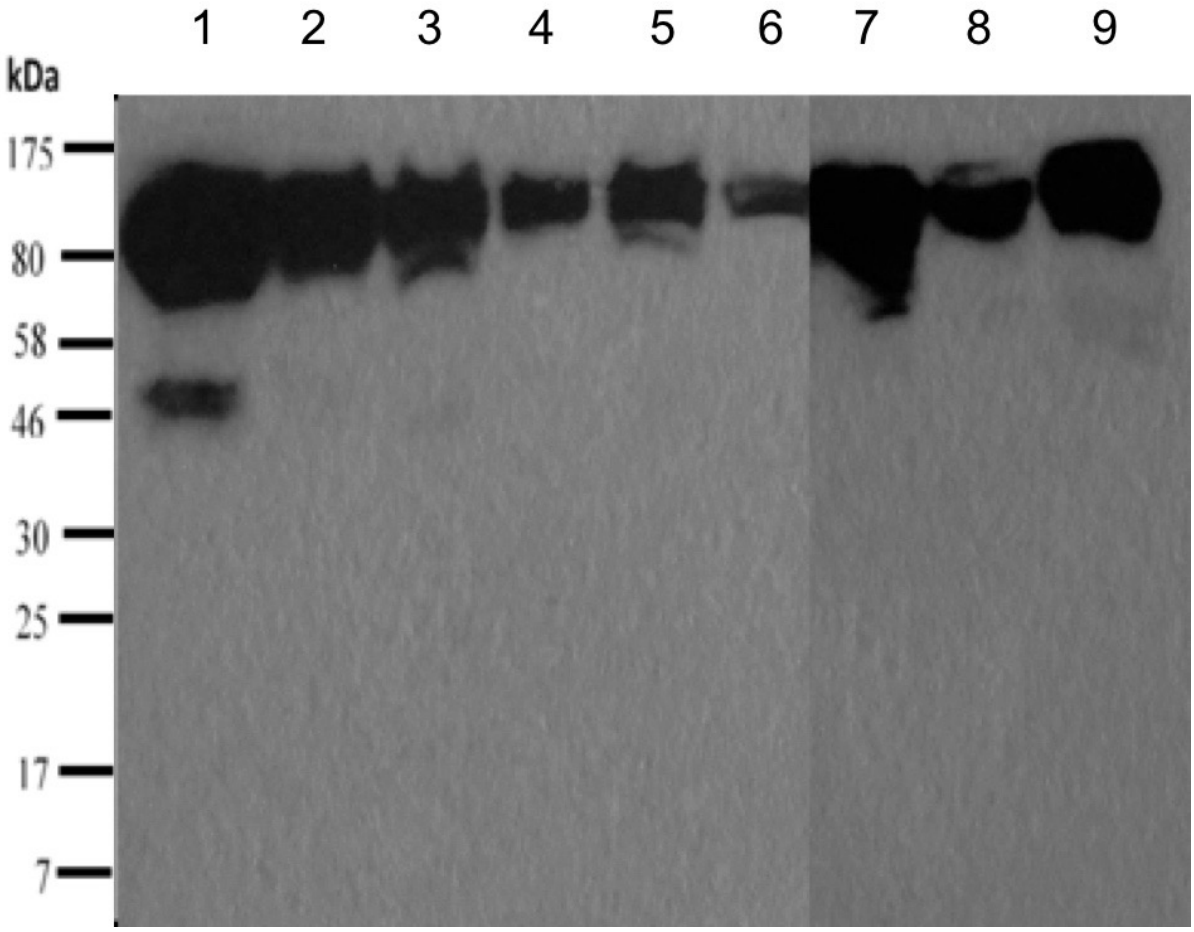


Figure 3.22 Immunoblotting of 1 × 10^8 *L. tarentolae* promastigotes to detect the effect of the presence of GSH on the expression of GFP-γGCS-His recombinant protein. Cell extract from three transfected *L. tarentolae* (*L.t L.don* γGCS, *L.t L.maj* γGCS, *L.t L.mex* γGCS) supplemented with GSH; *L.t L.maj* γGCS (0.5 mM lane 1, 1 mM lane 2 and 5 mM lane 3), *L.t L.don* γGCS (0.5 mM lane 4, 1 mM lane 5 and 5 mM lane 6), *L.t L.mex* γGCS (0.5mM lane 7, 1mM lane 8 and 5mM lane 9).

3.3.5. Purification of GFP-γGCS-his

Ideally a purification method is required that give full length recombinant protein without any contaminating proteins. However, studies using Coomassie blue staining of the proteins present in the eluate from the affinity column showed that multiple proteins were present. There was a dominant protein with a molecular weight of ~109KDa, which would correlate with the expected size for full length GFP-γGCS-his recombinant protein. To reduce the amount of contaminating proteins, the effect of changing the extraction conditions i.e. purification conditions i.e. buffer used, washing procedure and imidazole concentration, was determined.

Studies under native conditions showed that higher amounts of eluted protein were recovered if sodium phosphate buffer pH 7.4 instead of Tris buffer pH 7.4 was used i.e. 1.5 mg/ml eluted protein was recovered compared to 0.250 mg/ml from a 1L *L. tarentolae* culture containing 6 x 10^8 cells. Therefore, in subsequent studies sodium phosphate buffer pH 7.4 was used to isolate γGCS under native conditions. Figure 3.23 shows an example of the result was obtained for isolation of recombinant γGCS from transfected *L. tarentolae.*

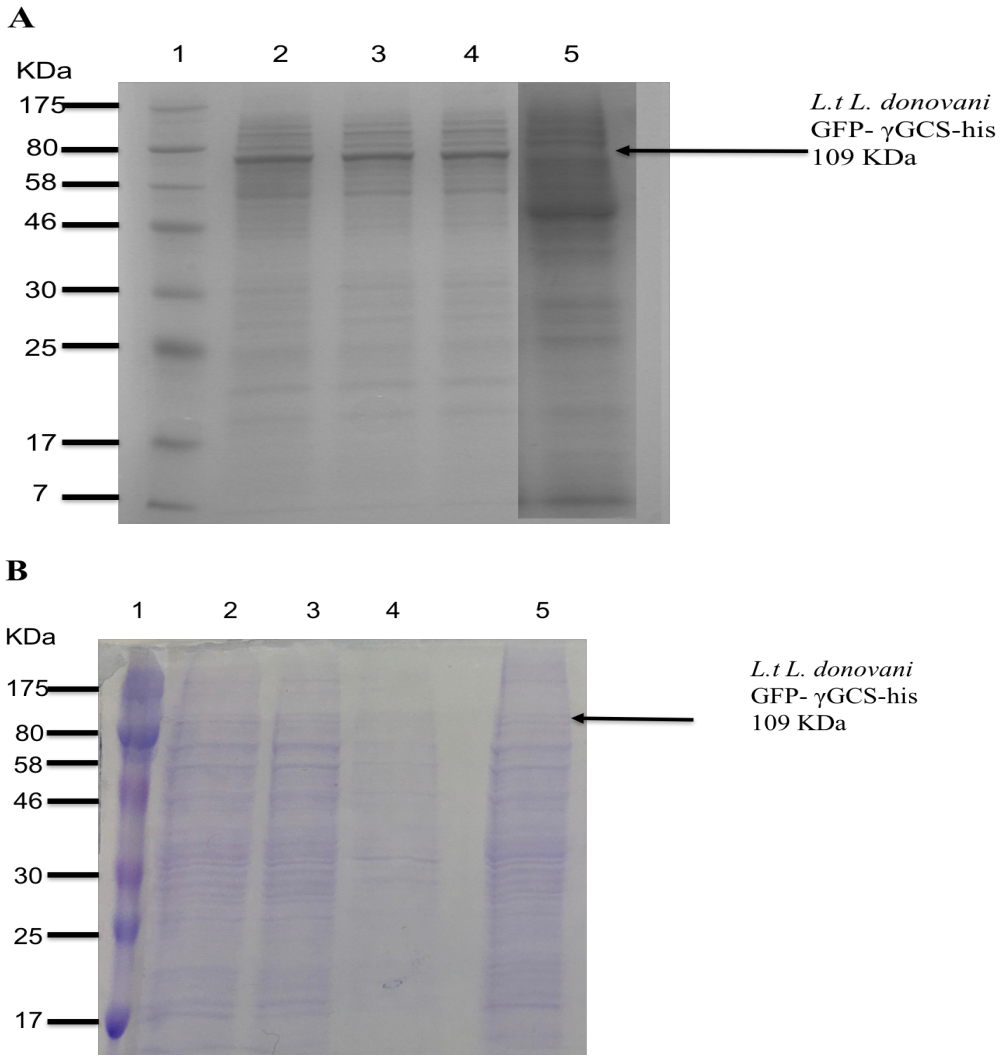


Figure 3.21 Picture of a commassie stained gel used to separate the proteins present in the eluate from the affinity column used to purify recombinant GFP-γGCS-his from transfected *L. tarentolae*. A 1L bulk culture of transfected *L. tarentolae* were concentrated by concentrated by centrifugation, resuspended in 10 ml phosphate buffer pH 7.4 (A) or Tris buffer pH 7.4 (B) and the soluble proteins present were extracted using sonication. The suspension was centrifuged at 15000 x g to remove particulate matter and the resulting solution of soluble protein was loaded on to a Ni-NTA affinity column. The proteins (50µg/lane) present in the resultant eluate from the affinity column or the original soluble protein were separated by SDS-PAGE and the the proteins present visualised by Coomassie blue staining. Total cytosolic soluble fraction *L.t L don* GFP- γGCS-his (lane 2), *L.t L maj* GFP- γGCS-his (lane 3), *L.t L mex* GFP- γGCS-his (lane 4) and WT (lane5). (B) Tris buffer used to purification of GFP- γGCS-his recombinant protein, 50 µg of total cytosolic soluble fraction *L.t L don* GFP- γGCS-his (lane 2), *L.t L maj* GFP- γGCS his (lane 3), *L.t L mex* GFP- γGCS-his (lane 4) and WT (lane5), molecular weight marker (lane 1).

3.3.6. Determine the optimum metal for metal affinity chromatography

Purification of His-tagged proteins using IMAC is based on the interactions between a transition metal ion immobilized on a matrix and specific amino acid side chains (Bornhorst and Falke, 2000). Expression and purification studies using Co^{2+} and Ni^{2} resins for protein purification showed that Ni^{2+} affinity column reduced the number of non-specific protein bands present after isolation. Washing the affinity column after the soluble protein had been passed down the affinity column showed that 100 mM imidazole in the buffer was enough to elute the bound protein from the Co^{2+} resin column, whereas elution from the Ni^{2+} resin column required buffer containing 400 mM (Fig. 3.24).

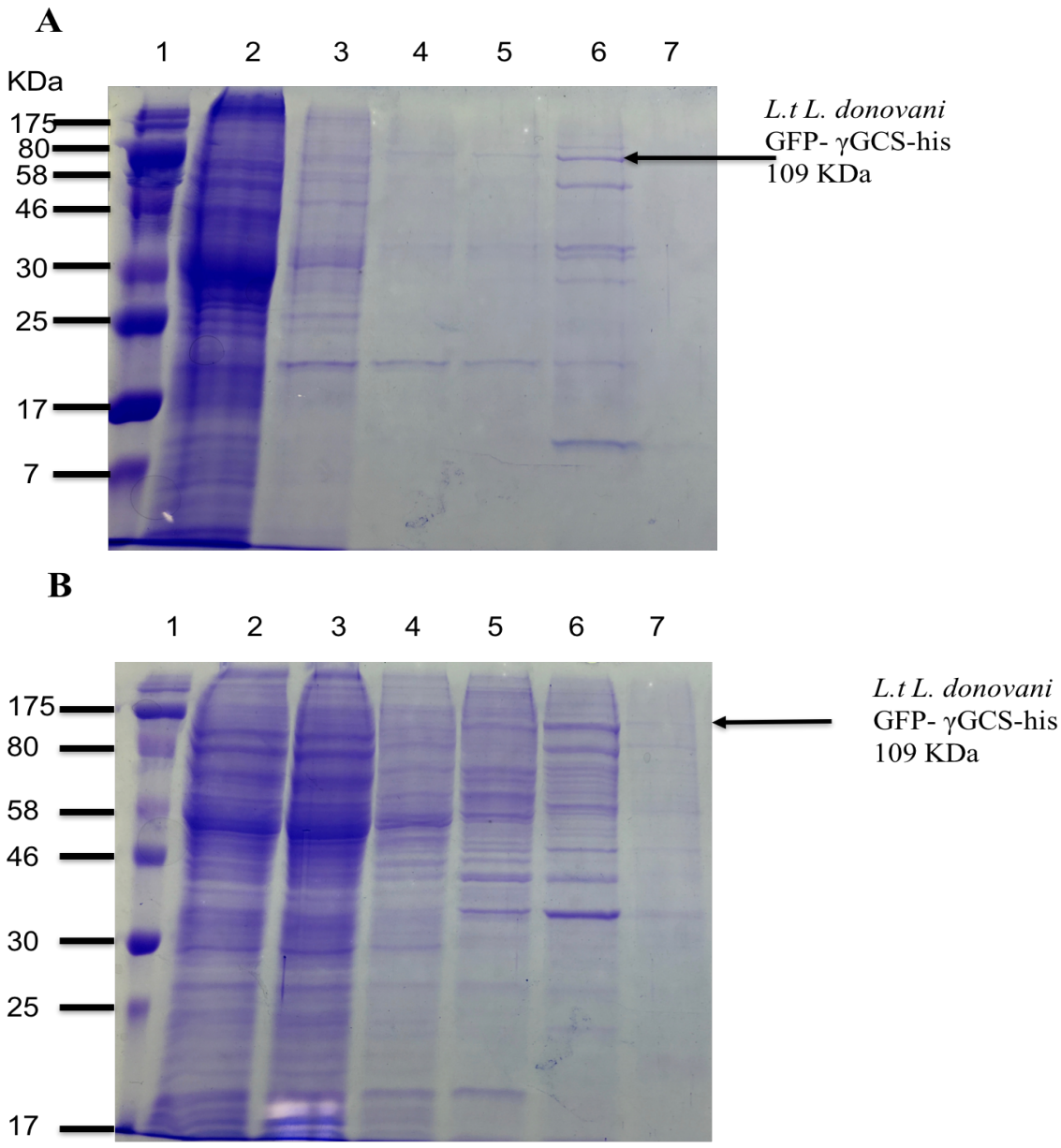


Figure 3.22 The effect of using different concentrations of imidazole in the elution buffer to isolate recombinant GFP-γGCS-his from different affinity columns. Soluble protein prepared from 6 x 10^8 cells/ml transfected parasites was passed down a Ni^{2+}-NTA or Co^{2+}-CMA affinity column and eluted using elution buffer containing different concentrations of imidazole. The eluted protein (50 µg/lane) was separated by separated by SDS-PAGE and the proteins present visualised by Coomassie blue staining. Total cytosolic soluble fraction (lane 2), washing 1 (20 mM imidazole, lane 3), washing 2 (50 mM imidazole, lane 4), washing 3 (100 mM imidazole, lane5) and eluted fractions (400 mM imidazole, lane 6 and 7), molecular weight marker (lane 1).

3.3.7. Ni+2-NTA resin vs. his-Trap column

Purification of GFP-γGCS-his using either the Ni^{2+}-NTA or Co^{2+}-CMA affinity columns still gave a recombinant protein that had additional nonspecific bands. Therefore, the effect of using a different Ni^{2+}based affinity column i.e. the His Trap® column his-Trap on purification of *L. L. don* GFP- γGCS-his was determined. A higher amount of GFP-γGCS-his was recovered using the Ni^{2+}-NTA affinity column and using the His Trap® column gave more non-specific protein bands under the same conditions (Figure 3.25).

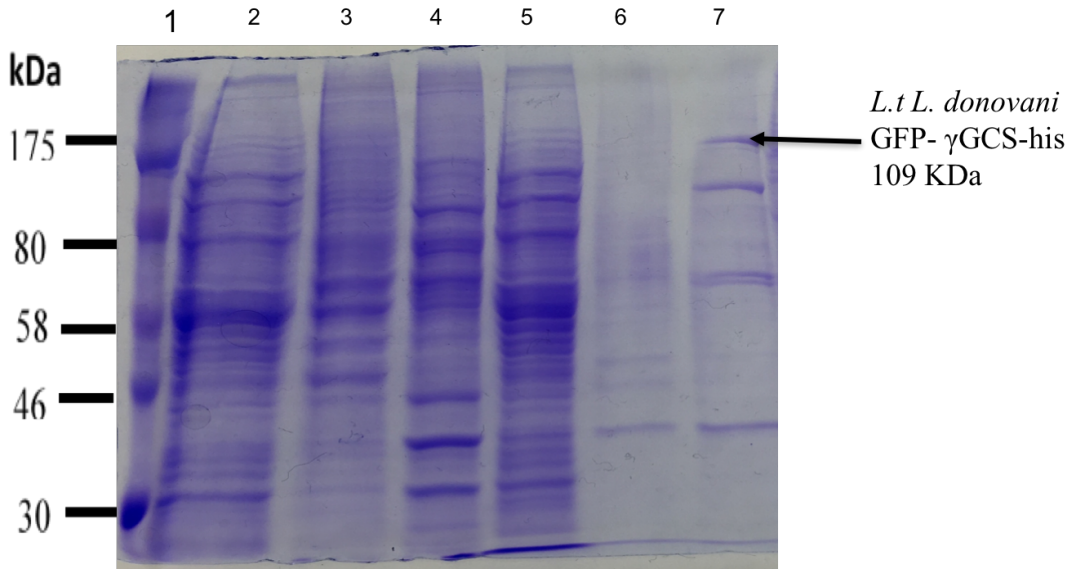


Figure 3.23 The effect of using different concentrations of imidazole in the elution buffer to isolate recombinant GFP-γGCS-his from different affinity columns. Soluble protein prepared from 6x 108 cells/ml transfected parasites was passed down a Ni^{2+}-NTA or his-Trap® column and eluted using elution buffer containing different concentrations of imidazole. The *L. tarentolae* culture was incubated at 25ºC and purified using sodium phosphate buffer. 50 µg of total cytosolic fraction (lane 2), washing (lane 3), and eluted (lane 4) soluble protein fraction sample using His-Trap column and total cytosolic fraction (lane 5), washing (lane 6), and eluted (lane 7) soluble protein fraction sample using Ni^{2+}-NTA affinity column of *L.t L. don*-GFP- γGCS-his cells were separated using SDS-PAGE gel and the proteins produced visualised using Coomassie blue stain. M (lane 1).

Western blot studies were carried out to show that if the additional proteins present after elution were due to degradation occurred during the purification steps. The protein with the molecular weight that correlated with full length GFP-γGCS-his protein was present in all soluble fraction samples and had a GFP-tag or his-tag (Fig. 3.26). However more than one protein band, with molecular weights lower that the full-length protein, were present in the flow through fraction samples. Therefore, the lower molecular weight proteins could be degraded products. Gradient elution using different concentration of imidazole was tested but this did not improve recovery.

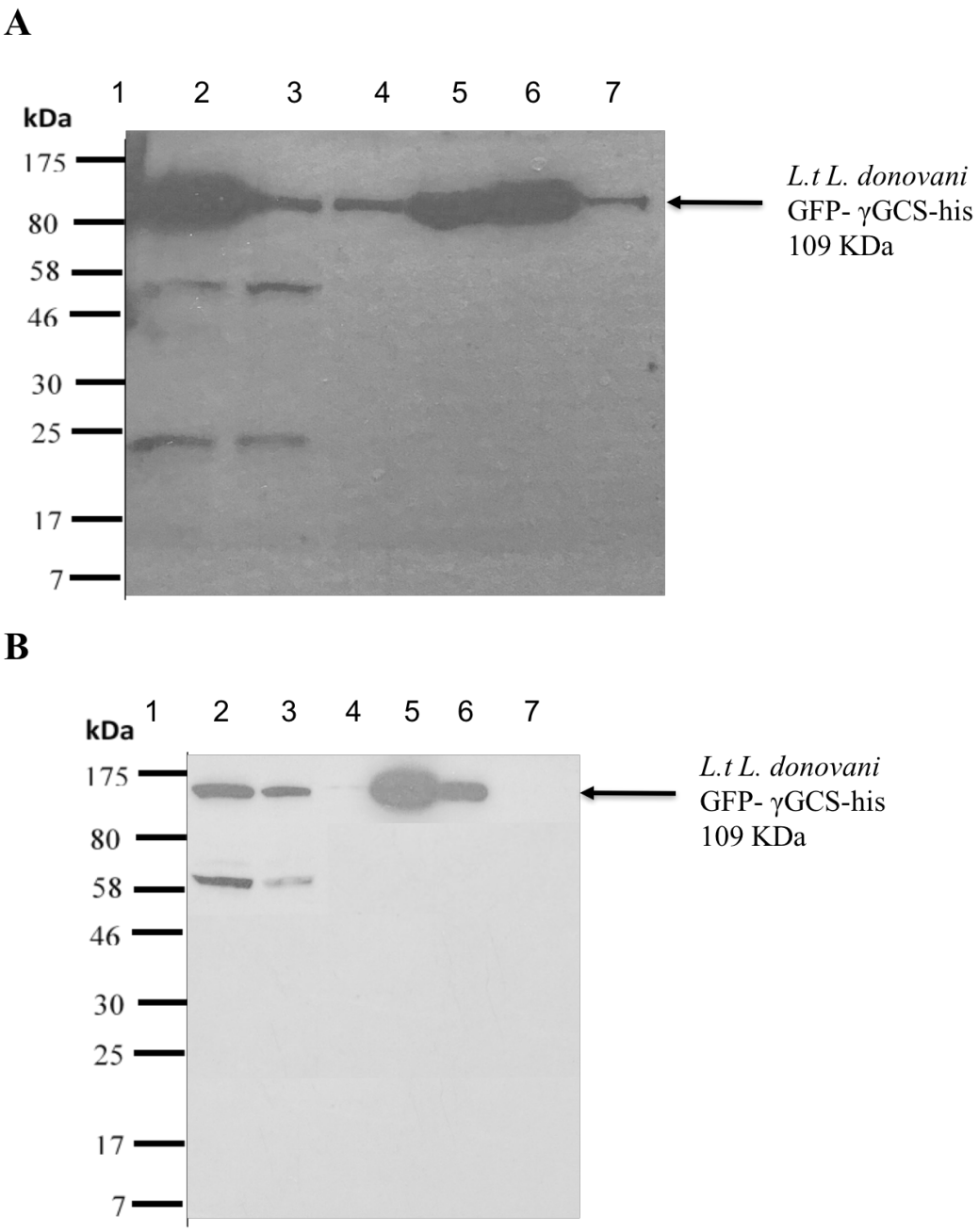


Figure 3.24 Results from Western blot studies to identify His-tag proteins present in *L. don* γGCS recombinant protein samples produced during purification using affinity column. Samples obtained from studies shown in Figs 5.4. Proteins present in samples obtained during affinity column purification of recombinant GFP-γGCS-his were separated by SDS gel electrophoresis ((50 µg/lane) and transferred to nitrocellulose membrane. The membrane was the probed with a GFP-Tag mouse mAB (A) or a His-Tag rabbit mAb HRP conjugate (B) and the presence of His tagged proteins was determined by chemiluminescence. Soluble fraction (2), flow through (3), washing (50 mM imidazole, 4), elution 1 (400 mM imidazole, 5), elution 2 (400 mM imidazole, 6) and elution 3 (400 mM imidazole, 7). The position of the molecular weight markers (1).

One of the factors that could have reduced the amount of recombinant γGCS from transfected *L. tarentolae* parasites was the presence of the native enzyme encoded by *L. tarentolae* γGCS gene sequence (appendix 3). The *L. tarentolae* γGCS gene sequence has considerable homology to γGCS gene sequence of the three pathogenic *Leishmania* spp. The homology between the γGCS of the three species (*L. don, L. maj.* and *L. mex)* and the γGCS of the *L. tarentolae* is 86%, 85% and 83% in respectively. This could allow formation of heterodomains between monomers of the different proteins (Park and Raines, 2000). Therefore, transfected recombinant γGCS could bind to the *L. tarentolae* WT γGCS and produce a heterodimer. Therefore, studies were carried out to delete the WT γGCS gene sequence from the transfected parasites by replacing part of the upstream of the gene with two different antibiotic resistance markers (hygromycin and blasticidin). Two different constructs were designed containing two different antibiotic markers as mentioned above. Deletion of the native γGCS was achieved in two steps. The first step involved replacing one of the two alleles with the hygromycin containing construct, and the second step used a positive clone from the first experiment to replace the second allele with the blasticidin containing construct. Positive cultures were transferred expanded in culture and used to obtain DNA for PCR studies using primers that were specific to *L. tarentolae*-WT γGCS. The WT γGCS gene sequence was successfully deleted from two of the three transfected *L. tarentolae* strains i.e. *L.t L. mex* still showed the presence of WT γGCS gene (Figure 3.27). Unfortunately, due to lack of time, studies to look at protein expression using these parasites was not possible.

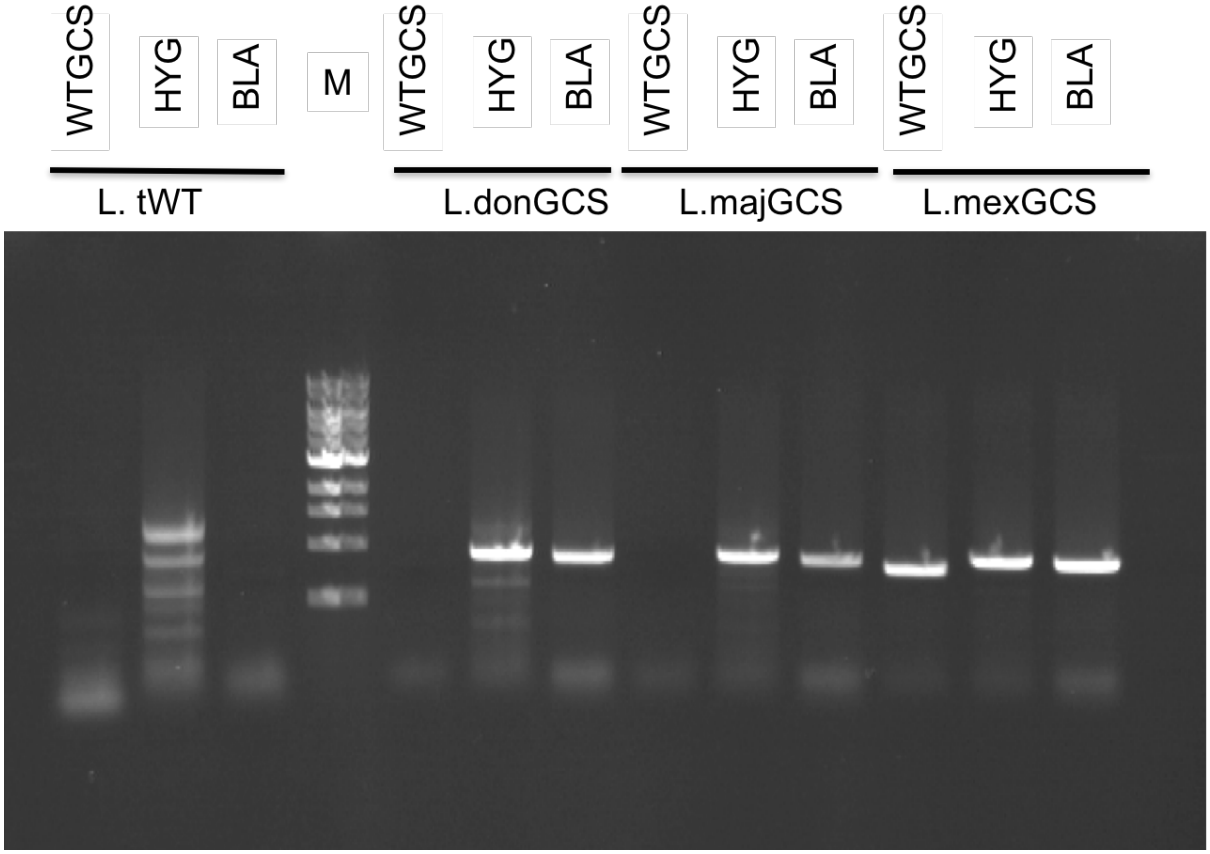


Figure 3.25 Picture of PCR amplified products for the three transfected *L. tarentolae* and *L. tarentolae* WT to show that the WT γGCS was successfully deleted from parasites transfected with *L.don* γGCS and *L. maj* γGCS but still shown with parasites transfected with *L. mex* γGCS. DNA isolated from WT and transfected parasites (*L.t L.don* γGCS, *L.t L. maj* γGCS or *L.t L. mex* γGCS) was used in a PCR reaction using primers for WT- γGCS, hygromycin B or blasticidin. The resulting PCR products were separated by agarose gel electrophoresis and DNA bands visualied by staining the gel with ethidium bromide. The PCR products bands the anticipated size of 920 bp WT γGCS (WTγGCS), 949 bp hygromycin (HYG), and 915 bp, blasticidin (BLA), molecular weight marker (M).

3.4. Discussion

The relevant gene sequence of γGCS for *L. major, L. mexicana* and *L. donovani* was successfully cloned into the *Leishmania* expression vector pSSU-int vector. In this study, the DNA sequence that encodes γGCS was cloned into a *Leishmania* plasmid vector, coordinated with genomic DNA of the *Leishmania* spp. The level of expression can vary in different stages of *Leishmania* and it is regulated by post-transcriptional modulation, which depends on the intergenic regions at the 3' of the coding sequences (Charest *et al.*, 1996). Therefore, including the well-characterised cysteine proteinase B 2.8 gene cluster (CPB 2.8) into the expression vector used to produce the recombinant protein ensured abundant expression of our gene sequence (Mottram *et al.*, 1997).

DNA constructs containing the coding regions for γGCS, puromycin and CPB 2.8 IR were introduced into the rDNA locus of *L. tarentolae*. Clones of transfected parasites were obtained using a dilution method and a number of positive samples were obtained for all three parasites i.e. *L. don,* 20 clones, *L. maj,* 25 clones and *L. mex,* 18 clones at a 1:40 dilution of the samples. Six random clones were selected and tested for the expression of the GFP marker. All selected tested clones expressed high amounts of the GFP marker. Integration into the rDNA locus was confirmed by PCR analysis using genomic DNA of the transfected parasites as template and primers complementary to sequences 597 bp upstream of the 5' end of the 18S rRNA gene and to the 3' end of the sequence containing the γGCS in pSSU-int. Thus, amplification of a 1.956 Kb fragment was only possible if the gene sequence integrated into the DNA cassette integrated into the a 18S rRNA gene occurred. The DNA construct for γGCS was tagged from both ends with reporting markers (GFP from N terminal and His-Tag from C terminal). The His tag allowed purification the recombinant protein produced and the GFP-tag would allow cell localisation studies for the protein to be completed (Seibel *et al.*, 2007).

The relevant gene sequence of γGCS was cloned into the expression vector through various steps to match the cloning sites of the restriction enzymes. The

vector was then used to transfect *E coli* and successfully transfected colonies were selected on the basis of antibiotic resistance. However, bacteria may not have contained the gene insert in the right orientation therefore enzyme digests were carried out to show the fragments of the expected size were obtained. Successful transfection was then confirmed by carrying out 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 five months to finish cloning the γGCS gene sequence into the expression vector pSSU-int. This period was required to prepare the expression vector pSSU-int with cloning sites that were compatible with the insertion of the γGCS gene sequence. The γGCS sequence also needed to be flanked with the EGFP reporting gene. This was obtained from another plasmid, and it was taken as a full fragment, and inserted into the MCS of the pSSU-int expression vector. In addition, three different constructs for the different γGCS gene from three *Leishmania* species were required, and some of the steps were repeated more than once to obtain inserts in the correct orientation. Three constructs without the TEV insertion were also constructed for the γGCS gene sequence from the three *Leishmania* species. Sequencing of the three constructs showed that the gene of interest were with correct orientation in the expression vector.

Despite completing the construct, still needed to be able to remove the tags from the original expressed protein (Parenky *et al.*, 2014). Therefore, introducing TEV protease cleavage site was done to allow removal of the GFP tag (Fox and Waugh, 2003, Kapust and Waugh, 2000). The fragment of a particular size range containing the TEV protease cleavage site sequence was 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). High fidelity polymerase was used to amplify the relevant fragment sequence of TEV protease, to reduce the chances of getting mutations introduced to the amplified PCR fragment. This enzyme is fused with a processivity-enhancing

domain, increasing the fidelity and copying speed with an error rate > 50-fold lower than that of *Tag* DNA polymerase (Bohlke *et al.*, 2000). The reason behind not including any cleavage site between the γGCS and the His-tag was the benefit of using carboxypeptidase B, which could remove the His residue from the N-terminus.

Alignment of the γGCS gene sequence for the three *Leishmania* species showed high identity (94%) for all three species. There was 96% identity between *L. donovani* and *L. major*, 93% identity between *L. donovani* and *L. mexicana* (see appendix 3).

Numerous types of reporter genes are currently available for protozoan parasites and GFP is the widespread used (Dube *et al.*, 2009) for protozoan parasites. There are several advantages of GFP based assays over other non-reporter gene based assays, including superior simplicity, easier kinetic detection, low cost and enhanced biosafety (Singh *et al.*, 2009). The GFP reporter gene has been used either in plasmid form or integrated into a defined locus beside other types of reporters been used on recombinant *Leishmania* species; such as luciferase-expressing *L. donovani*; integrative luciferase/-lactamase and episomal GFP/EGFP/ß-galactosidase- expressing *L. amazonensis*; episomal/integrative luciferase and integrative GFP/ ß-lactamase/ ß-galactosidase expressing *L. major*; and episomal/integrative GFP and integrative ß-galactosidase-expressing *L. mexicana* (Dube *et al.*, 2009, Chan *et al.*, 2003, Roy *et al.*, 2000). Using a GFP reporter gene on a plasmid has two drawbacks. First, depending on the copy number of plasmids per cell, the gene expression is extremely heterogenous in populations of transfected parasites. Second, in the absence of antibiotic resistance marker the host can easily lose the plasmid during passage of the parasites over time. Integration of the tagged recombinant protein permanently into the genomic DNA of the host overcomes these issues (Misslitz *et al.*, 2000). This vector has been used for the constitutive expression of other target proteins. In this study, directional cloning of GFP-γGCS-His into the rRNA locus was confirmed by diagnostic PCR using genomic DNA of transfected promastigotes as template. *L.tarentolae* promastigotes were transfected with γGCS from three

different *Leishmania* species i.e *L.donovani, L .major* or *L. mexicana* by electroporation using the linearized pSSU construct as described in Chapter 3. The dominant band observed on the agarose gel revealed that electroporation of the pSSU-GFP-γGCS-His construct was successfully integrated into the rDNA locus of the *L. tarentolae.* Stable expression of GFP-γGCS-His in transfected live parasites were evaluated and confirmed by epi-fluorescence microscopy and immunoblotting. Green fluorescent parasites images indicate that the GFP-γGCS-His gene was expressed successfully during the growth of transfected promastigotes. This study was conducted to establish an easy method for stable expression of recombinant protein through life stages of *Leishmania* parasites and monitoring infection of parasites in the host cells. EGFP expression has been studied to monitor infection of amastigotes in infected mice (Bolhassani *et al.*, 2011). Green fluorescent images of all three transfected promastigotes showed to have the same intensity of expression over the period of six months, which means the amount of recombinant protein expressed remained the same. Culturing transfected promastigotes without the presence of the antibiotic resistance selection marker puromycin showed that EGFP was expressed abundantly and stably in promastigotes of the three transfected *Leishmania* promastigotes. The fluorescence intensity pattern showed full-body distribution within promastigotes indicating a homogenous level of expression within the parasite. Microscope images showed that *L. tarentolae* parasites transfected with *L. mex*γGCS had lower EGFP expression intensity compared with *L.t L.don*γGCS or *L.t L.maj*γGCS promastigotes. Differences in the gene sequence of *L. mexicana* γGCS from the other two species could be responsible or perhaps it depends on the actual location of the integration of the foreign gene sequence into the specific parasite. That could be related to the expression level of the CPB 2.8 gene. Therefore, selecting a high expressing clone from a mixed culture could ensure high expression for all the transfected parasites.

Leishmania promastigotes were grown in two different media to determine the effect of using different media on the parasite growth. Growing *L. tarentolae* WT in yeast extract medium was gave significantly better growth than HOMEM ($P < 0.0001$). The yeast extract medium was supplemented with hemin rather than

FCS as FCS is more expensive than the hemin. Heme is essential growth factor (Pal and Joshi-Purandare, 2001) and the parasites lack the enzymes for its biosynthesis. Therefore, supplementing media with hemin is crucial for the duplication of promastigotes (Chang and Chang, 1985, Chang *et al.*, 1975). These studies showed that *Lt. L. don* γ, *Lt. L.maj* γGCS and *Lt. L. mex* parasites grew better in yeast extract medium.

These studies showed that parasite growth in yeast extract medium using a low inoculum was minimal, which probably reflects the fact that this medium is still not optimal for parasite growth and adding other supplements may improve parasite growth. Studies have shown that *L. tarentolae* promastigotes grown in media supplemented with L-proline, glutamate or aspartate improve oxygen uptake and that the parasite may have a proline-glutamate interconversion oxidation pathway such as proline oxidase metabolic pathway (Krassner, 1969). L-proline is used by *L. tarentolae* as the main source of carbon to produce alanine and recent studies have shown that L-proline can affect the irreversible conversion of gamma-glutamate semialdehyde (γGS) into L-glutamate and NADH pathway in *Trypanosoma brucei* (Mantilla *et al.*, 2017). The low concentration of proline and the absence of glutamate in the yeast extract medium could therefore have a detrimental effect on parasite growth.

Despite the being able to grow parasites, the number of *Leishmania* parasites never reached $1x10^9$ cells/ml reported by Fritche and colleagues (Fritsche *et al.*, 2007), the maximum mean cell number achieved was around $2.3\ x10^8$ cells/ml. Dr. Weise's group at Strathclyde Institute of Pharmacy and Biomedical Science have also tried using the yeast extract medium under the same conditions and have had the same issues. Another option would be to use SDM-79 medium, which is very rich medium compared to the yeast extract medium but is too expensive for large scale production of *L. tarentolae* promastigotes.

Culturing *Leishmania* promastigotes in yeast extract medium supplemented with hydrogen peroxide increased the number of the parasites/ml 10-fold ($1.9\text{-}4.5\ x\ 10^8$ cells). Hydrogen peroxide reported to have direct effect for differentiation of

promastigotes to amastigotes for *L. amazonensis* (Mittra *et al.*, 2013). Another study showed that hydrogen peroxide can induce apoptosis-like death in *L. donovani* (Nandi *et al.*, 2010). And hydrogen peroxide can kill both promastigotes and amastigotes when the parasites are exposed to hydrogen peroxide during phagocytosis (Hammoda *et al.*, 1996, Channon and Blackwell, 1985) or if the hydrogen peroxide is added exogenously *in vitro* (Murray and Nathan, 1988). Studies in have shown that high concentrations of H_2O_2 e.g. 4 mM can reduce the level of intracellular GSH within parasites, and this may adversely affect their ability to tolerate oxidative stress (Das *et al.*, 2001).

A DNA construct containing the coding regions for the recombinant protein, puromycin and CPB 2.8 IR was introduced into the rDNA locus of *L. tarentolae* and the expression level of the recombinant protein obtained was reproducibly high. Higher level of protein expression in *Leishmania* can be achieved by placing the gene of interest under the control of a rRNA promoter i.e. pol I (Gay *et al.*, 1996, Uliana *et al.*, 1996). Results from this study indicate that protein production can be driven by pol I in *L. tarentolae* (Misslitz *et al.*, 2000). However, the post-transcription process is IR-dependent which in this case is regulated by CPB 2.8, which flanked γGCS into the rRNA subunit. This protein is expressed highly expressed in both life cycle stages of *Leishmania* and the ratio for the mRNA levels of the CPB 2.8 genes is 1:6:33 for procyclic promastigotes: metacyclic promastigotes: amastigotes (Mottram *et al.*, 1997). MALDI studies would be confirm the identity of all separated proteins present.

Previous studies using *E. coli* to produce recombinant *Leishmania* γGCS showed that it was mainly expressed in inclusion bodies, and purification using urea to soluble the extracted proteins resulted in the production of both full length and truncated forms of γGCS (Henriquez *et al.*, 2010). In this study γGCS was expressed using a construct with a N-terminus GFP and C-terminus his-tag but again studies indicated that purification, without using urea, still produced full length and truncated forms of γGCS.

Using a tag which consists of six histidine residues is generally enough to yield high affinity interactions with a purification column (Bornhorst and Falke, 2000). However, 2% of all eukaryotic protein residues are histidine, as some cellular proteins contain two or more adjacent histidine residues (Schmitt *et al.*, 1993). In addition, disulfide bonds and nonspecific hydrophobic interactions can also cause some co-purification with the desired protein (Bornhorst and Falke, 2000). *Leishmania* is known to have a higher natural abundance of proteins containing histidine residues (Crowe *et al.*, 1994). Therefore, these proteins could bind to the metal resin and be responsible for the contamination with lower molecular weight proteins found in this study.

In this study, different affinity columns were used to purify the recombinant protein. IMAC columns use iminodiacetic acid (IDA) as a matrix to chelate transition metals through three coordination sites (Porath *et al.*, 1975). A problem with the use of IDA matrices is that the metal ion is only weakly bound to such a three-coordinate matrix. Metal leaching from the matrix during purification causes lowered yields and impure products (Crowe *et al.*, 1994). Ni^{2+} or Co^{2+} have a binding capacity of 5-10 mg protein/ml of matrix resin and a high binding affinity for six-residue poly-histidine tag at pH 8.0 (Schmitt *et al.*, 1993), and Co^{2+} has been reported to exhibit less non-specific protein binding than the Ni^{2+}. However, the results of this study showed that Ni^{2+} had higher affinity for the recombinant protein and required a 4 times higher concentration of imidazole in the wash buffer to elute the recombinant protein. A gravity Ni-NTA agarose resin column had higher affinity than using his-Trap® resin column. These results could be due to technique used to load samples on the Ni-NTA agarose column, as this depended on gravity to move the sample through the column whereas the his-Trap® column depends on a manual technique i.e. using a syringe. This may mean that the time that the protein need to bind with the ion sites on the column is important so reapplying the crude extract to the affinity column may improve the recombinant protein yield.

Deletion of the native γGCS gene from the transfected *l. tarentolae* was confirmed by PCR analysis using genomic DNA isolated from transgenic parasites as

template and primers complementary to sequences 679 bp upstream of the 5' end of the LtaP18 gene and to the 3' end of the sequence in the native γGCS. For *L.t L. don* γGCS 6 of 96, *L.t L. maj* γGCS 10 of 96 and *L.t L. mex* γGCS 4 of 96, clones grew in the presence of the resistance antibiotic. PCR studies showed that the native γGCS gene present in transfected *L. tarentolae* had been removed successfully and replaced with two different antibiotic markers (hygromycin and blasticidin) for *L. tarentolae* transfected with *L. donovani* γGCS or *L. major* γGCS. While *L. tarentolae* transfected with *L. mexicana* γGCS still had the native γGCS still present, although one allele had been deleted. Surprisingly the selected clone did not give a PCR product but it still grew in the presence of blasticidin. This indicates that they have integrated the resistance gene into the locus but did not delete the native γGCS totally, perhaps indicating that it is present in another place in the genomic DNA. Gamma GCS is an enzyme that *Leishmania* requires, therefore removing the native γGCS from the transfected parasites could enhance the expression of the recombinant γGCS by up to 50%. However, due to lack of time studies to confirm this could not be completed.

