University of Strathclyde

Strathclyde Institute of Pharmacy and Biomedical Sciences

# Development of a vaccine against leishmaniasis

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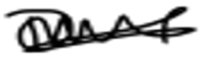
Signed: 

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| APC | Antigen presenting cell |
| bp | Base pair |
| BCG | Bacillus Calmette-Guerin |
| BLI | Bioluminescence |
| BSA | Bovine serum albumin |
| CL | Cutaneous Leishmaniasis |
| DC | Dendritic cell |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxyribonucleotide triphosphate |
| EDTA | Ethylenediaminetetraacetic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| FCS | Fetal calf serum |
| γGCS | Gamma Glutamyl Cysteine Synthetase |
| HRP | Horseradish peroxidase |
| IFN-γ | Interferon gamma |
| Ig | Immunoglobulin |
| IL | Interleukin |
| IPTG | Isopropyl β-D-1-thiogalactopyranoside |
| IVIS | *In vivo* imaging system |
| kDa | kilo Daltons |
| MCL | Mucocutaneous Leishmaniasis |
| MHC | Major histocompatibility complex |
| NK | Natural killer |
| NADPH | Nicotinamide Adenine Dinucleotide Phosphate Oxidase |
| NO | Nitric oxide |
| LdγGCS | *L. donovani* γGCS |
| LmγGCS | *L. mexicana* γGCS |
| LmjγGCS | *L. major* γGCS |
| PCR | Polymerase chain reaction |

| | |
|---|---|
| RNA | Ribonucleic acid |
| RNS | Reactive nitrogen species |
| ROS | Reactive oxygen species |
| SE | Standard error |
| Th | T-helper cells |
| TNF-α | Tumor necrosis factor-α |
| T reg | T regulatory |
| VL | Visceral Leishmaniasis |

## ABSTRACT

*Leishmania* parasites, protozoan parasites transmitted by female sand flies, cause the disease leishmaniasis. Leishmaniasis is responsible for 25,000–65,000 deaths and 0.7–1.0 million new cases per year. At present, there is no clinical vaccine available. Previous studies have indicated that recombinant gamma glutamylcysteine synthetase (γGCS) can protect against *L. donovani and L. major* infection in a murine model. However, these studies showed that production of full-length recombinant γGCS was problematic and only truncated forms of the protein could be isolated. Therefore, in this study new plasmid constructs were produced for expression of recombinant γGCS from *L. donovani, L. major* and *L. mexicana*. Molecular techniques were used to produce a series of plasmids that code for the full-length sequence of γGCS. Constructs were engineered to encode for an N-terminal T7-tag and a C-terminal His-tag, to facilitate isolation of pure and full-length γGCS protein from an *Escherichia coli* expression vector (pET21a(+)). Optimal expression occurred in transfected *E. coli* when bacteria were cultured at 18ºC after induction using 0.1 mM isopropyl β-D-1-thiogalactopyranoside. The recombinant protein was found to be present in the soluble fraction and a HisPur™ Ni-NTA spin column purification kit was used to obtain recombinant γGCS. Gel electrophoresis and western blot analysis showed that full-length γGCS protein was isolated for all three recombinant proteins. However, subsequent isolation using a T7•Tag® affinity purification kit resulted in insufficient amounts of protein.

Vaccine studies were completed using *L. tarentolae* promastigotes expressing γGCS. Mice were immunised on day 0 and 21 with parasites expressing γGCS from *L. donovani* alone or a mixture of parasites expressing γGCS from *L. donovani, L. major* or *L. mexicana* (triple vaccine). On day 42 mice were infected with *L. donovani* amastigotes and parasite burdens were determined 14 days later. Protection was associated with a mixed Th1/Th2 response based on specific IgG1 and IgG2a antibodies and on cytokines produced by antigen stimulated splenocytes used in *in vitro* proliferation assays.

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# Chapter 1 General Introduction

## *1.1 Leishmaniasis*

Leishmaniasis is one of the seven most important tropical and subtropical diseases in the world, which affects between 12-15 million people worldwide. There are approximately 2 million new cases every year and it is responsible for 70,000 deaths each year (Ornellas-Garcia *et al.*, 2023; Torres-Guerrero *et al.*, 2017). Leishmaniasis is caused by infection with the protozoan parasite *Leishmania*. It is transmitted from one host to another via the bite of the vector, a female sand fly (Bates *et al.*, 2004) but only 20 of 53 species of *Leishmania* infect people (Ikeogu *et al.*, 2020). It is a heterogeneous group of diseases, but it can be divided into 3 main types, depending on the clinical symptoms, i.e. visceral leishmaniasis (VL), cutaneous leishmaniasis (CL) or mucocutaneous leishmaniasis (MCL). The outcome of infection depends on parasite characteristics, host factors such as the host's immune responses and vector biology (Burza *et al.*, 2018; Tao and Jia, 2024). Table 1 shows the species responsible for causing different types of leishmaniasis and Figure 1.1 shows their geographical distribution.