Western blot studies showed that full length GFP-γGCS-His protein was present in all fractions of the cytosol samples. Anti-GFP blot studies showed the presence of three bands in the total and soluble cytosol fraction samples. The 109 KDa band correlated with the size of full length of the GFP-γGCS-His protein. The band of ~27 KDa could be anticipated the GFP cleaved protein and band ~50KDa could be related to incomplete translation of GFP-γGCS-His mRNA. The anti-his blot studies showed the presence of two bands in total and soluble cytosol fractions, which correlate to the size of full length GFP-γGCS-His protein and a truncated product. These truncated products were not present in the purified fractions from the affinity columns showing they did not contain the hexa-histidine tag. The presence of the recombinant GFP-γGCS-His protein bands in the soluble cytosolic and washing samples indicated that some of the recombinant GFP-γGCS-His protein became detached from the affinity column, which means the tag did not bind strongly enough to the affinity column to prevent loss of the recombinant GFP-γGCS-His protein during purification.

In summary results from these studies showed that:

1. produced a γGCS gene sequence of *L. donovani, L. major* or *L. mexicana* construct which is flanked with a EGFP-tag (N terminal) and his-tag (C terminal) and a TEV protease cleavage site was introduced into the EGFP-γGCS-His construct, between EGFP and γGCS sequence.
2. The EGFP-γGCS-His cons truct was inserted into the expression vector pSSU-INT and used to transfect *L. tarentolae.* Parasites integrated with GFP-γGCS-His gene gave stable expression of the fusion protein for 6 months without the requirement for antibiotics to maintain expression of the gene insert.
3. Imaging studies showed that the γGCS was localized within the cytoplasm of the promastigotes which was expected for this enzyme.
4. Supplementing the culture medium with H_2O_2 increased the intensity of expression of γGCS recombinant protein on Western blot studies, with 12 μM of H_2O_2 supplement being the most appropriate concentration to use in future studies.
5. Expression of the recombinant GFP-γGCS-His protein produced full length protein and truncated protein but after isolation from the affinity column it was impossible to produce pure full-length protein for all three transfected parasites.

CHAPTER 4. EXPRESSION OF γGCS AS AN EXCRETED SOLUBLE PROTEIN FROM *L. TARENTOLAE*

4.1. Introduction

L. tarentolae can produce heterologous proteins either intracellularly or secreted into their external environment. *L. tarentolae* culture medium contains little protein and the parasite produces very low levels of endogenous secretory proteins. Therefore producing transfected parasites secretes a recombinant protein should allow an efficient method to obtain high levels of a recombinant protein (Basile and Peticca, 2009). *L. mexicana* produces secreted acid phosphatase (SAP), a protein complex which forms filaments, from its flagella pocket. SAP consists of two different glycosylated polypeptide chains, SAP 1 (SAP1) and SAP 2 (SAP2) (Wiese *et al.*, 1995). The SAP1 open reading frame has 537 amino acids encoding by the gene *lmsap1.* SAP2 is encoded by the gene *lmsap2,* and has an open reading frame of 888 amino acids. SAP1 and SAP2 consists of three sub domains, an N-terminal domain containing 428 amino acids, forming a globular acid phosphatase, and C-terminal region consisting of 54 amino acids. The third sub domain, linking the N and C-terminal domains, contains 33 amino acids in SAP1 and 384 amino acids in SAP2 (Wiese *et al.*, 1995). The linking region in both SAP1 and SAP2 consists of serine and threonine rich amino acid sequences that represent O-glycosylation sites.

Secreted SAP filaments can be produced in the parasites even if one of the *lmsap1* or *lmsap2* genes are deleted (Wiese *et al.*, 1995). This enzyme has the ability to be expressed and secreted to the medium if the gene sequence is transfected into *L. major* (Wiese *et al.*, 1999). An integration cassette on the pSSU-int allows successful integration of homologous gene into the S18 rRNA locus of *Leishmania* cells by homologous recombinant (Misslitz *et al.*, 2000). The human erythropoietin protein was expressed in *L. tarentolae* cells as secreted protein. This was achieved by replacing the signal peptide sequence from human erythropoietin with the *L. mexicana* SAP signal peptide (Breitling *et al.*, 2002). *L. tarentolae* has also been used in an inducible system where a T7 polymerase cassette which depends on tetracycline as repressor was used to give control over transcription induction (Kushnir *et al.*, 2005). Therefore, in this study constructs were tested for their ability to produce a recombinant γGCS protein which could be harvested from *L. tarentolae* culture medium. This would give a

simple continuous manufacture method for recombinant γGCS. Two different constructs of SAP1 and SAP2 were cloned into the plasmid vector pSSU-int combined with the γGCS to be secreted into the growth medium. Figure 4.1 explain the process for cloning the γGCS with SAP1 and SAP2 into the expression vector.

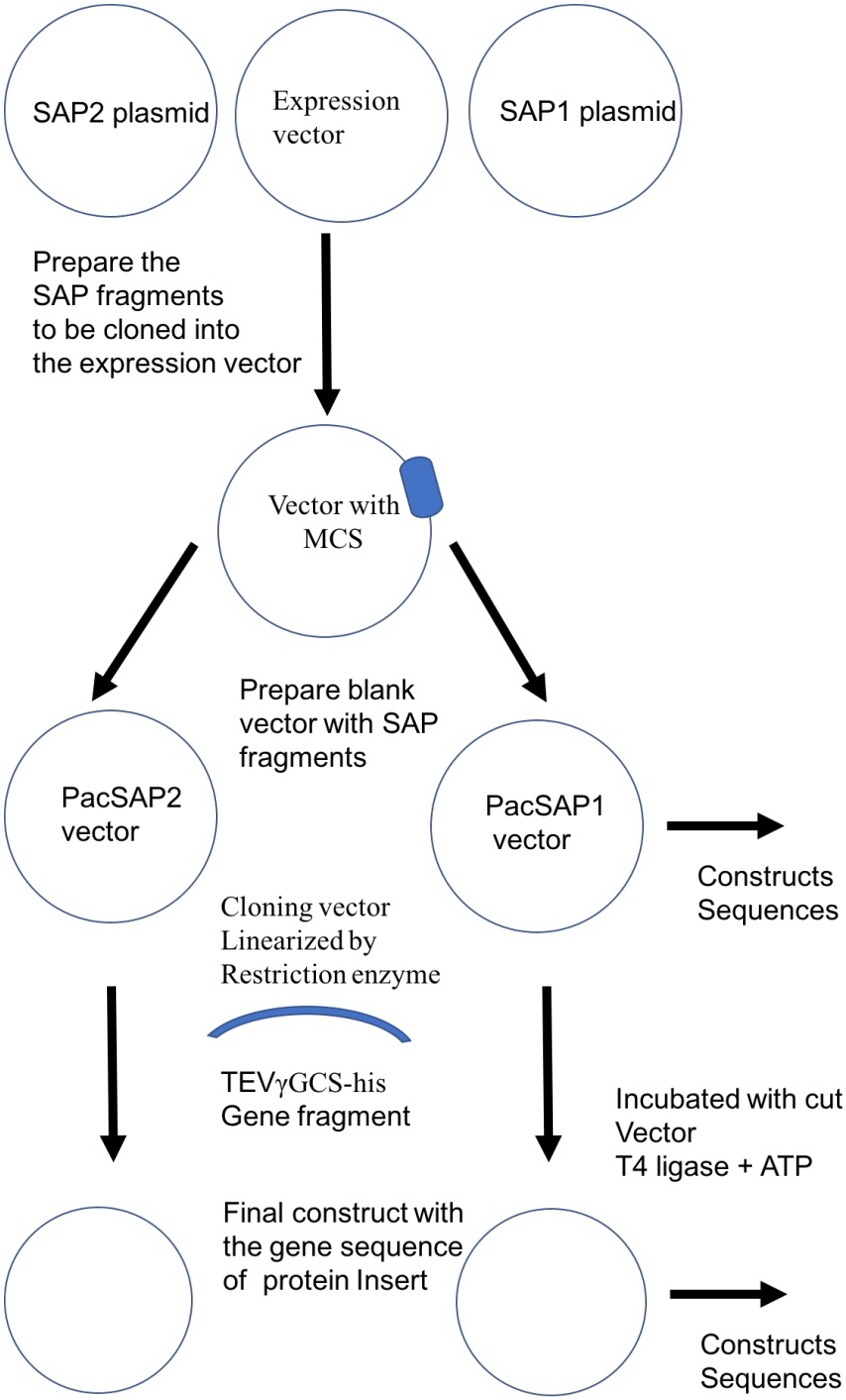


Figure 4.1 Diagram to show plasmids used to prepare SAP1 and SAP2 with multiple cloning sites (MCS) and the insertion of TEVγGCS gene sequence.

The plasmid construct cassette contained a eukaryote selection marker, a genomic integration locus, a secreted acid phosphatase and an insertion site for the protein of interest. The genomic integration 18S rRNA locus and hygromycin eukaryote selection marker was obtained from plasmid pSSUintlmcpb2. The second puromycin eukaryote selection marker was obtained from plasmid pSSUmcs. SAP1 with multiple restriction enzyme sites attached was obtained from plasmid pAseSAP1modTet and SAP2 with multiple clone sites was obtained from pAsbSAP2modTet. Multiple restriction sites allow incorporation of protein(s) of interest into the vector. Two blank vectors pSSUPacSAP1 and pSSUPacSAP2 were constructed and γGCS, along with TEV cleavage site, was inserted into the blank vectors using *HpaI* and *BamHI* restriction sites. A hexa-histidine tag was also added to the C-terminal of the open reading frames for both proteins for purification purposes. The aims of the study were to:

1. Create plasmid constructs for γGCS expression in *L. tarentolae* to produce fusion proteins with SAP from *L. mexicana* and this generate proteins SAP1/γGCS and SAP2/γGCS.
2. Monitor success SAP1/γGCS and SAP2/γGCS by carrying out western blot studies using expression using anti-SAP monoclonal antibody mAb LT8.2
3. Monitor success SAP1/γGCS and SAP2/γGCS by out enzyme assays to determine SAP activity of the expressed protein.

4.2. Results

4.2.1. Construction of plasmid pSSUPacSAP2

*Xho*I/*Xba*I digested fragments from plasmids pSSUmcs 7358 bp, pAsbSAP2CmodTet 3197 bp were ligated and transformed into *E. coli* DH5α. Six *E. coli* DH5α pSSUPacSAP2 clones were used to prepare plasmid DNA using a miniprep and digested with *Eco*RV/*Nde*I (Figure 4.2). Clone 1, 3 and 5 matched the expected *Eco*RV/*Nde*I fragmentation pattern of 1954 bp, 4302 bp and 4299 bp. A band of double intensity was observed between 4 kb and 5 kb, which corresponded with the bands of 4299 bp and 4302 bp. Similar studies using *Bam*HI, *Nco*I and *Sma*I (Figure 4.3), matched the expected fragmentation pattern for *Bam*HI, *Nco*I and restriction enzyme digests.

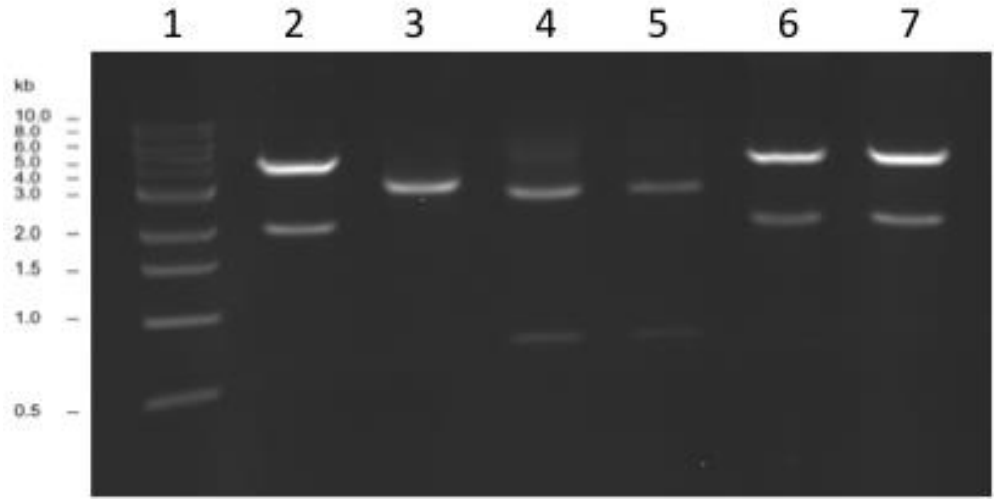


Figure 4.2 Results of digest of pSSUPacSAP2 with EcoRV/NdeI restriction enzymes. The expected fragment sizes are 1954 bp, 4299 bp and 4302 bp. Lane 1 1 kb marker, lane 2 clone 1 positive, lane 3 clone 2 negative, lane 4 clone 3 negative, lane 5 clone 4 negative, lane 6 clone 5 positive and lane 7 clone 6 positive.

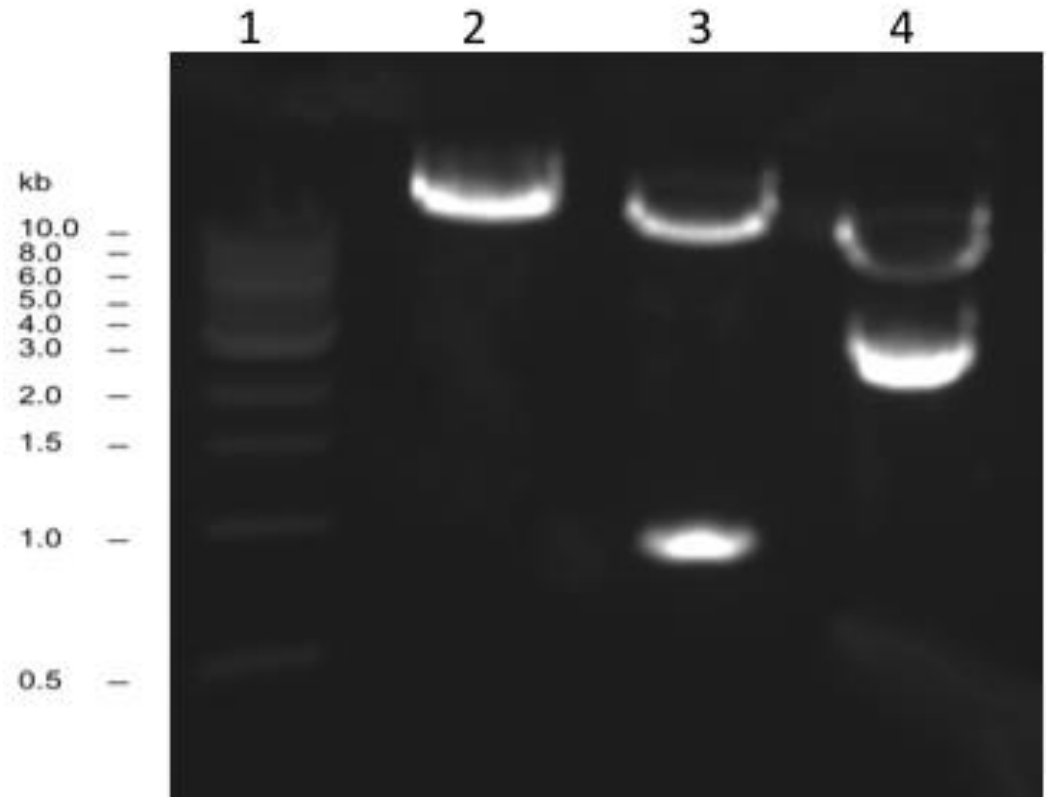


Figure 4.3 Results of digest of plasmid pSSUPacSAP2 with *BamHI, NcoI* or *SmaI*. Lane 1: 1kb marker, Lane 2: *BamHI*, expected fragmentation 10555 bp, Lane 3: *NcoI*, expected fragmentation 868 bp, 9226 bp, Lane 4: SmaI, expected fragmentation 2800 bp, 7755 bp.

4.2.2. Construction of plasmid pSSUPacSAP1

Plasmids pSSUPacSAP2 and pAseSAP1CmodTet were cut at *Not*I/*Xba*I restriction sites to produce fragments 7862 bp and 1640 bp for ligation. Test digests from six *E. coli* DH5α pSSUPacSAP1 clones were run (Figure 4.4) and all six clones were positive for the expected *Eco*RV/*Nde*I fragmentation pattern of 4299 bp, 3248 bp and 1954 bp. Plasmid pSSUPacSAP2 was purified from clone 3 and digested using *Eco*RV / *Nde*I, *Bam*HI, *Kpn*I and *SmaI*. It gave the expected sized fractions (Figure 4.11).

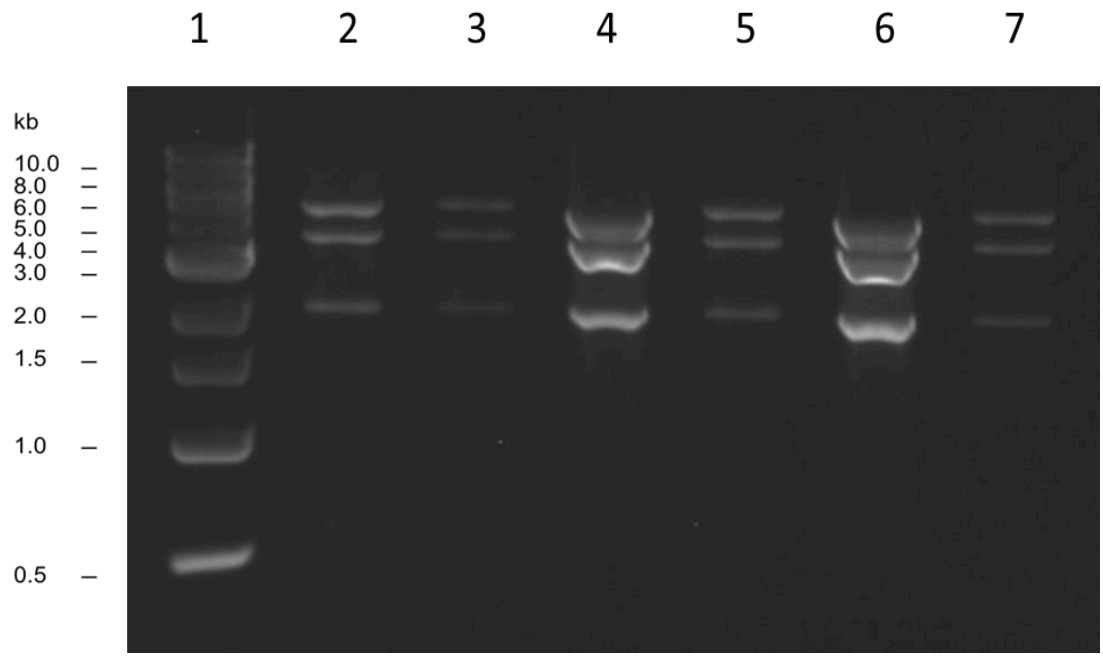


Figure 4.4 Results of test digest of pSSUPacSAP1 with EcoRV / Nde I restriction enzymes. The expected fragmentation of pSSUPacSAP1 4299 bp, 3248 bp and 1954 bp. Lane 1 1 kb marker, Lane 2 clone 1, Lane 3 clone 2, Lane 4 clone 3, Lane 5 clone 4, Lane 6 clone 5 and Lane 7 clone 6. All clones six clone have positive fragmentation patterns. Positive clone 3.

4.2.3. Construction of plasmid pSSUHygSAP1

pSSUPacSAP1 *Xho*I/*Hpa*I digest product of 2130 bp and pSSUintlmcbp2 *Xho*I/*Sma*I digest product of 7794 bp was ligated and used to transform *E. coli* DH5α to produce *E. coli* DH5α pSSUHygSAP1. Six clones from *E. coli* DH5α pSSUHygSAP1 matched the expected *Nde*I/ *Not*I fragmentation pattern i.e. fragments of molecular weight 1175 bp, 3438 bp and 5312 bp (Figure 4.5). Clone 1 was selected for further analysis and digested using *Spe*I / *Xho*I c (Figure 4.6). This showed that clone 1 was positive and had the expected fragmentation pattern i.e. it gave fragments with size of 3485bp, 6440 bp. Plasmid DNA isolated from pSSUPHygSAP1 clone 1 was then digested using *Nde*I, *Sma*I, *BamH*1 / *XbaI* and *Xho*I / *Spe*I and the resulting fraction pattern showed it was the correct plasmid (Figure 4.7).

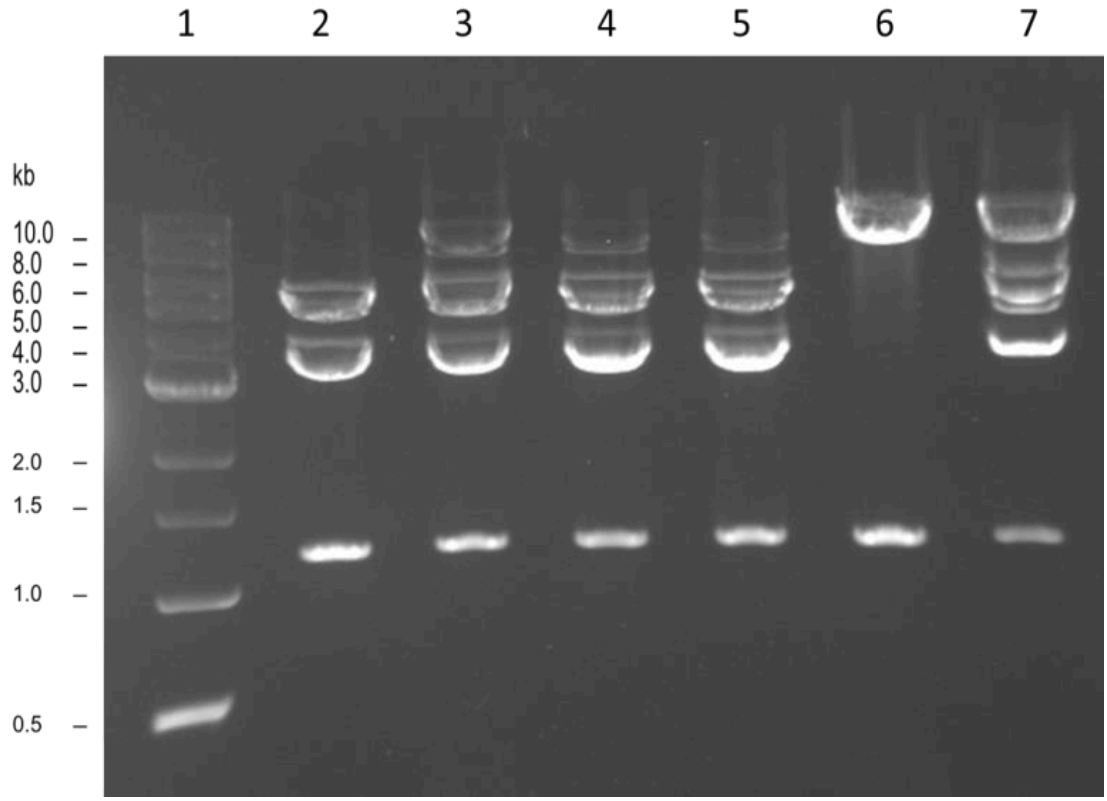


Figure 4.5 Results of *NdeI/NotI* test digest of pSSUHygSAP1. It should give fragment sizes 1175bp, 3438 bp and 5312 bp Lane 1: 1kb marker, Lane 2 to 7: clones 1 to 6. Clone 1 identified as positive combine with *SpeI/XhoI* test digest data.

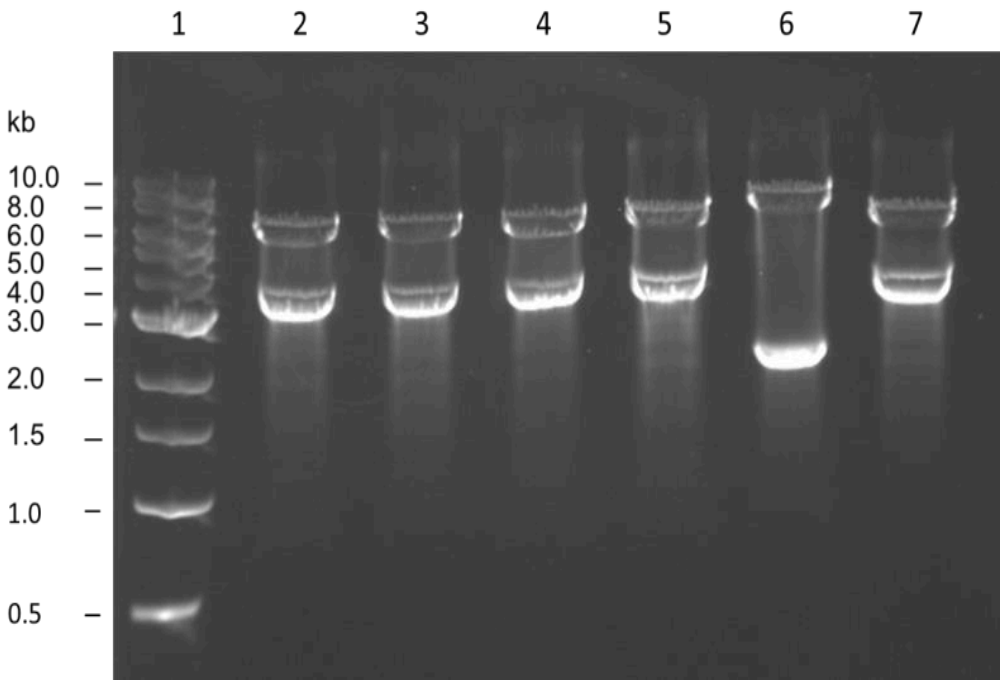


Figure 4.6 Results of SpeI/XhoI digest of SSUHygSAP1. It should give fragment sizes of 3485 bp and 6440 bp. Lane 1: 1 kb marker, Lane 2 to 7: clones 1 to 6. Clone 1 identified as positive combined with *NdeI/NotI* test digest data.

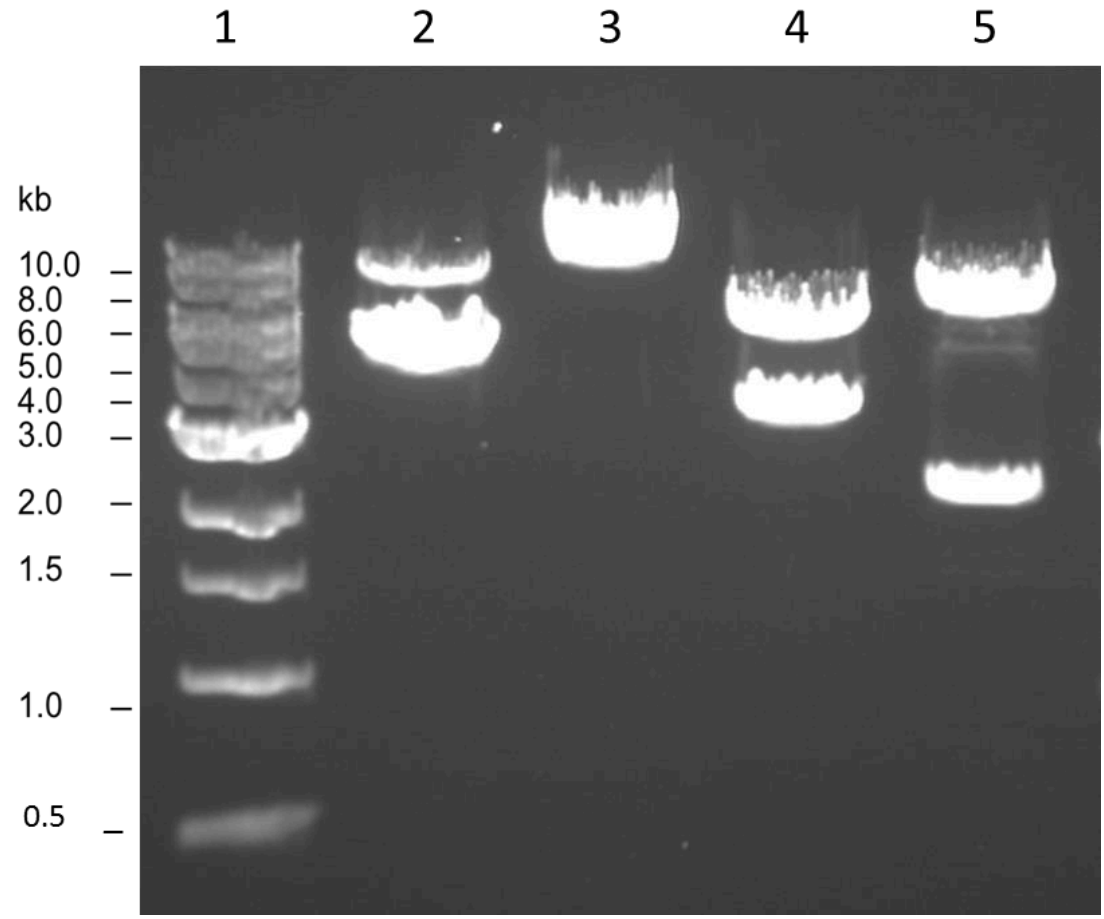


Figure 4.7 Results of test digest of pSSUhygSAP1 with NdeI, SmaI, XhoI and SpeI, or BamHI and XbaI. Lane 1 1 kb marker, Lane 2 *NdeI* digest fragment size, 4613 bp, 5312 bp, Lane 3 *SmaI* digest fragment size, 9925 bp, Lane 4 *XhoI*, *SpeI* digest fragment size 3485 bp, 5585 bp, Lane 5 *BamH*I, *Xba*I digest fragment size 78 bp (not observed), 2386 bp, 7461 bp.

4.2.4. Construction of plasmid pSSUHygSAP2

pAsbSAP2Cmod was fragmented using the restriction enzymes *Xho*I/*Sma*I to give a 3184 bp fragment that was ligated with a 7794 bp product from plasmid pSSUintlmcbp2. The resulting plasmid was then used to transform *E. coli* DH5α. Test digests using *Nde*I / *Not*I of plasmid DNA isolated from six of the *E. coli* DH5α pSSUHygSAP2 clones, gave fragments of the expected size i.e. 1175 bp, 4491 bp and 5312 bp (Figure 4.8). Plasmid DNA isolated from pSSUHygSAP2 clone 3 was digested with *Apa*I, *Not*I / *Nde*I and *Not*I / *Eco*RV and gave the predicted fragmentation pattern (Figure 4.9).

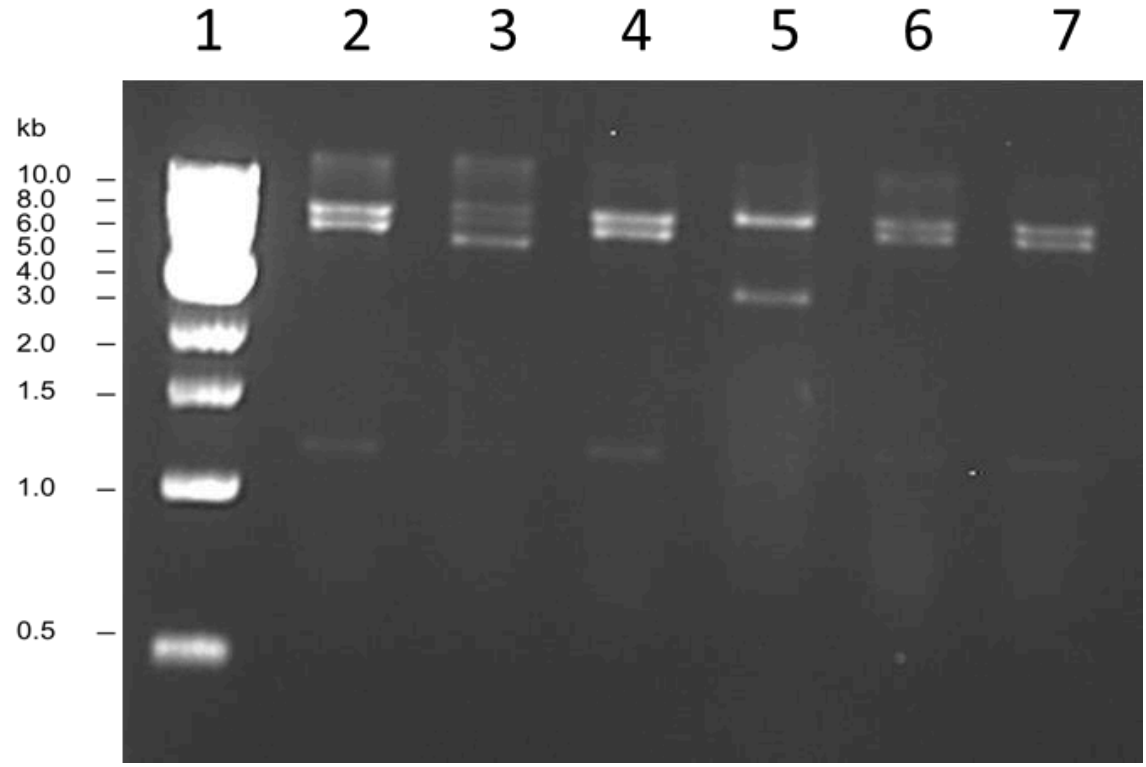


Figure 4.8 Results of *NdeI /NotI* enzyme digest of plasmid pSSUHygSAP2. The expected fragmentation size was 1175 bp, 4491 bp and 5312 bp. Lane 1: 1 kb marker, Lane 2: clone 1 positive, Lane 3: clone 2 negative, Lane 4: clone 3 positive, Lane 5: clone 4 negative, Lane 6: clone 5 positive, Lane 7: clone 7 positive.

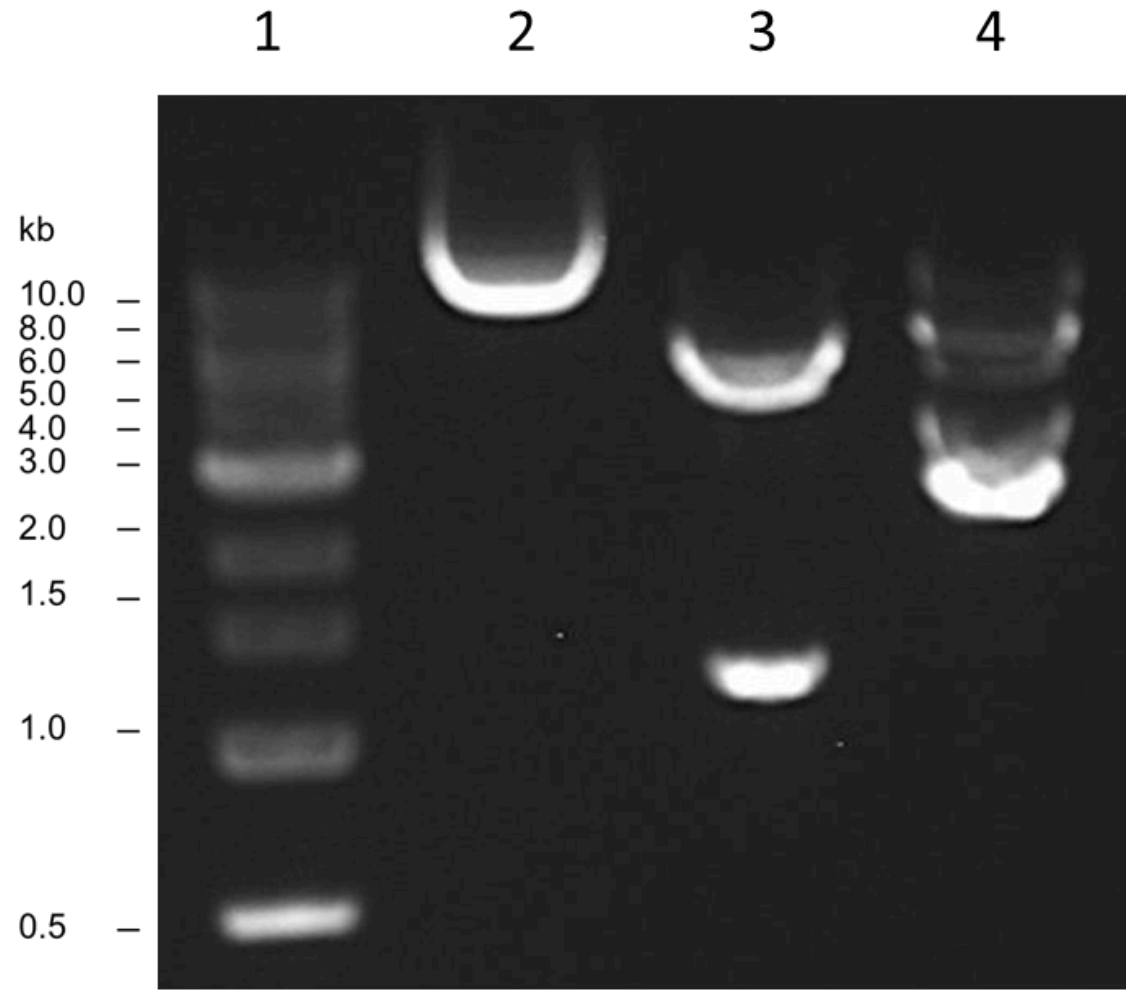


Figure 4.9 Results of digest of plasmid pSSUHygSAP2 with restriction enzymes *ApaI, NotI/ NdeI,* or *NotI/EcoRV*. Lane 1 marker, Lane 2 ApaI digest fragmentation 10555 bp, Lane 3 *NotI/NdeI* digest fragmentation 1175 bp, 4491 bp, 5312 bp, Lane 4 *NotI/EcoRV* digest fragmentation 3123 bp, 2380 bp, 5475 bp.

4.2.5. Construction of plasmid pSSUHSAP2SAG1

*Avr*II/*Nru*I fragments from plasmid pSSUHygSAP2 10972 bp and pUCCYCSAGSAP 763 bp were ligated to produce plasmid pSSUHSAP2SAG1. This plasmid was used to transform *E. coli* DH5α and DNA from pSSUHSAP2SAG1 clones were prepared using a miniprep. The resulting DNA was digested using *Bam*HI and clone 6 had the expected fragmentation pattern i.e. fragments of 828 bp and 10900 bp (Figure 4.10). Plasmid DNA from this clone was then digested with *Eco*RV/*Nde*I, *Bst*BI/*Xba*I or *Bam*HI and the expected fragmentation pattern was obtained to confirm its identity (Figure 4.11).

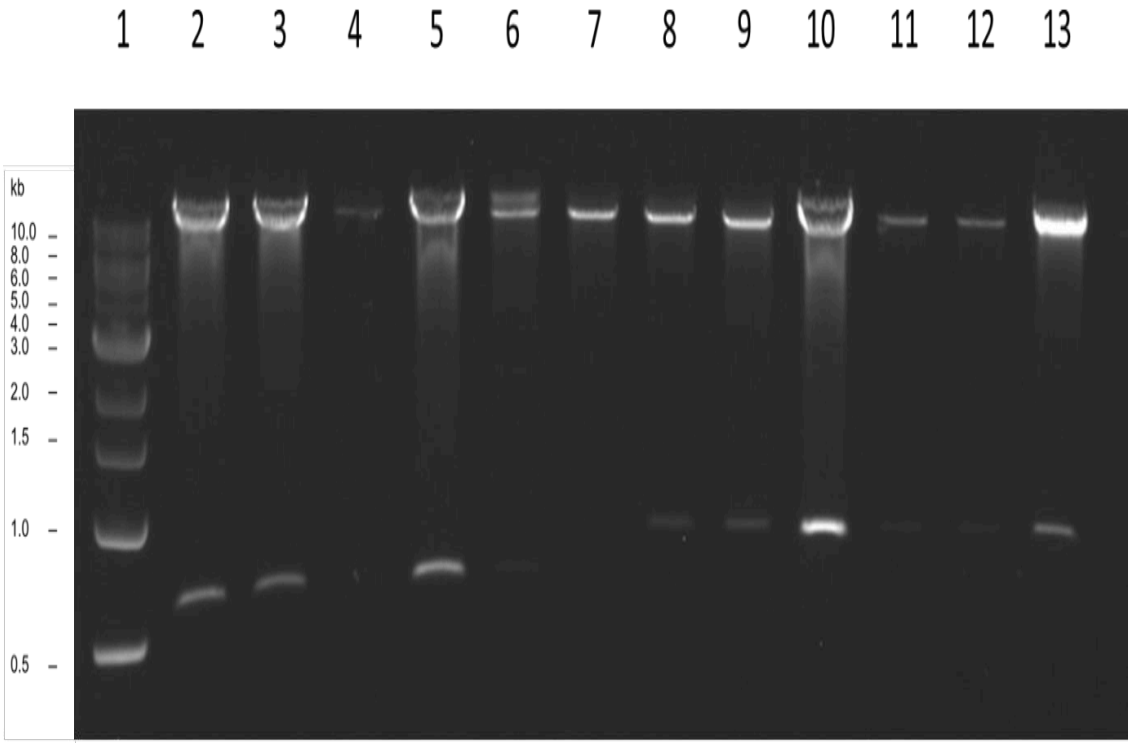


Figure 4.10 Results of digest of plasmids pSSUHSAP2CYC18 and pSSUHSAP2SAG1 with *BamHI* restriction enzyme. Lane 1: 1 kb marker Lane 2 to 7: pSSUHSAP2CYC18 clones 1-6 expected fragmentation 606 bp and 10900 bp. Clones 1,2,4 and 5 are positive for 541 bp CYC18 fragment. pSSUHSAP2CYC18 positive clone 1 selected for inoculation of midiprep culture. Lane 8 to 13: pSSUHSAP2SAG1 clones 1-6 expected fragmentation 828 bp and 10900 bp. All clones are positive for 841 bp SAG1fragment.

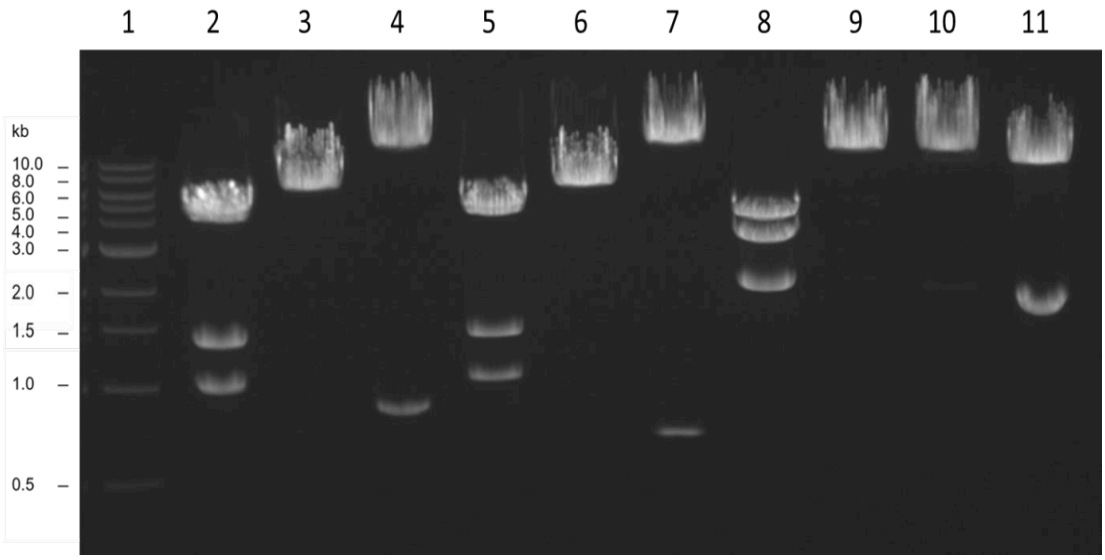


Figure 4.11 Results of enzyme digest of plasmids pSSUHSAP2SAG1, pSSUHSAP2CYC18 and pSSUPacSAP1 with *EcoRV/NdeI, BstBI/XbaI, BamHI, KpnI* or *SmaI*. Lane 1 1 kb marker, Lane 2 pSSUHSAP2SAG1 *EcoRV / NdeI* expected fragments 1012 bp, 1368 bp, 4300 bp, 5048 bp, Lane 3 pSSUHSAP2SAG1 *Bst*BI/*Xba*I expected fragments 5722 bp, 6006 bp, Lane 4 pSSUHSAP2SAG1 *Bam*HI expected fragment 828 bp, 10900 bp, Lane 5 pSSUHSAP2CYC18 *Eco*RV/*Nde*I expected fragment 1012 bp, 1368 bp, 4300 bp, 5826 bp. Lane 6 pSSUHSAP2CYC18 *Bst*BI/*Xba*I fragments 5722 bp, 5784 bp. Lane 7 pSSUHSAP2CYC18 *Bam*HI expected fragments 606 bp, 10900 bp. Lane 8 pSSUPACSAP1 *Eco*RV / *Nde*I fragments 1954 bp, 3248 bp, 4300 bp, Lane 9 pSUSPACSAP1 *Bam*HI linear fragment 9502 bp, Lane 10 pSSUPACSAP1 *Kpn*I 27 bp, Lane 11 pSSUPACSAP1 *Sma*I fragment 1747 bp, 7755 bp.

4.2.6. Construction of plasmid pSSUPSAP1TEVγGCS

HpaI/*BamHI* were used to digest plasmid pSSUPacSAP1 to give a 9487 bp which was ligated with a 2103 bp produced by *EcoRV*/*BamHI* digest of pSSUGFPTEVγGCS. The resulting product was used to transform into *E. coli* DH5α to give pSSUPSAP1TEVγGCS plasmid. Clones of the bacteria were grown and plasmid DNA extracted and digested with *BamHI*/*EcoRV* (Figure 4.12). Clone 6 gave the expected fragmentation pattern, with products of 1049 bp, 1954 bp and 7027 bp produced. Plasmid DNA from this clone was digested with *Kpn*I, *Sma*I, *BamH*I/ *Eco*RV or *Xho*I/*Xba*I and gave the expected fragmentation pattern (Figure 4.13)

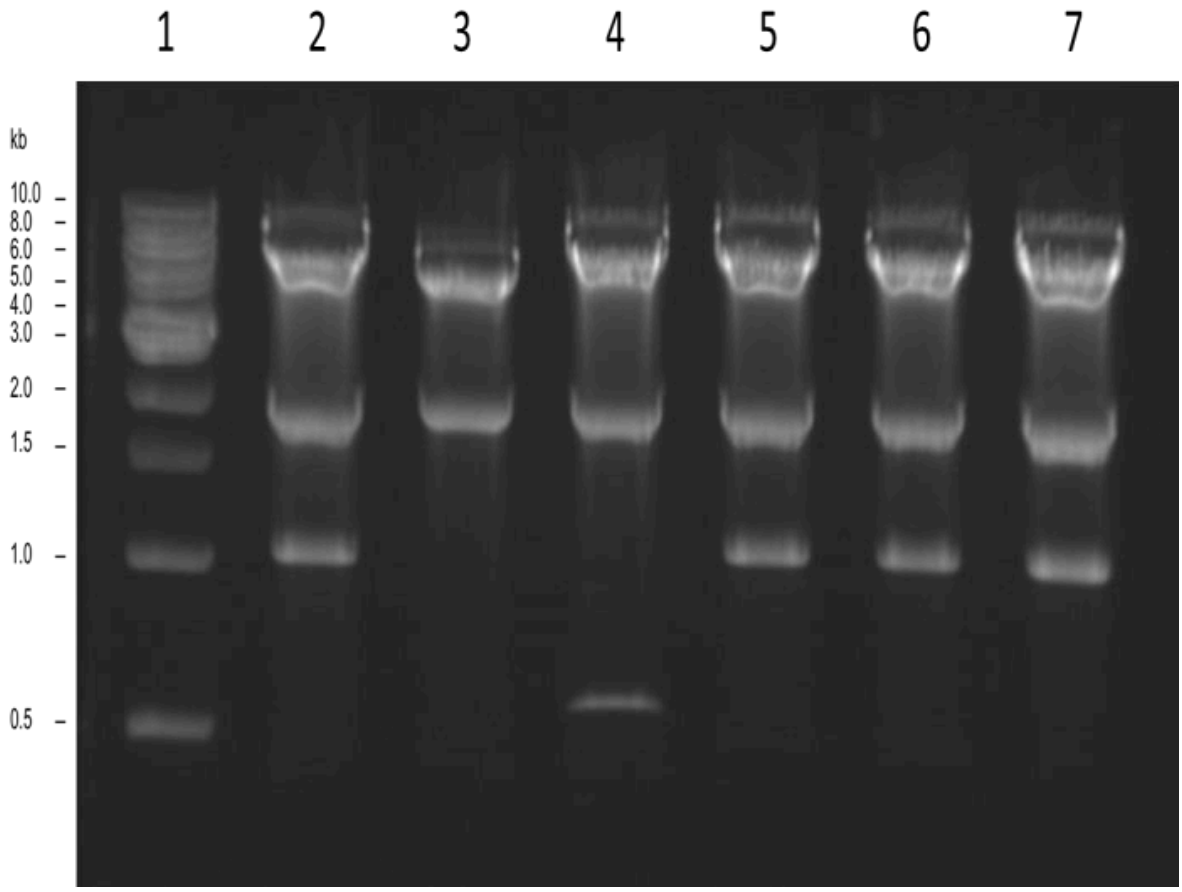


Figure 4.12 Results of digest of pSSUPSAP1TEVγGCS using *BamHI/EcoRV* restriction enzymes. The expected fragment size was 1047 bp, 1954 bp, 7029 bp, Lane 1 kb marker, Lane 2 clone 1 positive, Lane 3 clone 2 negative, Lane 4 clone 3 negative, Lane 5 clone 4 positive, Lane 6 clone 5 positive, Lane 7 clone 6 positive.

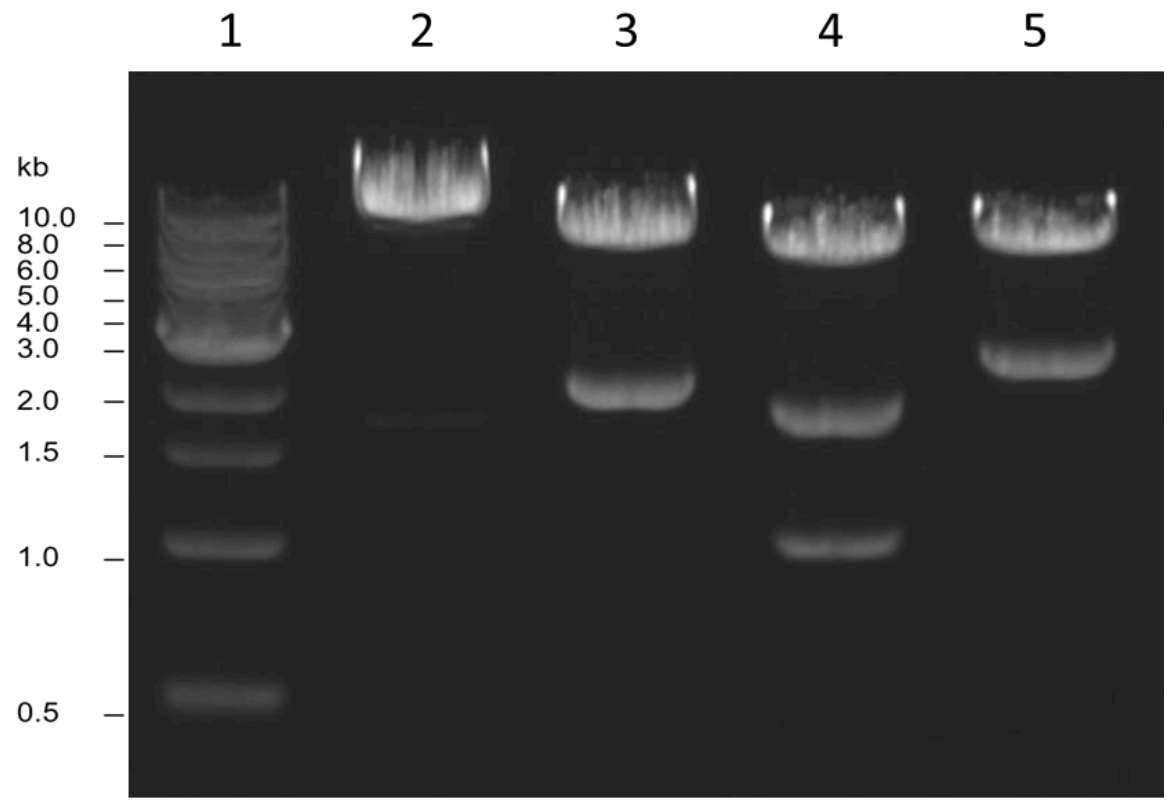


Figure 4.13 Results of enzyme digest of plasmid pSSUPSAP1TEVγGCS with Kpn1, Sma1, BamH1/EcoRV or Xho1/Xba1. Lane 1 1kb marker, Lane 2 Kpn1 fragment size 27bp, 10003bp, Lane 3 Sma1 fragment size 2275bp, 7755bp, Lane 4 BamH1/EcoRV fragment size 1047bp, 1954bp, 7029bp, Lane 5 *Xho*1/*Xba*1 fragment size 2672bp, 7358bp.

4.2.7. Construction of plasmid pSSUPSAP2TEVγGCS

*Avr*II/*Nru*I was used to digest plasmid pSSUPacSAP2 to give a 10540bp and this was ligated with plasmid pSSUGFPTEVγGCS (2103bp). The resulting plasmid (pSSUPSAP2TEVγGCS) was used to transform into *E. coli* DH5α. DNA from pSSUPSAP2TEVγGCS clone 5 was digested with *Bam*HI/*Eco*RV and gave the predicted restriction digest fragments of 1749bp and 9334bp (Figure 4.14). Plasmid DNA from this clone also gave the expected digest fragmentation patterns after digestion with *NdeI, NotI, SmaI or NruI* (Figure 4.15). Constructs pSSUPSAP1TEVγGCS and pSSUPSAP2TEVγGCS were used to transfect *L. tarentolae* promastigotes for secreted recombinant protein studies.

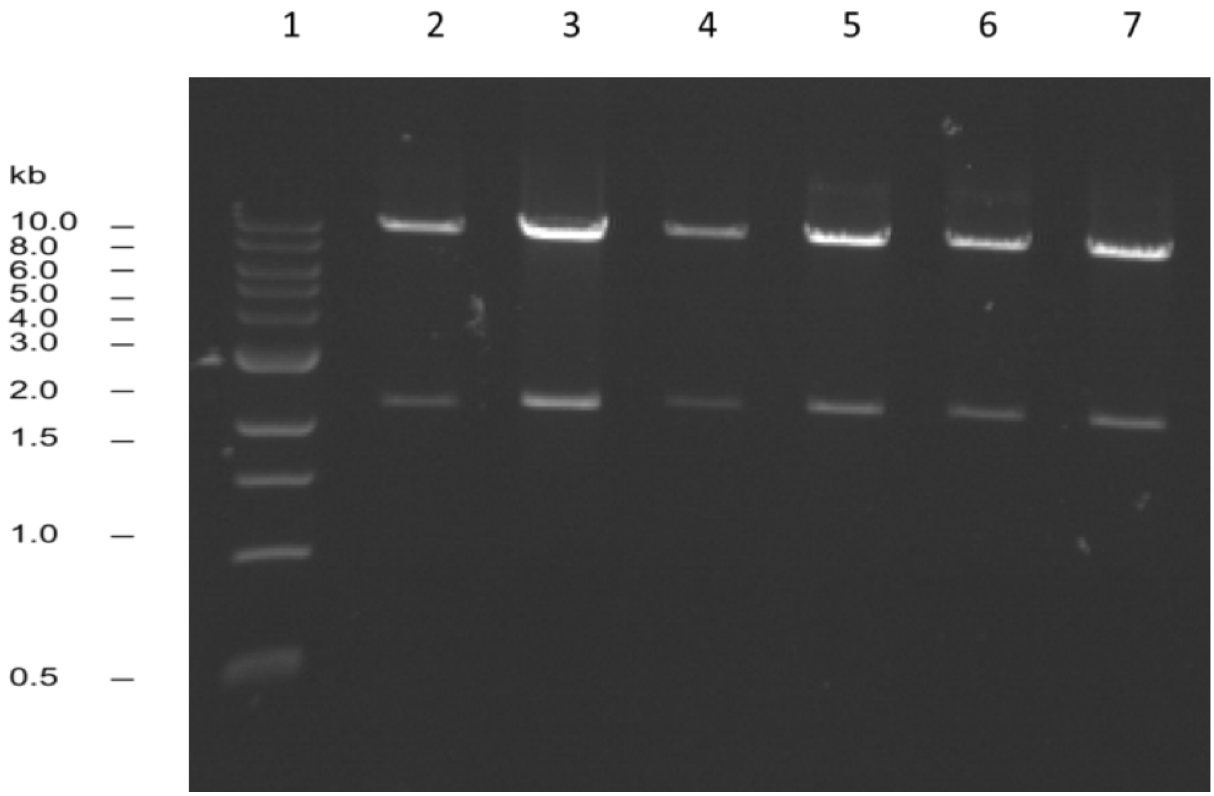


Figure 4.14 Results of digest of pSSUPSAP2TEVγGCS using *BamHI/EcoRV* restriction enzymes. The expected fragment size was 1749 bp and 9334 bp, Lane 1 kb marker, all tested clones were positive.

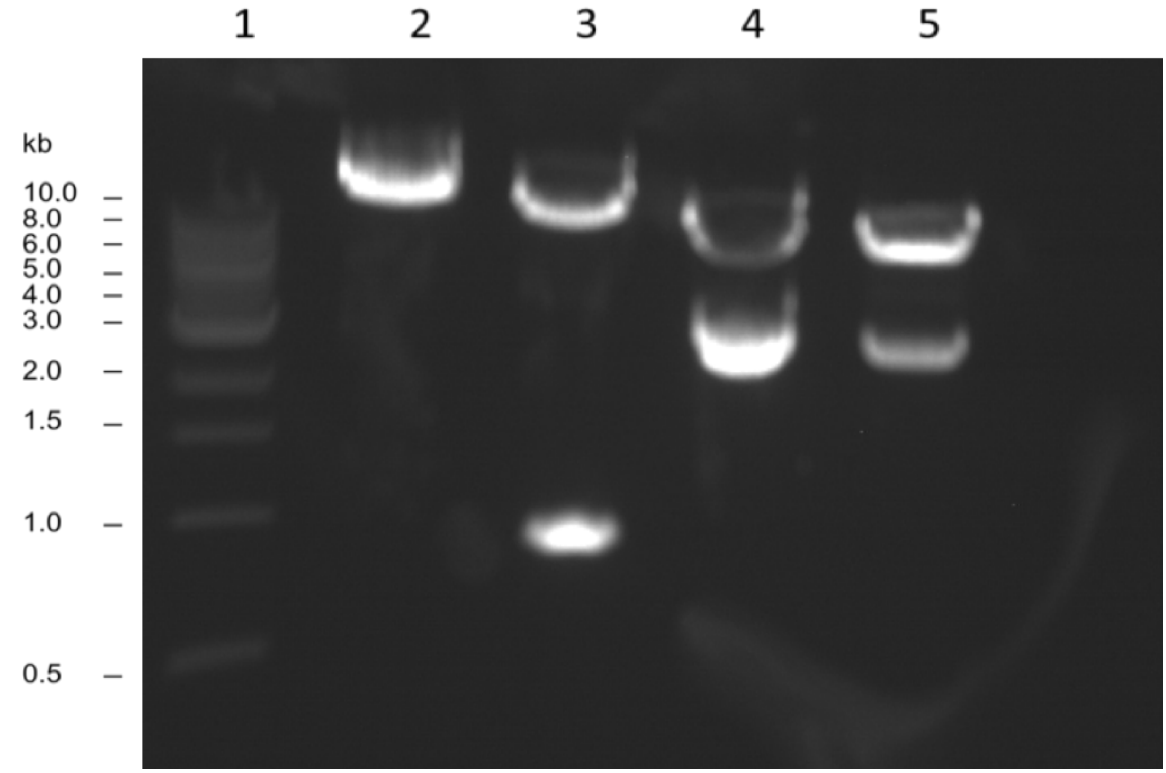


Figure 4.15 Results of enzyme digest of plasmid pSSUPSAP2TEVγGCS with NdeI, NotI, SmaI or NruI. Lane 1 1kb marker, Lane 2 NdeI fragment size 11083 bp, Lane 3 NotI fragment size 990 bp, 10093 bp, Lane 4 SmaI fragment size 2950 bp, 8133 bp, Lane 5 NruI fragment size 2935 bp, 8148 bp.

4.2.8. Detecting Recombinant protein expression by transfected *L. tarentolae* clones using SAP activity assay

SAP enzyme activity was determined using the substrate (*p*-nitrophenylphosphate) and a qualitative photometric assay. The absorbance of harvested supernatant from transfected *L. tarentolae* at 405 nm was determined in triplicate. Supernatants harvested from WT *L. tarentolae* were used as the negative control and supernatant from WT *L. mexicana* was used as the positive control.

Three clones of each transfected cell lines were grown and tested for SAP activity (Figure 4.16). Both the negative and positive controls gave the expected result. The highest SAP activity observed for parasites transfected with the vector

pSSUPSAP2TEVγGCS but the clones had low activity compared to the positive control. This indicated that transfected clones did not secrete a lot of γGCS /SAP1 or γGCS /SAP2 protein. Western blot studies showed that bands equivalent to the size of SAP1(100 KDa) and SAP2 (180 KDa) fragments alone were present but a band equivalent to the size of γGCS /SAP1 (209 KDa) or γGCS /SAP2 (289 KDa) fusion protein were absent (Figure 4.17). Therefore, studies were not completed and further work on these parasites was terminated as the studies indicated that γGCS /SAP1 or γGCS /SAP2 fusion proteins were not expressed by transfected parasites.

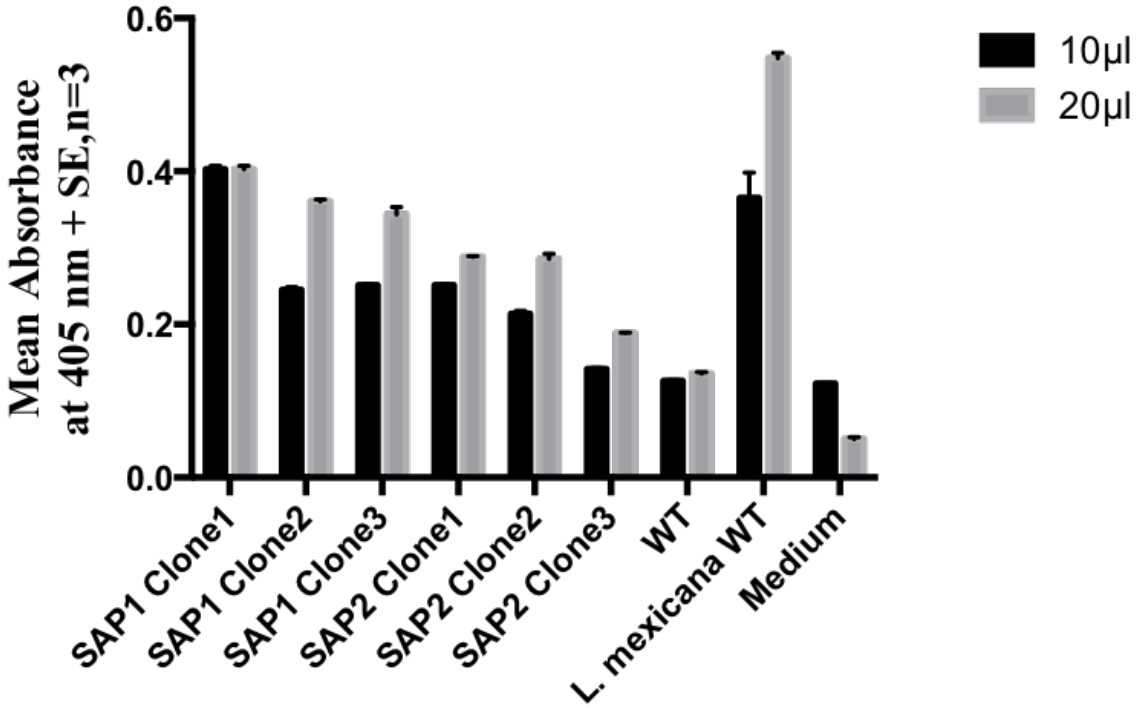


Figure 4.16 SAP activity of *L. tarentolae* parasites expressed a fusion protein of γGCS and SAP1 or SAP2. Supernatants from $2x10^8$ cells.10 µl and 20 µl supernatant samples. phosphatase assay of these clones incubated at 37°C for 30 min. *L. tarentolae* (WT) as negative control and *L. mexicana* as positive control.

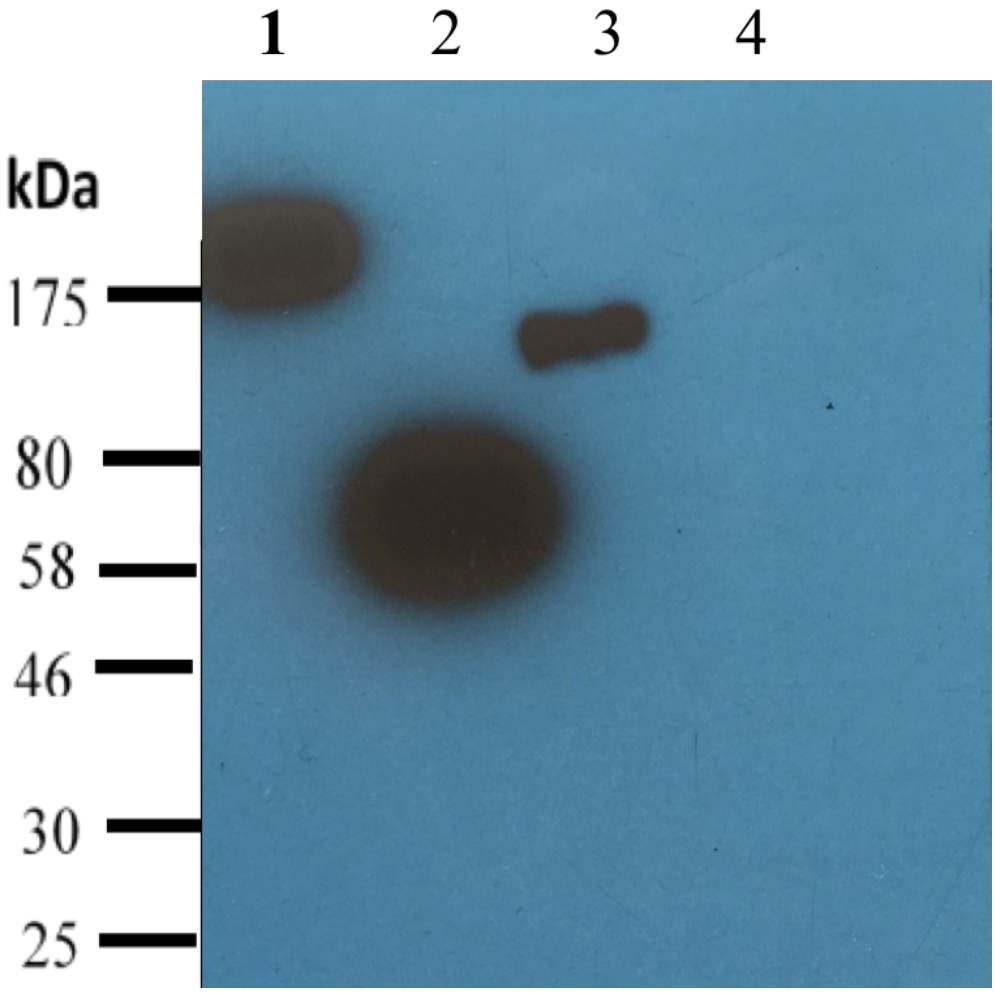


Figure 4.17 Western Blot of secreted products of *L. tarentolae* parasites expressed a fusion protein of γGCS and SAP1 or SAP2 with mAb LT8.2. Lane 1 SAP1SAG1 (surface antigen 1), lane 2 SAP1TEVγGCS, lane 3 SAP2TEVγGCS and *L. tarentolae* WT. concentrated culture supernatants of promastigotes (2x 10^8 cells/ml) cultured for 72 h in serum-free medium were subjected to SDS-PAGE and blotted to nitrocellulose membrane.

4.3. Discussion

The construction of two genomic integrative plasmid vectors that contained *L. mexicana* SAP open reading frame, multiple cloning sites and selective puromycin resistance marker was completed. The blank vector multiple cloning sites allowed for ease of construction of eight fusion protein vectors by cleaving of restriction sites with endonucleases to allow insertion of the protein of interest. As SAP secretion in WT *L. mexicana* occurs in the promastigote stage of the culture (Wiese *et al*, 1995) the *L. tarentolae* fusion SAP protein allows for antibiotic selection pressure to be applied at culture inoculation without use of external induction of protein expression at a specific phase in culture growth as with bacterial expression systems (Dortay *et al.*, 2011).

Electroporation of WT *L. tarentolae* resulted in successful transfection, producing all six of the six cell lines required. Cloning studies using a 1:40 dilutions of transfected parasites showed that not all of the clones produced were positive and often low concentrations of the linearized plasmid was produced. Cloning the TEVγGCS coding sequence into the SAP1 and SAP2 cloning vector took more than six months. The issues behind this delay were caused because the plasmids used to prepare the cloning sites were old and there were no electronic versions of these plasmids. The first steps of cloning ligation of the inserted gene fragment into the vector included identical blunt ends of the same enzyme, which meant that successful insertion of the gene fragment had to be repeated more than twice and colonies had to checked to ensure that the insertion of the DNA sequence occurred in the correct orientation.

An advantage of using a SAP fusion protein is that protein expression can be detected using a phosphatase reporter assay. This allows results to be obtained a few hours after harvesting the supernatant from cell cultures. Comparison of the qualitative phosphatase activity for all single transfected cell lines showed there was a difference in expression levels between cell lines expressing SAP1 and SAP2. The SAP1 cell lines consistently show higher levels of expression than SAP2 (Figure 4.16).

The *lmsap* locus comprises two tandemly arranged genes, *lmsap1* and *lmsap2*, which differ only in the length of the predicted Ser/thr-rich domains. Both *lmsap1* and *lmsap2* are transcribed in the promastigote stage of the parasite resulting in approximately equal amounts of two corresponding mRNAs, which may be derived from polycistronic precursor by trans-splicing and polyadenylation (Stierhof *et al.*, 1998). According to the ratio of SAP1 to SAP2 found in the promastigote culture supernatant, the *lmsap1* mRNA is translated more efficiently than the *lmsap2* message. These results were also obtained in the original study (Wiese *et al.*, 1995). However, western blot studies showed that the secreted filamentous SAP1 and SAP2 failed to carry the γGCS expressed protein and secrete it outside the promastigotes cells. This could be because cleavage occurred for the translated protein and the γGCS recombinant protein remained inside the promastigote, or partially expression occurred and the promastigotes failed to express the γGCS along with *lmsap* locus. More studies are required to determine which occurs.

However, purification of recombinant SAP filaments from the supernatant was carried out to determine if γGCS protein was present. The SAP filaments have previously been purified from harvested supernatant by centrifugation (Stierhof *et al.*, 1998). The limitation of the techniques used in project is the ability to detect only the SAP protein by enzyme assay and LT 8.2 antibody (Stierhof *et al.*, 1998). To determine if γGCS is attached to the SAP filaments immunoblot studies using mAb LT 8.2 antibody could be used to compare the band sizes of SAP protein expressed from blank vectors with expressed SAP fusion proteins (Stierhof *et al.*, 1998). Comparing SAP from the blank vector with SAP fusion protein is an indirect method of detection of the co-expressed protein. Development of a PCR protocol for detecting genomic integration of strains or use of quantitative PCR could be used to monitor gene expression in transfected parasites (Yuan *et al.*, 2014). Another option that may improve expression of the γGCS /SAP1 or SAP2 fusion protein would be to replace the secreted acid phosphatase signal in the plasmid vector containing T7 polymerase in the downstream 3' to obtain an expression vector which can inducible secreted protein (Breitling *et al.*, 2002).

In conclusion, this study showed that:

1. It was possible to construct an expression vector with the *lmsap* locus and the γGCS gene sequence of *L. donovani.*
2. Secretion of recombinant γGCS into the medium as a fusion protein with SAP1 or SAP2 did not occur.

CHAPTER 5. EFFECT OF USING TRANSFECTED *L. TARENTOLAE* PARASITES AS A LIVE VACCINE AGAINST CUTANEOUS LEISHMANIASIS

5.1. Introduction

Leishmaniasis is a disease that is caused by infection with different *Leishmania* species. To date, there is no recommended clinical vaccine to prevent *Leishmania* infections. The oldest vaccination strategy against CL or VL is to inoculate people with virulent *Leishmania* promastigotes to stimulate immunity in a process known as 'leishmanization' (Okwor and Uzonna, 2009, Mccall *et al.*, 2013). This strategy induces strong immunity after recovery from infection, but can cause permanent scarring (Okwor and Uzonna, 2009). Vaccination with killed parasites alone, or killed parasites given with BCG as an adjuvant failed to protect against infection (Momeni *et al.*, 1999). Therefore, attenuated *Leishmania* strains, which do not cause infection, but can stimulate the appropriate immune responses to protect against a subsequent infection, have been developed (Uzonna *et al.*, 2004, Papadopoulou *et al.*, 2002, Alexander *et al.*, 1998). This approach has given partial protection against wild-type virulent parasites (Belkaid *et al.*, 2002, Uzonna *et al.*, 2001). In some cases there is a risk that the attenuated parasite may develop virulence (Handman, 2001). Studies have shown that the presence of a small number of parasites is required for maintenance of immunity against *Leishmania* and development of *Leishmania* specific effector cells. However, central memory T cells ($CD62L^{high}$, $IL\text{-}2^{pos}$, $IFN\text{-}\gamma^{neg}$) can develop in the absence of parasites (Zaph *et al.*, 2004). *L. tarentolae* is non-pathogenic to humans (Breton *et al.*, 2005), but it can differentiate to the amastigote form within mammalian MØ. *L. tarentolae* can also target DCs, causing DC activation, induction of T cell proliferation and the production of IFN-γ (Breton *et al.*, 2005). *L. tarentolae* has been used as a vaccine vector candidate in a number of studies. For example, *L. tarentolae* transfected with cysteine proteinases (type I and II, CPA/CPB) and sandfly salivary antigen (PpSP15) from *Phlebotomus papatasi,* as a combined live vaccine, was used to immunise against *L. major* infection (Zahedifard *et al.*, 2014). This study indicated that antigen stimulated spleen cells produced both Th1 and Th2-type responses, and resulted in lower parasites burden in compared to controls. Another study using *L. tarentolae* expressing the *L. donovani* A2 antigen and cysteine proteinases A (CPA and CPB without its unusual C-terminal extension (CPB^{-CTE}) was used to immunise against VL caused by *L. donovani* infection (Saljoughian *et al.*, 2013).

Vaccination induced stronger antigen-specific Th1-type responses and no parasites were found in the liver or spleen at 4th week post-challenge in vaccinated groups. Protective immunity was associated with significantly higher levels of IFN-γ production by antigen stimulated splenocytes before and after challenge. In addition, antigen stimulated splenocytes from immunised mice produced significantly lower levels of IL-10 after challenge compared to cells from infected controls. This vaccine also protected outbreed dogs (Shahbazi *et al.*, 2015) against *L. infantum*, resulting in significantly lower parasite burdens in vaccinated dogs compared to controls, and significantly higher levels of specific IgG2 but not IgG1 antibodies. In addition, antigen stimulated spleen cells produced significantly higher levels of IFN-γ and TNF-α, but lower levels of IL-10, which indicated that the immune profile was altered to Th1-type response. More recently, vaccination of mice with *L. tarentolae* expressing lipophosphoglycan 3 against *L. infantum* infection (Pirdel and Farajnia, 2017) resulted in significantly higher levels of IFN-γ and lower levels of IL-10 by antigen stimulated spleen cells compared to mice similarly treated with wild type parasites. This live vaccine also induced higher levels of parasite-specific IgG2a compared to IgG1 in vaccinated mice compared to controls. However, the transfected *L. tarentolae* only gave partial protection against *L. infantum*.

Previous studies have shown that vaccination by an injectable route with a plasmid containing the gene sequence of *L. donovani* γGCS (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.*, 2012). However, in these studies sterile immunity was not achieved. It is possible that *L. tarentolae*, transfected with the gene sequence γGCS from pathogenic species may give a higher level of protection.

Therefore, studies were carried out to:

1. Determine if vaccination with live *L. tarentolae* parasites transfected with γGCS from three different pathogenic species of *Leishmania* i.e. *L. don* γGCS, *L. maj* γGCS and *L. mex* γGCS could protect against infection with *L. major.*

2. Characterise the immune responses induced by vaccination with live *L. tarentolae* parasites transfected with γGCS, before and after challenge with *L. major.*

5.2. Results

5.2.1. Expt 1: studies to determine if vaccination with transfected *l. tarentolae* can protect against *L. major* infection and characterisation of the immune responses induced.

The effect of immunising with WT *L. tarentolae or L. tarentolae* transfected with EGFP-γGCS-His from *L. donovani* (*L. don* γGCS), *L. major* (*L. maj* γGCS) or *L. mexicana* (*L. mex* γGCS), or a combination of all three transfected parasites on the outcome of *L. major* was determined (Fig 5.1). Thus, mice were immunised by the subcutaneous route into the rump on days 0 and 14 with:

(i) PBS alone - control

(ii) *L. tarentolae*-WT parasites – 1 x 10^7 cells/dose (WT).

(iii) *L.t L.don* EGFP-γGCS-His – 1 x 10^7 cells/dose (*L.t L.don* γGCS).

(iv) *L.t L.maj* EGFP-γGCS-His – 1 x 10^7 cells/dose (*L.t L.maj* γGCS)

(v) *L.t L.mex* EGFP-γGCS-His – 1 x 10^7 cells/dose (*L.t L.mex* γGCS)

(vi) mixture of *L.t L.don* γGCS, *L.t L.maj* γGCS and *L.t L.mex* γGCS 1:1:1 ratio – 1 x 10^7 cells/dose (Triple vaccine)

On day 28 vaccinated and control mice were infected with 1 x 10^7 luciferase-expressing *L. major* promastigotes by subcutaneous injection via the footpad. Mice vaccinated with the triple vaccine had a higher level of protection against infection with *L. major*, demonstrated as a significant reduction in the both footpad thickness and light emitted from the site of infection compared to both single vaccine groups and infected control ($p < 0.0001$, Figure 5.2 and Table 5.1). The amount of BLI expressed/amastigote using amastigotes recovered from footpad lesions was similar for all 6 groups of mice (Table 5.1), but it was lower that the BLI emitted by promastigotes used at infection.

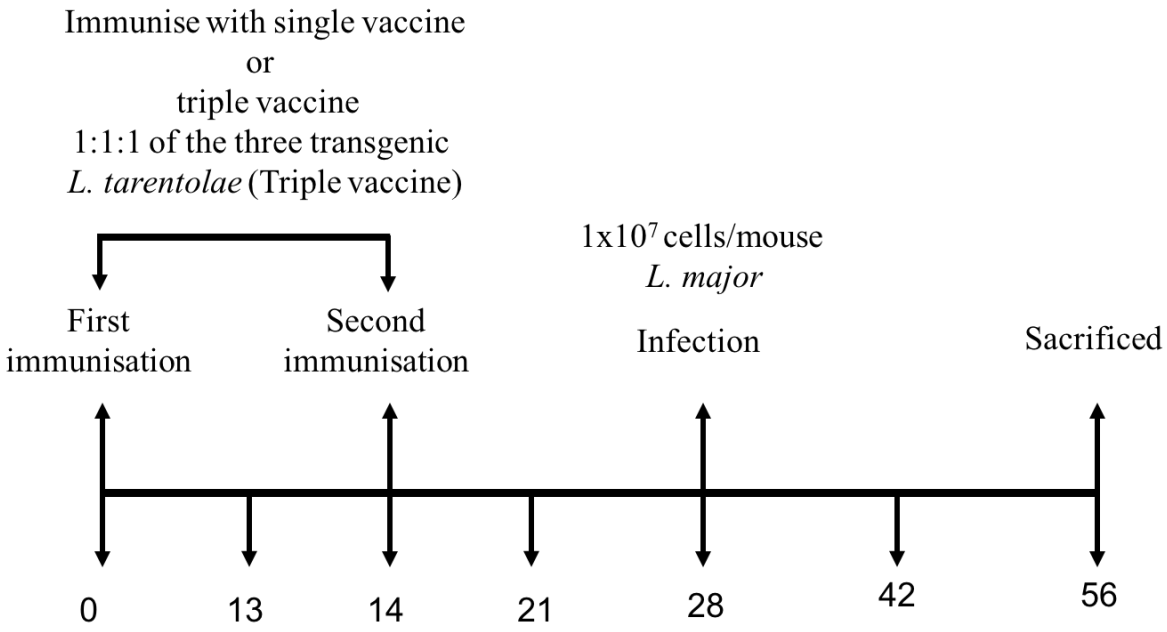


Figure 5.1 The vaccination protocol used in experiments. Mice were immunised on day 0 and 14 with 1x10^7 *L.tarentolae*-WT, *L. tarentolae* transfected with the gene sequence for *L. don* γGCS, *L. maj* γGCS aor *L. mex* γGCS) as single vaccine or as mixture of all three types of transfected parasites (1;1:1 ratio, triple vaccine). Blood samples were collected on days 13, 21, 42 and 56 of the experiment. On day 28 post-infection i.e. day 56, mice were sacrificed and spleens and popliteal lymph nodes were collected and used in lymphocyte proliferation assays.

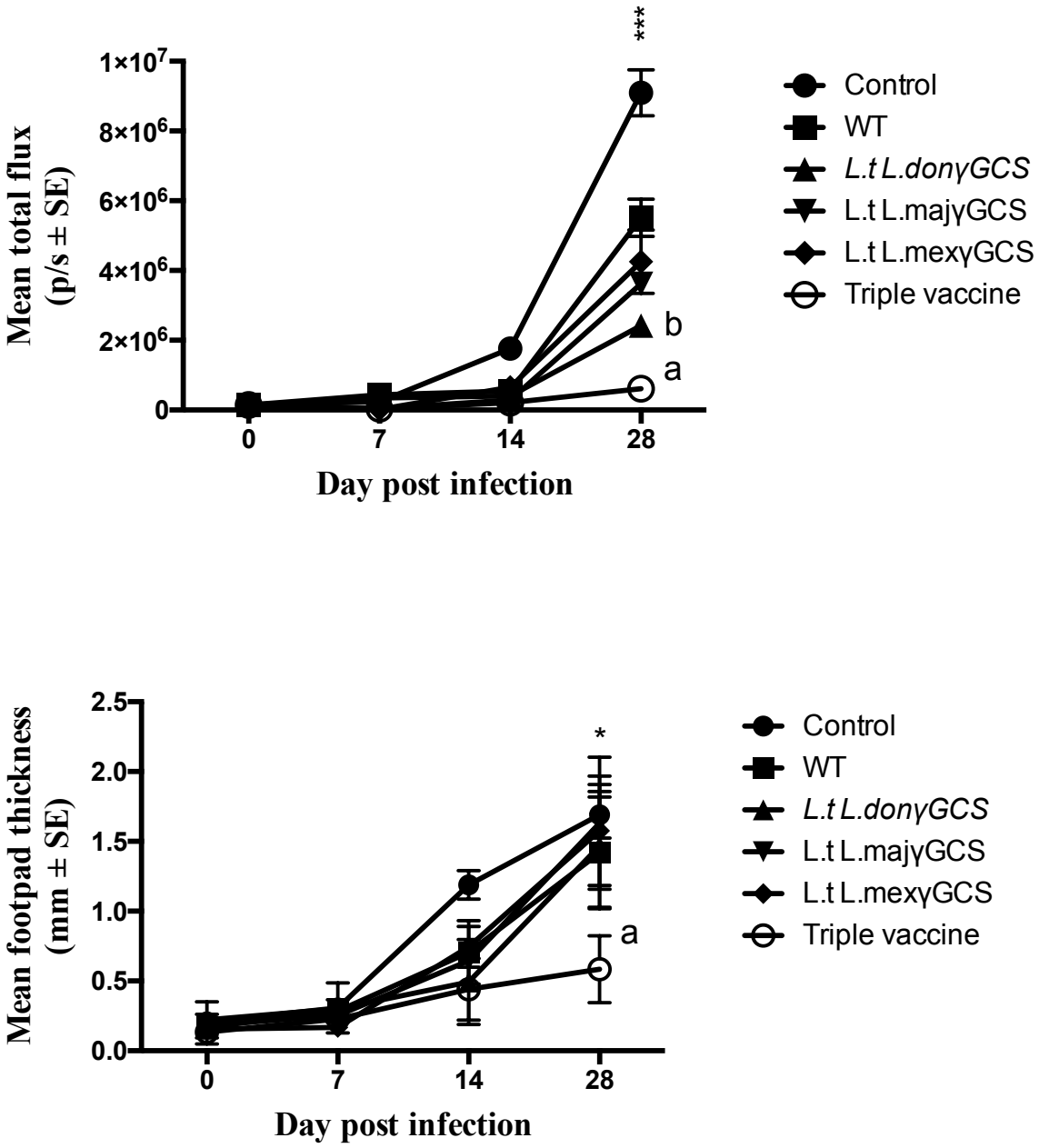


Figure 5.2 The effect of different vaccines on the outcome of *L. major* infection. Mice (n=5/ treatment) were immunised on days 0 and 14 using the protocol shown in Fig. 7.1 with PBS alone (control) or $1x10^7$ *L. tarentolae* promastigotes (WT, *L.t L.don* γGCS, *L.t L.maj* γGCS, *L.t L.mex* γGCS or a mixture of all three [triple vaccine]. On day 28 all the mice were infected with $1x10^7$ *L. major* promastigotes by subcutaneous injection intro the footpad (*Lmaj*Luc strain). Parasite burdens were determined by assessing footpad thickness of the infected footpad relative to the uninfected footpad over the course of infection. The experiment was terminated on day 28 post-infection i.e. day 56 of the experiment. ***$p < 0.0001$ triple vaccine compared to infected control, *$p < 0.05$ triple vaccine compared to infected control $^a p < 0.05$ triple vaccine compared to WT

Table 5.1 The effect of vaccination on the parasite burdens of *L. major* infected mice. Mice (n= 5/treatment) were immunised with transfected live *L. tarentolae* or *L. tarentolae*-WT ($1x10^7$ cells/ml) on day 0 and 14. On day 28 mice were infected with 1×10^7 *L. major* promastigotes by subcutaneous injection into the footpad. On day 28 post-infection (i.e. day 56) the parasite burden of infected mice was determined by direct counting of the number of parasite/ml present in footpad lesions or by determining the amount of bioluminescence (BLI) emitted from each footpad. The mean reduction in parasite burdens ± SE compared to the infected control is shown in parentheses. *p < 0.05, ***p < 0.001 compared to infected controls, [a]p < 0.05 compared to WT. BLI emitted/*L. major* parasite i.e. promastigotes pre-infection: amastigote post-infection

	Mean total FLUX (p/s ± SE x10⁶)	**Mean parasite number/ml (± SE x10⁶)**	**Mean BLI/parasite ± SE**
Pre-infection			2.2 ± 0.3
Infected Control	2.98 ± 0.98	26 ± 0.58	0.23 ± 0.17
WT vaccine	1.66 ± 0.95 (44% ± 0.18)	16 ± 0.84 (38% ± 0.11)	0.12 ± 0.06
L.t L.don γGCS vaccine	0.8 ± 0.55* (74% ± 0.02)	0.96 ± 0.14* (96% ± 0.01)	0.8 ± 0.59
L.t L.maj γGCS vaccine	1.02 ± 0.09* (86% ± 0.01)	0.99 ± 0.2* (94% ± 0.02)	1.32 ± 0.44
L.t L.mex γGCS vaccine	1.27 ± 0.72 (72% ± 0.19)	1 ± 0.26 (96% ± 0.01)	0.49 ± 0.21
Triple vaccine	0.2 ± 0.17***a (92% ± 0.03)	0.68 ± 0.28*a (98% ± 0.01)	0.19 ± 0.15

Blood samples were collected over the course of the study to determine if immunity was associated with differences in specific IgG1 and IgG2a antibody responses compared to infected controls. Specific IgG1 and IgG2a antibody responses to *L. major* antigen was determine as this would also show the amount of cross-immunity induced by the different vaccines. Vaccination with the triple vaccine induced significant specific IgG2a and IgG1 antibodies by day 7 after the second vaccine dose compared to controls and the other vaccine groups ($p < 0.05$). After infection, specific IgG1 antibody levels were similar to infected controls whereas IgG2a were significantly higher than infected controls and the

other vaccine groups ($p < 0.01$, Figure 5.3). The ratio of specific IgG2a/IgG1 levels for the different groups on day 56 were (0.08, <1) infected control, (0.66, <1) WT vaccines, (1.6, >1) *L.t L.don* γGCS vaccine, (1.5, >1) *L.t L.maj* γGCS vaccine, (1.1, >1) *L.t L.mex* γGCS vaccine, (11.52, >1) triple vaccine. This indicated that vaccination with the triple vaccine induced a predominant Th1 antibody response.