VL also known as kala-azar, occurs in more than 60 countries e.g. Brazil, Ethiopia, India, Kenya, Somalia, South Sudan, and Sudan. *L. donovani* causes VL in Africa and Asia and *L. infantum* causes VL in the Middle East, South America, and Central America (Burza *et al.*, 2018). Parasites reside mainly in the spleen, bone marrow and liver. Clinical symptoms include low-grade fever, weight loss, muscle loss, anaemia, and leukopenia, with hepatosplenomegaly being one of the most serious symptoms. The mortality rate for VL is nearly 100% if it is left untreated (Sundar and Singh, 2018). VL can have a short (2 weeks) or long (8 months) incubation period and its clinical symptoms can be more severe if the host's immune system is

immunosuppressed (Burza *et al.*, 2018). CL is caused by infection with a number *Leishmania* species e.g. *Leishmania tropica* and *L. major* in Africa, Europe and Asia, whereas *L. braziliensis* and *L. mexicana* cause CL in South and Central America (Stamper *et al.*, 2019). CL is not life threatening and can be self-healing, but it can cause disfiguring scarring, which leads to adverse psychological effects on the person infected and can result in social stigma (Nuwangi *et al.*, 2023). Cutaneous lesions form at the site of the sand fly's bite, and these can lead to single or multiple papules weeks to months later. A papule can develop into an ulcer in some cases (Burza *et al.*, 2018) and increase in body temperature is one of the most frequent symptoms for CL (Torres-Guerrero *et al.*, 2017).

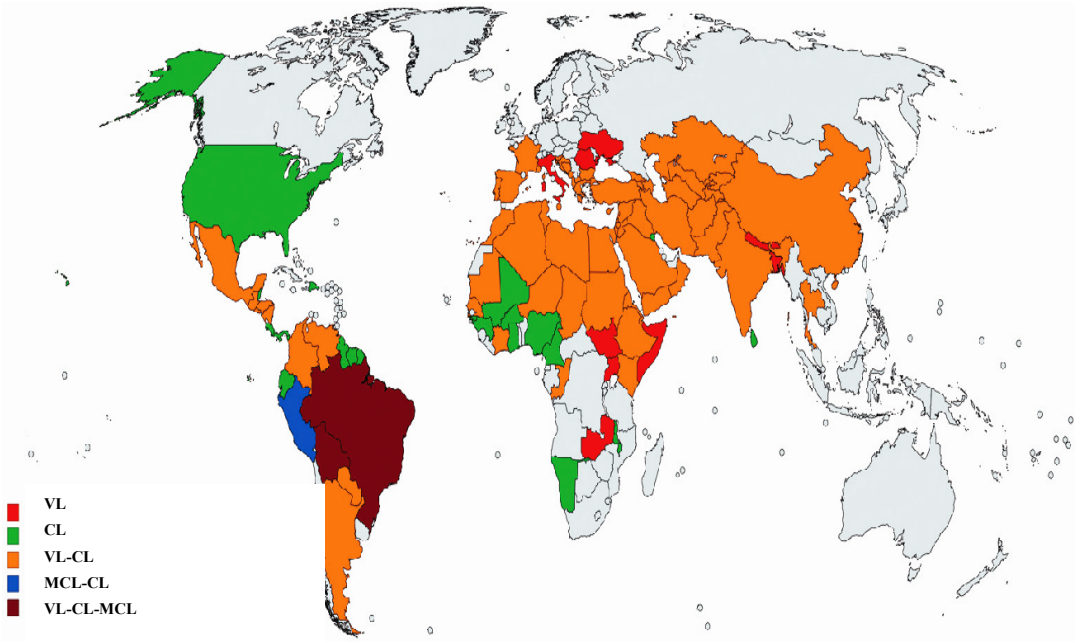


**Figure 1.1 Global distribution of different types of leishmaniasis.** The map shows the distribution of different types of leishmaniasis around the world. VL– visceral leishmaniasis, CL – cutaneous leishmaniasis, and MCL – mucocutaneous leishmaniasis [adapted from (Esteves *et al.*, 2018)].

MCL generally occurs after cutaneous infection and results in the damage to oral-nasal cavities, which can be very disfiguring (Ghazanfar and Malik, 2016). This can appear 2 years after infection and can occur over a 30-year period. MCL is mostly caused by infection with *L. braziliensis* but infection with *L. amazonensis, L. guyanensis* and *L. panamensis* can also cause MCL (Handler *et al.*, 2015). The majority of cases (90%) occur in Bolivia, Brazil, Ethiopia and Peru (Esteves *et al.*, 2018).

## *1.2 The life cycle of* Leishmania

The genus *Leishmania* is divided into 4 subgenera; *Leishmania*, *Viannia, Sauroleishmania* and *Mundinia* depending on taxonomic and nomenclatural systems and the most common and clinically curial species belong to *Leishmania* and *Viannia* subgenera (Mathison and Bradley, 2023) (Table 1.1). *L. tarentolae is a Sauroleishmania* species. It is a protozoan parasite of the gecko and it is not considered pathogenic to humans. *L. tarentolae* is extensively studied for a wide variety of biotechnological applications (Mendoza- Roldan *et al.*, 2022).