The effect of vaccination on the immune responses of vaccinated and infected control mice was also assessed by determining the levels of cytokine and nitrite produced by *in vitro* stimulated popliteal lymph node cells or spleen cells taken from immunised or control mice on day 56 i.e. day 28 post-infection (Figs. 5.4, 5.5, 5.6 and 5.7). Immunisation with the WT *L. tarentolae* did not confer any significant protection based on BLI or lesion size. Antigen stimulated lymph node cells from these animals made significantly higher levels of IFN-γ ($p < 0.05$) whereas spleen cells from the same mice made similar amounts of IFN-γ as splenocytes from infected controls. Antigen stimulation of both type of cells from the mice immunised with the triple vaccine produced significantly higher levels of IFN-γ, nitrite and IL-5 (lymph node cells only), but similar levels of IL-10, compared to infected control values (Figure 5.4, 5.5, 5.6 and 5.7). Con A stimulation of cells from mice immunised with the triple vaccine induced significantly greater production of IFN-γ, nitrite, IL-5 but not IL-10 for both cell populations compared to infected controls ($p < 0.05$, Figure 5.4, 5.5, 5.6 and 5.7). It was also apparent that higher amounts of IFN-γ were produced by antigen stimulated cells from the lymph node cells compared to the spleen cells for all the mice. Overall the results of this experiment indicated that the triple vaccine gave the highest level of protection and the greatest enhancement in immune responses compared to controls. Therefore, this vaccine was used in a second study.

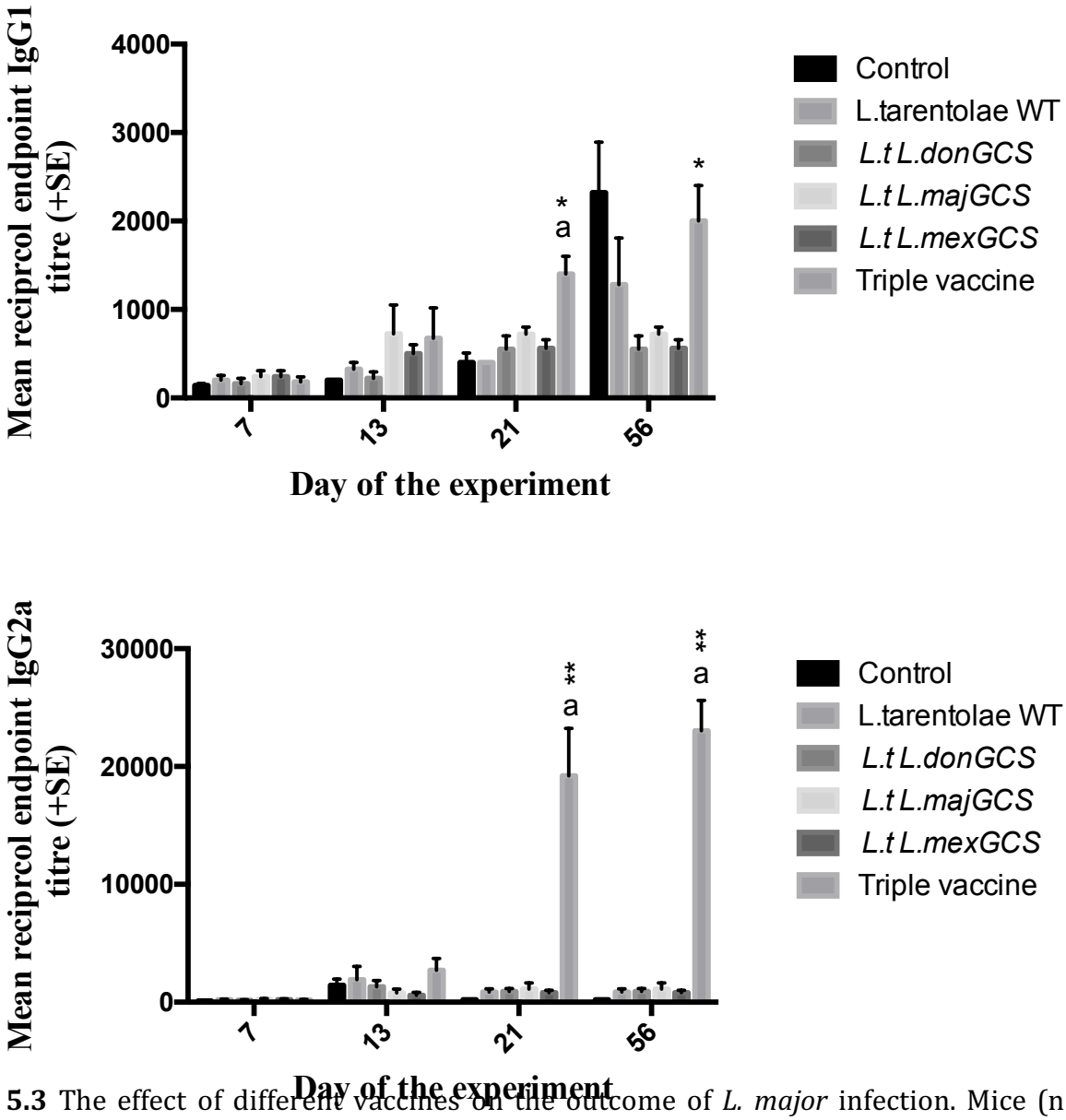


Figure 5.3 The effect of different vaccines on the outcome of *L. major* infection. Mice (n = 5/ treatment) were immunised on days 0 and 14 using the protocol shown in Fig. 7.1 with PBS alone (control) or $1x10^7$ *L. tarentolae* promastigotes (WT, *L.t L.don* γGCS, *L.t L.maj* γGCS, *L.t L.mex* γGCS or a mixture of all three [triple vaccine]). On day 28 all the mice were infected with $1x10^7$ *L. major* promastigotes by subcutaneous injection intro the footpad (*Lmaj*Luc strain). Mice bleeding on day pre-infection (13, 21) and 42, 56 post-infection to detect specific antibody titre. **p < 0.001 compared to infected control, *p <0.001 compared to infected control, ^{a}p < 0.05 compared to single vaccines.

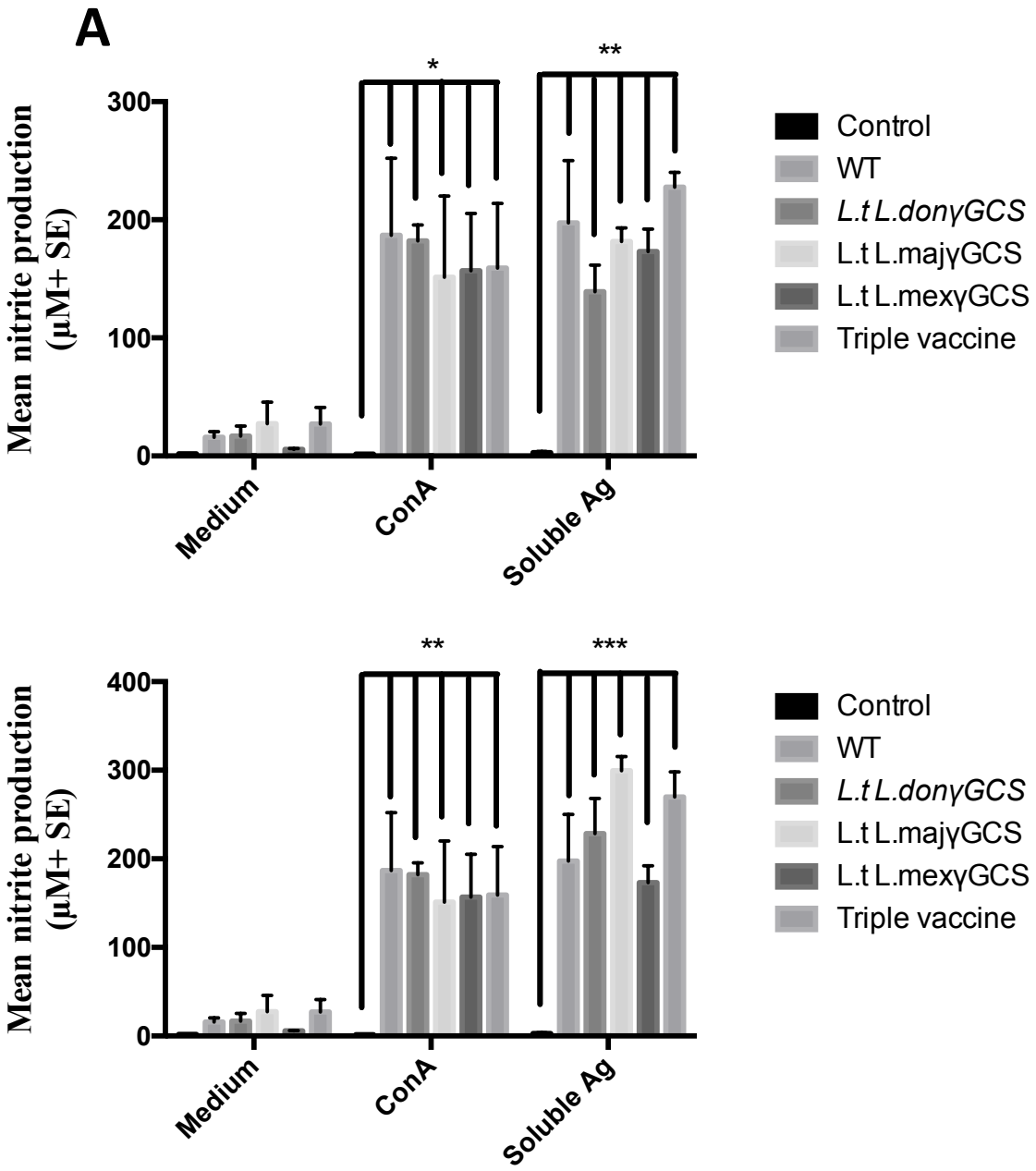


Figure 5.4 Mean nitrite (A) production by splenocytes or popliteal lymph node cells from mice shown in Table 7.1. Splenocytes (5 x 10^5/ml, A) or popliteal lymph node cells (5 x 10^5/ml, B) were incubated with medium alone (controls), ConA (5 µg/ml) or *L. major* soluble antigen (25 µg/ml) for 72 hr. Nitrite levels in cell supernatants were determined using a Greiss assay. * $p < 0.05$ compared to control, ** $p < 0.001$ compared to control, *** $p < 0.0001$ compared to control.

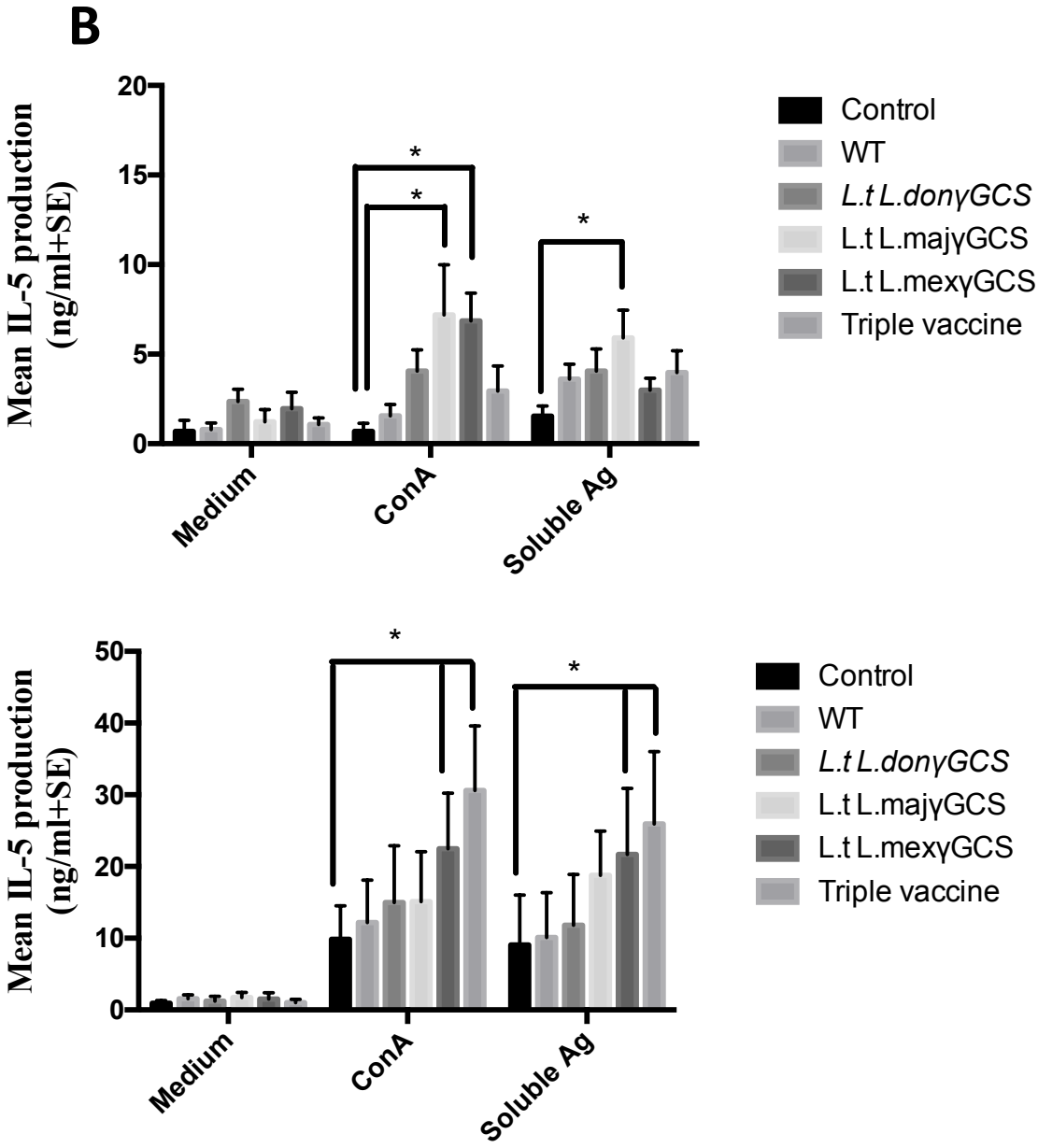


Figure 5.5 Mean IL-5 (B) production by splenocytes or popliteal lymph node cells from mice shown in Table 7.1. Splenocytes (5 x 10^5/ml, A) or popliteal lymph node cells (5 x 10^5/ml, B) were incubated with medium alone (controls), ConA (5 µg/ml) or *L. major* soluble antigen (25 µg/ml) for 72 hr. Nitrite levels in cell supernatants were determined using a Greiss assay. * p< 0.05 compared to control, ** p< 0.001 compared to control, *** p< 0.0001 compared to control

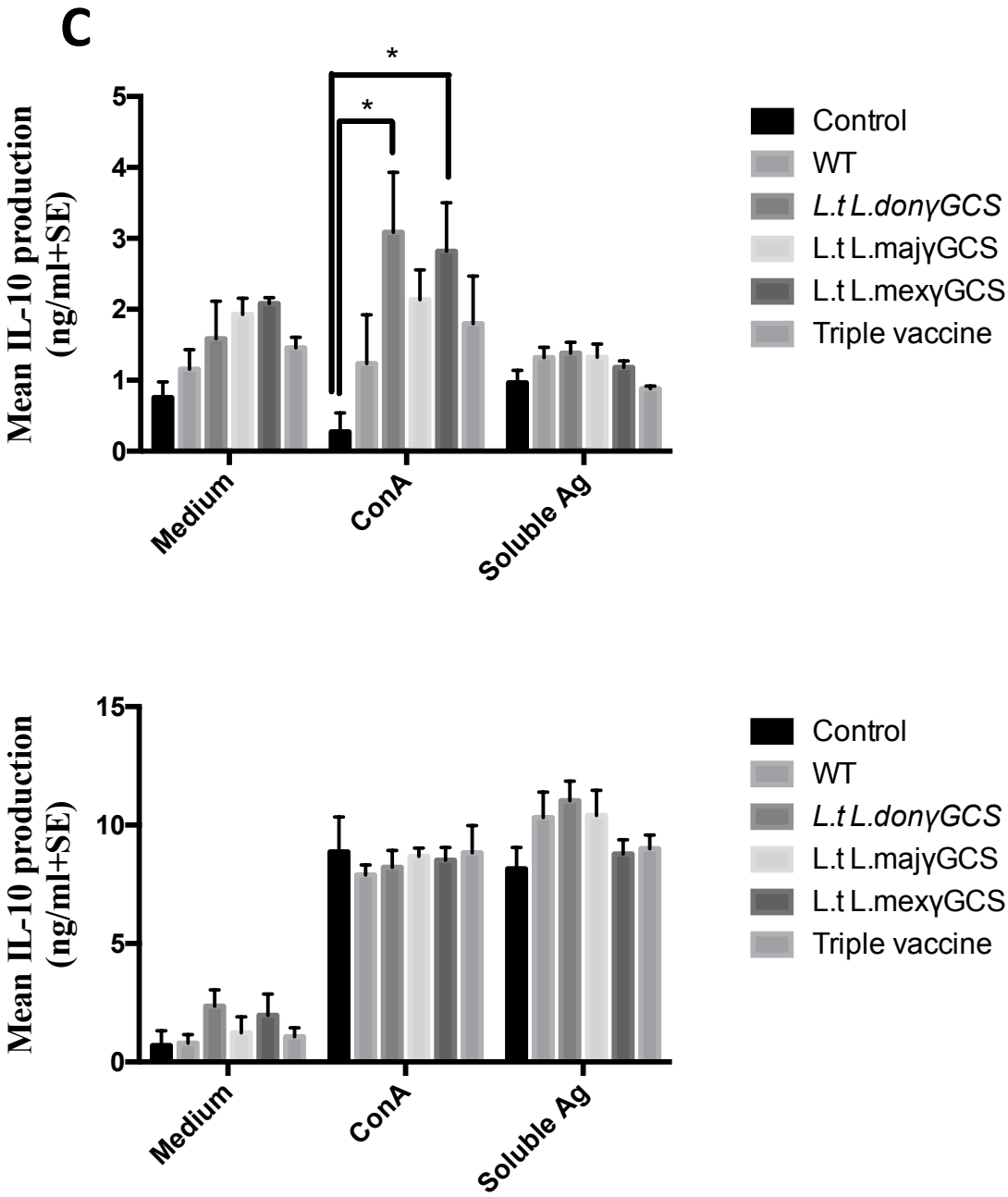


Figure 5.6 Mean IL-10 (C) production by splenocytes or popliteal lymph node cells from mice shown in Table 7.1. Splenocytes (5 x 10^5/ml, A) or popliteal lymph node cells (5 x 10^5/ml, B) were incubated with medium alone (controls), ConA (5 µg/ml) or *L. major* soluble antigen (25 µg/ml) for 72 hr. Nitrite levels in cell supernatants were determined using a Greiss assay. * p< 0.05 compared to control, ** p< 0.001 compared to control, *** p< 0.0001 compared to control

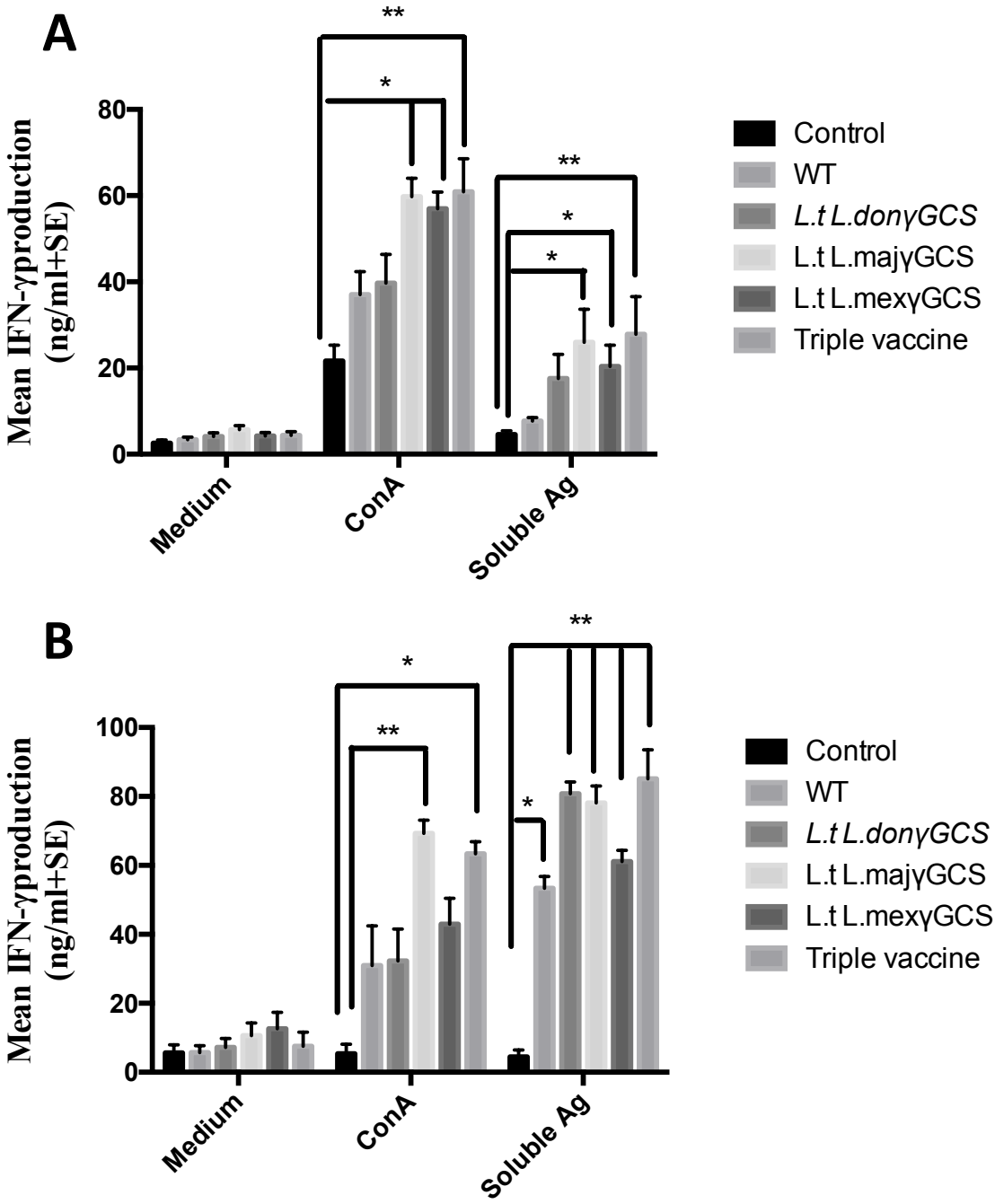


Figure 5.7 Mean IFN-γ production by splenocytes or popliteal lymph node from mice shown in Table 7.1. Splenocytes (5 x 10^5/ml, A) or popliteal lymph node cells (5 x 10^5/ml, B) were incubated with medium alone (controls), ConA (5 μg/ml) or *L. major* soluble antigen (25 μg/ml) for 72 hr. Nitrite levels in cell supernatants were determined using a Greiss assay * $p < 0.05$ compared to control, ** $p < 0.001$ compared to control, *** $p < 0.0001$ compared to control

5.2.2. Expt 2: The effect of immunisation with the triple vaccine on immunity to *L. major* infection

Similar results to that obtained in experiment 1 were obtained. Thus, immunisation with the triple vaccine significantly reduced parasite burdens in infected animals compared to controls based on changes in footpad thickness, BLI and direct counting of parasites present in footpad lesions ($p < 0.001$, Figure 5.8, Table 5.2). This study also showed that *L. major* amastigotes have lower BLI/parasite compared to promastigotes.

The type of immune response induced by the two vaccines was assessed to determine if a particular type of immune response was associated with protection against *L. major* infection.

Specific IgG1 and IgG2a antibody responses to *L. major* antigen was determined as this would also show the amount of cross-immunity induced by the different vaccines. Vaccination with the triple vaccine induced significant specific IgG2a antibodies against the *L. major* soluble antigen by day 7 after the second vaccine dose compared to controls and the WT vaccine group ($p < 0.05$), but all three groups had similar specific IgG1 responses. After infection with *L. major*, mice given the triple vaccine had significantly higher IgG2a levels compared to infected controls and mice given the WT vaccine ($p < 0.001$). In contrast, all three groups had similar specific IgG1 antibody levels were similar (Figure 5.9). This response associated with a predominant Th1-type response based on the ratio of IgG2a/IgG1 responses. Thus, the ratio of specific IgG2a/IgG1 levels on day 56 were 0.25 for the infected control, 2.11 for the WT vaccine, and 11.29 for the triple vaccine.

Stimulation of cells from mice given the triple vaccine with *L. major* soluble antigen resulted in significantly higher levels of IFN-γ, nitrite and IL-5 ($p < 0.05$), but not IL-10 for both cell populations compared to infected controls (Figures 5.10, 5.11 and 5.12). Comparison of the specific responses of splenocytes from the vaccine groups showed that splenocytes from mice given the triple vaccine produced significantly higher levels of nitrite and IL-5 ($p < 0.01$) but similar

amount of IFN-γ and IL-10. In contrast, similar levels of IFN-γ, nitrite, and IL-10 were produced by antigen stimulated lymph nodes cells obtained from both groups (Figures 5.10-5.13). Stimulation with Con A resulted in the production of similar levels of IFN-γ by lymph node cells and splenocytes from all three groups of mice. However significantly higher amounts of nitrite were produced by both populations of cells from mice given the triple vaccine and IL-5 by splenocytes ($p < 0.05$) compared to cells from the other two groups. Whereas more IL-10 was produced by splenocytes from mice immunised with WT *L. tarentolae* compared to cells from the other two groups of mice ($p < 0.05$, Figures 5.10, 5.11 and 5.13). Results for lymph nodes cells also showed the same trend apparent in the first experiment i.e. they produced more IFN-γ than splenocytes. And in this experiment protection by the triple vaccine was also associated with a predominant Th1 response.

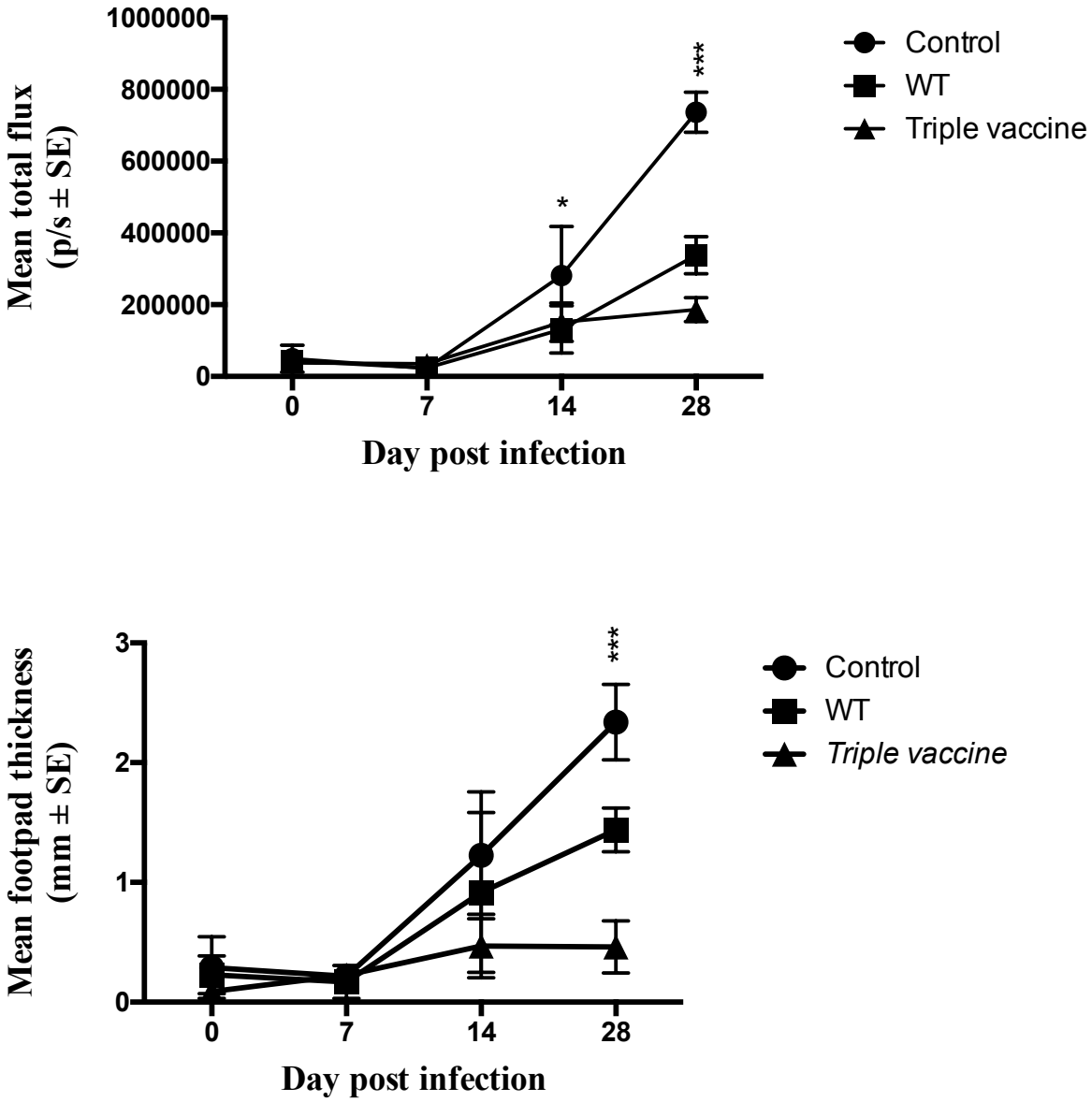


Figure 5.8 The effect of different vaccines on the outcome of *L. major* infection. Mice (n = 5/ treatment) were immunised on days 0 and 14 using the protocol shown in Fig. 7.1 with PBS alone (control) or 1 x 10^7 *L. tarentolae* promastigotes (WT or a mixture of all three {triple vaccine]). On day 28 all the mice were infected with 1 x 10^7 *L. major* promastigotes by subcutaneous injection into the footpad (*Lmaj*Luc strain). Parasite burdens was determined by assessing footpad thickness of the infected footpad relative to the uninfected footpad over the course of infection. The experiment was terminated on day 28 post-infection i.e. day 56 of the experiment. ***$p < 0.0001$ triple vaccine compared to infected control.

Table 5.2 The effect of vaccination on the parasite burdens of *L. major* infected mice. Mice (n = 5/ treatment) were immunised with transfected live triple vaccine or *L. tarentolae*-WT (1 x 10^7 cells/ml) on day 0 and 14. On day 28 mice were infected with 1x10^7 *L. major* promastigotes by subcutaneous injection into the footpad. On day 28 post-infection (i.e. day 56) the parasite burdens of infected mice were determined by direct counting of the number of parasite/ml present in footpad lesions or by determining the amount of bioluminescence (BLI) emitted from each footpad. The mean reduction in parasite burdens ± SE compared to the infected control is shown in parentheses. **p < 0.001 compared to infected control. BLI emitted/*L. major* parasite i.e. promastigotes pre-infection: amastigote post-infection.

	Total flux (p/s ± SE x 10^6)	**Parasite burden parasite/ml x 10^6 ± SE**	**BLI/parasite ratio**
pre-infection			0.45 ± 0.73
infected control	2.79 ± 0.95	26.6 ± 1.2	0.15 ± 0.75
***L. tarentolae* WT**	2.04 ± 0.85 (48.96% ± 0.10)	14.1± 3.81 (46% ± 0.15)	0.17 ± 0.82
Triple vaccine	0.32 ± 0.12** (90.41% ± 0.03)	0.70 ± 0.64** (94.31 ± 0.01)	0.32 ± 0.15

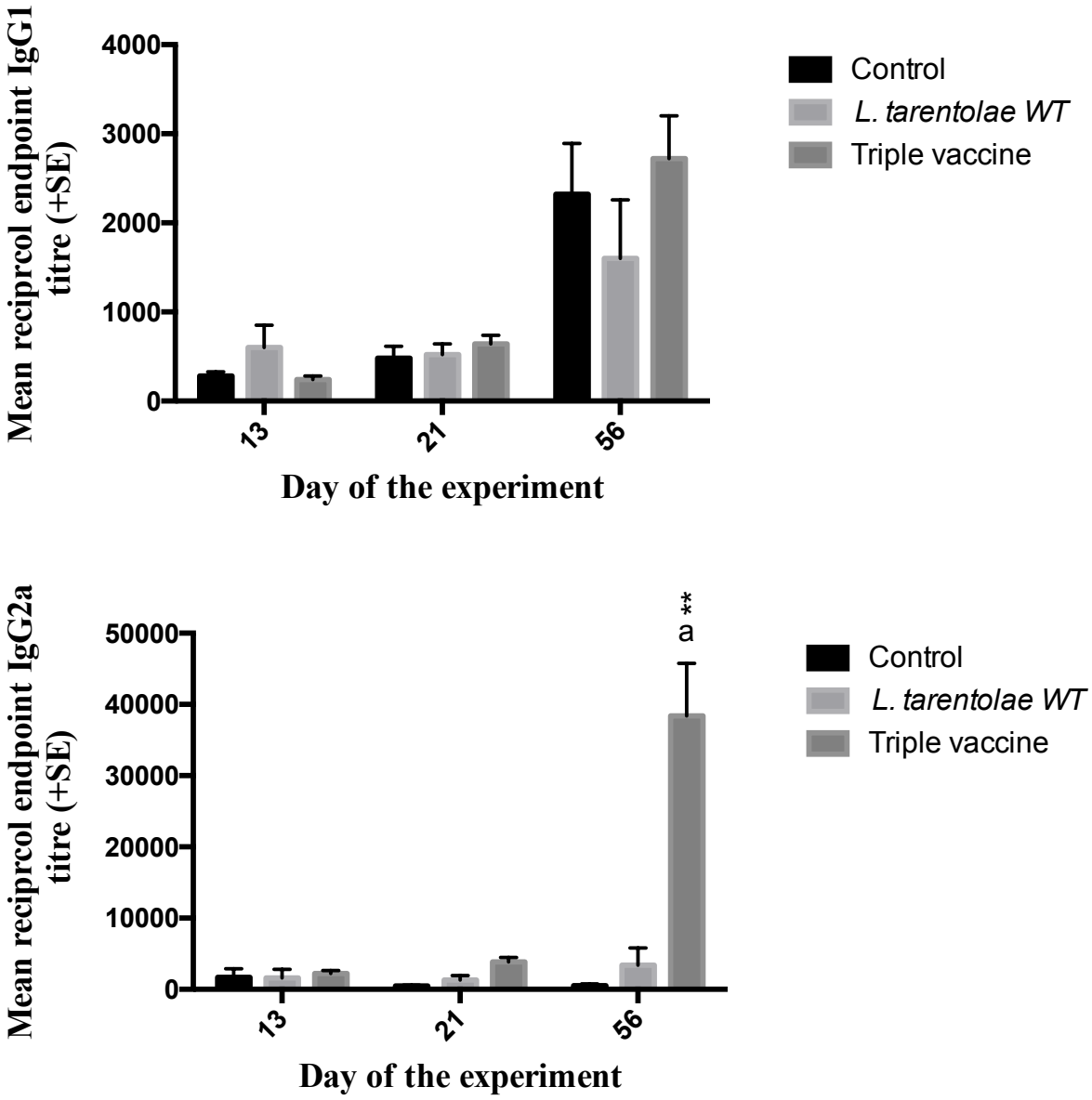


Figure 5.9 The effect of immunisation with different vaccines on the outcome of *L. major* infection. Mice (n = 5/treatment) were immunised on days 0 and 14 using the protocol shown in Fig. 7.1 with PBS alone (control) or $1x10^7$ *L. tarentolae* promastigotes (WT or a mixture of all three transfected *L. tarentolae* [triple vaccine]). On day 28 all the mice were infected with 1×10^7 *L. major* promastigotes by subcutaneous injection intro the footpad (*Lmaj*Luc strain). Mice bleeding on day pre-infection (13, 21) and 42, 56 post-infection to detect specific antibody titre. **p < 0.001 compared to infected control, *p <0.001 compared to infected control, $^a p < 0.05$ compared to single vaccines.

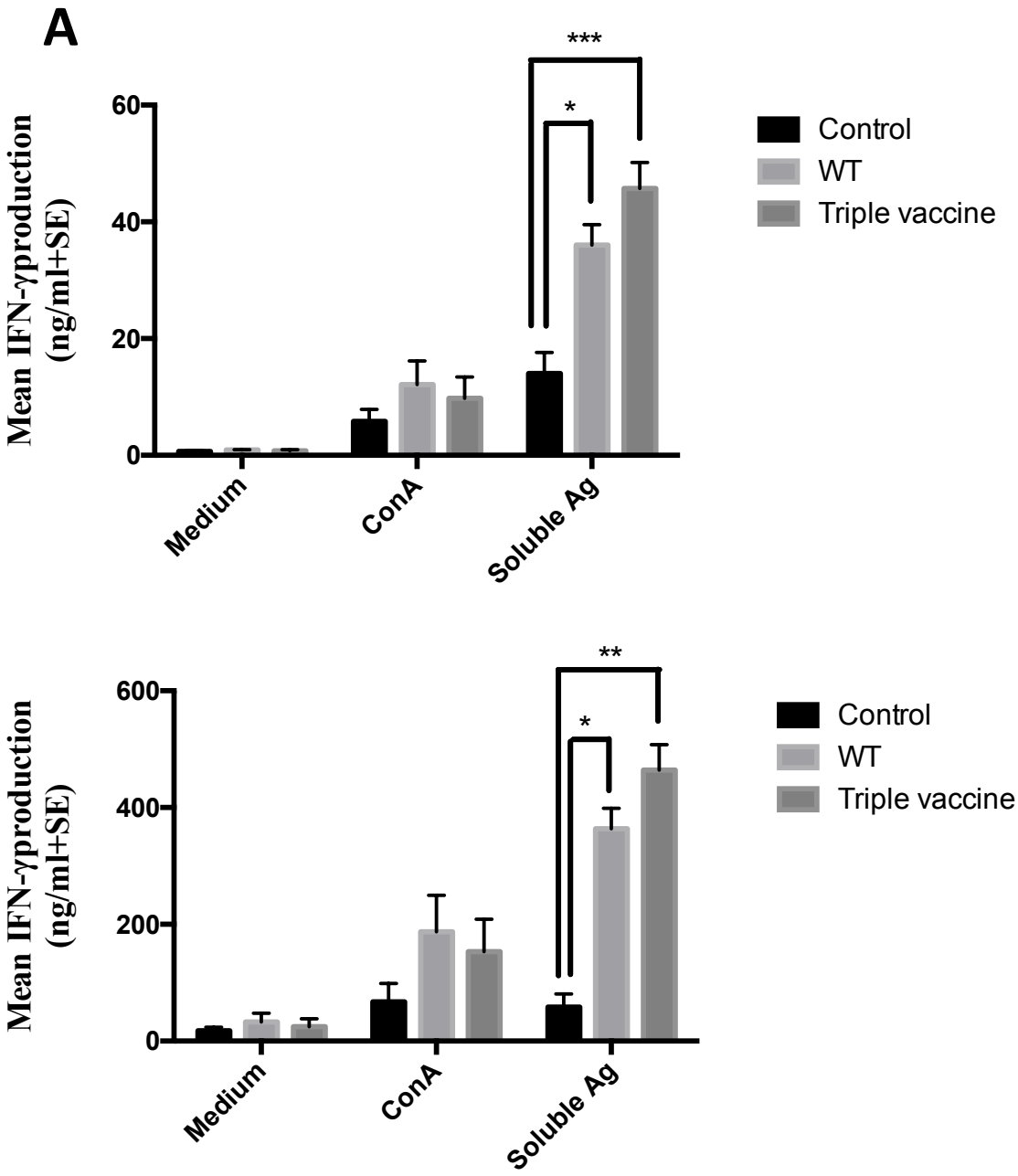


Figure 5.10 Mean IFN-γ (A) production by splenocytes or popliteal lymph node cells from mice shown in Table 7.1 Splenocytes (5 x 105/ml, A) or popliteal lymph node cells (5 x 105/ml, B) were incubated with medium alone (controls), ConA (5 µg/ml) or L. major soluble

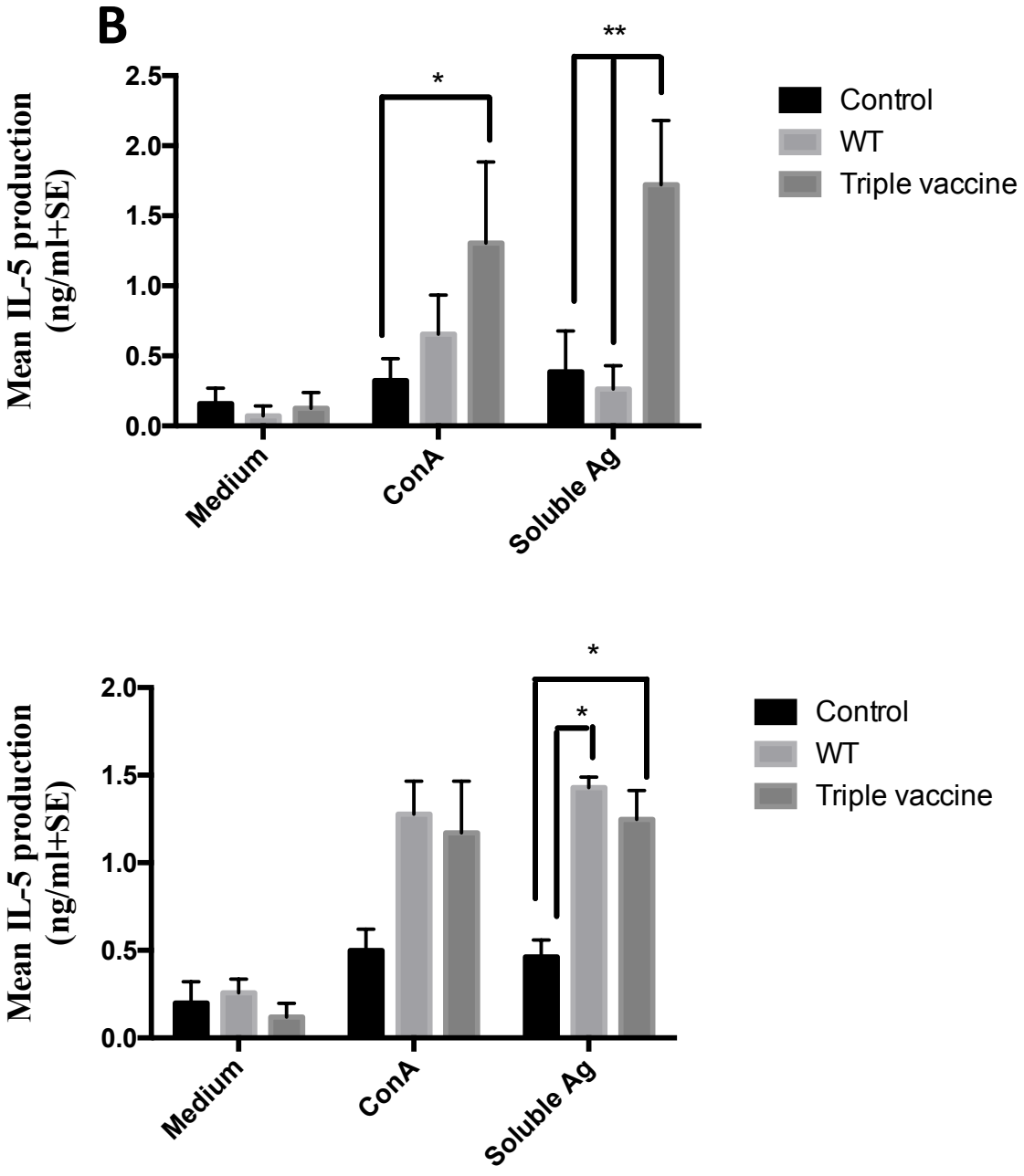


Figure 5.11 Mean IL-5 (B) production by splenocytes or popliteal lymph node cells from mice shown in Table 7.1 Splenocytes (5 x 10^5/ml, A) or popliteal lymph node cells (5 x 10^5/ml, B) were incubated with medium alone (controls), ConA (5 µg/ml) or *L. major* soluble antigen (25 µg/ml) for 72 hr. * $p < 0.05$ compared to control

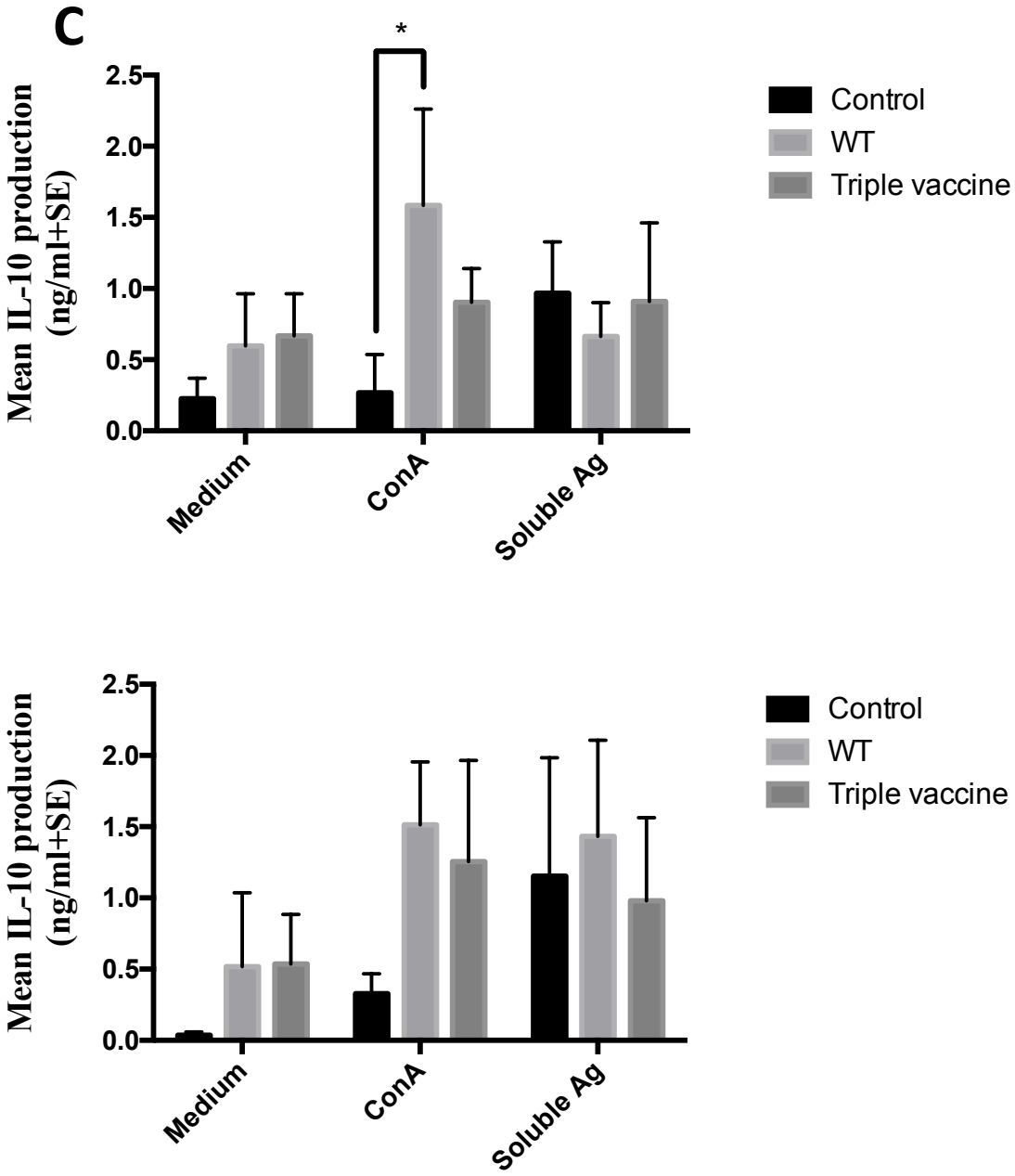


Figure 5.12 Mean IL-10 (C) production by splenocytes or popliteal lymph node cells from mice shown in Table 7.1 Splenocytes (5 x 10^5/ml, A) or popliteal lymph node cells (5 x 10^5/ml, B) were incubated with medium alone (controls), ConA (5 µg/ml) or *L. major* soluble antigen (25 µg/ml) for 72 hr. * p< 0.05 compared to control, ** p< 0.001 compared to control, *** p< 0.0001 compared to control

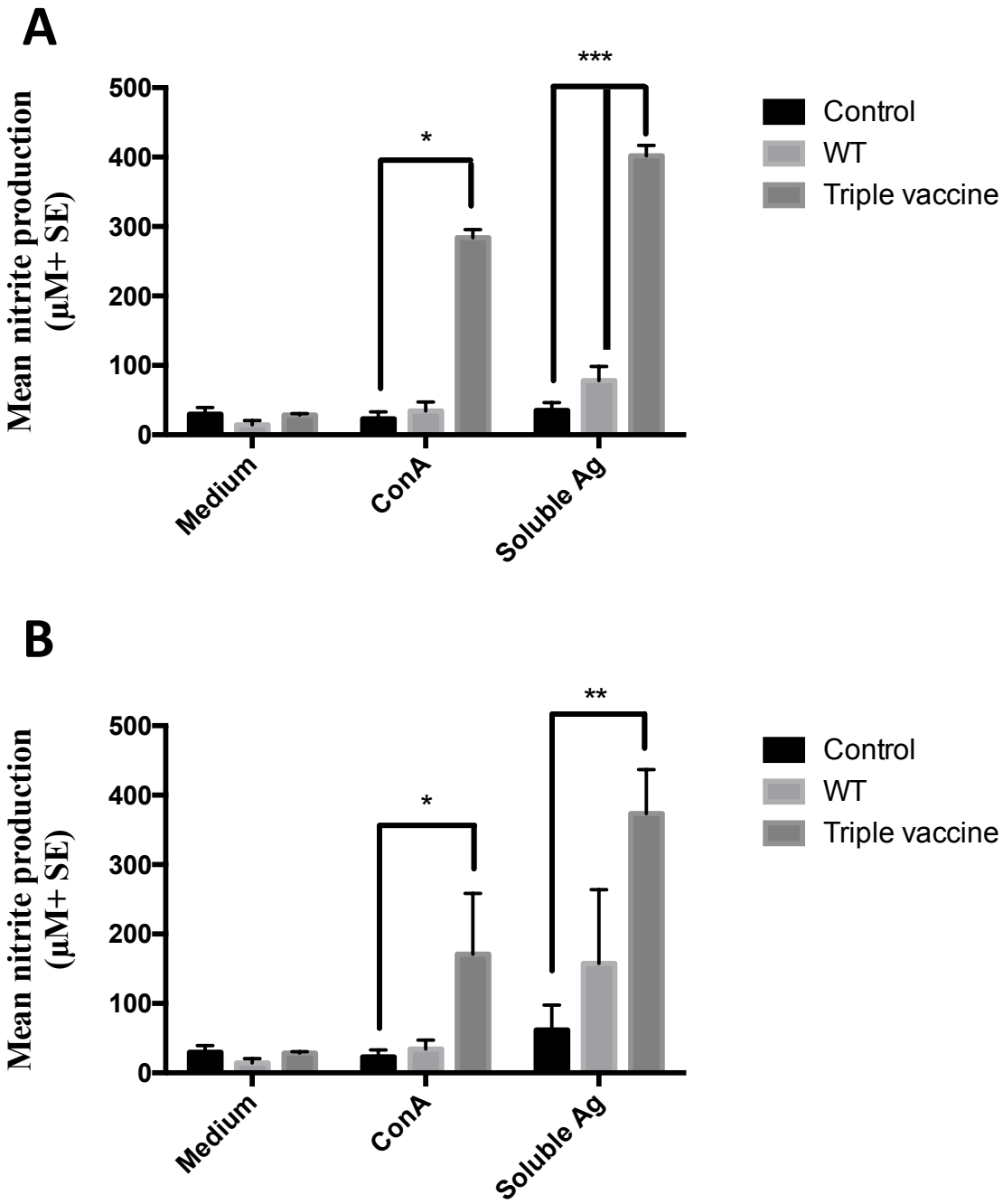


Figure 5.13 Mean nitrite production by splenocytes (A) and popliteal lymph node cells lymphocyte (B) from vaccinated mice stimulated *in vitro* on day 28 of infection. Mice were immunised by subcutaneous injection with PBS (controls), *L. tarentolae* (WT) (1 x 10^7 cells/dose) or triple vaccine (1 x 10^7 cells/dose) on day -28 and -14. Mice cells were incubated with medium alone (control), ConA (5 µg/ml) or *L. maj soluble Ag* (25 µg/ml) and 72 hr. Nitrite levels in cell supernatants were determined using a Greiss assay * $p< 0.05$ compared to control, ** $p< 0.001$ compared to control, *** $p< 0.0001$ compared to control, n=3/ treatment.

5.3. Discussion

The results of this study demonstrated that the triple vaccine gave the highest level of protection against *L. major* infection compared to the other live vaccines tested, but all of the *L. tarentolae* live vaccine gave significant protection against infection. Thus, vaccination with *L.t L.maj* EGFP-γGCS-His parasites resulted in an 86% suppression in parasite burden compared to controls, vaccination with *L. tarentolae*-WT parasites caused a 44% suppression in parasite burden compared to controls; and the triple vaccine caused a 94% reduction in parasite burdens compared to controls. This is perhaps not unexpected as previous studies have shown that vaccination with *L. tarentolae* expressing a protein from a pathogenic *Leishmania* species protected against *Leishmania*. For example, *L. tarentolae* transfected with cysteine proteinases type I and II (CPB and CPA) elicited an increased expression of IFN-γ mRNA and a strong Th1-type response against parasite challenge (Rafati *et al.*, 2005). Protection was associated with increased IFN-γ and low IL-10 levels in vaccinated study groups before and after challenge (Fernandes *et al.*, 2008). And protection was associated with a predominant Th1 response based on the specific IgG2a/IgG1 ratios and cytokine production. Results from this study also showed that protective immunity was associated with Th1-type response and cytokine levels.

Sterile immunity was not achieved in this study but this may be improved by expressing more than one protein from pathogenic *Leishmania* in *L. tarentolae* or using a different vaccination protocol. For example, a prime-boost vaccine approach was used to induce protection but not sterile immunity against *L. major* where mice were primed with DNA encoding for the sand fly salivary PpSP15 protein and then boosted by immunising with the DNA vaccine and live *l. tarentolae* expressing cysteine proteinases (Taheri *et al.,* 2014). In future studies a 'prime and boost' approach could be used where mice are immunised with the triple vaccine and then boosted with recombinant γGCS protein or primed with protein and then boosted with live parasites. And the effect of increasing the number of vaccine doses given on protection should be determined.

In this study *L. major* promastigotes had higher BLI/parasite compared to amastigotes. 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). Similarly, it been shown that the amount of BLI emitted by *L. infantum* luciferase amastigotes depends on the activity of the amastigotes (Michel *et al.*, 2011).

The results of this study demonstrated that vaccination with *L. tarentolae* or transfected *L. tarentolae* transfected with γGCS from *L. major*, *L. mexicana* or *L. donovani* induced a specific IgG antibody response against the soluble *L. major* antigen produced from promastigotes, indicating that immunisation induced antibodies that recognized *L. major*-specific proteins. Studies have demonstrated that antibodies can interfere with binding and/or internalization of promastigotes by macrophages as well as parasites survival and multiplication (Deplazes *et al.*, 1995). On day 7 post-infection infected control and vaccinated mice had similar sized footpads and similar amounts of BLI emitted from their footpads. Therefore, antibodies present pre-infection in vaccinated mice did not appear to inhibit parasite growth. The reduction in parasite burdens observed in this study are therefore a result of immune responses post-infection. In CL susceptibility is associated with elevated IgG1 levels and elevated production of IL-10 levels by macrophages (Kane and Mosser, 2001, Buxbaum and Scott, 2005). IL-10 is a regulatory cytokine that has suppressive effects on immune responses function, targeting multiple activation and antigen presentation pathways of macrophages and dendritic cells (Mcfarlane *et al.*, 2011). In this study, there was no significant difference in the level of IL-10 for vaccinated groups and the infected control, indicating that down regulation of IL-10 was not associated with protection. Protection in this study was however associated with enhanced production of IFN-γ by antigen stimulated splenocytes and popliteal lymph node cells, with lymph node cells producing the higher amounts. IFN-γ can stimulated a number of immunological responses that can protect against *Leishmania*. Production of IFN-γ induces the expression of inducible nitric oxide synthase

(iNOS or NOS_2) by macrophages (Horta *et al.*, 2012). Neutrophils and macrophages produce NO in response to IFN-γ production and a second signal delivered by a pathogenic-associated molecular patterns (PAMP) ligand or IFN-γ which iNOS indorses the oxidation of the guanidine nitrogen of L-arginine, resulting in the production of NO and citrulline (Nathan and Shiloh, 2000). NO and superoxide are produced in activated macrophages and generate peroxynitrite ($ONOO^-$), a free radical that indicated to be highly toxic to *Leishmania* infection (Radi *et al.*, 2001). The higher levels of IFN-γ would also explain the enhanced specific IgG2a levels in vaccinated mice, and the predominant Th1 response, especially in moce given the triple vaccine where the ratio of IgG2a/IgG1 was well in excess of 1. A higher ratio of IgG2a/IgG1 was associated with protective immunity against *L. major* by other researchers (Rostamian *et al.*, 2017).

IL-5 production can be used as an indicator of Th2-type response (Cummings *et al.*, 2010), which is associated with susceptibility to *L. major* infection (Matthews *et al.*, 2000). In this study vaccination with the triple vaccine was associated with enhanced Il-5 production by popliteal lymph nodes cells/splenocytes in response to specific antigen in lymphocyte proliferation assays. However IL-5 can also have a protective role as IL-5 stimulates eosinophil maturation and migration, and these cells can suppress lesion development and cause parasite killing (Rodriguez and Wilson, 2014).

The results of this study showed that local immune responses were more predominant than systematic immune response based on the level of cytokines produced by splenocytes and lymph node cells in proliferation assays. *L. major* infection is known to attenuate T cell stimulatory functions of dendritic cells and suppress local immune response against parasite antigen (Makala *et al.*, 2011). Lymph node cells from with *L. major* infected mice have increased expression of indoleamine 2,3 dioxygenase (IDO), which reduces the host proinflammatory immune responses and can enhance parasite burdens. Therefore, local immune response is the most important in resistance to *L. major.* Futures studies should determine the roles of IL-4, IL-17 and the IDO in the triple vaccine model.

Vaccination with live *L. tarentolae* transfected with triple vaccine can be persist in the host by monitoring the parasites through the course infection by measuring the fluorescence intensity due to the GFP production. This method can indicate how long the parasites can stay alive inside the host.

In conclusion, this study showed that:

1. Vaccination with transgenic *L. tarentolae*- EGFP-γGCS- protected mice against *L. major* infection. Vaccination with triple vaccine gave the highest significant protection

2. Protection was associated with an enhanced Th1 response.

CHAPTER 6. CONCLUSIONS AND FUTURE WORK

Leishmaniasis is an infectious disease caused by the protozoan parasite *Leishmania*. At present, there are a limited number of drugs available for the treatment of Leishmaniasis and ideally a vaccine is required to prevent infection is required. Previous studies in our laboratory have shown that intramuscular immunisation with a plasmid containing the *L. donovani* gene sequence for γGCS 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), *L. major* and *L. mexicana* (Campbell *et al.*, 2012). However, they did not give sterile immunity against infection. Therefore, this study was carried out to determine if vaccination with recombinant γGCS produced using *L. tarentolae* would result in the production of full-length γGCS, of sufficient purity for a vaccine candidate.

The γGCS gene sequences of *L. donovani*, L*. major* and *L. mexicana*, tagged with GFP at the N-terminus and His at the C-terminus, were successfully cloned into the pSSU 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 GFP-γGCS-his expression cassettes for *L. donovani, L. mexicana and L. major* were integrated to the small locus of the 18S rRNA of the non-pathogenic species *L. tarentolae*. In this study, recombinant GFP-γGCS-his expression was driven by the endogenous RNA pol I promoter, which is based upstream of the 18S rRNA locus. Previous studies using pol I promoters have shown it to be highly expressed, resulting in high expression of the protein of interest (Misslitz *et al.*, 2000). One way to overcome this problem in this study would be to use foreign RNA polymerase e.g. T7 polymerase. This polymerase was 10 times more powerful than pol I in driving protein expression in Trypanosomatidae (Wirtz *et al.*, 1999). This promotor can also be controlled by a strong repressor such as tetracycline (TET) (Kushnir *et al.*, 2005). This could allow the development of an inducible system, where protein expression in *L. tarentolae* could be 'switched on' once the parasites reach a certain density. This would be similar to the *E. coli* system where protein is induced by beta galactosidase (Dalbow and Young, 1975) or isopropyl β-D-1-

thiogalactopyranoside (Marbach and Bettenbrock, 2012) treatment. Another alternative is to transfect the parasites again so that they contain additional copies of the foreign γGCS gene. This approach has been used in other study where a simultaneous electroporation approach to obtain double transfected cell lines was devised using a multiple simultaneous plasmid cassette to integrate into the genome of Saccharomyces *cerevisiae* (Yuan *et al.*, 2014).

Growing a large number of parasites may be another way to compensate for low recombinant protein production within transfected parasites. Studies were carried to identify a medium that would allow production of parasite cultures that would give 1-10 x 10^8 parasites/ml. Other researchers have suggested that this would be possible using a yeast extract medium, which is reported to give 1x 10^9 cells/ml (Fritsche *et al.*, 2007). This would have been ideal as this would have been a cheap medium for large scale production of parasites to produce the recombinant protein. However, it proved difficult to reproduce these studies and the best growth was obtained using yeast extract medium supplemented with 1% (v/v) FCS and 2.5 mg/ml of hemin, but this still did not give the high parasite densities required for recombinant protein expression. Improved growth could be obtained by supplementing the yeast extract medium with other additives e.g. proline, cysteine and glutamate. Studies have shown that *L. tarentolae* promastigotes grown in media supplemented with L-proline, glutamate or aspartate have improved oxygen uptake and that the parasite may have a proline-glutamate interconversion oxidation pathway such as proline oxidase metabolic pathway (Krassner, 1969). L-proline is used by *L. tarentolae* as the main source of carbon to produce alanine and recent studies have shown that L-proline can affect the irreversible conversion of gamma-glutamate semialdehyde into L-glutamate and NADH pathway in *Trypanosoma brucei* (Mantilla *et al.*, 2017).

GFP as a fusion partner, has been widely used as reporter molecule to track the expression or localization of recombinant proteins both *in vitro* and *in vivo*. For example, GFP gene expression from two inducible promoters was detected by flow cytometric measurement using GFP fluorescent intensity (Soboleski *et al.*, 2005). Similarly, assessment of GFP levels was used to determine the presence of

lentivector in the lymph nodes of mice and the ability of the virus to transdue cells was analysed using FACS (Esslinger *et al.*, 2003). Studies could be carried out to track antigen presentation and tracking recombinant protein uptake if a high enough concentration of pure GFP-TEV-γGCS-his protein was produced. It may also be possible to study the effect of supplement on protein expression by monitoring changes in fluorescence within parasites using a 96 well plate format and IVIS imaging or a plate reader. In future studies the effect of BSO, GSH or H_2O_2, treatment on γGCS expression by transfected *L. taerentolae* could be monitored using changes in fluorescence if a high enough parasite density was obtained. And assessing GFP expression could be a useful way to quality control parasites as good/poor producers of γGCS and identification of the best day for harvesting the cells for recombinant protein production. Differences in protein production for batches of parasites is highly likely to occur as studies in this project using luciferase-expressing *Leishmania* spp showed that the BLI signal varied over time.

Purification studies to isolate recombinant γGCS showed that the Ni-NTA column was the best affinity column for recovering soluble recombinant GFP-TEV-γGCS-his protein. It was possible to obtain full-length GFP-TEV-γGCS-his protein, but it was contaminated with truncated GFP-TEV-γGCS-his protein and other lower molecular weight his-tagged proteins. One possible way to prevent degradation of the GFP-TEV-γGCS-his would be to use bath sonication instead of the probe sonication, as this may decrease protein cleavage and increase the recovery of the protein during solubilising the proteins (Pchelintsev *et al.*, 2016). Another option is to make a new expression plasmid by removing the GFP tag from gene sequence so that the expressed protein only has the his-tag. This would reduce the size of the recombinant fusion protein to 80 KDa and could facilitate production of full length of recombinant γGCS. Other methods that could be tested to improve the recovery of full length soluble *Leishmania* γGCS would be to include ion exchange methods or gel filtration after using the nickel column affinity purification. If it is possible to obtain full length *Leishmania* γGCS then the next step would be to carry out crystallisation studies so that 3-D modelling could be used to identify the active site of the enzyme. This would allow modelling of a

specific inhibitor, based on BSO, that would inhibit *Leishmania* γGCS but not human γGCS. This parasite-specific inhibitor would be a rationale drug candidate for the treatment of Leishmaniasis because it is an essential enzyme for the parasites as it allows them to survive within activated MØ (Oza *et al*., 2005) as it helps parasites by protecting against oxidative stress (Mukherjee *et al.*, 2009). The study of Mukherjee and co-workers demonstrated that it was no possible to generate GSH1 null mutants of *L. infantum,* and that parasites generated an extra copy of the GSH1 gene. However, supplementing the parasites with a rescue plasmid containing GSH1 allowed generation of null chromosomal GSH1 mutants. This study revealed that the parasites were able to maintain the plasmid even in the absence of antibiotic selection. It may be possible to increase γGCS production by transfecting parasites with a plasmid containing the γGCS gene sequence.

Another way to improve *Leishmania* γGCS expression that could be tested is to change the affinity tag e.g. glutathione S-transferase (Young *et al*., 2012), c-Myc or Strep-tag®. These tags may give better protein recovery as they may give higher affinity binding to their respective affinity columns than the his-tag. This could be a single or double change e.g. by combining the Step-tag® with 8 histidine residues instead of the 6 used in this study (Kobayashi *et al.*, 2008).

Studies to produce of a secreted fusion protein containing γGCS and SAP1 or SAP 2 from *L. mexicana,* which could be harvested from the parasite culture medium, failed. However, replacing the SAP signal in the plasmid vector containing T7 polymerase in the downstream 3' is promising expression vector to produce inducible secreted protein (Breitling *et al.*, 2002).

Although *L. tarentolae* is phylogenetically related to pathogenic *Leishmania* species, it proved difficult to obtain enough recombinant γGCS protein to allow vaccine studies. Therefore, the transfected *L. tarentolae* parasites produced in this study were tested as a live vaccine against *L. major.* Vaccination with the triple vaccine gave the best protection against *L. major* infection but did not give sterile immunity against the parasites. However, this may be possible if a higher vaccine does was given e.g. 2×10^7 promastigotes/dose instead of 1×10^7/dose.

Or the number of vaccination could be increased from 2 to 3 or a 'prime and boost' schedule could be used (Pirdel and Farajnia, 2017, Katebi *et al.*, 2015, Zahedifard *et al.*, 2014). Thus, mice could be immunised with the live vaccine and then boosted with the recombinant DNA vaccine or recombinant protein or combinations from these three approaches. This type of approach has been used by Zahedifard and colleagues (Zahedifard *et al.*, 2014) where a *L. tarentolae* expressing cysteine proteinases (type I and II, CPA/CPB) live vaccine was combined with the sand fly salivary molecule PpSP15 as a DNA vaccine to protect mice against L. infantum infection. Instead of immunising with a mixture of parasites, using parasites transfected with the γGCS gene sequence of different pathogenic *Leishmania*, it would be possible to use parasites transfected with all three γGCS gene sequences since the 18S rRNA of the recipient parasite can be loaded with more than one gene. Thus, transfecting *L. tarentolae* with the DNA cassettes that code for the different γGCS for all three species may give a better live vaccine and one parasite could produce γGCS protein from all three species. Moreover, the triple vaccine can be tested against different type of *Leishmania* infections to prove that this vaccine is a potential universal vaccine against *Leishmania* infection. In addition, monitoring the infection by carrying out a time course study would give an idea of the length of time the parasites survival inside the host, if the parasites can induce infection in the host, or simply act by delivering the recombinant antigens for activation of protective immune responses.

The aim of this study was to produce full length γGCS, which is a relatively large protein. It may be easy to produce subunits of the protein, which could still be used as a vaccine, as it is often easier to express smaller recombinant proteins. Therefore, studies to identify T cell epitopes are required to identify polypeptide sequences that would drive the induction of protective memory T cells. For example, Ventura and colleagues investigated which peptide sequences of CMV glycoprotein G acted as the epitope responsible for stimulating the $CD4^+$ T cell and antibody responses (Ventura *et al.*, 2012).

In summary, this project identified a promising live vaccine candidate to protect against *Leishmania* infection. In addition, the plasmids and transfected parasites produced in this study could also be used to produce recombinant *Leishmania* γGCS in future studies and a number of additional avenues of research were identified.

REFERENCES

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.

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.

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.

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.

Ansari, N. A., Kumar, R., Gautam, S., Nylen, S., Singh, O. P., Sundar, S. & Sacks, D. 2011. IL-27 and IL-21 are associated with T cell IL-10 responses in human visceral leishmaniasis. *J Immunol,* 186**,** 3977-85.

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.

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.

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.

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.

Bajenoff, M., Breart, B., Huang, A. Y., Qi, H., Cazareth, J., Braud, V. M., Germain, R. N. & Glaichenhaus, N. 2006. Natural killer cell behavior in lymph nodes revealed by static and real-time imaging. *J Exp Med,* 203**,** 619-31.

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

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

Basak, A., Shervani, N. J., Mbikay, M. & Kolajova, M. 2008. Recombinant proprotein convertase 4 (PC4) from Leishmania tarentolae expression system: purification, biochemical study and inhibitor design. *Protein Expr Purif,* 60**,** 117-26.

Basile, G. & Peticca, M. 2009. Recombinant protein expression in Leishmania tarentolae. *Mol Biotechnol,* 43**,** 273-8.
Basu, M. K. & Ray, M. 2005. Macrophage and Leishmania: an unacceptable coexistence. *Crit Rev Microbiol,* 31**,** 145-54.
Beattie, L. & Kaye, P. M. 2011. Leishmania-host interactions: what has imaging taught us? *Cell Microbiol,* 13**,** 1659-67.
Belkaid, Y., Piccirillo, C. A., Mendez, S., Shevach, E. M. & Sacks, D. L. 2002. CD4+CD25+ regulatory T cells control Leishmania major persistence and immunity. *Nature,* 420**,** 502-7.
Bern, C. & Chowdhury, R. 2006. The epidemiology of visceral leishmaniasis in Bangladesh: prospects for improved control. *Indian J Med Res,* 123**,** 275-88.
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.
Besteiro, S., Williams, R. A., Coombs, G. H. & Mottram, J. C. 2007. Protein turnover and differentiation in Leishmania. *Int J Parasitol,* 37**,** 1063-75.
Bhandari, V., Kulshrestha, A., Deep, D. K., Stark, O., Prajapati, V. K., Ramesh, V., Sundar, S., Schonian, G., Dujardin, J. C. & Salotra, P. 2012. Drug susceptibility in Leishmania isolates following miltefosine treatment in cases of visceral leishmaniasis and post kala-azar dermal leishmaniasis. *PLoS Negl Trop Dis,* 6**,** e1657.
Birnbaum, R. & Craft, N. 2011. Innate immunity and Leishmania vaccination strategies. *Dermatol Clin,* 29**,** 89-102.
Boes, M., Cerny, J., Massol, R., Op Den Brouw, M., Kirchhausen, T., Chen, J. & Ploegh, H. L. 2002. T-cell engagement of dendritic cells rapidly rearranges MHC class II transport. *Nature,* 418**,** 983-8.
Bogdan, C. 2012. Natural killer cells in experimental and human leishmaniasis. *Front Cell Infect Microbiol,* 2**,** 69.
Bohlke, K., Pisani, F. M., Vorgias, C. E., Frey, B., Sobek, H., Rossi, M. & Antranikian, G. 2000. PCR performance of the B-type DNA polymerase from the thermophilic euryarchaeon Thermococcus aggregans improved by mutations in the Y-GG/A motif. *Nucleic Acids Res,* 28**,** 3910-7.
Bolhassani, A., Taheri, T., Taslimi, Y., Zamanilui, S., Zahedifard, F., Seyed, N., Torkashvand, F., Vaziri, B. & Rafati, S. 2011. Fluorescent Leishmania species: development of stable GFP expression and its application for in vitro and in vivo studies. *Exp Parasitol,* 127**,** 637-45.
Bornhorst, J. A. & Falke, J. J. 2000. Purification of proteins using polyhistidine affinity tags. *Methods Enzymol,* 326**,** 245-54.
Breitling, R., Klingner, S., Callewaert, N., Pietrucha, R., Geyer, A., Ehrlich, G., Hartung, R., Muller, A., Contreras, R., Beverley, S. M. & Alexandrov, K. 2002. Non-pathogenic trypanosomatid protozoa as a platform for protein research and production. *Protein Expr Purif,* 25**,** 209-18.
Breton, M., Tremblay, M. J., Ouellette, M. & Papadopoulou, B. 2005. Live nonpathogenic parasitic vector as a candidate vaccine against visceral leishmaniasis. *Infect Immun,* 73**,** 6372-82.

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.

Bryson, K. J., Wei, X. Q. & Alexander, J. 2008. Interleukin-18 enhances a Th2 biased response and susceptibility to Leishmania mexicana in BALB/c mice. *Microbes Infect,* 10**,** 834-9.

Bunn, P. T., Stanley, A. C., De Labastida Rivera, F., Mulherin, A., Sheel, M., Alexander, C. E., Faleiro, R. J., Amante, F. H., Montes De Oca, M., Best, S. E., James, K. R., Kaye, P. M., Haque, A. & Engwerda, C. R. 2014. Tissue requirements for establishing long-term CD4+ T cell-mediated immunity following Leishmania donovani infection. *J Immunol,* 192**,** 3709-18.

Burchmore, R. J. & Barrett, M. P. 2001. Life in vacuoles--nutrient acquisition by Leishmania amastigotes. *Int J Parasitol,* 31**,** 1311-20.

Buxbaum, L. U. & Scott, P. 2005. Interleukin 10- and Fcgamma receptor-deficient mice resolve Leishmania mexicana lesions. *Infect Immun,* 73**,** 2101-8.

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. 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**,** 1357-63.

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., 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.

Chaga, G., Hopp, J. & Nelson, P. 1999. Immobilized metal ion affinity chromatography on Co2+-carboxymethylaspartate-agarose Superflow, as demonstrated by one-step purification of lactate dehydrogenase from chicken breast muscle. *Biotechnol Appl Biochem,* 29 (Pt 1)**,** 19-24.

Chan, M. M., Bulinski, J. C., Chang, K. P. & Fong, D. 2003. A microplate assay for Leishmania amazonensis promastigotes expressing multimeric green fluorescent protein. *Parasitol Res,* 89**,** 266-71.

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.

Chang, C. S. & Chang, K. P. 1985. Heme requirement and acquisition by extracellular and intracellular stages of Leishmania mexicana amazonensis. *Mol Biochem Parasitol,* 16**,** 267-76.
Chang, K. P., Chang, C. S. & Sassa, S. 1975. Heme biosynthesis in bacterium-protozoon symbioses: enzymic defects in host hemoflagellates and complemental role of their intracellular symbiotes. *Proc Natl Acad Sci U S A,* 72**,** 2979-83.
Channon, J. Y. & Blackwell, J. M. 1985. A study of the sensitivity of Leishmania donovani promastigotes and amastigotes to hydrogen peroxide. I. Differences in sensitivity correlate with parasite-mediated removal of hydrogen peroxide. *Parasitology,* 91 (Pt 2)**,** 197-206.
Charest, H., Zhang, W. W. & Matlashewski, G. 1996. The developmental expression of Leishmania donovani A2 amastigote-specific genes is post-transcriptionally mediated and involves elements located in the 3'-untranslated region. *J Biol Chem,* 271**,** 17081-90.
Chavez-Fumagalli, M. A., Ribeiro, T. G., Castilho, R. O., Fernandes, S. O., Cardoso, V. N., Coelho, C. S., Mendonca, D. V., Soto, M., Tavares, C. A., Faraco, A. A. & Coelho, E. A. 2015. New delivery systems for amphotericin B applied to the improvement of leishmaniasis treatment. *Rev Soc Bras Med Trop,* 48**,** 235-42.
Chen, Z., He, Y., Shi, B. & Yang, D. 2013. Human serum albumin from recombinant DNA technology: challenges and strategies. *Biochim Biophys Acta,* 1830**,** 5515-25.
Cirillo, D. M., Valdivia, R. H., Monack, D. M. & Falkow, S. 1998. Macrophage-dependent induction of the Salmonella pathogenicity island 2 type III secretion system and its role in intracellular survival. *Mol Microbiol,* 30**,** 175-88.
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.
Clayton, C. E. 1999. Genetic manipulation of kinetoplastida. *Parasitol Today,* 15**,** 372-8.
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**,** 2216-21.
Coler, R. N. & Reed, S. G. 2005. Second-generation vaccines against leishmaniasis. *Trends Parasitol,* 21**,** 244-9.
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.

Crowe, J., Dobeli, H., Gentz, R., Hochuli, E., Stuber, D. & Henco, K. 1994. 6xHis-Ni-NTA chromatography as a superior technique in recombinant protein expression/purification. *Methods Mol Biol,* 31**,** 371-87.

Cummings, H. E., Tuladhar, R. & Satoskar, A. R. 2010. Cytokines and their STATs in cutaneous and visceral leishmaniasis. *J Biomed Biotechnol,* 2010**,** 294389.

D'aiuto, L., Robison, C. S., Gigante, M., Nwanegbo, E., Shaffer, B., Sukhwani, M., Castro, C. A. & Chaillet, J. R. 2008. Human IL-12 p40 as a reporter gene for high-throughput screening of engineered mouse embryonic stem cells. *BMC Biotechnol,* 8**,** 52.

Dahl, F., Gullberg, M., Stenberg, J., Landegren, U. & Nilsson, M. 2005. Multiplex amplification enabled by selective circularization of large sets of genomic DNA fragments. *Nucleic Acids Res,* 33**,** e71.

Dalbow, D. G. & Young, R. 1975. Synthesis time of beta-galactosidase in Escherichia coli B/r as a function of growth rate. *Biochem J,* 150**,** 13-20.

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.

Darocha, W. D., Silva, R. A., Bartholomeu, D. C., Pires, S. F., Freitas, J. M., Macedo, A. M., Vazquez, M. P., Levin, M. J. & Teixeira, S. M. 2004. Expression of exogenous genes in Trypanosoma cruzi: improving vectors and electroporation protocols. *Parasitol Res,* 92**,** 113-20.

Das, M., Mukherjee, S. B. & Shaha, C. 2001. Hydrogen peroxide induces apoptosis-like death in Leishmania donovani promastigotes. *J Cell Sci,* 114**,** 2461-9.

Das, V. N., Siddiqui, N. A., Pandey, K., Singh, V. P., Topno, R. K., Singh, D., Verma, R. B., Ranjan, A., Sinha, P. K. & Das, P. 2009. A controlled, randomized nonblinded clinical trial to assess the efficacy of amphotericin B deoxycholate as compared to pentamidine for the treatment of antimony unresponsive visceral leishmaniasis cases in Bihar, India. *Ther Clin Risk Manag,* 5**,** 117-24.

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

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

De Menezes, J. P., Saraiva, E. M. & Da Rocha-Azevedo, B. 2016. The site of the bite: Leishmania interaction with macrophages, neutrophils and the extracellular matrix in the dermis. *Parasit Vectors,* 9**,** 264.

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.

Deplazes, P., Smith, N. C., Arnold, P., Lutz, H. & Eckert, J. 1995. Specific IgG1 and IgG2 antibody responses of dogs to Leishmania infantum and other parasites. *Parasite Immunol,* 17**,** 451-8.

Dominguez, M., Moreno, I., Aizpurua, C. & Torano, A. 2003. Early mechanisms of Leishmania infection in human blood. *Microbes Infect,* 5**,** 507-13.

Dorlo, T. P., Balasegaram, M., Beijnen, J. H. & De Vries, P. J. 2012. Miltefosine: a review of its pharmacology and therapeutic efficacy in the treatment of leishmaniasis. *J Antimicrob Chemother,* 67**,** 2576-97.
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.
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. *Cell Microbiol,* 2**,** 365-77.
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.
Elliott, G. & O'hare, P. 1999. Live-cell analysis of a green fluorescent protein-tagged herpes simplex virus infection. *J Virol,* 73**,** 4110-9.
Engwerda, C. R., Ato, M. & Kaye, P. M. 2004. Macrophages, pathology and parasite persistence in experimental visceral leishmaniasis. *Trends Parasitol,* 20**,** 524-30.
Esslinger, C., Chapatte, L., Finke, D., Miconnet, I., Guillaume, P., Levy, F. & Macdonald, H. R. 2003. In vivo administration of a lentiviral vaccine targets DCs and induces efficient CD8(+) T cell responses. *J Clin Invest,* 111**,** 1673-81.
Evans, K. J. & Kedzierski, L. 2012. Development of Vaccines against Visceral Leishmaniasis. *J Trop Med,* 2012**,** 892817.
Faleiro, R. J., Kumar, R., Hafner, L. M. & Engwerda, C. R. 2014. Immune regulation during chronic visceral leishmaniasis. *PLoS Negl Trop Dis,* 8**,** e2914.
Fang, A., Gerson, D. F. & Demain, A. L. 2006. Menstrum for culture preservation and medium for seed preparation in a tetanus toxin production process containing no animal or dairy products. *Lett Appl Microbiol,* 43**,** 360-3.
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.
Ferguson, M. A. 1997. The surface glycoconjugates of trypanosomatid parasites. *Philos Trans R Soc Lond B Biol Sci,* 352**,** 1295-302.
Fernandes, A. P., Costa, M. M., Coelho, E. A., Michalick, M. S., De Freitas, E., Melo, M. N., Luiz Tafuri, W., Resende Dde, M., Hermont, V., Abrantes Cde, F. & Gazzinelli, R. T. 2008. Protective immunity against challenge with Leishmania (Leishmania) chagasi in beagle dogs vaccinated with recombinant A2 protein. *Vaccine,* 26**,** 5888-95.
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.
Forestier, C. L., Machu, C., Loussert, C., Pescher, P. & Spath, G. F. 2011. Imaging host cell-Leishmania interaction dynamics implicates parasite motility, lysosome recruitment, and host cell wounding in the infection process. *Cell Host Microbe,* 9**,** 319-30.
Fox, J. D. & Waugh, D. S. 2003. Maltose-binding protein as a solubility enhancer. *Methods Mol Biol,* 205**,** 99-117.
Frearson, J. A., Wyatt, P. G., Gilbert, I. H. & Fairlamb, A. H. 2007. Target assessment for antiparasitic drug discovery. *Trends Parasitol,* 23**,** 589-95.
Frezard, F., Demicheli, C. & Ribeiro, R. R. 2009. Pentavalent antimonials: new perspectives for old drugs. *Molecules,* 14**,** 2317-36.
Fritsche, C., Sitz, M., Weiland, N., Breitling, R. & Pohl, H. D. 2007. Characterization of the growth behavior of Leishmania tarentolae: a new expression system for recombinant proteins. *J Basic Microbiol,* 47**,** 384-93.
Fumarola, L., Spinelli, R. & Brandonisio, O. 2004. In vitro assays for evaluation of drug activity against Leishmania spp. *Res Microbiol,* 155**,** 224-30.
Ganguly, S., Das, N. K., Barbhuiya, J. N. & Chatterjee, M. 2010. Post-kala-azar dermal leishmaniasis--an overview. *Int J Dermatol,* 49**,** 921-31.
Gay, L. S., Wilson, M. E. & Donelson, J. E. 1996. The promoter for the ribosomal RNA genes of Leishmania chagasi. *Mol Biochem Parasitol,* 77**,** 193-200.
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.
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. & Lauletta Lindoso, J. A. 2012. Cutaneous and mucocutaneous leishmaniasis. *Infect Dis Clin North Am,* 26**,** 293-307.
Gupta, G., Oghumu, S. & Satoskar, A. R. 2013. Mechanisms of immune evasion in leishmaniasis. *Adv Appl Microbiol,* 82**,** 155-84.
Gurung, P. & Kanneganti, T. D. 2015. Innate immunity against Leishmania infections. *Cell Microbiol,* 17**,** 1286-94.
Halford, W. P., Balliet, J. W. & Gebhardt, B. M. 2004. Re-evaluating natural resistance to herpes simplex virus type 1. *J Virol,* 78**,** 10086-95.
Hammoda, N. A., Negm, A. Y., Hussein, E. D., El-Temsahy, M. M. & Rashwan, E. A. 1996. Leishmania major: leishmanicidal activity of activated macrophages by interferon gamma or tumour necrosis factor. *J Egypt Soc Parasitol,* 26**,** 555-66.
Handman, E. 2001. Leishmaniasis: current status of vaccine development. *Clin Microbiol Rev,* 14**,** 229-43.
Harhay, M. O., Olliaro, P. L., Costa, D. L. & Costa, C. H. 2011. Urban parasitology: visceral leishmaniasis in Brazil. *Trends Parasitol,* 27**,** 403-9.

Hemayatkar, M., Mahboudi, F., Majidzadeh, A. K., Davami, F., Vaziri, B., Barkhordari, F., Adeli, A., Mahdian, R. & Davoudi, N. 2010. Increased expression of recombinant human tissue plasminogen activator in Leishmania tarentolae. *Biotechnol J,* 5**,** 1198-206.

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.

Hochuli, E., Dobeli, H. & Schacher, A. 1987. New metal chelate adsorbent selective for proteins and peptides containing neighbouring histidine residues. *J Chromatogr,* 411**,** 177-84.

Horta, M. F., Mendes, B. P., Roma, E. H., Noronha, F. S., Macedo, J. P., Oliveira, L. S., Duarte, M. M. & Vieira, L. Q. 2012. Reactive oxygen species and nitric oxide in cutaneous leishmaniasis. *J Parasitol Res,* 2012**,** 203818.

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.

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.

Jongco, A. M., Ting, L. M., Thathy, V., Mota, M. M. & Kim, K. 2006. Improved transfection and new selectable markers for the rodent malaria parasite Plasmodium yoelii. *Mol Biochem Parasitol,* 146**,** 242-50.

Joshua M Mutiso, J. C. M., Thomas M Kariuki, Michael M Gicheru 2009. Montanide ISA 720 is more effective than BCG as an adjuvant for Leishmania killed vaccine in BALB/c mice. *International Journal of Integrative Biology*.

Kane, M. M. & Mosser, D. M. 2001. The role of IL-10 in promoting disease progression in leishmaniasis. *J Immunol,* 166**,** 1141-7.

Kapust, R. B. & Waugh, D. S. 2000. Controlled intracellular processing of fusion proteins by TEV protease. *Protein Expr Purif,* 19**,** 312-8.

Kashif, M., Manna, P. P., Akhter, Y., Alaidarous, M. & Rub, A. 2016. The Screening of novel inhibitors against Leishmania donovani Calcium ion channel to fight Leishmaniasis. *Infect Disord Drug Targets*.

Katebi, A., Gholami, E., Taheri, T., Zahedifard, F., Habibzadeh, S., Taslimi, Y., Shokri, F., Papadopoulou, B., Kamhawi, S., Valenzuela, J. G. & Rafati, S. 2015. Leishmania tarentolae secreting the sand fly salivary antigen PpSP15 confers protection against Leishmania major infection in a susceptible BALB/c mice model. *Mol Immunol,* 67**,** 501-11.

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.

Kaye, P. & Scott, P. 2011. Leishmaniasis: complexity at the host-pathogen interface. *Nat Rev Microbiol,* 9**,** 604-15.

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.
Khamesipour, A., Rafati, S., Davoudi, N., Maboudi, F. & Modabber, F. 2006. Leishmaniasis vaccine candidates for development: a global overview. *Indian J Med Res,* 123, 423-38.
Kima, P. E. & Soong, L. 2013. Interferon gamma in leishmaniasis. *Front Immunol,* 4, 156.
Kimura, A. & Kishimoto, T. 2010. IL-6: regulator of Treg/Th17 balance. *Eur J Immunol,* 40, 1830-5.
Kobayashi, T., Morone, N., Kashiyama, T., Oyamada, H., Kurebayashi, N. & Murayama, T. 2008. Engineering a novel multifunctional green fluorescent protein tag for a wide variety of protein research. *PLoS One,* 3, e3822.
Kobets, T., Grekov, I. & Lipoldova, M. 2012. Leishmaniasis: prevention, parasite detection and treatment. *Curr Med Chem,* 19, 1443-74.
Krassner, S. M. 1969. Proline metabolism in Leishmania tarentolae. *Exp Parasitol,* 24, 348-63.
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.
Kuchimaru, T., Iwano, S., Kiyama, M., Mitsumata, S., Kadonosono, T., Niwa, H., Maki, S. & Kizaka-Kondoh, S. 2016. A luciferin analogue generating near-infrared bioluminescence achieves highly sensitive deep-tissue imaging. *Nat Commun,* 7, 11856.
Kushnir, S., Gase, K., Breitling, R. & Alexandrov, K. 2005. Development of an inducible protein expression system based on the protozoan host Leishmania tarentolae. *Protein Expr Purif,* 42, 37-46.
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.
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.
Li, Y. 2011. Recombinant production of antimicrobial peptides in Escherichia coli: a review. *Protein Expr Purif,* 80, 260-7.
Lu, S. C. 2009. Regulation of glutathione synthesis. *Mol Aspects Med,* 30, 42-59.
Makala, L. H., Baban, B., Lemos, H., El-Awady, A. R., Chandler, P. R., Hou, D. Y., Munn, D. H. & Mellor, A. L. 2011. Leishmania major attenuates host immunity by stimulating local indoleamine 2,3-dioxygenase expression. *J Infect Dis,* 203, 715-25.
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.
Mantilla, B. S., Marchese, L., Casas-Sanchez, A., Dyer, N. A., Ejeh, N., Biran, M., Bringaud, F., Lehane, M. J., Acosta-Serrano, A. & Silber, A. M. 2017. Proline

Metabolism is Essential for Trypanosoma brucei brucei Survival in the Tsetse Vector. *PLoS Pathog,* 13**,** e1006158.
Marbach, A. & Bettenbrock, K. 2012. lac operon induction in Escherichia coli: Systematic comparison of IPTG and TMG induction and influence of the transacetylase LacA. *J Biotechnol,* 157**,** 82-8.
Matthews, D. J., Emson, C. L., Mckenzie, G. J., Jolin, H. E., Blackwell, J. M. & Mckenzie, A. N. 2000. IL-13 is a susceptibility factor for Leishmania major infection. *J Immunol,* 164**,** 1458-62.
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.
Mccall, L. I., Zhang, W. W., Ranasinghe, S. & Matlashewski, G. 2013. Leishmanization revisited: immunization with a naturally attenuated cutaneous Leishmania donovani isolate from Sri Lanka protects against visceral leishmaniasis. *Vaccine,* 31**,** 1420-5.
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. 2014. Leishmaniasis: clinical syndromes and treatment. *QJM,* 107**,** 7-14.
Meehan, H. A., Lundberg, R. A. & Connell, G. J. 2000. A trypanosomatid protein specifically interacts with a mammalian iron-responsive element. *Parasitol Res,* 86**,** 109-14.
Merlen, T., Sereno, D., Brajon, N., Rostand, F. & Lemesre, J. L. 1999. Leishmania spp: completely defined medium without serum and macromolecules (CDM/LP) for the continuous in vitro cultivation of infective promastigote forms. *Am J Trop Med Hyg,* 60**,** 41-50.
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.
Misslitz, A., Mottram, J. C., Overath, P. & Aebischer, T. 2000. Targeted integration into a rRNA locus results in uniform and high level expression of transgenes in Leishmania amastigotes. *Mol Biochem Parasitol,* 107**,** 251-61.
Mittra, B., Cortez, M., Haydock, A., Ramasamy, G., Myler, P. J. & Andrews, N. W. 2013. Iron uptake controls the generation of Leishmania infective forms through regulation of ROS levels. *J Exp Med,* 210**,** 401-16.
Modabber, F. 2010. Leishmaniasis vaccines: past, present and future. *Int J Antimicrob Agents,* 36 Suppl 1**,** S58-61.
Mollinedo, F., Janssen, H., De La Iglesia-Vicente, J., Villa-Pulgarin, J. A. & Calafat, J. 2010. Selective fusion of azurophilic granules with Leishmania-containing phagosomes in human neutrophils. *J Biol Chem,* 285**,** 34528-36.

Momeni, A. Z., Jalayer, T., Emamjomeh, M., Khamesipour, A., Zicker, F., Ghassemi, R. L., Dowlati, Y., Sharifi, I., Aminjavaheri, M., Shafiei, A., Alimohammadian, M. H., Hashemi-Fesharki, R., Nasseri, K., Godal, T., Smith, P. G. & Modabber, F. 1999. A randomised, double-blind, controlled trial of a killed L. major vaccine plus BCG against zoonotic cutaneous leishmaniasis in Iran. *Vaccine,* 17**,** 466-72.

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.

Moravej, A., Rasouli, M., Asaei, S., Kalani, M. & Mansoori, Y. 2013. Association of interleukin-18 gene variants with susceptibility to visceral leishmaniasis in Iranian population. *Mol Biol Rep,* 40**,** 4009-14.

Moskaug, J. O., Carlsen, H., Myhrstad, M. C. & Blomhoff, R. 2005. Polyphenols and glutathione synthesis regulation. *Am J Clin Nutr,* 81**,** 277S-283S.

Mottram, J. C., Frame, M. J., Brooks, D. R., Tetley, L., Hutchison, J. E., Souza, A. E. & Coombs, G. H. 1997. The multiple cpb cysteine proteinase genes of Leishmania mexicana encode isoenzymes that differ in their stage regulation and substrate preferences. *J Biol Chem,* 272**,** 14285-93.

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., 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.

Mullen, A. B., Lawrence, C. E., Mcfarlane, E., Wei, X. Q. & Carter, K. C. 2006. Endogenous interleukin-18 is involved in immunity to Leishmania donovani but its absence does not adversely influence the therapeutic activity of sodium stibogluconate. *Immunology,* 119**,** 348-54.

Mureev, S., Kovtun, O., Nguyen, U. T. & Alexandrov, K. 2009. Species-independent translational leaders facilitate cell-free expression. *Nat Biotechnol,* 27**,** 747-52.

Mureev, S., Kushnir, S., Kolesnikov, A. A., Breitling, R. & Alexandrov, K. 2007. Construction and analysis of Leishmania tarentolae transgenic strains free of selection markers. *Mol Biochem Parasitol,* 155**,** 71-83.

Murray, H. W. & Nathan, C. F. 1988. In vivo killing of intracellular visceral Leishmania donovani by a macrophage-targeted hydrogen peroxide-generating system. *J Infect Dis,* 158**,** 1372-5.

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.

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.
Nandi, N., Sen, A., Banerjee, R., Kumar, S., Kumar, V., Ghosh, A. N. & Das, P. 2010. Hydrogen peroxide induces apoptosis-like death in Entamoeba histolytica trophozoites. *Microbiology,* 156**,** 1926-41.
Nascimento, I. P. & Leite, L. C. 2012. Recombinant vaccines and the development of new vaccine strategies. *Braz J Med Biol Res,* 45**,** 1102-11.
Nathan, C. & Shiloh, M. U. 2000. Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. *Proc Natl Acad Sci U S A,* 97**,** 8841-8.
Navin, T. R., Krug, E. C. & Pearson, R. D. 1989. Effect of immunoglobulin M from normal human serum on Leishmania donovani promastigote agglutination, complement-mediated killing, and phagocytosis by human monocytes. *Infect Immun,* 57**,** 1343-6.
Neuber, H. 2008. Leishmaniasis. *J Dtsch Dermatol Ges,* 6**,** 754-65.
Niculae, A., Bayer, P., Cirstea, I., Bergbrede, T., Pietrucha, R., Gruen, M., Breitling, R. & Alexandrov, K. 2006. Isotopic labeling of recombinant proteins expressed in the protozoan host Leishmania tarentolae. *Protein Expr Purif,* 48**,** 167-72.
Nylen, S. & Eidsmo, L. 2012. Tissue damage and immunity in cutaneous leishmaniasis. *Parasite Immunol,* 34**,** 551-61.
Nylen, S. & Sacks, D. 2007. Interleukin-10 and the pathogenesis of human visceral leishmaniasis. *Trends Immunol,* 28**,** 378-84.
O'daly, J. A. & Rodriguez, M. B. 1988. Differential growth requirements of several Leishmania spp. in chemically defined culture media. *Acta Trop,* 45**,** 109-26.
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. & Uzonna, J. 2009. Vaccines and vaccination strategies against human cutaneous leishmaniasis. *Hum Vaccin,* 5**,** 291-301.
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.
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.
Pal, J. K. & Joshi-Purandare, M. 2001. Dose-dependent differential effect of hemin on protein synthesis and cell proliferation in Leishmania donovani promastigotes cultured in vitro. *J Biosci,* 26**,** 225-31.
Papadopoulou, B., Roy, G., Breton, M., Kundig, C., Dumas, C., Fillion, I., Singh, A. K., Olivier, M. & Ouellette, M. 2002. Reduced infectivity of a Leishmania donovani biopterin transporter genetic mutant and its use as an attenuated strain for vaccination. *Infect Immun,* 70**,** 62-8.

Pappalardo, F., Pennisi, M., Castiglione, F. & Motta, S. 2010. Vaccine protocols optimization: in silico experiences. *Biotechnol Adv,* 28**,** 82-93.
Parenky, A., Myler, H., Amaravadi, L., Bechtold-Peters, K., Rosenberg, A., Kirshner, S. & Quarmby, V. 2014. New FDA draft guidance on immunogenicity. *AAPS J,* 16**,** 499-503.
Park, C. & Raines, R. T. 2000. Dimer formation by a "monomeric" protein. *Protein Sci,* 9**,** 2026-33.
Parodi, A. J. 1993. N-glycosylation in trypanosomatid protozoa. *Glycobiology,* 3**,** 193-9.
Pavli, A. & Maltezou, H. C. 2010. Leishmaniasis, an emerging infection in travelers. *Int J Infect Dis,* 14**,** e1032-9.
Pchelintsev, N. A., Adams, P. D. & Nelson, D. M. 2016. Critical Parameters for Efficient Sonication and Improved Chromatin Immunoprecipitation of High Molecular Weight Proteins. *PLoS One,* 11**,** e0148023.
Pearson, R. D. & Steigbigel, R. T. 1980. Mechanism of lethal effect of human serum upon Leishmania donovani. *J Immunol,* 125**,** 2195-201.
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.
Phan, H. P., Sugino, M. & Niimi, T. 2009. The production of recombinant human laminin-332 in a Leishmania tarentolae expression system. *Protein Expr Purif,* 68**,** 79-84.
Pingoud, A. & Jeltsch, A. 2001. Structure and function of type II restriction endonucleases. *Nucleic Acids Res,* 29**,** 3705-27.
Pirdel, L. & Farajnia, S. 2017. A Non-pathogenic Recombinant Leishmania Expressing Lipophosphoglycan 3 Against Experimental Infection with Leishmania infantum. *Scand J Immunol,* 86**,** 15-22.
Porath, J., Carlsson, J., Olsson, I. & Belfrage, G. 1975. Metal chelate affinity chromatography, a new approach to protein fractionation. *Nature,* 258**,** 598-9.
Radi, R., Peluffo, G., Alvarez, M. N., Naviliat, M. & Cayota, A. 2001. Unraveling peroxynitrite formation in biological systems. *Free Radic Biol Med,* 30**,** 463-88.
Rafati, S., Nakhaee, A., Taheri, T., Taslimi, Y., Darabi, H., Eravani, D., Sanos, S., Kaye, P., Taghikhani, M., Jamshidi, S. & Rad, M. A. 2005. Protective vaccination against experimental canine visceral leishmaniasis using a combination of DNA and protein immunization with cysteine proteinases type I and II of L. infantum. *Vaccine,* 23**,** 3716-25.
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.
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.
Raymond, F., Boisvert, S., Roy, G., Ritt, J. F., Legare, D., Isnard, A., Stanke, M., Olivier, M., Tremblay, M. J., Papadopoulou, B., Ouellette, M. & Corbeil, J. 2012. Genome sequencing of the lizard parasite Leishmania tarentolae

reveals loss of genes associated to the intracellular stage of human pathogenic species. *Nucleic Acids Res,* 40**,** 1131-47.
Reiner, S. L. & Locksley, R. M. 1995. The regulation of immunity to Leishmania major. *Annu Rev Immunol,* 13**,** 151-77.
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.
Ricardo-Carter, C., Favila, M., Polando, R. E., Cotton, R. N., Bogard Horner, K., Condon, D., Ballhorn, W., Whitcomb, J. P., Yadav, M., Geister, R. L., Schorey, J. S. & Mcdowell, M. A. 2013. Leishmania major inhibits IL-12 in macrophages by signalling through CR3 (CD11b/CD18) and down-regulation of ETS-mediated transcription. *Parasite Immunol,* 35**,** 409-20.
Rodriguez, N. E. & Wilson, M. E. 2014. Eosinophils and mast cells in leishmaniasis. *Immunol Res,* 59**,** 129-41.
Rodriguez-Pinto, D., Navas, A., Blanco, V. M., Ramirez, L., Garcerant, D., Cruz, A., Craft, N. & Saravia, N. G. 2012. Regulatory T cells in the pathogenesis and healing of chronic human dermal leishmaniasis caused by Leishmania (Viannia) species. *PLoS Negl Trop Dis,* 6**,** e1627.
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., Tovar, J., Fonseca, S. G., Moraes, R. H., Cruz, A. K., Hothersall, J. S., Noronha-Dutra, A. A., Ferreira, S. H. & Cunha, F. Q. 2006. 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.
Rostamian, M., Sohrabi, S., Kavosifard, H. & Niknam, H. M. 2017. Lower levels of IgG1 in comparison with IgG2a are associated with protective immunity against Leishmania tropica infection in BALB/c mice. *J Microbiol Immunol Infect,* 50**,** 160-166.
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**,** e00383-13.
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.
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.
Saljoughian, N., Taheri, T., Zahedifard, F., Taslimi, Y., Doustdari, F., Bolhassani, A., Doroud, D., Azizi, H., Heidari, K., Vasei, M., Namvar Asl, N., Papadopoulou, B. & Rafati, S. 2013. Development of novel prime-boost strategies based on a tri-gene fusion recombinant L. tarentolae vaccine against

experimental murine visceral leishmaniasis. *PLoS Negl Trop Dis,* 7**,** e2174.
Schmitt, J., Hess, H. & Stunnenberg, H. G. 1993. Affinity purification of histidine-tagged proteins. *Mol Biol Rep,* 18**,** 223-30.
Seibel, N. M., Eljouni, J., Nalaskowski, M. M. & Hampe, W. 2007. Nuclear localization of enhanced green fluorescent protein homomultimers. *Anal Biochem,* 368**,** 95-9.
Shahbazi, M., Zahedifard, F., Taheri, T., Taslimi, Y., Jamshidi, S., Shirian, S., Mahdavi, N., Hassankhani, M., Daneshbod, Y., Zarkesh-Esfahani, S. H., Papadopoulou, B. & Rafati, S. 2015. Evaluation of Live Recombinant Nonpathogenic Leishmania tarentolae Expressing Cysteine Proteinase and A2 Genes as a Candidate Vaccine against Experimental Canine Visceral Leishmaniasis. *PLoS One,* 10**,** e0132794.
Sharifi, I., Aflatoonian, M. R., Fekri, A. R., Hakimi Parizi, M., Aghaei Afshar, A., Khosravi, A., Sharifi, F., Aflatoonian, B., Khamesipour, A., Dowlati, Y., Modabber, F. & Nadim, A. 2015. A comprehensive review of cutaneous leishmaniasis in kerman province, southeastern iran-narrative review article. *Iran J Public Health,* 44**,** 299-307.
Sharma, U. & Singh, S. 2009. Immunobiology of leishmaniasis. *Indian J Exp Biol,* 47**,** 412-23.
Simpson, L., Frech, G. C. & Maslov, D. A. 1996. RNA editing in trypanosomatid mitochondria. *Methods Enzymol,* 264**,** 99-121.
Singh, B. & Sundar, S. 2012. Leishmaniasis: vaccine candidates and perspectives. *Vaccine,* 30**,** 3834-42.
Singh, N., Gupta, R., Jaiswal, A. K., Sundar, S. & Dube, A. 2009. Transgenic Leishmania donovani clinical isolates expressing green fluorescent protein constitutively for rapid and reliable ex vivo drug screening. *J Antimicrob Chemother,* 64**,** 370-4.
Singh, R. K., Srivastava, A. & Singh, N. 2012. Toll-like receptor signaling: a perspective to develop vaccine against leishmaniasis. *Microbiol Res,* 167**,** 445-51.
Singh, S., Sharma, U. & Mishra, J. 2011. Post-kala-azar dermal leishmaniasis: recent developments. *Int J Dermatol,* 50**,** 1099-108.
Sinha, P. K., Van Griensven, J., Pandey, K., Kumar, N., Verma, N., Mahajan, R., Kumar, P., Kumar, R., Das, P., Mitra, G., Flevaud, L., Ferreyra, C., Remartinez, D., Pece, M. & Palma, P. P. 2011. Liposomal amphotericin B for visceral leishmaniasis in human immunodeficiency virus-coinfected patients: 2-year treatment outcomes in Bihar, India. *Clin Infect Dis,* 53**,** e91-8.
Soboleski, M. R., Oaks, J. & Halford, W. P. 2005. Green fluorescent protein is a quantitative reporter of gene expression in individual eukaryotic cells. *Faseb j,* 19**,** 440-2.
Sodoyer, R. 2004. Expression systems for the production of recombinant pharmaceuticals. *BioDrugs,* 18**,** 51-62.
Solbach, W. & Laskay, T. 2000. The host response to Leishmania infection. *Adv Immunol,* 74**,** 275-317.
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. 2008. Modulation of dendritic cell function by Leishmania parasites. *J Immunol,* 180**,** 4355-60.
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.
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.
Stierhof, Y. D., Wiese, M., Ilg, T., Overath, P., Haner, M. & Aebi, U. 1998. Structure of a filamentous phosphoglycoprotein polymer: the secreted acid phosphatase of Leishmania mexicana. *J Mol Biol,* 282**,** 137-48.
Striepen, B., He, C. Y., Matrajt, M., Soldati, D. & Roos, D. S. 1998. Expression, selection, and organellar targeting of the green fluorescent protein in Toxoplasma gondii. *Mol Biochem Parasitol,* 92**,** 325-38.
Sundar, S. & Chakravarty, J. 2010. Liposomal amphotericin B and leishmaniasis: dose and response. *J Glob Infect Dis,* 2**,** 159-66.
Sundar, S. & Chatterjee, M. 2006. Visceral leishmaniasis - current therapeutic modalities. *Indian J Med Res,* 123**,** 345-52.
Sundar, S. & Olliaro, P. L. 2007. Miltefosine in the treatment of leishmaniasis: Clinical evidence for informed clinical risk management. *Ther Clin Risk Manag,* 3**,** 733-40.
Taheri, T., Seyed, N. & Rafati, S. 2016. DNA Integration in Leishmania Genome: An Application for Vaccine Development and Drug Screening. *Methods Mol Biol,* 1403**,** 603-22.
Teixeira, S. M. 1998. Control of gene expression in Trypanosomatidae. *Braz J Med Biol Res,* 31**,** 1503-16.
Torti, D. C. & Feldman, S. R. 2007. Interleukin-12, interleukin-23, and psoriasis: current prospects. *J Am Acad Dermatol,* 57**,** 1059-68.
Trinchieri, G. 1998. Interleukin-12: a cytokine at the interface of inflammation and immunity. *Adv Immunol,* 70**,** 83-243.
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.
Uliana, S. R., Fischer, W., Stempliuk, V. A. & Floeter-Winter, L. M. 1996. Structural and functional characterization of the Leishmania amazonensis ribosomal RNA promoter. *Mol Biochem Parasitol,* 76**,** 245-55.
Uzonna, J. E., Spath, G. F., Beverley, S. M. & Scott, P. 2004. Vaccination with phosphoglycan-deficient Leishmania major protects highly susceptible mice from virulent challenge without inducing a strong Th1 response. *J Immunol,* 172**,** 3793-7.
Uzonna, J. E., Wei, G., Yurkowski, D. & Bretscher, P. 2001. Immune elimination of Leishmania major in mice: implications for immune memory, vaccination, and reactivation disease. *J Immunol,* 167**,** 6967-74.
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.
Ventura, C., Bisceglia, H., Girerd-Chambaz, Y., Burdin, N. & Chaux, P. 2012. HLA-DR and HLA-DP restricted epitopes from human cytomegalovirus glycoprotein B recognized by CD4+ T-cell clones from chronically infected individuals. *J Clin Immunol,* 32**,** 1305-16.
Watts, T. H. 2005. TNF/TNFR family members in costimulation of T cell responses. *Annu Rev Immunol,* 23**,** 23-68.
Waugh, D. S. 2011. An overview of enzymatic reagents for the removal of affinity tags. *Protein Expr Purif,* 80**,** 283-93.
Wiese, M., Gorcke, I. & Overath, P. 1999. Expression and species-specific glycosylation of Leishmania mexicana secreted acid phosphatase in Leishmania major. *Mol Biochem Parasitol,* 102**,** 325-9.
Wiese, M., Ilg, T., Lottspeich, F. & Overath, P. 1995. Ser/Thr-rich repetitive motifs as targets for phosphoglycan modifications in Leishmania mexicana secreted acid phosphatase. *EMBO J,* 14**,** 1067-74.
Wirtz, E., Leal, S., Ochatt, C. & Cross, G. A. 1999. A tightly regulated inducible expression system for conditional gene knock-outs and dominant-negative genetics in Trypanosoma brucei. *Mol Biochem Parasitol,* 99**,** 89-101.
Wiwanitkit, V. 2012. Interest in paromomycin for the treatment of visceral leishmaniasis (kala-azar). *Ther Clin Risk Manag,* 8**,** 323-8.
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.
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.
Yuan, C., Gao, J., Guo, J., Bai, L., Marshall, C., Cai, Z., Wang, L. & Xiao, M. 2014. Dimethyl sulfoxide damages mitochondrial integrity and membrane potential in cultured astrocytes. *PLoS One,* 9**,** e107447.
Zahedifard, F., Gholami, E., Taheri, T., Taslimi, Y., Doustdari, F., Seyed, N., Torkashvand, F., Meneses, C., Papadopoulou, B., Kamhawi, S., Valenzuela, J. G. & Rafati, S. 2014. Enhanced protective efficacy of nonpathogenic recombinant leishmania tarentolae expressing cysteine proteinases combined with a sand fly salivary antigen. *PLoS Negl Trop Dis,* 8**,** e2751.
Zaph, C., Uzonna, J., Beverley, S. M. & Scott, P. 2004. Central memory T cells mediate long-term immunity to Leishmania major in the absence of persistent parasites. *Nat Med,* 10**,** 1104-10.
Zhang, T., He, X., Tsang, T. C. & Harris, D. T. 2004. SING: a novel strategy for identifying tumor-specific, CTL-recognized tumor antigens. *Faseb j,* 18**,** 600-2.
Zhang, W. W., Charest, H. & Matlashewski, G. 1995. The expression of biologically active human p53 in Leishmania cells: a novel eukaryotic system to produce recombinant proteins. *Nucleic Acids Res,* 23**,** 4073-80.

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.

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., 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.

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.

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.

Ansari, N. A., Kumar, R., Gautam, S., Nylen, S., Singh, O. P., Sundar, S. & Sacks, D. 2011. IL-27 and IL-21 are associated with T cell IL-10 responses in human visceral Leishmaniasis. *J Immunol,* 186**,** 3977-85.

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.

Bajenoff, M., Breart, B., Huang, A. Y., Qi, H., Cazareth, J., Braud, V. M., Germain, R. N. & Glaichenhaus, N. 2006. Natural killer cell behavior in lymph nodes revealed by static and real-time imaging. *J Exp Med,* 203**,** 619-31.

Barbieri, C. L. 2006. Immunology of canine Leishmaniasis. *Parasite Immunol,* 28**,** 329-37.

Basak, A., Shervani, N. J., Mbikay, M. & Kolajova, M. 2008. Recombinant proprotein convertase 4 (PC4) from Leishmania tarentolae expression system: purification, biochemical study and inhibitor design. *Protein Expr Purif,* 60**,** 117-26.

Basile, G. & Peticca, M. 2009. Recombinant protein expression in Leishmania tarentolae. *Mol Biotechnol,* 43**,** 273-8.

Basu, M. K. & Ray, M. 2005. Macrophage and Leishmania: an unacceptable coexistence. *Crit Rev Microbiol,* 31**,** 145-54.

Beattie, L. & Kaye, P. M. 2011. Leishmania-host interactions: what has imaging taught us? *Cell Microbiol,* 13**,** 1659-67.

Belkaid, Y., Piccirillo, C. A., Mendez, S., Shevach, E. M. & Sacks, D. L. 2002. CD4+CD25+ regulatory T cells control Leishmania major persistence and immunity. *Nature,* 420**,** 502-7.

Bern, C. & Chowdhury, R. 2006. The epidemiology of visceral Leishmaniasis in Bangladesh: prospects for improved control. *Indian J Med Res,* 123**,** 275-88.

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.

Bhandari, V., Kulshrestha, A., Deep, D. K., Stark, O., Prajapati, V. K., Ramesh, V., Sundar, S., Schonian, G., Dujardin, J. C. & Salotra, P. 2012. Drug susceptibility in Leishmania isolates following miltefosine treatment in cases of visceral Leishmaniasis and post kala-azar dermal Leishmaniasis. *PLoS Negl Trop Dis,* 6**,** e1657.

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

Boes, M., Cerny, J., Massol, R., Op Den Brouw, M., Kirchhausen, T., Chen, J. & Ploegh, H. L. 2002. T-cell engagement of dendritic cells rapidly rearranges MHC class II transport. *Nature,* 418**,** 983-8.

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

Bohlke, K., Pisani, F. M., Vorgias, C. E., Frey, B., Sobek, H., Rossi, M. & Antranikian, G. 2000. PCR performance of the B-type DNA polymerase from the thermophilic euryarchaeon Thermococcus aggregans improved by mutations in the Y-GG/A motif. *Nucleic Acids Res,* 28**,** 3910-7.

Bolhassani, A., Taheri, T., Taslimi, Y., Zamanilui, S., Zahedifard, F., Seyed, N., Torkashvand, F., Vaziri, B. & Rafati, S. 2011. Fluorescent Leishmania species: development of stable GFP expression and its application for in vitro and in vivo studies. *Exp Parasitol,* 127**,** 637-45.

Bornhorst, J. A. & Falke, J. J. 2000. Purification of proteins using polyhistidine affinity tags. *Methods Enzymol,* 326**,** 245-54.

Breitling, R., Klingner, S., Callewaert, N., Pietrucha, R., Geyer, A., Ehrlich, G., Hartung, R., Muller, A., Contreras, R., Beverley, S. M. & Alexandrov, K. 2002. Non-pathogenic trypanosomatid protozoa as a platform for protein research and production. *Protein Expr Purif,* 25**,** 209-18.

Breton, M., Tremblay, M. J., Ouellette, M. & Papadopoulou, B. 2005. Live nonpathogenic parasitic vector as a candidate vaccine against visceral Leishmaniasis. *Infect Immun,* 73**,** 6372-82.

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.

Bunn, P. T., Stanley, A. C., De Labastida Rivera, F., Mulherin, A., Sheel, M., Alexander, C. E., Faleiro, R. J., Amante, F. H., Montes De Oca, M., Best, S. E., James, K. R., Kaye, P. M., Haque, A. & Engwerda, C. R. 2014. Tissue requirements for establishing long-term CD4+ T cell-mediated immunity following Leishmania donovani infection. *J Immunol,* 192**,** 3709-18.

Burchmore, R. J. & Barrett, M. P. 2001. Life in vacuoles--nutrient acquisition by Leishmania amastigotes. *Int J Parasitol,* 31**,** 1311-20.

Buxbaum, L. U. & Scott, P. 2005. Interleukin 10- and Fcgamma receptor-deficient mice resolve Leishmania mexicana lesions. *Infect Immun,* 73**,** 2101-8.

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. 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**,** 1357-63.

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., 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.

Chaga, G., Hopp, J. & Nelson, P. 1999. Immobilized metal ion affinity chromatography on Co2+-carboxymethylaspartate-agarose Superflow, as demonstrated by one-step purification of lactate dehydrogenase from chicken breast muscle. *Biotechnol Appl Biochem,* 29 (Pt 1)**,** 19-24.

Chan, M. M., Bulinski, J. C., Chang, K. P. & Fong, D. 2003. A microplate assay for Leishmania amazonensis promastigotes expressing multimeric green fluorescent protein. *Parasitol Res,* 89**,** 266-71.

Chang, C. S. & Chang, K. P. 1985. Heme requirement and acquisition by extracellular and intracellular stages of Leishmania mexicana amazonensis. *Mol Biochem Parasitol,* 16**,** 267-76.

Chang, K. P., Chang, C. S. & Sassa, S. 1975. Heme biosynthesis in bacterium-protozoon symbioses: enzymic defects in host hemoflagellates and complemental role of their intracellular symbiotes. *Proc Natl Acad Sci U S A,* 72**,** 2979-83.

Channon, J. Y. & Blackwell, J. M. 1985. A study of the sensitivity of Leishmania donovani promastigotes and amastigotes to hydrogen peroxide. I. Differences in sensitivity correlate with parasite-mediated removal of hydrogen peroxide. *Parasitology,* 91 (Pt 2)**,** 197-206.

Charest, H., Zhang, W. W. & Matlashewski, G. 1996. The developmental expression of Leishmania donovani A2 amastigote-specific genes is post-transcriptionally mediated and involves elements located in the 3'-untranslated region. *J Biol Chem,* 271**,** 17081-90.

Chavez-Fumagalli, M. A., Ribeiro, T. G., Castilho, R. O., Fernandes, S. O., Cardoso, V. N., Coelho, C. S., Mendonca, D. V., Soto, M., Tavares, C. A., Faraco, A. A. & Coelho, E. A. 2015. New delivery systems for amphotericin B applied to the improvement of Leishmaniasis treatment. *Rev Soc Bras Med Trop,* 48**,** 235-42.

Chen, Z., He, Y., Shi, B. & Yang, D. 2013. Human serum albumin from recombinant DNA technology: challenges and strategies. *Biochim Biophys Acta,* 1830**,** 5515-25.

Cirillo, D. M., Valdivia, R. H., Monack, D. M. & Falkow, S. 1998. Macrophage-dependent induction of the Salmonella pathogenicity island 2 type III secretion system and its role in intracellular survival. *Mol Microbiol,* 30**,** 175-88.

Clayton, C. E. 1999. Genetic manipulation of kinetoplastida. *Parasitol Today,* 15**,** 372-8.

Coler, R. N. & Reed, S. G. 2005. Second-generation vaccines against Leishmaniasis. *Trends Parasitol,* 21**,** 244-9.
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.
Crowe, J., Dobeli, H., Gentz, R., Hochuli, E., Stuber, D. & Henco, K. 1994. 6xHis-Ni-NTA chromatography as a superior technique in recombinant protein expression/purification. *Methods Mol Biol,* 31**,** 371-87.
Cummings, H. E., Tuladhar, R. & Satoskar, A. R. 2010. Cytokines and their STATs in cutaneous and visceral Leishmaniasis. *J Biomed Biotechnol,* 2010**,** 294389.
D'aiuto, L., Robison, C. S., Gigante, M., Nwanegbo, E., Shaffer, B., Sukhwani, M., Castro, C. A. & Chaillet, J. R. 2008. Human IL-12 p40 as a reporter gene for high-throughput screening of engineered mouse embryonic stem cells. *BMC Biotechnol,* 8**,** 52.
Dahl, F., Gullberg, M., Stenberg, J., Landegren, U. & Nilsson, M. 2005. Multiplex amplification enabled by selective circularization of large sets of genomic DNA fragments. *Nucleic Acids Res,* 33**,** e71.
Dalbow, D. G. & Young, R. 1975. Synthesis time of beta-galactosidase in Escherichia coli B/r as a function of growth rate. *Biochem J,* 150**,** 13-20.
Darocha, W. D., Silva, R. A., Bartholomeu, D. C., Pires, S. F., Freitas, J. M., Macedo, A. M., Vazquez, M. P., Levin, M. J. & Teixeira, S. M. 2004. Expression of exogenous genes in Trypanosoma cruzi: improving vectors and electroporation protocols. *Parasitol Res,* 92**,** 113-20.
Das, M., Mukherjee, S. B. & Shaha, C. 2001. Hydrogen peroxide induces apoptosis-like death in Leishmania donovani promastigotes. *J Cell Sci,* 114**,** 2461-9.
Das, V. N., Siddiqui, N. A., Pandey, K., Singh, V. P., Topno, R. K., Singh, D., Verma, R. B., Ranjan, A., Sinha, P. K. & Das, P. 2009. A controlled, randomized nonblinded clinical trial to assess the efficacy of amphotericin B deoxycholate as compared to pentamidine for the treatment of antimony unresponsive visceral Leishmaniasis cases in Bihar, India. *Ther Clin Risk Manag,* 5**,** 117-24.
David, C. V. & Craft, N. 2009. Cutaneous and mucocutaneous Leishmaniasis. *Dermatol Ther,* 22**,** 491-502.
De Menezes, J. P., Saraiva, E. M. & Da Rocha-Azevedo, B. 2016. The site of the bite: Leishmania interaction with macrophages, neutrophils and the extracellular matrix in the dermis. *Parasit Vectors,* 9**,** 264.
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.
Deplazes, P., Smith, N. C., Arnold, P., Lutz, H. & Eckert, J. 1995. Specific IgG1 and IgG2 antibody responses of dogs to Leishmania infantum and other parasites. *Parasite Immunol,* 17**,** 451-8.
Dominguez, M., Moreno, I., Aizpurua, C. & Torano, A. 2003. Early mechanisms of Leishmania infection in human blood. *Microbes Infect,* 5**,** 507-13.

Dorlo, T. P., Balasegaram, M., Beijnen, J. H. & De Vries, P. J. 2012. Miltefosine: a review of its pharmacology and therapeutic efficacy in the treatment of Leishmaniasis. *J Antimicrob Chemother,* 67**,** 2576-97.
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.
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. *Cell Microbiol,* 2**,** 365-77.
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.
Elliott, G. & O'hare, P. 1999. Live-cell analysis of a green fluorescent protein-tagged herpes simplex virus infection. *J Virol,* 73**,** 4110-9.
Engwerda, C. R., Ato, M. & Kaye, P. M. 2004. Macrophages, pathology and parasite persistence in experimental visceral Leishmaniasis. *Trends Parasitol,* 20**,** 524-30.
Esslinger, C., Chapatte, L., Finke, D., Miconnet, I., Guillaume, P., Levy, F. & Macdonald, H. R. 2003. In vivo administration of a lentiviral vaccine targets DCs and induces efficient CD8(+) T cell responses. *J Clin Invest,* 111**,** 1673-81.
Evans, K. J. & Kedzierski, L. 2012. Development of Vaccines against Visceral Leishmaniasis. *J Trop Med,* 2012**,** 892817.
Faleiro, R. J., Kumar, R., Hafner, L. M. & Engwerda, C. R. 2014. Immune regulation during chronic visceral Leishmaniasis. *PLoS Negl Trop Dis,* 8**,** e2914.
Fang, A., Gerson, D. F. & Demain, A. L. 2006. Menstrum for culture preservation and medium for seed preparation in a tetanus toxin production process containing no animal or dairy products. *Lett Appl Microbiol,* 43**,** 360-3.
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.
Ferguson, M. A. 1997. The surface glycoconjugates of trypanosomatid parasites. *Philos Trans R Soc Lond B Biol Sci,* 352**,** 1295-302.
Fernandes, A. P., Costa, M. M., Coelho, E. A., Michalick, M. S., De Freitas, E., Melo, M. N., Luiz Tafuri, W., Resende Dde, M., Hermont, V., Abrantes Cde, F. & Gazzinelli, R. T. 2008. Protective immunity against challenge with Leishmania (Leishmania) chagasi in beagle dogs vaccinated with recombinant A2 protein. *Vaccine,* 26**,** 5888-95.
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.
Fox, J. D. & Waugh, D. S. 2003. Maltose-binding protein as a solubility enhancer. *Methods Mol Biol,* 205**,** 99-117.
Frezard, F., Demicheli, C. & Ribeiro, R. R. 2009. Pentavalent antimonials: new perspectives for old drugs. *Molecules,* 14**,** 2317-36.

Fritsche, C., Sitz, M., Weiland, N., Breitling, R. & Pohl, H. D. 2007. Characterization of the growth behavior of Leishmania tarentolae: a new expression system for recombinant proteins. *J Basic Microbiol,* 47**,** 384-93.
Fumarola, L., Spinelli, R. & Brandonisio, O. 2004. In vitro assays for evaluation of drug activity against Leishmania spp. *Res Microbiol,* 155**,** 224-30.
Ganguly, S., Das, N. K., Barbhuiya, J. N. & Chatterjee, M. 2010. Post-kala-azar dermal Leishmaniasis--an overview. *Int J Dermatol,* 49**,** 921-31.
Gay, L. S., Wilson, M. E. & Donelson, J. E. 1996. The promoter for the ribosomal RNA genes of Leishmania chagasi. *Mol Biochem Parasitol,* 77**,** 193-200.
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.
Goto, H. & Lauletta Lindoso, J. A. 2012. Cutaneous and mucocutaneous Leishmaniasis. *Infect Dis Clin North Am,* 26**,** 293-307.
Gupta, G., Oghumu, S. & Satoskar, A. R. 2013. Mechanisms of immune evasion in Leishmaniasis. *Adv Appl Microbiol,* 82**,** 155-84.
Gurung, P. & Kanneganti, T. D. 2015. Innate immunity against Leishmania infections. *Cell Microbiol,* 17**,** 1286-94.
Halford, W. P., Balliet, J. W. & Gebhardt, B. M. 2004. Re-evaluating natural resistance to herpes simplex virus type 1. *J Virol,* 78**,** 10086-95.
Hammoda, N. A., Negm, A. Y., Hussein, E. D., El-Temsahy, M. M. & Rashwan, E. A. 1996. Leishmania major: leishmanicidal activity of activated macrophages by interferon gamma or tumour necrosis factor. *J Egypt Soc Parasitol,* 26**,** 555-66.
Handman, E. 2001. Leishmaniasis: current status of vaccine development. *Clin Microbiol Rev,* 14**,** 229-43.
Hemayatkar, M., Mahboudi, F., Majidzadeh, A. K., Davami, F., Vaziri, B., Barkhordari, F., Adeli, A., Mahdian, R. & Davoudi, N. 2010. Increased expression of recombinant human tissue plasminogen activator in Leishmania tarentolae. *Biotechnol J,* 5**,** 1198-206.
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.
Hochuli, E., Dobeli, H. & Schacher, A. 1987. New metal chelate adsorbent selective for proteins and peptides containing neighbouring histidine residues. *J Chromatogr,* 411**,** 177-84.
Horta, M. F., Mendes, B. P., Roma, E. H., Noronha, F. S., Macedo, J. P., Oliveira, L. S., Duarte, M. M. & Vieira, L. Q. 2012. Reactive oxygen species and nitric oxide in cutaneous Leishmaniasis. *J Parasitol Res,* 2012**,** 203818.
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.

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.
Jongco, A. M., Ting, L. M., Thathy, V., Mota, M. M. & Kim, K. 2006. Improved transfection and new selectable markers for the rodent malaria parasite Plasmodium yoelii. *Mol Biochem Parasitol,* 146**,** 242-50.
Kane, M. M. & Mosser, D. M. 2001. The role of IL-10 in promoting disease progression in Leishmaniasis. *J Immunol,* 166**,** 1141-7.
Kapust, R. B. & Waugh, D. S. 2000. Controlled intracellular processing of fusion proteins by TEV protease. *Protein Expr Purif,* 19**,** 312-8.
Kashif, M., Manna, P. P., Akhter, Y., Alaidarous, M. & Rub, A. 2016. The Screening of novel inhibitors against Leishmania donovani Calcium ion channel to fight Leishmaniasis. *Infect Disord Drug Targets*.
Katebi, A., Gholami, E., Taheri, T., Zahedifard, F., Habibzadeh, S., Taslimi, Y., Shokri, F., Papadopoulou, B., Kamhawi, S., Valenzuela, J. G. & Rafati, S. 2015. Leishmania tarentolae secreting the sand fly salivary antigen PpSP15 confers protection against Leishmania major infection in a susceptible BALB/c mice model. *Mol Immunol,* 67**,** 501-11.
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.
Khamesipour, A., Rafati, S., Davoudi, N., Maboudi, F. & Modabber, F. 2006. Leishmaniasis vaccine candidates for development: a global overview. *Indian J Med Res,* 123**,** 423-38.
Kobayashi, T., Morone, N., Kashiyama, T., Oyamada, H., Kurebayashi, N. & Murayama, T. 2008. Engineering a novel multifunctional green fluorescent protein tag for a wide variety of protein research. *PLoS One,* 3**,** e3822.
Krassner, S. M. 1969. Proline metabolism in Leishmania tarentolae. *Exp Parasitol,* 24**,** 348-63.
Kuchimaru, T., Iwano, S., Kiyama, M., Mitsumata, S., Kadonosono, T., Niwa, H., Maki, S. & Kizaka-Kondoh, S. 2016. A luciferin analogue generating near-infrared bioluminescence achieves highly sensitive deep-tissue imaging. *Nat Commun,* 7**,** 11856.
Kushnir, S., Gase, K., Breitling, R. & Alexandrov, K. 2005. Development of an inducible protein expression system based on the protozoan host Leishmania tarentolae. *Protein Expr Purif,* 42**,** 37-46.
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.
Li, Y. 2011. Recombinant production of antimicrobial peptides in Escherichia coli: a review. *Protein Expr Purif,* 80**,** 260-7.
Makala, L. H., Baban, B., Lemos, H., El-Awady, A. R., Chandler, P. R., Hou, D. Y., Munn, D. H. & Mellor, A. L. 2011. Leishmania major attenuates host immunity by stimulating local indoleamine 2,3-dioxygenase expression. *J Infect Dis,* 203**,** 715-25.
Mantilla, B. S., Marchese, L., Casas-Sanchez, A., Dyer, N. A., Ejeh, N., Biran, M., Bringaud, F., Lehane, M. J., Acosta-Serrano, A. & Silber, A. M. 2017. Proline

Metabolism is Essential for Trypanosoma brucei brucei Survival in the Tsetse Vector. *PLoS Pathog,* 13**,** e1006158.
Marbach, A. & Bettenbrock, K. 2012. lac operon induction in Escherichia coli: Systematic comparison of IPTG and TMG induction and influence of the transacetylase LacA. *J Biotechnol,* 157**,** 82-8.
Matthews, D. J., Emson, C. L., Mckenzie, G. J., Jolin, H. E., Blackwell, J. M. & Mckenzie, A. N. 2000. IL-13 is a susceptibility factor for Leishmania major infection. *J Immunol,* 164**,** 1458-62.
Mccall, L. I., Zhang, W. W., Ranasinghe, S. & Matlashewski, G. 2013. Leishmanization revisited: immunization with a naturally attenuated cutaneous Leishmania donovani isolate from Sri Lanka protects against visceral Leishmaniasis. *Vaccine,* 31**,** 1420-5.
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. 2014. Leishmaniasis: clinical syndromes and treatment. *QJM,* 107**,** 7-14.
Meehan, H. A., Lundberg, R. A. & Connell, G. J. 2000. A trypanosomatid protein specifically interacts with a mammalian iron-responsive element. *Parasitol Res,* 86**,** 109-14.
Merlen, T., Sereno, D., Brajon, N., Rostand, F. & Lemesre, J. L. 1999. Leishmania spp: completely defined medium without serum and macromolecules (CDM/LP) for the continuous in vitro cultivation of infective promastigote forms. *Am J Trop Med Hyg,* 60**,** 41-50.
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.
Misslitz, A., Mottram, J. C., Overath, P. & Aebischer, T. 2000. Targeted integration into a rRNA locus results in uniform and high level expression of transgenes in Leishmania amastigotes. *Mol Biochem Parasitol,* 107**,** 251-61.
Mittra, B., Cortez, M., Haydock, A., Ramasamy, G., Myler, P. J. & Andrews, N. W. 2013. Iron uptake controls the generation of Leishmania infective forms through regulation of ROS levels. *J Exp Med,* 210**,** 401-16.
Mollinedo, F., Janssen, H., De La Iglesia-Vicente, J., Villa-Pulgarin, J. A. & Calafat, J. 2010. Selective fusion of azurophilic granules with Leishmania-containing phagosomes in human neutrophils. *J Biol Chem,* 285**,** 34528-36.
Momeni, A. Z., Jalayer, T., Emamjomeh, M., Khamesipour, A., Zicker, F., Ghassemi, R. L., Dowlati, Y., Sharifi, I., Aminjavaheri, M., Shafiei, A., Alimohammadian, M. H., Hashemi-Fesharki, R., Nasseri, K., Godal, T., Smith, P. G. & Modabber, F. 1999. A randomised, double-blind, controlled trial of a killed L. major vaccine plus BCG against zoonotic cutaneous Leishmaniasis in Iran. *Vaccine,* 17**,** 466-72.

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.
Moskaug, J. O., Carlsen, H., Myhrstad, M. C. & Blomhoff, R. 2005. Polyphenols and glutathione synthesis regulation. *Am J Clin Nutr,* 81**,** 277S-283S.
Mottram, J. C., Frame, M. J., Brooks, D. R., Tetley, L., Hutchison, J. E., Souza, A. E. & Coombs, G. H. 1997. The multiple cpb cysteine proteinase genes of Leishmania mexicana encode isoenzymes that differ in their stage regulation and substrate preferences. *J Biol Chem,* 272**,** 14285-93.
Mougneau, E., Bihl, F. & Glaichenhaus, N. 2011. Cell biology and immunology of Leishmania. *Immunol Rev,* 240**,** 286-96.
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.
Mureev, S., Kovtun, O., Nguyen, U. T. & Alexandrov, K. 2009. Species-independent translational leaders facilitate cell-free expression. *Nat Biotechnol,* 27**,** 747-52.
Mureev, S., Kushnir, S., Kolesnikov, A. A., Breitling, R. & Alexandrov, K. 2007. Construction and analysis of Leishmania tarentolae transgenic strains free of selection markers. *Mol Biochem Parasitol,* 155**,** 71-83.
Murray, H. W. & Nathan, C. F. 1988. In vivo killing of intracellular visceral Leishmania donovani by a macrophage-targeted hydrogen peroxide-generating system. *J Infect Dis,* 158**,** 1372-5.
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.
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.
Nandi, N., Sen, A., Banerjee, R., Kumar, S., Kumar, V., Ghosh, A. N. & Das, P. 2010. Hydrogen peroxide induces apoptosis-like death in Entamoeba histolytica trophozoites. *Microbiology,* 156**,** 1926-41.
Nathan, C. & Shiloh, M. U. 2000. Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. *Proc Natl Acad Sci U S A,* 97**,** 8841-8.
Navin, T. R., Krug, E. C. & Pearson, R. D. 1989. Effect of immunoglobulin M from normal human serum on Leishmania donovani promastigote agglutination, complement-mediated killing, and phagocytosis by human monocytes. *Infect Immun,* 57**,** 1343-6.
Neuber, H. 2008. Leishmaniasis. *J Dtsch Dermatol Ges,* 6**,** 754-65.
Niculae, A., Bayer, P., Cirstea, I., Bergbrede, T., Pietrucha, R., Gruen, M., Breitling, R. & Alexandrov, K. 2006. Isotopic labeling of recombinant proteins expressed in the protozoan host Leishmania tarentolae. *Protein Expr Purif,* 48**,** 167-72.
Nylen, S. & Eidsmo, L. 2012. Tissue damage and immunity in cutaneous Leishmaniasis. *Parasite Immunol,* 34**,** 551-61.

Nylen, S. & Sacks, D. 2007. Interleukin-10 and the pathogenesis of human visceral Leishmaniasis. *Trends Immunol,* 28**,** 378-84.
O'Daly – change thisO'daly, J. A. & Rodriguez, M. B. 1988. Differential growth requirements of several Leishmania spp. in chemically defined culture media. *Acta Trop,* 45**,** 109-26.
Okwor, I. & Uzonna, J. 2009. Vaccines and vaccination strategies against human cutaneous Leishmaniasis. *Hum Vaccin,* 5**,** 291-301.
Okwor, I. & Uzonna, J. E. 2013. The immunology of Leishmania/HIV co-infection. *Immunol Res,* 56**,** 163-71.
Pal, J. K. & Joshi-Purandare, M. 2001. Dose-dependent differential effect of hemin on protein synthesis and cell proliferation in Leishmania donovani promastigotes cultured in vitro. *J Biosci,* 26**,** 225-31.
Papadopoulou, B., Roy, G., Breton, M., Kundig, C., Dumas, C., Fillion, I., Singh, A. K., Olivier, M. & Ouellette, M. 2002. Reduced infectivity of a Leishmania donovani biopterin transporter genetic mutant and its use as an attenuated strain for vaccination. *Infect Immun,* 70**,** 62-8.
Pappalardo, F., Pennisi, M., Castiglione, F. & Motta, S. 2010. Vaccine protocols optimization: in silico experiences. *Biotechnol Adv,* 28**,** 82-93.
Parenky, A., Myler, H., Amaravadi, L., Bechtold-Peters, K., Rosenberg, A., Kirshner, S. & Quarmby, V. 2014. New FDA draft guidance on immunogenicity. *AAPS J,* 16**,** 499-503.
Park, C. & Raines, R. T. 2000. Dimer formation by a "monomeric" protein. *Protein Sci,* 9**,** 2026-33.
Parodi, A. J. 1993. N-glycosylation in trypanosomatid protozoa. *Glycobiology,* 3**,** 193-9.
Pavli, A. & Maltezou, H. C. 2010. Leishmaniasis, an emerging infection in travelers. *Int J Infect Dis,* 14**,** e1032-9.
Pchelintsev, N. A., Adams, P. D. & Nelson, D. M. 2016. Critical Parameters for Efficient Sonication and Improved Chromatin Immunoprecipitation of High Molecular Weight Proteins. *PLoS One,* 11**,** e0148023.
Pearson, R. D. & Steigbigel, R. T. 1980. Mechanism of lethal effect of human serum upon Leishmania donovani. *J Immunol,* 125**,** 2195-201.
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.
Phan, H. P., Sugino, M. & Niimi, T. 2009. The production of recombinant human laminin-332 in a Leishmania tarentolae expression system. *Protein Expr Purif,* 68**,** 79-84.
Pingoud, A. & Jeltsch, A. 2001. Structure and function of type II restriction endonucleases. *Nucleic Acids Res,* 29**,** 3705-27.
Pirdel, L. & Farajnia, S. 2017. A Non-pathogenic Recombinant Leishmania Expressing Lipophosphoglycan 3 Against Experimental Infection with Leishmania infantum. *Scand J Immunol,* 86**,** 15-22.
Porath, J., Carlsson, J., Olsson, I. & Belfrage, G. 1975. Metal chelate affinity chromatography, a new approach to protein fractionation. *Nature,* 258**,** 598-9.

Radi, R., Peluffo, G., Alvarez, M. N., Naviliat, M. & Cayota, A. 2001. Unraveling peroxynitrite formation in biological systems. *Free Radic Biol Med,* 30**,** 463-88.

Rafati, S., Nakhaee, A., Taheri, T., Taslimi, Y., Darabi, H., Eravani, D., Sanos, S., Kaye, P., Taghikhani, M., Jamshidi, S. & Rad, M. A. 2005. Protective vaccination against experimental canine visceral Leishmaniasis using a combination of DNA and protein immunization with cysteine proteinases type I and II of L. infantum. *Vaccine,* 23**,** 3716-25.

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.

Reiner, S. L. & Locksley, R. M. 1995. The regulation of immunity to Leishmania major. *Annu Rev Immunol,* 13**,** 151-77.

Ricardo-Carter, C., Favila, M., Polando, R. E., Cotton, R. N., Bogard Horner, K., Condon, D., Ballhorn, W., Whitcomb, J. P., Yadav, M., Geister, R. L., Schorey, J. S. & Mcdowell, M. A. 2013. Leishmania major inhibits IL-12 in macrophages by signalling through CR3 (CD11b/CD18) and down-regulation of ETS-mediated transcription. *Parasite Immunol,* 35**,** 409-20.

Rodriguez, N. E. & Wilson, M. E. 2014. Eosinophils and mast cells in Leishmaniasis. *Immunol Res,* 59**,** 129-41.

Rodriguez-Pinto, D., Navas, A., Blanco, V. M., Ramirez, L., Garcerant, D., Cruz, A., Craft, N. & Saravia, N. G. 2012. Regulatory T cells in the pathogenesis and healing of chronic human dermal Leishmaniasis caused by Leishmania (Viannia) species. *PLoS Negl Trop Dis,* 6**,** e1627.

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. 2006. 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.

Rostamian, M., Sohrabi, S., Kavosifard, H. & Niknam, H. M. 2017. Lower levels of IgG1 in comparison with IgG2a are associated with protective immunity against Leishmania tropica infection in BALB/c mice. *J Microbiol Immunol Infect,* 50**,** 160-166.

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.

Saljoughian, N., Taheri, T., Zahedifard, F., Taslimi, Y., Doustdari, F., Bolhassani, A., Doroud, D., Azizi, H., Heidari, K., Vasei, M., Namvar Asl, N., Papadopoulou, B. & Rafati, S. 2013. Development of novel prime-boost strategies based on a tri-gene fusion recombinant L. tarentolae vaccine against experimental murine visceral Leishmaniasis. *PLoS Negl Trop Dis,* 7**,** e2174.

Schmitt, J., Hess, H. & Stunnenberg, H. G. 1993. Affinity purification of histidine-tagged proteins. *Mol Biol Rep,* 18**,** 223-30.

Seibel, N. M., Eljouni, J., Nalaskowski, M. M. & Hampe, W. 2007. Nuclear localization of enhanced green fluorescent protein homomultimers. *Anal Biochem,* 368**,** 95-9.

Shahbazi, M., Zahedifard, F., Taheri, T., Taslimi, Y., Jamshidi, S., Shirian, S., Mahdavi, N., Hassankhani, M., Daneshbod, Y., Zarkesh-Esfahani, S. H., Papadopoulou, B. & Rafati, S. 2015. Evaluation of Live Recombinant Nonpathogenic Leishmania tarentolae Expressing Cysteine Proteinase and A2 Genes as a Candidate Vaccine against Experimental Canine Visceral Leishmaniasis. *PLoS One,* 10**,** e0132794.

Sharifi, I., Aflatoonian, M. R., Fekri, A. R., Hakimi Parizi, M., Aghaei Afshar, A., Khosravi, A., Sharifi, F., Aflatoonian, B., Khamesipour, A., Dowlati, Y., Modabber, F. & Nadim, A. 2015. A comprehensive review of cutaneous Leishmaniasis in kerman province, southeastern iran-narrative review article. *Iran J Public Health,* 44**,** 299-307.

Sharma, U. & Singh, S. 2009. Immunobiology of Leishmaniasis. *Indian J Exp Biol,* 47**,** 412-23.

Simpson, L., Frech, G. C. & Maslov, D. A. 1996. RNA editing in trypanosomatid mitochondria. *Methods Enzymol,* 264**,** 99-121.

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

Singh, N., Gupta, R., Jaiswal, A. K., Sundar, S. & Dube, A. 2009. Transgenic Leishmania donovani clinical isolates expressing green fluorescent protein constitutively for rapid and reliable ex vivo drug screening. *J Antimicrob Chemother,* 64**,** 370-4.

Singh, R. K., Srivastava, A. & Singh, N. 2012. Toll-like receptor signaling: a perspective to develop vaccine against Leishmaniasis. *Microbiol Res,* 167**,** 445-51.

Singh, S., Sharma, U. & Mishra, J. 2011. Post-kala-azar dermal Leishmaniasis: recent developments. *Int J Dermatol,* 50**,** 1099-108.

Sinha, P. K., Van Griensven, J., Pandey, K., Kumar, N., Verma, N., Mahajan, R., Kumar, P., Kumar, R., Das, P., Mitra, G., Flevaud, L., Ferreyra, C., Remartinez, D., Pece, M. & Palma, P. P. 2011. Liposomal amphotericin B for visceral Leishmaniasis in human immunodeficiency virus-coinfected patients: 2-year treatment outcomes in Bihar, India. *Clin Infect Dis,* 53**,** e91-8.

Soboleski, M. R., Oaks, J. & Halford, W. P. 2005. Green fluorescent protein is a quantitative reporter of gene expression in individual eukaryotic cells. *Faseb j,* 19**,** 440-2.

Sodoyer, R. 2004. Expression systems for the production of recombinant pharmaceuticals. *BioDrugs,* 18**,** 51-62.

Soong, L. 2008. Modulation of dendritic cell function by Leishmania parasites. *J Immunol,* 180**,** 4355-60.

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.

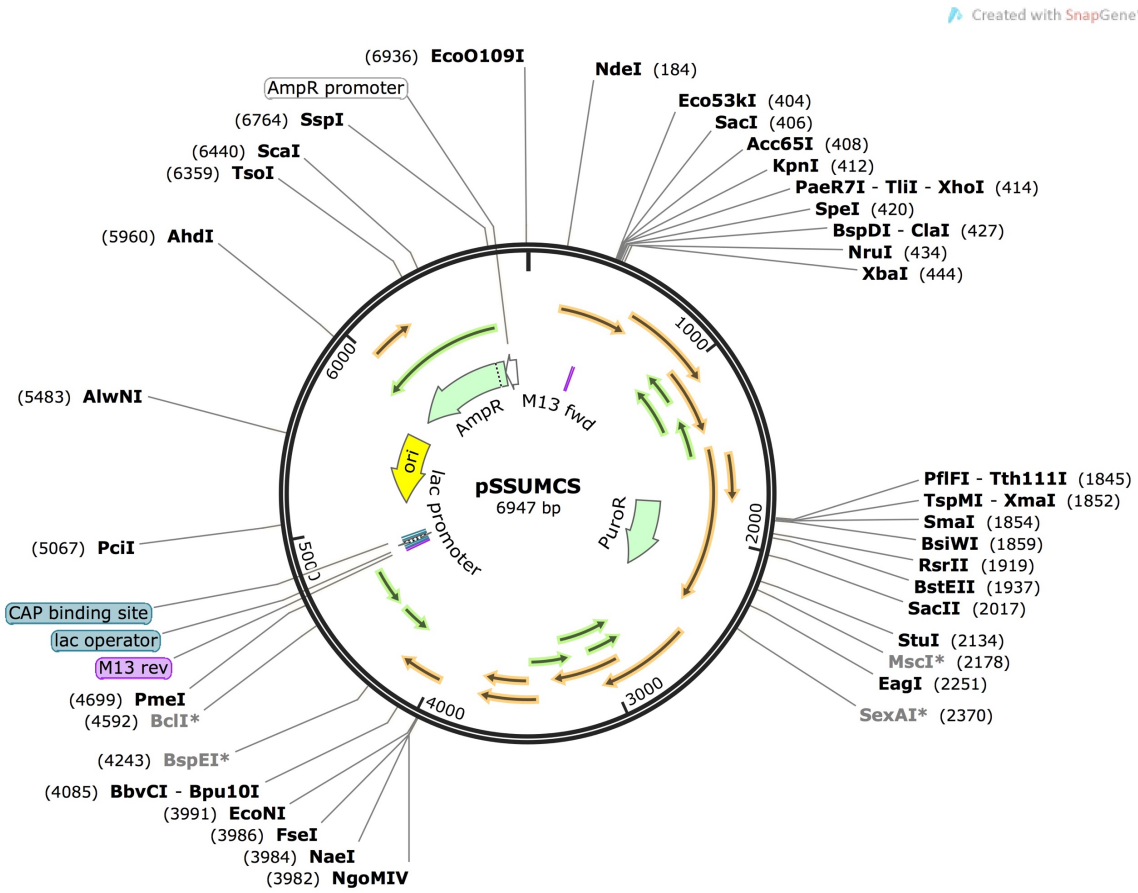
Spath, G. F., Epstein, L., Leader, B., Singer, S. M., Avila, H. A., Turco, S. J. & Beverley, S. M. 2000. Lipophosphoglycan is a virulence factor distinct from related glycoconjugates in the protozoan parasite Leishmania major. *Proc Natl Acad Sci U S A,* 97**,** 9258-63.

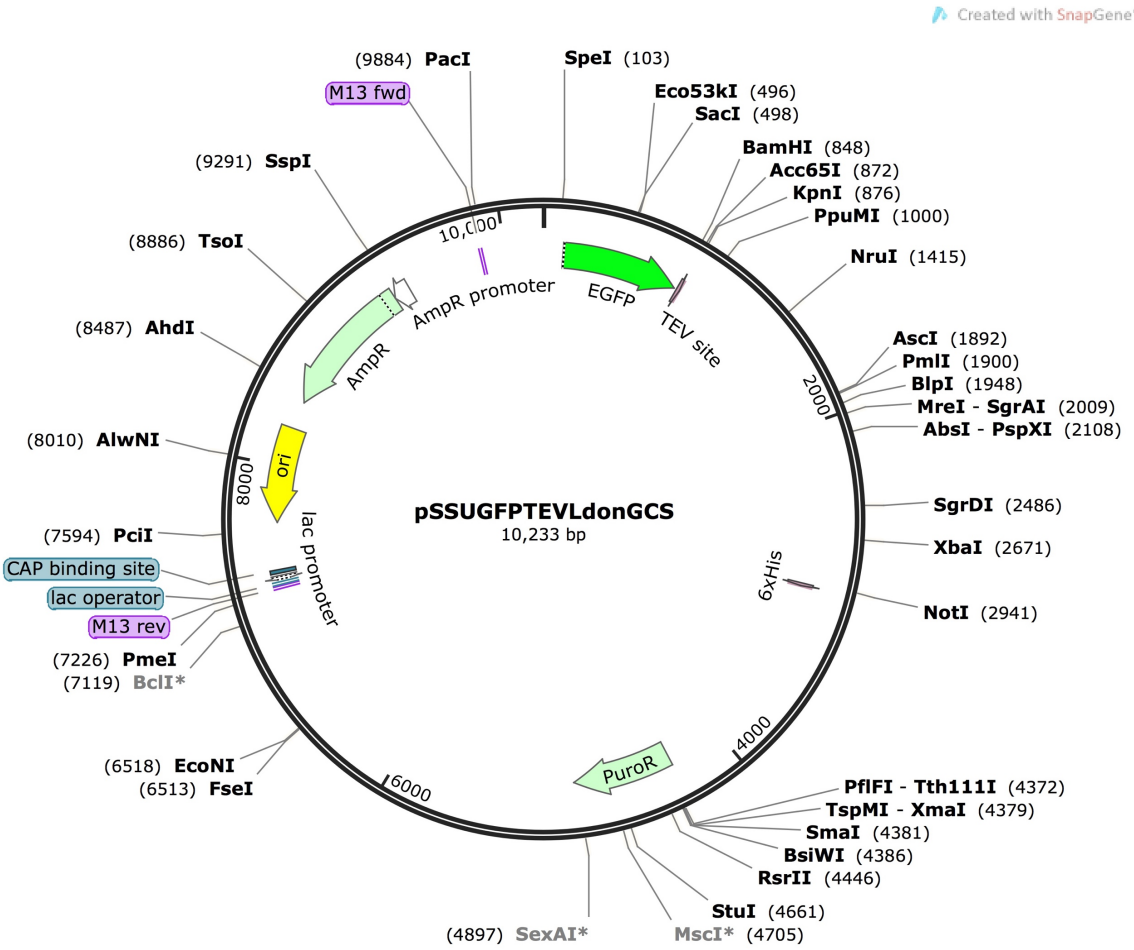
Stierhof, Y. D., Wiese, M., Ilg, T., Overath, P., Haner, M. & Aebi, U. 1998. Structure of a filamentous phosphoglycoprotein polymer: the secreted acid phosphatase of Leishmania mexicana. *J Mol Biol,* 282**,** 137-48.
Striepen, B., He, C. Y., Matrajt, M., Soldati, D. & Roos, D. S. 1998. Expression, selection, and organellar targeting of the green fluorescent protein in Toxoplasma gondii. *Mol Biochem Parasitol,* 92**,** 325-38.
Sundar, S. & Chakravarty, J. 2010. Liposomal amphotericin B and Leishmaniasis: dose and response. *J Glob Infect Dis,* 2**,** 159-66.
Sundar, S. & Chatterjee, M. 2006. Visceral Leishmaniasis - current therapeutic modalities. *Indian J Med Res,* 123**,** 345-52.
Sundar, S. & Olliaro, P. L. 2007. Miltefosine in the treatment of Leishmaniasis: Clinical evidence for informed clinical risk management. *Ther Clin Risk Manag,* 3**,** 733-40.
Taheri, T., Seyed, N. & Rafati, S. 2016. DNA Integration in Leishmania Genome: An Application for Vaccine Development and Drug Screening. *Methods Mol Biol,* 1403**,** 603-22.
Teixeira, S. M. 1998. Control of gene expression in Trypanosomatidae. *Braz J Med Biol Res,* 31**,** 1503-16.
Trinchieri, G. 1998. Interleukin-12: a cytokine at the interface of inflammation and immunity. *Adv Immunol,* 70**,** 83-243.
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.
Uliana, S. R., Fischer, W., Stempliuk, V. A. & Floeter-Winter, L. M. 1996. Structural and functional characterization of the Leishmania amazonensis ribosomal RNA promoter. *Mol Biochem Parasitol,* 76**,** 245-55.
Uzonna, J. E., Spath, G. F., Beverley, S. M. & Scott, P. 2004. Vaccination with phosphoglycan-deficient Leishmania major protects highly susceptible mice from virulent challenge without inducing a strong Th1 response. *J Immunol,* 172**,** 3793-7.
Uzonna, J. E., Wei, G., Yurkowski, D. & Bretscher, P. 2001. Immune elimination of Leishmania major in mice: implications for immune memory, vaccination, and reactivation disease. *J Immunol,* 167**,** 6967-74.
Van Griensven, J. & Diro, E. 2012. Visceral Leishmaniasis. *Infect Dis Clin North Am,* 26**,** 309-22.
Ventura, C., Bisceglia, H., Girerd-Chambaz, Y., Burdin, N. & Chaux, P. 2012. HLA-DR and HLA-DP restricted epitopes from human cytomegalovirus glycoprotein B recognized by CD4+ T-cell clones from chronically infected individuals. *J Clin Immunol,* 32**,** 1305-16.
Waugh, D. S. 2011. An overview of enzymatic reagents for the removal of affinity tags. *Protein Expr Purif,* 80**,** 283-93.
Wiese, M., Gorcke, I. & Overath, P. 1999. Expression and species-specific glycosylation of Leishmania mexicana secreted acid phosphatase in Leishmania major. *Mol Biochem Parasitol,* 102**,** 325-9.
Wiese, M., Ilg, T., Lottspeich, F. & Overath, P. 1995. Ser/Thr-rich repetitive motifs as targets for phosphoglycan modifications in Leishmania mexicana secreted acid phosphatase. *EMBO J,* 14**,** 1067-74.
Wirtz, E., Leal, S., Ochatt, C. & Cross, G. A. 1999. A tightly regulated inducible expression system for conditional gene knock-outs and dominant-

negative genetics in Trypanosoma brucei. *Mol Biochem Parasitol,* 99**,** 89-101.
Wiwanitkit, V. 2012. Interest in paromomycin for the treatment of visceral Leishmaniasis (kala-azar). *Ther Clin Risk Manag,* 8**,** 323-8.
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.
Yuan, C., Gao, J., Guo, J., Bai, L., Marshall, C., Cai, Z., Wang, L. & Xiao, M. 2014. Dimethyl sulfoxide damages mitochondrial integrity and membrane potential in cultured astrocytes. *PLoS One,* 9**,** e107447.
Zahedifard, F., Gholami, E., Taheri, T., Taslimi, Y., Doustdari, F., Seyed, N., Torkashvand, F., Meneses, C., Papadopoulou, B., Kamhawi, S., Valenzuela, J. G. & Rafati, S. 2014. Enhanced protective efficacy of nonpathogenic recombinant Leishmania tarentolae expressing cysteine proteinases combined with a sand fly salivary antigen. *PLoS Negl Trop Dis,* 8**,** e2751.
Zaph, C., Uzonna, J., Beverley, S. M. & Scott, P. 2004. Central memory T cells mediate long-term immunity to Leishmania major in the absence of persistent parasites. *Nat Med,* 10**,** 1104-10.
Zhang, T., He, X., Tsang, T. C. & Harris, D. T. 2004. SING: a novel strategy for identifying tumor-specific, CTL-recognized tumor antigens. *Faseb j,* 18**,** 600-2.
Zhang, W. W., Charest, H. & Matlashewski, G. 1995. The expression of biologically active human p53 in Leishmania cells: a novel eukaryotic system to produce recombinant proteins. *Nucleic Acids Res,* 23**,** 4073-80.

APPENDIXES

Appendix 1 pSSU-INTMKK *Leishmania* cloning vector

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PpuMI (1000)
NruI (1415)
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PmlI (1900)
BlpI (1948)
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XbaI (2671)
NotI (2941)
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BsiWI (4386)
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(7226) PmeI
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lac operator
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(8487) AhdI
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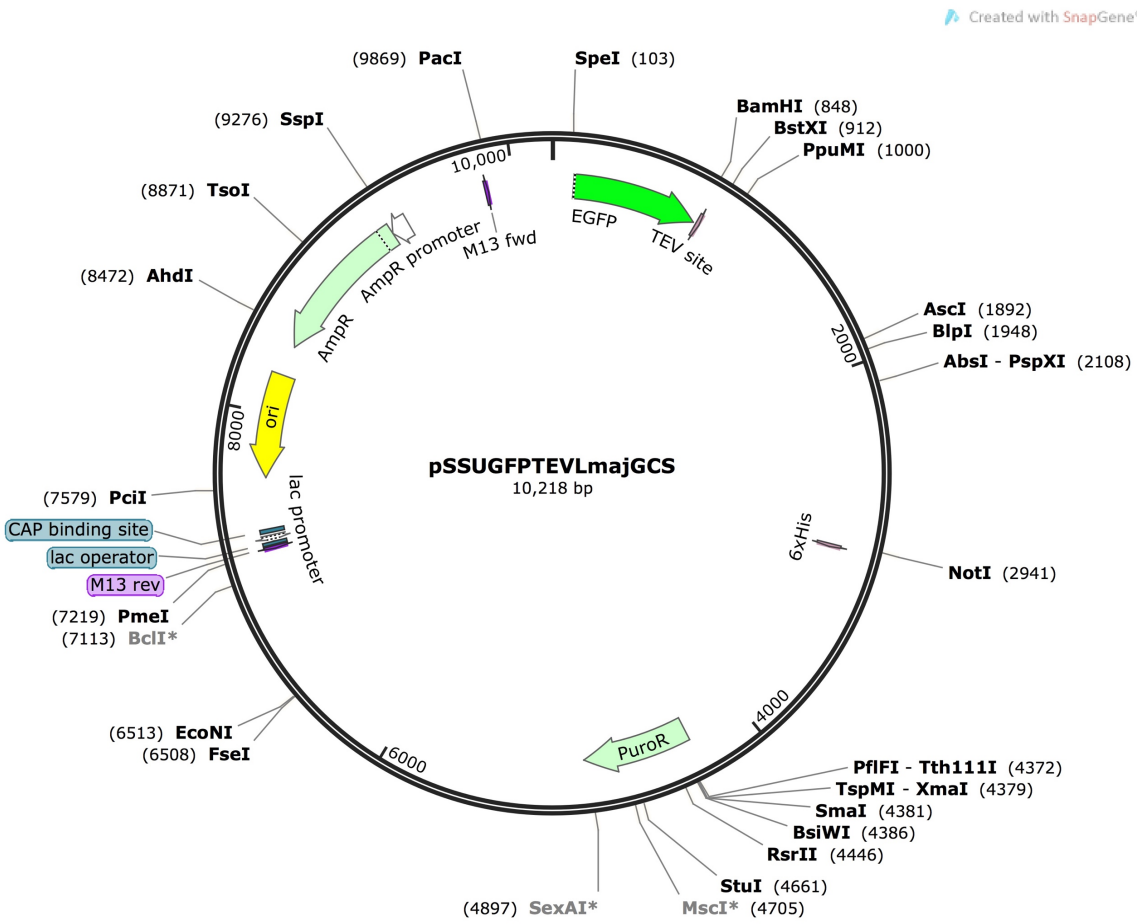
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AmpR
ori
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BlpI (1948)
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lac promoter
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PuroR
4000
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TspMI - XmaI (4379)
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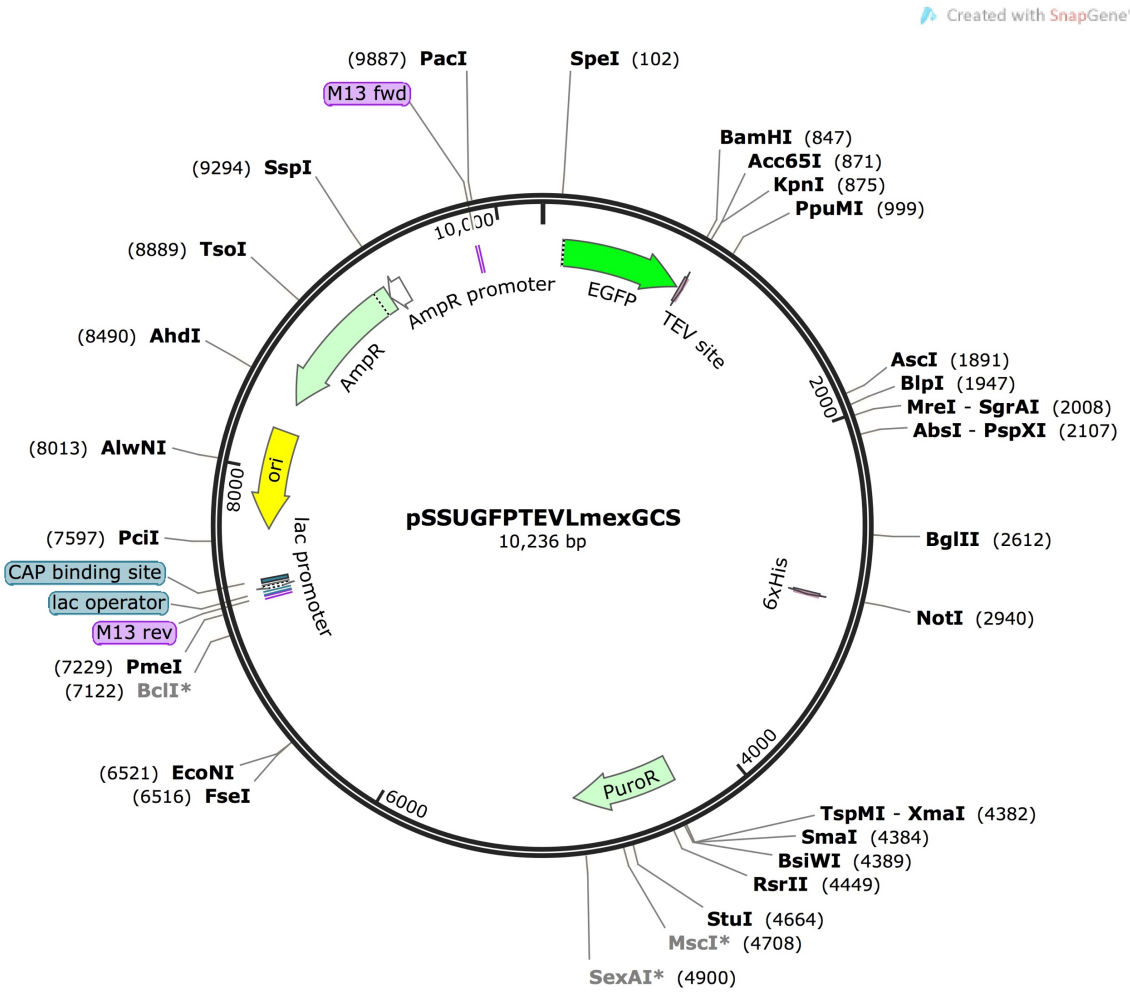
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EGFP
TEV site
6xHis
PuroR
lac promoter
ori
2000
4000
6000
8000

Appendix 2 The gene sequence for *Leishmania* γGCS tagged with EGFP

***L. donovani* γGCS**

ATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCT
GGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGC
CACCTACGGCAAGCTTACCCTCAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCC
TGGCCCACCCTCGTGACCACCTTCACCTACGGCGTGCAGTGCTTTAGCCGCTACCCCG
ACCATATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGG
AGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGT
TCGAGGGCGACACCCTGGTGAACCGCATCGAGCTCAAGGGCATCGATTTCAAGGAGG
ACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATA
TCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACA
TCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCG
ACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAA
AGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGG
GATCA**CTCTCGGCATGGACGAGCTGTACAAGCAATTG**GACGGATCCGAGAACCTG
TACTTCCAGGGTACCATGGGGCTCTTGACGACTGGCGGCGCCCCGATACAGTGGGGC
ACCGATGCAAATAGAAAGGCCATTCCGCACGTCAGAGAGCACGGCATTCAGCAGTTC
CTCAACGTTTTCAAGAACAAAAAGGACCTCCATGGTATGCCGTTTCTCTGGGGAGAG
GAGCTGGAGCACCAGCTAATCCAGATCCACGATAACACGGTTACCCTCAGCACGGAA
AGTGCGATGGTAATGAACAAGCTGAGGGCGCGTCCTGACAACTGCGCCGTGTGGAAC
CCCGAATATGGAAGCTTCATGATCGAAAGCACGCCAGACCACCCGTACAGTCTGTCG
GTGGAGAGCCTCGACTCGGTGCAGGACAACATCGAGCGGCGGTACGACATGCTCAAC
AAGGAGGCACCACCCGGCGTGGTCGGCACCACCTTTGTGACTTTCCCACTCATGGGC
CAGGGCAACTTTGTCCACTGCAGTGATAAGAGTTCTCCGTACTCGCAGTCGCTTTTT
GTTCCTGATGCGTGCATCAACCAAACGCATCCGCGCTTCGCGAACCTGACGGCAAAC
ATTCGCCTGCGCCGCGGTCAAAAGGTTTGCGTCCTGGTGCCTCTGTACATGGACTCC
CGTACAATGCAGGACACGGTGGACCCCCAACTAAACATTGACCTGACTCCACACAAC
AAGGACATTTTTTACTCCATGAGAGAAAACGGCAGGAACATGACCGACGAACTCTA
CGCGGAGACGGACGCGTCTGCCGCTCTGCTAGTGCCCAGCAGCTCTCTCGATCCACG
CGAGGACTACCCTGTCACCGAGACGCTGAAGCAGCTCTTCACCCCTGCTACGCTCTA
CTACTACGCACAGTACTTCACGGGACAGCGCCGCGAGCATATGCAGGAACGCTACAA
CGCGTGTAACTGCCCCGTAACCTTGGTCAGCCACCCGTGCATCTACATGGACTGCAT
GGCCTTTGGCATGGGTAACAGCGCTCTGCAAGTGACGATGCAGCTGGACAACATTCA
CGAGGCGCGCCACGTGTACGACCAGCTCGCCATCTTGTGCCCGGCATTTCTGGCTCTC
AGCTCAGCCACGCCGTTCCAAAAGGGTCTTCTTTGCGACACCGATGTGCGCTGGCTG
ACTATCGCCGGCGCTGTCGACGACCGCCGCGTGGAGGA**GGTGCCGCGTATTCT**CAA
GTCTCGCTACGACTCCATCTCCGTCTTCATCAGCGACAGAACCGAAAACCTCGAGGA
ATTCAACGATTCACAAATAGCGATAAACCGCTCGTACTGTGAACTTCTGAAGGACTC
CGGTGTGGACGTGCGGTTGGCGAACCACATTGCACATTTGTTCATTCGAGATCCGCT
TGTGATGTACGACAAGATGATCGACATCGATGACACGACGCACACGGAGCACTTTGA
CAACATCCAGTCCACTAACTGGCAGACAGTGCGCTTCAAGCCTCCGCCGATAGGAAA
CGACATTGGCTGGCGCGTTGAGTTCCGCGTGATGGATATTCAGCCAACACCGTTCGA
AAACGCCGCCTTCGCCGTCTTCATTCCGCTTCTCACCAAGGCCATCATCACCTACAAG
CCCTGCTTTTACACCAAGATCTCCATCGTCGACGAGAATATGGGCCGCGCACATCGC
ATCAACCCGTGTGGAGAACAATACATTATGCGCAAGGACATTTTCGCCGACAAGTGC
ACCGCCAGCGACGAGGAGACGGCGAGGATGAGCATTGACGAGATCTTCAACGGCAA

GGAGGGCGGCTTCTATGGACTCATCCCCCTCGTGTGCCGCTATCTAGACGACGAGGG
GAAGCGAAGTCCCCTCGTAAACTCCTACCTGAAGTTCCTGTCAATGCGCGCCTCTGG
CCGCATTCCCACACCTGCGCAGTACATGCGCAAGTTTGTCACGACACACCCCGACTA
CAAGCACGACTCACGCCTCACCGACAGCATCGCGCGTGACCTTGTGCAGCGCATGCA
CGGCCTGGCTGCGAATCAGATCCACGACGATGACTACCTTCCCATGAGCTTTTTCAC
GGCCGATACAGTAGAGAGCACCAAGA**GCGGCCGC**GATATCCACCATCACCATCATCA
C**TAG**

***L. major* γGCS**

ATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTG
GACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCC
ACCTACGGCAAGCTTACCCTCAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCT
GGCCCACCCTCGTGACCACCTTCACCTACGGCGTGCAGTGCTTTAGCCGCTACCCCGA
CCATATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGA
GCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTT
CGAGGGCGACACCCTGGTGAACCGCATCGAGCTCAAGGGCATCGATTTCAAGGAGGA
CGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATAT
CATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACAT
CGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGA
CGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAA
GACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGG
ATCACTCTCGGCATGGACGAGCTGTACAAGCAATTGGACGGATCCGAGAACCTGTAC
TTCCAGGGTACCATGGGGCTCTTGACGACTGGCGGCGCCCCAATACAATGGGGCACC
GATGCAAATAGCAAAGCCATTCCGCACGTCAGAGAGCACGGCATTCAACAGTTCCTC
AACGTTTTCAAAAGCAAAAAGGACCTCCATGGTATGCCGTTTTTCTGGGGAGAGGA
GCTGGAGCACCAGCTGATCCAGCTCCACGATGACACGGTTACCCTCAGCACAGAAGG
TGCGGAGGTAATGAACAAGCTGAGGGCGCGTCCTGACAACTGTGCCGTGTGGAATCC
CGAATATGGAAGCTTCATGGTCGAAAGCACGCCAGACCACCCTTACACTCTGTCGGT
GGAGAGCCTCGACTCGGTGCAGGACAACATCGCGCGGCGGTACCACATGCTCAACGA
GGAGGCGCCACCCGGCGTGGTCGGCACCACCTTTGTGACTTTCCCACTCATGGGCCA
GGGTAACTTTGTACACTGCAGTGATAAGAGCTCTCCGTACTCGCAGTCGCTGTTTGT
TCCTGATGCGTGCATCAACCAAACGCATCCGCGCTTTGCGAACCTGACGGCAAaCAT
TCGCCTGCGCCGCGGTCAAAAGGTTTGCATCCTGGTGCCTCTGTACGTGGACTCCCG
AACAATGCAGGACACGGTGGACCCCCGACTAAACATTGACCTGACTCCACACAACAA
GGACATTTTTCACTCCAGGAGAGAAAACGGCAGGAGCATGACCGACGAACTCTACGC
GCACACGGACGCGTCTGCCGCTCTGCTAGTGCCGAGTAGCTCTCTCGACCCACGCGA
GGACTACCCTGTCACCGAGACTCTGAAGCAGCTCTTCACCCCTGCTGCGCTCTACTAC
TACGCACAGTACTTCACGGGACAGCACCGCGAGCATATGCAGGAGCGCTACAACGCG
TGTAACTACCCCGTAACCTTGGTCAGCCACCCGTGCATCTACATGGACTGCATGGCC
TTTGGCATGGGTAACAGCGCTCTGCAAGTGACGATGCAGCTGGACAACATTCACGAG
GCGCGCCACGTGTACGACCAGCTCGCCATCTTGTGCCCGGCGCTTCTGGCTCTCAGCT
CAGCCACGCCGTTCCAGAAGGGTCTTCTTTGCGACACCGATGTGCGCTGGCTGACTA
TTGCCGGCGCCGTCGACGACCGCCGCGTGGAGGAGGTGCCGCATATCCTCAAGTCTC
GCTACGACTCCATCTCCGTCTTCATCAGCGACAGAACCGAAAACCTCGAGGAATTCA
ACGATTCACAGATAGCGATAAACCGCTCGTACTATGAACTCCTGAAGGACTCCGGTG
TCGACGTGCGGTTGGCGAACCACATTGCACATCTGTTCATTCGAGATCCGCTTGTGA
TGTACGACAAGATGATCGACATCGATGACACGACGCACACAGAGCACTTTGACAACA
TCCAGTCCACTAACTGGCAGACAGTGCGCTTCAAGCCTCCGCCGCTAGGCAACGACA

TTGGCTGGCGCGTTGAGTTCCGCGTGATGGATATTCAGCCAACGCCGTTCGAGAACG
CCGCCTTCGCCGTCTTCATTCCGCTTCTCACCAAGGCCATCGTCAACTACAAGCCCTG
CTTTTACACCAAGATCTCCATCGTCGACGAGAATATGGGCCGCGCACATCGCATCAA
CCCATGTGGAGAACAATACATTATGCGCAAGGACATTTTCGCCCACAAGTGCACCGC
CAGTGACGAGGAGACGGCGAGGATGAGCATTGACGAGATCTTCAACGGCAAGGAGG
GCGGATTCTATGGACTCATCCCCCTCGTGTGCCGCTATCTCGACGACGAGGGGAAGC
GAAGTCCCCTCGTAAACTCCTACTTGAAGTTCCTGTCAATGCGCGCCTCTGGCCGCA
TTCCCACACCTGCGCAGTACATGCGAAAGTTTGTCACGACACATCCCGACTACAAAC
ACGACTCACGCCTCACCGACAGCATCGCACGTGACCTTGTGCAGCGCATGCACGGTC
TGGCTTCGAATCAGATCCACGACGATGACTACCTTCCCATAAGCGTCTTCAAGGCCA
CCACAAGAGAGAGTGTCAAGAGCGGCCGCGATATCCACCATCACCATCATCAC**TAG**

***L. mexicana* γGCS**

ATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTG
GACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCC
ACCTACGGCAAGCTTACCCTCAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCT
GGCCCACCCTCGTGACCACCTTCACCTACGGCGTGCAGTGCTTTAGCCGCTACCCCGA
CCATATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGA
GCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTT
CGAGGGCGACACCCTGGTGAACCGCATCGAGCTCAAGGGCATCGATTTCAAGGAGGA
CGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATAT
CATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACAT
CGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGA
CGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAA
GACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGG
ATCACTCTCGGCATGGACGAGCTGTACAAGCAATTGGAC**GGATCC**GAGAACCTGTA
CTTCCAGGGTACCATGGTATTCTTGACGGATGGCGGCGCCGCGATTCAGTGGGGCAC
CGACGCACATAGCAAGGCCATTCCGCACGTCAGAGAGCATGGCATTCAGCAGTTCCT
CAACGTTTTCAAGAACAAGAAGGACCTACATGGCATGCCGTTTCTCTGGGGCGAGGA
GGTGGAACACCAGCTGATCCAGATCCACGATAACACGGTTACCCTCAGCACAGAAAG
TGAGATGGTAATTAACAAGCTGAGAGCGCGTCCTGACAGCTGCGCCGTGTGGAACTT
CGAATATGGCAGCTTCATGGTAGAAAGCACGCCAGACCACCCGTACAATCTGTCAGT
GGAGAGCCTCGACTCAGTGCAGGACAACATCGCGCGACGGTACGACATGCTCAACAA
GGAGGCGCCACCTGGCGTGGTCGGCACCACCTTTGTGACTTTCCCACTCATGGGCCA
GGGTAACTTTGTCCACTGCAGTAGCAAGAGCTCTCCGTACTCTCAGTCGCTTTTTGT
TCCCGATGCGTGCATCAACCAAACGCATCCGCGCTTCGCGAACCTGACGGCAAACAT
TCGCTTGCGCCGCGGCCAAAAGGTTTGCATCCTGGTGCCTCTGTACATGGACACACG
TACAATGGAGAACACGGTGGACCCTCGACTGAACATTGACCTGACTCCACGCAACAA
TGACATTTTTTACTCCATGAGAGAAAACGGCAGGAATACCACCGACGAGCTCTACGC
GGAGACGGACGCGTTTGCCGCTCCTCTAGTGCCCAGGAGCTCTATCGATCCACGCGA
GGACTACCCGGCCACCGAGACGCTGAGGCAACTCTTCACCCCTGCCACACTCCGCTAC
TACGCACAGTACTTCACGGAAGAGCACCGCGAGCATATGCAGGAACTCTACAACGCG
TGTCCCTGCCCTGTACCCTTGGTGAGCCACCCGTGCATCTACATGGACTGCATGGCC
TTTGGCATGGGTAGCAGCGCTCTGCAAGTGACGATGCAGCTGGACAACATTCACGAG
GCGCGCCACGTGTATGACCAGCTCGCCATCTTGTGCCCGGCATTTCTGGCTCTCAGC
TCAGCCACGCCGTTTCAGAAGGGTCTTCTTTGCGACACCGATGTGCGCTGGCTGACT
ATCGCCGGCGCTGTGGACGACCGCCGCGCCGAGGAGGTGCCGCGTATTCTCAAGTCG
CGCTACGACTCCATCTCCGTCTTCATCAGTGACAGAACCGAAAACCTCGAGGAGTTC

AACGATTCACACATAGAGGTGAACCGCTCGTACTGTGAACTTCTGAAGGACTCCGGT
GTGGACGTGCGGTTGGCGAACCACATTGCACATCTGTTTATTCGCGATCCCCTTGTG
ATGTACGACAAGATGATCGACATCGATGACACGACGCACACGGAGCACTTTGATAAC
ATCCAGTCCACTAATTGGCAGACAATGCGCTTCAAGCCTCCGCCGATAGGCAGCGAC
ATTGGCTGGCGCGTTGAGTTTCGAGTGATGGATATTCAGCCAACACCATTCGAGAAC
GCCGCCTTCGCTGTCTTCATTCCGCTTCTCACCAAGGCCATCGTCAACTACAAGCCCT
GCTTTTACACCAAAATCTCCATCGTCGAGGAGAATATGAGTCGCGCACATCGCATCA
ACCCCTGTGGAGAACAATACGTTATGCGTAAGGACATTTTTGCCAACAAGTGCACCG
CCAGCGACGAGGAGACAGCGAGGATGAGCATTGACGAGATCTTCAACGGCAAGGAG
GACGGCTTCTATGGACTCATCCCCCTCGTGTGCCGCTATCTCGACAGCGAGGGAAAG
CGAAGTCCCCTCATAAACTCCTACTTGAAGCTCCTGTCAATGCGCGCCTCTGGCAGC
ATTCCCACACCTGCGCAGTACATGCGCAGGTTTGTCACAACGCACCCCGACTACAAG
CACGACTCACGCCTCACCGACAGTATCGCACGTGACCTTGTCCAGCACATGCACAGC
CTGGCCTCGAATCAGATCCACGACGATGACTATCTTCCGATGAGCATCTTCAGGGTC
GATTCAGTAGAAAGCACCAAGAGCGGCCGCGATATCCACCATCACCATCATCACTAG

BamH1 = GGATCC

NOT1 = GCGGCCG

His Tag: CACCATCACCATCATCACTAG

Appendix 3 Alignment for γGCS sequence from *L. donovani, L. major and L. mexicana.*

The nucleotides identity was *L. don* / *L. major* 96%, *L. don*/ *L. mexicana* 93%, *L. major*/ *L. mexicana* 91%.

Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand
3341 bits(1809)	0.0	1987/2076(96%)	0/2076(0%)	Plus/Plus

```
L.donovani  1    ATGGGGCTCTTGACGACTGGCGGCGCCCCGATACAGTGGGGCACCGATGCAAATAGAAAG 60
                 |||||||||||||||||||||||||||||| ||||| |||||||||||||||||||| ||
L.major1    ATGGGGCTCTTGACGACTGGCGGCGCCCCAATACAATGGGGCACCGATGCAAATAGCAAA 60

L.donovani  61   GCCATTCCGCACGTCAGAGAGCACGGCATTCAGCAGTTCCTCAACGTTTTCAAGAACAAA 120
                 |||||||||||||||||||||||||||||||| |||||||||||||||||||| | ||||
L.major61   GCCATTCCGCACGTCAGAGAGCACGGCATTCAACAGTTCCTCAACGTTTTCAAAAGCAAA 120

L.donovani  121  AAGGACCTCCATGGTATGCCGTTTCTCTGGGGAGAGGAGCTGGAGCACCAGCTAATCCAG 18
0
                 |||||||||||||||||||||||| |||||||||||||||||||||||||||||| ||||||
L.major121  AAGGACCTCCATGGTATGCCGTTTTTTCTGGGGAGAGGAGCTGGAGCACCAGCTGATCCAG 180

L.donovani  181  ATCCACGATAACACGGTTACCCTCAGCACGGAAAGTGCGATGGTAATGAACAAGCTGAGG 24
0
                  |||||||| |||||||||||||||||| ||| ||||| ||||||||||||||||||
L.major181  CTCCACGATGACACGGTTACCCTCAGCACAGAAGGTGCGGAGGTAATGAACAAGCTGAGG 240

L.donovani  241  GCGCGTCCTGACAACTGCGCCGTGTGGAACCCCGAATATGGAAGCTTCATGATCGAAAGC 30
0
                 ||||||||||||||||| |||||||||| |||||||||||||||||||| ||||||||
L.major241  GCGCGTCCTGACAACTGTGCCGTGTGGAATCCCGAATATGGAAGCTTCATGGTCGAAAGC 300

L.donovani  301  ACGCCAGACCACCCGTACAGTCTGTCGGTGGAGAGCCTCGACTCGGTGCAGGACAACATC 360
                 |||||||||||||| |||| ||||||||||||||||||||||||||||||||||||||||
L.major301  ACGCCAGACCACCCTTACACTCTGTCGGTGGAGAGCCTCGACTCGGTGCAGGACAACATC 360

L.donovani  361  GAGCGGCGGTACGACATGCTCAACAAGGAGGCACCACCCGGCGTGGTCGGCACCACCTTT 420
                 | |||||||||| |||||||||| ||||||| ||||||||||||||||||||||||||
L.major361  GCGCGGCGGTACCACATGCTCAACGAGGAGGCGCCACCCGGCGTGGTCGGCACCACCTTT 420

L.donovani  421  GTGACTTTCCCACTCATGGGCCAGGGCAACTTTGTCCACTGCAGTGATAAGAGTTCTCCG 480
                 ||||||||||||||||||||||||| |||||||| |||||||||||||||| ||||||
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L.major421 GTGACTTTCCCACTCATGGGCCAGGGTAACTTTGTACACTGCAGTGATAAGAGCTCTCCG 480

L.donovani 481 TACTCGCAGTCGCTTTTTGTTCCTGATGCGTGCATCAACCAAACGCATCCGCGCTTCGCG 540
|||||||||||||| |||||||||||||||||||||||||||||||||||||||||||||| |||
L.major481 TACTCGCAGTCGCTGTTTGTTCCTGATGCGTGCATCAACCAAACGCATCCGCGCTTTGCG 540

L.donovani 541 AACCTGACGGCAAACATTCGCCTGCGCCGCGGTCAAAAGGTTTGCGTCCTGGTGCCTCTG 600
|||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||
L.major541 AACCTGACGGCAAACATTCGCCTGCGCCGCGGTCAAAAGGTTTGCATCCTGGTGCCTCTG 600

L.donovani 601 TACATGGACTCCCGTACAATGCAGGACACGGTGGACCCCCAACTAAACATTGACCTGACT 660
||| |||||||||| |||||||||||||||||||||||| ||||||||||||||||||
L.major601 TACGTGGACTCCCGAACAATGCAGGACACGGTGGACCCCCGACTAAACATTGACCTGACT 660

L.donovani 661 CCACACAACAAGGACATTTTTTACTCCATGAGAGAAAACGGCAGGAACATGACCGACGAA 72
0
|||||||||||||||||||| |||||| |||||||||||||||| ||||||||||||
L.major661 CCACACAACAAGGACATTTTTCACTCCAGGAGAGAAAACGGCAGGAGCATGACCGACGAA 720

L.donovani 721 CTCTACGCGGAGACGGACGCGTCTGCCGCTCTGCTAGTGCCCAGCAGCTCTCTCGATCCA 780
||||||||| | |||||||||||||||||||||||||||| || |||||||||| |||
L.major721 CTCTACGCGCACACGGACGCGTCTGCCGCTCTGCTAGTGCCGAGTAGCTCTCTCGACCCA 780

L.donovani 781 CGCGAGGACTACCCTGTCACCGAGACGCTGAAGCAGCTCTTCACCCCTGCTACGCTCTAC 840
|||||||||||||||||||||||||| ||||||||||||||||||||||| ||||||||
L.major781 CGCGAGGACTACCCTGTCACCGAGACTCTGAAGCAGCTCTTCACCCCTGCTGCGCTCTAC 840

L.donovani 841 TACTACGCACAGTACTTCACGGGACAGCGCCGCGAGCATATGCAGGAACGCTACAACGCG 900
|||||||||||||||||||||||||||| |||||||||||||||| ||||||||||||
L.major841 TACTACGCACAGTACTTCACGGGACAGCACCGCGAGCATATGCAGGAGCGCTACAACGCG 900

L.donovani 901 TGTAACTGCCCCGTAACCTTGGTCAGCCACCCGTGCATCTACATGGACTGCATGGCCTTT 960
||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
L.major901 TGTAACTACCCCGTAACCTTGGTCAGCCACCCGTGCATCTACATGGACTGCATGGCCTTT 960

L.donovani 961 GGCATGGGTAACAGCGCTCTGCAAGTGACGATGCAGCTGGACAACATTCACGAGGCGCGC 10
20
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
L.major961 GGCATGGGTAACAGCGCTCTGCAAGTGACGATGCAGCTGGACAACATTCACGAGGCGCGC 1020

L.donovani 1021 CACGTGTACGACCAGCTCGCCATCTTGTGCCCGGCATTTCTGGCTCTCAGCTCAGCCACG 108
0
|||||||||||||||||||||||||||||||||| ||||||||||||||||||||||||
L.major1021 CACGTGTACGACCAGCTCGCCATCTTGTGCCCGGCGCTTCTGGCTCTCAGCTCAGCCACG 1080

L.donovani 1081 CCGTTCCAAAAGGGTCTTCTTTGCGACACCGATGTGCGCTGGCTGACTATCGCCGGCGCT 11
40

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L.major1081 CCGTTCCAGAAGGGTCTTCTTTGCGACACCGATGTGCGCTGGCTGACTATTGCCGGCGCC 1140

L.donovani 1141 GTCGACGACCGCCGCGTGGAGGAGGTGCCGCGTATTCTCAAGTCTCGCTACGACTCCATC 12
00

|||||||||||||||||||||||||||||| ||| |||||||||||||||||||||||||

L.major1141 GTCGACGACCGCCGCGTGGAGGAGGTGCCGCATATCCTCAAGTCTCGCTACGACTCCATC 1200

L.donovani 1201 TCCGTCTTCATCAGCGACAGAACCGAAAACCTCGAGGAATTCAACGATTCACAAATAGCG 12
60

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L.major1201 TCCGTCTTCATCAGCGACAGAACCGAAAACCTCGAGGAATTCAACGATTCACAGATAGCG 1260

L.donovani 1261 ATAAACCGCTCGTACTGTGAACTTCTGAAGGACTCCGGTGTGGACGTGCGGTTGGCGAAC 13
20

|||||||||||||||| |||||| |||||||||||||||| ||||||||||||||||||

L.major1261 ATAAACCGCTCGTACTATGAACTCCTGAAGGACTCCGGTGTCGACGTGCGGTTGGCGAAC 1320

L.donovani 1321 CACATTGCACATTTGTTCATTCGAGATCCGCTTGTGATGTACGACAAGATGATCGACATC 13
80

|||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||

L.major1321 CACATTGCACATCTGTTCATTCGAGATCCGCTTGTGATGTACGACAAGATGATCGACATC 1380

L.donovani 1381 GATGACACGACGCACACGGAGCACTTTGACAACATCCAGTCCACTAACTGGCAGACAGTG 14
40

|||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||

L.major1381 GATGACACGACGCACACAGAGCACTTTGACAACATCCAGTCCACTAACTGGCAGACAGTG 1440

L.donovani 1441 CGCTTCAAGCCTCCGCCGATAGGAAACGACATTGGCTGGCGCGTTGAGTTCCGCGTGATG 15
00

|||||||||||||||||| |||| ||||||||||||||||||||||||||||||||||||

L.major1441 CGCTTCAAGCCTCCGCCGCTAGGCAACGACATTGGCTGGCGCGTTGAGTTCCGCGTGATG 1500

L.donovani 1501 GATATTCAGCCAACACCGTTCGAAAACGCCGCCTTCGCCGTCTTCATTCCGCTTCTCACC 156
0

|||||||||||||| |||||||| ||||||||||||||||||||||||||||||||||||

L.major1501 GATATTCAGCCAACGCCGTTCGAGAACGCCGCCTTCGCCGTCTTCATTCCGCTTCTCACC 1560

L.donovani 1561 AAGGCCATCATCACCTACAAGCCCTGCTTTTACACCAAGATCTCCATCGTCGACGAGAAT 16
20

||||||||| ||| ||||||||||||||||||||||||||||||||||||||||||||||||

L.major1561 AAGGCCATCGTCAACTACAAGCCCTGCTTTTACACCAAGATCTCCATCGTCGACGAGAAT 1620

L.donovani 1621 ATGGGCCGCGCACATCGCATCAACCCGTGTGGAGAACAATACATTATGCGCAAGGACATT 1680

L.major1621 ATGGGCCGCGCACATCGCATCAACCCATGTGGAGAACAATACATTATGCGCAAGGACATT 1680

L.donovani 1681 TTCGCCGACAAGTGCACCGCCAGCGACGAGGAGACGGCGAGGATGAGCATTGACGAGATC 1740

L.major1681 TTCGCCCACAAGTGCACCGCCAGTGACGAGGAGACGGCGAGGATGAGCATTGACGAGATC 1740

L.donovani 1741 TTCAACGGCAAGGAGGGCGGCTTCTATGGACTCATCCCCCTCGTGTGCCGCTATCTAGAC 1800

L.major1741 TTCAACGGCAAGGAGGGCGGATTCTATGGACTCATCCCCCTCGTGTGCCGCTATCTCGAC 1800

L.donovani 1801 GACGAGGGGAAGCGAAGTCCCCTCGTAAACTCCTACCTGAAGTTCCTGTCAATGCGCGCC 1860

L.major1801 GACGAGGGGAAGCGAAGTCCCCTCGTAAACTCCTACTTGAAGTTCCTGTCAATGCGCGCC 1860

L.donovani 1861 TCTGGCCGCATTCCCACACCTGCGCAGTACATGCGCAAGTTTGTCACGACACACCCCGAC 1920

L.major1861 TCTGGCCGCATTCCCACACCTGCGCAGTACATGCGAAAGTTTGTCACGACACATCCCGAC 1920

L.donovani 1921 TACAAGCACGACTCACGCCTCACCGACAGCATCGCGCGTGACCTTGTGCAGCGCATGCAC 1980

L.major1921 TACAAACACGACTCACGCCTCACCGACAGCATCGCACGTGACCTTGTGCAGCGCATGCAC 1980

L.donovani 1981 GGCCTGGCTGCGAATCAGATCCACGACGATGACTACCTTCCCATGAGCTTTTTCACGGCC 2040

L.major1981 GGTCTGGCTTCGAATCAGATCCACGACGATGACTACCTTCCCATAAGCGTCTTCAAGGCC 2040

L.donovani 2041 GATACAGTAGAGAGCACCAAGAGCGGCCGCGATATC 2076

L.major2041 ACCACAAGAGAGAGTGTCAAGAGCGGCCGCGATATC 2076

Alignment statistics for match #2

Score	Expect	Identities	Gaps	Strand
3024 bits(1637)	0.0	1925/2069(93%)	0/2069(0%)	Plus/Plus

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L.donovani  8    TCTTGACGACTGGCGGCGCCCCGATACAGTGGGGCACCGATGCAAATAGAAAGGCCATTC 67
                 ||||||||  |||||||||| |||| ||||||||||||||| ||| |||| ||||||||||
L.mexicana2084 TCTTGACGGATGGCGGCGCCGCGATTCAGTGGGGCACCGACGCACATAGCAAGGCCATTC 214
3

L.donovani  68   CGCACGTCAGAGAGCACGGCATTCAGCAGTTCCTCAACGTTTTCAAGAACAAAAAGGACC 127
                 ||||||||||||||| |||||||||||||||||||||||||||||||||||| |||||||
L.mexicana2144 CGCACGTCAGAGAGCATGGCATTCAGCAGTTCCTCAACGTTTTCAAGAACAAGAAGGACC 220
3

L.donovani  128  TCCATGGTATGCCGTTTCTCTGGGGAGAGGAGCTGGAGCACCAGCTAATCCAGATCCACG 187
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L.mexicana2204 TACATGGCATGCCGTTTCTCTGGGGCGAGGAGGTGGAACACCAGCTGATCCAGATCCACG 226
3

L.donovani  188  ATAACACGGTTACCCTCAGCACGGAAAGTGCGATGGTAATGAACAAGCTGAGGGCGCGTC 24
7
                 |||||||||||||||||||| ||||||| ||||||||| |||||||||| |||||||
L.mexicana2264 ATAACACGGTTACCCTCAGCACAGAAAGTGAGATGGTAATTAACAAGCTGAGAGCGCGTC 23
23

L.donovani  248  CTGACAACTGCGCCGTGTGGAACCCCGAATATGGAAGCTTCATGATCGAAAGCACGCCAG 30
7
                 |||||| |||||||||||||||  ||||||||| ||||||||| | ||||||||||||
L.mexicana2324 CTGACAGCTGCGCCGTGTGGAACTTCGAATATGGCAGCTTCATGGTAGAAAGCACGCCAG 238
3

L.donovani  308  ACCACCCGTACAGTCTGTCGGTGGAGAGCCTCGACTCGGTGCAGGACAACATCGAGCGGC 367
                 |||||||||||| |||||| |||||||||||||||| |||||||||||||||| ||| |
L.mexicana2384 ACCACCCGTACAATCTGTCAGTGGAGAGCCTCGACTCAGTGCAGGACAACATCGCGCGAC 244
3

L.donovani  368  GGTACGACATGCTCAACAAGGAGGCACCACCCGGCGTGGTCGGCACCACCTTTGTGACTT 427
                 ||||||||||||||||||||||||| ||||| |||||||||||||||||||||||||||
L.mexicana2444 GGTACGACATGCTCAACAAGGAGGCGCCACCTGGCGTGGTCGGCACCACCTTTGTGACTT 250
3

L.donovani  428  TCCCACTCATGGGCCAGGGCAACTTTGTCCACTGCAGTGATAAGAGTTCTCCGTACTCGC 487
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L.mexicana2504 TCCCACTCATGGGCCAGGGTAACTTTGTCCACTGCAGTAGCAAGAGCTCTCCGTACTCTC 256
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L.donovani 488 AGTCGCTTTTTGTTCCTGATGCGTGCATCAACCAAACGCATCCGCGCTTCGCGAACCTGA 547
|||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
L.mexicana2564 AGTCGCTTTTTGTTCCCGATGCGTGCATCAACCAAACGCATCCGCGCTTCGCGAACCTGA 262
3

L.donovani 548 CGGCAAACATTCGCCTGCGCCGCGGTCAAAAGGTTTGCGTCCTGGTGCCTCTGTACATGG 607
|||||||||||||| |||||||||| |||||||||||| ||||||||||||||||||||||
L.mexicana2624 CGGCAAACATTCGCTTGCGCCGCGGCCAAAAGGTTTGCATCCTGGTGCCTCTGTACATGG 268
3

L.donovani 608 ACTCCCGTACAATGCAGGACACGGTGGACCCCCAACTAAACATTGACCTGACTCCACACA 667
|| | ||||||||| || |||||||||||| | ||| ||||||||||||||||||| ||
L.mexicana2684 ACACACGTACAATGGAGAACACGGTGGACCCTCGACTGAACATTGACCTGACTCCACGCA 274
3

L.donovani 668 ACAAGGACATTTTTTACTCCATGAGAGAAAACGGCAGGAACATGACCGACGAACTCTACG 72
7
|||| |||||||||||||||||||||||||||||||||| | |||||||| |||||||
L.mexicana2744 ACAATGACATTTTTTACTCCATGAGAGAAAACGGCAGGAATACCACCGACGAGCTCTACG 280
3

L.donovani 728 CGGAGACGGACGCGTCTGCCGCTCTGCTAGTGCCCAGCAGCTCTCTCGATCCACGCGAGG 787
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L.mexicana2804 CGGAGACGGACGCGTTTGCCGCTCCTCTAGTGCCCAGGAGCTCTATCGATCCACGCGAGG 286
3

L.donovani 788 ACTACCCTGTCACCGAGACGCTGAAGCAGCTCTTCACCCCTGCTACGCTCTACTACTACG 847
||||||| | |||||||||||||| ||| |||||||||||||| || ||| ||||||||
L.mexicana2864 ACTACCCGGCCACCGAGACGCTGAGGCAACTCTTCACCCCTGCCACACTCCGCTACTACG 2923

L.donovani 848 CACAGTACTTCACGGGACAGCGCCGCGAGCATATGCAGGAACGCTACAACGCGTGTAACT 90
7
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L.mexicana2924 CACAGTACTTCACGGAAGAGCACCGCGAGCATATGCAGGAACTCTACAACGCGTGTCCCT 298
3

L.donovani 908 GCCCCGTAACCTTGGTCAGCCACCCGTGCATCTACATGGACTGCATGGCCTTTGGCATGG 967
|||| ||| ||||||| ||||||||||||||||||||||||||||||||||||||||||||||
L.mexicana2984 GCCCTGTACCCTTGGTGAGCCACCCGTGCATCTACATGGACTGCATGGCCTTTGGCATGG 304
3

L.donovani 968 GTAACAGCGCTCTGCAAGTGACGATGCAGCTGGACAACATTCACGAGGCGCGCCACGTGT 10
27

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L.mexicana3044 GTAGCAGCGCTCTGCAAGTGACGATGCAGCTGGACAACATTCACGAGGCGCGCCACGTGT 310
3

L.donovani 1028 ACGACCAGCTCGCCATCTTGTGCCCGGCATTTCTGGCTCTCAGCTCAGCCACGCCGTTCC 108
7

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L.mexicana3104 ATGACCAGCTCGCCATCTTGTGCCCGGCATTTCTGGCTCTCAGCTCAGCCACGCCGTTTC 3163

L.donovani 1088 AAAAGGGTCTTCTTTGCGACACCGATGTGCGCTGGCTGACTATCGCCGGCGCTGTCGACG 11
47

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L.mexicana3164 AGAAGGGTCTTCTTTGCGACACCGATGTGCGCTGGCTGACTATCGCCGGCGCTGTGGACG 322
3

L.donovani 1148 ACCGCCGCGTGGAGGAGGTGCCGCGTATTCTCAAGTCTCGCTACGACTCCATCTCCGTCT 12
07

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L.mexicana3224 ACCGCCGCGCCGAGGAGGTGCCGCGTATTCTCAAGTCGCGCTACGACTCCATCTCCGTCT 3283

L.donovani 1208 TCATCAGCGACAGAACCGAAAACCTCGAGGAATTCAACGATTCACAAATAGCGATAAACC 12
67

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L.mexicana3284 TCATCAGTGACAGAACCGAAAACCTCGAGGAGTTCAACGATTCACACATAGAGGTGAACC 334
3

L.donovani 1268 GCTCGTACTGTGAACTTCTGAAGGACTCCGGTGTGGACGTGCGGTTGGCGAACCACATTG 13
27

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L.mexicana3344 GCTCGTACTGTGAACTTCTGAAGGACTCCGGTGTGGACGTGCGGTTGGCGAACCACATTG 340
3

L.donovani 1328 CACATTTGTTCATTCGAGATCCGCTTGTGATGTACGACAAGATGATCGACATCGATGACA 13
87

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L.mexicana3404 CACATCTGTTTATTCGCGATCCCCTTGTGATGTACGACAAGATGATCGACATCGATGACA 346
3

L.donovani 1388 CGACGCACACGGAGCACTTTGACAACATCCAGTCCACTAACTGGCAGACAGTGCGCTTCA 14
47

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L.mexicana3464 CGACGCACACGGAGCACTTTGATAACATCCAGTCCACTAATTGGCAGACAATGCGCTTCA 352
3

L.donovani 1448 AGCCTCCGCCGATAGGAAACGACATTGGCTGGCGCGTTGAGTTCCGCGTGATGGATATTC 15
07
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L.mexicana3524 AGCCTCCGCCGATAGGCAGCGACATTGGCTGGCGCGTTGAGTTTCGAGTGATGGATATTC 358
3

L.donovani 1508 AGCCAACACCGTTCGAAAACGCCGCCTTCGCCGTCTTCATTCCGCTTCTCACCAAGGCCA 156
7
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L.mexicana3584 AGCCAACACCATTCGAGAACGCCGCCTTCGCTGTCTTCATTCCGCTTCTCACCAAGGCCA 3643

L.donovani 1568 TCATCACCTACAAGCCCTGCTTTTACACCAAGATCTCCATCGTCGACGAGAATATGGGCC 16
27
|| ||| |||||||||||||||||||||||| |||||||||||||| ||||||||| | |
L.mexicana3644 TCGTCAACTACAAGCCCTGCTTTTACACCAAAATCTCCATCGTCGAGGAGAATATGAGTC 370
3

L.donovani 1628 GCGCACATCGCATCAACCCGTGTGGAGAACAATACATTATGCGCAAGGACATTTTCGCCG 16
87
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L.mexicana3704 GCGCACATCGCATCAACCCCTGTGGAGAACAATACGTTATGCGTAAGGACATTTTTGCCA 376
3

L.donovani 1688 ACAAGTGCACCGCCAGCGACGAGGAGACGGCGAGGATGAGCATTGACGAGATCTTCAACG 17
47
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L.mexicana3764 ACAAGTGCACCGCCAGCGACGAGGAGACAGCGAGGATGAGCATTGACGAGATCTTCAACG 382
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L.donovani 1748 GCAAGGAGGGCGGCTTCTATGGACTCATCCCCCTCGTGTGCCGCTATCTAGACGACGAGG 18
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L.mexicana3824 GCAAGGAGGACGGCTTCTATGGACTCATCCCCCTCGTGTGCCGCTATCTCGACAGCGAGG 388
3

L.donovani 1808 GGAAGCGAAGTCCCCTCGTAAACTCCTACCTGAAGTTCCTGTCAATGCGCGCCTCTGGCC 18
67
| |||||||||||||| |||||||||||| ||||| |||||||||||||||||||||||
L.mexicana3884 GAAAGCGAAGTCCCCTCATAAACTCCTACTTGAAGCTCCTGTCAATGCGCGCCTCTGGCA 394
3

L.donovani 1868 GCATTCCCACACCTGCGCAGTACATGCGCAAGTTTGTCACGACACACCCCGACTACAAGC 1927

L.mexicana3944 GCATTCCCACACCTGCGCAGTACATGCGCAGGTTTGTCACAACGCACCCCGACTACAAGC 4003

L.donovani 1928 ACGACTCACGCCTCACCGACAGCATCGCGCGTGACCTTGTGCAGCGCATGCACGGCCTGG 1987

L.mexicana4004 ACGACTCACGCCTCACCGACAGTATCGCACGTGACCTTGTCCAGCACATGCACAGCCTGG 4063

L.donovani 1988 CTGCGAATCAGATCCACGACGATGACTACCTTCCCATGAGCTTTTTCACGGCCGATACAG 2047

L.mexicana4064 CCTCGAATCAGATCCACGACGATGACTATCTTCCGATGAGCATCTTCAGGGTCGATTCAG 4123

L.donovani 2048 TAGAGAGCACCAAGAGCGGCCGCGATATC 2076

L.mexicana4124 TAGAAAGCACCAAGAGCGGCCGCGATATC 4152

Score	Expect	Identities	Gaps	Strand
2813 bits(1523)	0.0	1889/2071(91%)	4/2071(0%)	Plus/Plus

Query 8 TCTTGACGACTGGCGGCGCCCCAATACAATGGGGCACCGATGCAAATAGCAAAGCCATTC 67

Sbjct 8 TCTTGACGGATGGCGGCGCCGCGATTCAGTGGGGCACCGACGCACATAGCAAGGCCATTC 67

Query 68 CGCACGTCAGAGAGCACGGCATTCAACAGTTCCTCAACGTTTTCAAAAGCAAAAAGGACC 127

Sbjct 68 CGCACGTCAGAGAGCATGGCATTCAGCAGTTCCTCAACGTTTTCAAGAACAAGAAGGACC 127

Query 128 TCCATGGTATGCCGTTTTTCTGGGGAGAGGAGCTGGAGCACCAGCTGATCCAGCTCCACG 187

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Sbjct 128  TACATGGCATGCCGTTTCTCTGGGGCGAGGAGGTGGAACACCAGCTGATCCAGATCCACG 187

Query 188  ATGACACGGTTACCCTCAGCACAGAAGGTGCGGAGGTAATGAACAAGCTGAGGGCGCGTC 247
           || ||||||||||||||||||||||| ||| |  |||||| |||||||||||| |||||||
Sbjct 188  ATAACACGGTTACCCTCAGCACAGAAAGTGAGATGGTAATTAACAAGCTGAGAGCGCGTC 247

Query 248  CTGACAACTGTGCCGTGTGGAATCCCGAATATGGAAGCTTCATGGTCGAAAGCACGCCAG 307
           |||||| ||| ||||||||||  ||||||||| |||||||||| ||||||||||||
Sbjct 248  CTGACAGCTGCGCCGTGTGGAACTTCGAATATGGCAGCTTCATGGTAGAAAGCACGCCAG 307

Query 308  ACCACCCTTACACTCTGTCGGTGGAGAGCCTCGACTCGGTGCAGGACAACATCGCGCGGC 367
           ||||||| |||| |||||| |||||||||||||||| |||||||||||||||||||| |
Sbjct 308  ACCACCCGTACAATCTGTCAGTGGAGAGCCTCGACTCAGTGCAGGACAACATCGCGCGAC 367

Query 368  GGTACCACATGCTCAACGAGGAGGCGCCACCCGGCGTGGTCGGCACCACCTTTGTGACTT 427
           ||||| |||||||||| |||||||||||| ||||||||||||||||||||||||||||||
Sbjct 368  GGTACGACATGCTCAACAAGGAGGCGCCACCTGGCGTGGTCGGCACCACCTTTGTGACTT 427

Query 428  TCCCACTCATGGGCCAGGGTAACTTTGTACACTGCAGTGATAAGAGCTCTCCGTACTCGC 487
           |||||||||||||||||||||||||| |||||||||  |||||||||||||||| |
Sbjct 428  TCCCACTCATGGGCCAGGGTAACTTTGTCCACTGCAGTAGCAAGAGCTCTCCGTACTCTC 487

Query 488  AGTCGCTGTTTGTTCCTGATGCGTGCATCAACCAAACGCATCCGCGCTTTGCGAACCTGA 547
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Sbjct 488  AGTCGCTTTTTGTTCCCGATGCGTGCATCAACCAAACGCATCCGCGCTTCGCGAACCTGA 547

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Sbjct 548  CGGCAAACATTCGCTTGCGCCGCGGCCAAAAGGTTTGCATCCTGGTGCCTCTGTACATGG 607

Query 608  ACTCCCGAACAATGCAGGACACGGTGGACCCCCGACTAAACATTGACCTGACTCCACACA 667
           || | || |||||| || |||||||||||| ||||| |||||||||||||||||| ||
Sbjct 608  ACACACGTACAATGGAGAACACGGTGGACCCTCGACTGAACATTGACCTGACTCCACGCA 667

Query 668  ACAAGGACATTTTTCACTCCAGGAGAGAAAACGGCAGGAGCATGACCGACGAACTCTACG 727
           |||| ||||||||| |||||| |||||||||||||||  |  |||||||| |||||||
Sbjct 668  ACAATGACATTTTTTACTCCATGAGAGAAAACGGCAGGAATACCACCGACGAGCTCTACG 727

Query 728  CGCACACGGACGCGTCTGCCGCTCTGCTAGTGCCGAGTAGCTCTCTCGACCCACGCGAGG 787
           || | |||||||||| ||||||||  |||||||| || |||||| |||| ||||||||||
Sbjct 728  CGGAGACGGACGCGTTTGCCGCTCCTCTAGTGCCCAGGAGCTCTATCGATCCACGCGAGG 787

Query 788  ACTACCCTGTCACCGAGACTCTGAAGCAGCTCTTCACCCCTGCTGCGCTCTACTACTACG 847
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Sbjct 788  ACTACCCGGCCACCGAGACGCTGAGGCAACTCTTCACCCCTGCCACACTCCGCTACTACG 847

Query 848  CACAGTACTTCACGGGACAGCACCGCGAGCATATGCAGGAGCGCTACAACGCGTGTAACT 907
           |||||||||||||| | |||||||||||||||||||||| | ||||||||||||  ||
Sbjct 848  CACAGTACTTCACGGAAGAGCACCGCGAGCATATGCAGGAACTCTACAACGCGTGTCCCT 907

Query 908  ACCCCGTAACCTTGGTCAGCCACCCGTGCATCTACATGGACTGCATGGCCTTTGGCATGG 967
            ||| ||| ||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct 908  GCCCTGTACCCTTGGTGAGCCACCCGTGCATCTACATGGACTGCATGGCCTTTGGCATGG 967

Query 968  GTAACAGCGCTCTGCAAGTGACGATGCAGCTGGACAACATTCACGAGGCGCGCCACGTGT 1027
           ||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 968  GTAGCAGCGCTCTGCAAGTGACGATGCAGCTGGACAACATTCACGAGGCGCGCCACGTGT 1027

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           | ||||||||||||||||||||||||  |||||||||||||||||||||||||||||| |
Sbjct 1028 ATGACCAGCTCGCCATCTTGTGCCCGGCATTTCTGGCTCTCAGCTCAGCCACGCCGTTTC 1087

Query 1088 AGAAGGGTCTTCTTTGCGACACCGATGTGCGCTGGCTGACTATTGCCGGCGCCGTCGACG 1147
           |||||||||||||||||||||||||||||||||||||||||||| |||||||| || ||||
Sbjct 1088 AGAAGGGTCTTCTTTGCGACACCGATGTGCGCTGGCTGACTATCGCCGGCGCTGTGGACG 1147

Query 1148 ACCGCCGCGTGGAGGAGGTGCCGCATATCCTCAAGTCTCGCTACGACTCCATCTCCGTCT 1207
           |||||||||  |||||||||||||| ||| |||||||| ||||||||||||||||||||||
Sbjct 1148 ACCGCCGCGCCGAGGAGGTGCCGCGTATTCTCAAGTCGCGCTACGACTCCATCTCCGTCT 1207

Query 1208 TCATCAGCGACAGAACCGAAAACCTCGAGGAATTCAACGATTCACAGATAGCGATAAACC 1267
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Sbjct 1208 TCATCAGTGACAGAACCGAAAACCTCGAGGAGTTCAACGATTCACACATAGAGGTGAACC 1267

Query 1268 GCTCGTACTATGAACTCCTGAAGGACTCCGGTGTCGACGTGCGGTTGGCGAACCACATTG 1327
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Sbjct 1268 GCTCGTACTGTGAACTTCTGAAGGACTCCGGTGTGGACGTGCGGTTGGCGAACCACATTG 1327

Query 1328 CACATCTGTTCATTCGAGATCCGCTTGTGATGTACGACAAGATGATCGACATCGATGACA 1387
           |||||||||| ||||| ||||| ||||||||||||||||||||||||||||||||||||||
Sbjct 1328 CACATCTGTTTATTCGCGATCCCCTTGTGATGTACGACAAGATGATCGACATCGATGACA 1387

Query 1388 CGACGCACACAGAGCACTTTGACAACATCCAGTCCACTAACTGGCAGACAGTGCGCTTCA 1447
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Sbjct 1388 CGACGCACACGGAGCACTTTGATAACATCCAGTCCACTAATTGGCAGACAATGCGCTTCA 1447

Query 1448 AGCCTCCGCCGCTAGGCAACGACATTGGCTGGCGCGTTGAGTTCCGCGTGATGGATATTC 1507
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Sbjct 1448 AGCCTCCGCCGATAGGCAGCGACATTGGCTGGCGCGTTGAGTTTCGAGTGATGGATATTC 1507

Query 1508 AGCCAACGCCGTTCGAGAACGCCGCCTTCGCCGTCTTCATTCCGCTTCTCACCAAGGCCA 1567
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Sbjct 1508 AGCCAACACCATTCGAGAACGCCGCCTTCGCTGTCTTCATTCCGCTTCTCACCAAGGCCA 1567

Query 1568 TCGTCAACTACAAGCCCTGCTTTTACACCAAGATCTCCATCGTCGACGAGAATATGGGCC 1627
|||||||||||||||||||||||||||||||| |||||||||||||| ||||||||| | |
Sbjct 1568 TCGTCAACTACAAGCCCTGCTTTTACACCAAAATCTCCATCGTCGAGGAGAATATGAGTC 1627

Query 1628 GCGCACATCGCATCAACCCATGTGGAGAACAATACATTATGCGCAAGGACATTTTCGCCC 1687
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Sbjct 1628 GCGCACATCGCATCAACCCCTGTGGAGAACAATACGTTATGCGTAAGGACATTTTTGCCA 1687

Query 1688 ACAAGTGCACCGCCAGTGACGAGGAGACGGCGAGGATGAGCATTGACGAGATCTTCAACG 1747
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Sbjct 1688 ACAAGTGCACCGCCAGCGACGAGGAGACAGCGAGGATGAGCATTGACGAGATCTTCAACG 1747

Query 1748 GCAAGGAGGGCGGATTCTATGGACTCATCCCCCTCGTGTGCCGCTATCTCGACGACGAGG 1807
||||||||| ||| |||||||||||||||||||||||||||||||||||||||||| |||||
Sbjct 1748 GCAAGGAGGACGGCTTCTATGGACTCATCCCCCTCGTGTGCCGCTATCTCGACAGCGAGG 1807

Query 1808 GGAAGCGAAGTCCCCTCGTAAACTCCTACTTGAAGTTCCTGTCAATGCGCGCCTCTGGCC 1867
| |||||||||||||||| |||||||||||||||| |||||||||||||||||||||||||
Sbjct 1808 GAAAGCGAAGTCCCCTCATAAACTCCTACTTGAAGCTCCTGTCAATGCGCGCCTCTGGCA 1867

Query 1868 GCATTCCCACACCTGCGCAGTACATGCGAAAGTTTGTCACGACACATCCCGACTACAAAC 1927
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Sbjct 1868 GCATTCCCACACCTGCGCAGTACATGCGCAGGTTTGTCACAACGCACCCCGACTACAAGC 1927

Query 1928 ACGACTCACGCCTCACCGACAGCATCGCACGTGACCTTGTGCAGCGCATGCACGGTCTGG 1987
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Sbjct 1928 ACGACTCACGCCTCACCGACAGTATCGCACGTGACCTTGTCCAGCACATGCACAGCCTGG 1987

Query 1988 CTTCGAATCAGATCCACGACGATGACTACCTTCCCATAAGCGTCTTCAAGGCC-AC-CAC 2045
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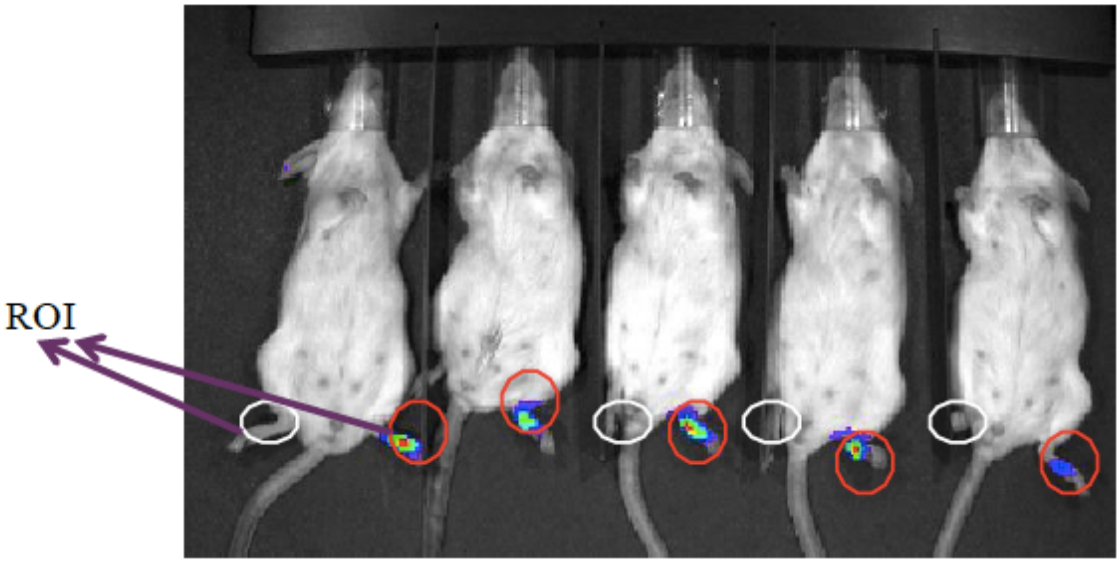
Sbjct 1988 CCTCGAATCAGATCCACGACGATGACTATCTTCCGATGAGCATCTTCAGGGTCGATTCAG 2047

Query 2046 AAGAGAGAGTGTCAAGAGCGGCCGCGATATC 2076
||| | || ||||||||||||||||||
Sbjct 2048 TAGA-A-AGCACCAAGAGCGGCCGCGATATC 2076

Appendix 4 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 luciferase expression Leishmania.

The formula used to calculate percentage of suppression

% Suppression = [(Mean control- sample value) /Mean control] x100