There are two hosts in *Leishmania's* life cycle: a mammalian host and an insect vector (Figure 1.2) and it involves different life stages, adapted to survive in their specific environments. The two main morphologies are the intracellular amastigote stage, which occurs in the mammalian host, and the promastigote stage, which is found in the female sand fly vector. Sand flies can transmit a single or multiple species of *Leishmania* (Sunter and Gull, 2017). When a female sand fly takes a blood meal from the host it deposits metacyclic promastigotes into the dermis, and this starts the infection (Figure 1.2). The outcome of infection depends on the number of metacyclic promastigotes deposited by the sand fly and how long the vector has been infected, the

infecting species and host factors (Ikeogu *et al.*, 2020). Immune responses vary over the course of infection and depend on genetic background, host immunity, host nutrition status, vector, species of parasite and social factors (Costa-da-Silva *et al.*, 2022). Additionally, if patients have another infection such as HIV, it can cause a higher level of morbidity and mortality (Cunha *et al.*, 2020), a weaker treatment response longer healing duration and rapid parasite spread (Maksoud and El Hokayem, 2023).

**Table 1.1 List of *Leishmania* species associated with different forms of leishmaniasis**. Each *Leishmania* species was associated with different etiological, epidemiological aspects with main reservoirs [adapted from (Cecílio *et al.*, 2022)].

| Genus / Sub Genera | Species | Clinical Form | Main Reservoir | Main Clinical Features |
|---|---|---|---|---|
| *Leishmania Leishmania* | *L. donovani* | VL,PKDL | Human | Fever, weight loss |
| | *L. tropica* | CL | Humans | Painless, ulcer |
| | *L. major* | CL | Rodents | Severe inflammation, rapid necrosis |
| | *L. aethiopica* | CL | Hyraxes | Nodular lesions |
| | *L. infantum* | VL,CL | Dogs, Rodents, Human | Fever |
| | *L. mexicana* | CL | Rodents | Single or multiple ulcer lesions |
| | *L. venezuelensis* | CL | Rodents | Ulcerating lesions |
| | *L. amazonensis* | CL | Rodents | Single or multiple ulcer lesions |
| *Leishmania Viannia* | *L. guyanensis* | CL | Sloths | Ulcer lesion may progress to mucocutaneous form |
| | *L. panamensis* | CL | Dogs | Ulcer lesion |
| | *L. peruviana* | CL | Unknown | Ulcer lesion may progress to mucocutaneous form |

*Leishmania* can migrate within the skin as metacyclic promastigotes using their flagellum, so phagocytosis can occur at a distance from the bite site. Membrane injury and loss of integrity can also occur at the phagocyte's surface due to interaction of the parasite's moving flagellum with the cell membrane, and this damage can increase the rate of success of infection. The promastigote stage transforms into the smaller amastigote form inside the macrophage, which has a short flagellum and a shorter body (Sunter and Gull, 2017). There are many factors, which can affect this transformation including lack of oxygen, low pH and nutritional depletion of tetrahydrobiopterin. In addition, changes in molecular processes within the parasite are also associated with these morphological changes (Besteiro *et al.*, 2007). The *Leishmania* parasite may even be able to sense the health status of its host macrophage via its flagellum and select healthy cells for infection, so that it can increase its chances of completing its life cycle in the host (Sunter and Gull, 2017). Macrophages can migrate to different tissues within the body and this may have a key role in spreading the *Leishmania* infection within the host's body (Loría-Cervera and Andrade-Narvaez, 2020). The parasite is transmitted to a new sand fly when the sand fly takes a blood meal from an infected mammalian host. Infected macrophages or free amastigotes present in the blood are taken up as part of the blood meal and the parasites are released into the sand fly's gut. The parasites change from the non-motile amastigote stage into the motile promastigote form and differentiation is influenced by factors such as change in pH and or ambient temperature (Sunter and Gull, 2017). Four life forms have been identified in the sand fly (Figure 1.2). Early promastigote forms have a short and ovoid body and little motility and are called procyclic promastigotes. Amastigotes transform to this form 12-18 hours after uptake by the sand fly (Inbar *et al.*, 2017). The length of

cell body of procyclic promastigotes can be between 6.5-11.5 μm and their flagellum is shorter than their body length (Sunter and Gull, 2017). By 36 to 60 hours post-infection, procyclic promastigotes convert to the nectomonad form, which has a longer cell body length of 12 μm. Enormous numbers of nectomonad promastigotes are formed in the abdominal midgut by 60 to 72 hours post-infection and many of them are attached to an epithelial cell by their flagellum. In the next 7-10 days, the promastigotes migrate towards the foregut and differentiate into leptomonad promastigotes. The last phase is named metacyclogenesis and this occurs in the thoracic midgut and stomodeal valve. Leptomonad promastigotes transform into metacyclic promastigotes, which are highly motile, have a short body, and their flagellum is longer than their body length (Inbar *et al.*, 2017).

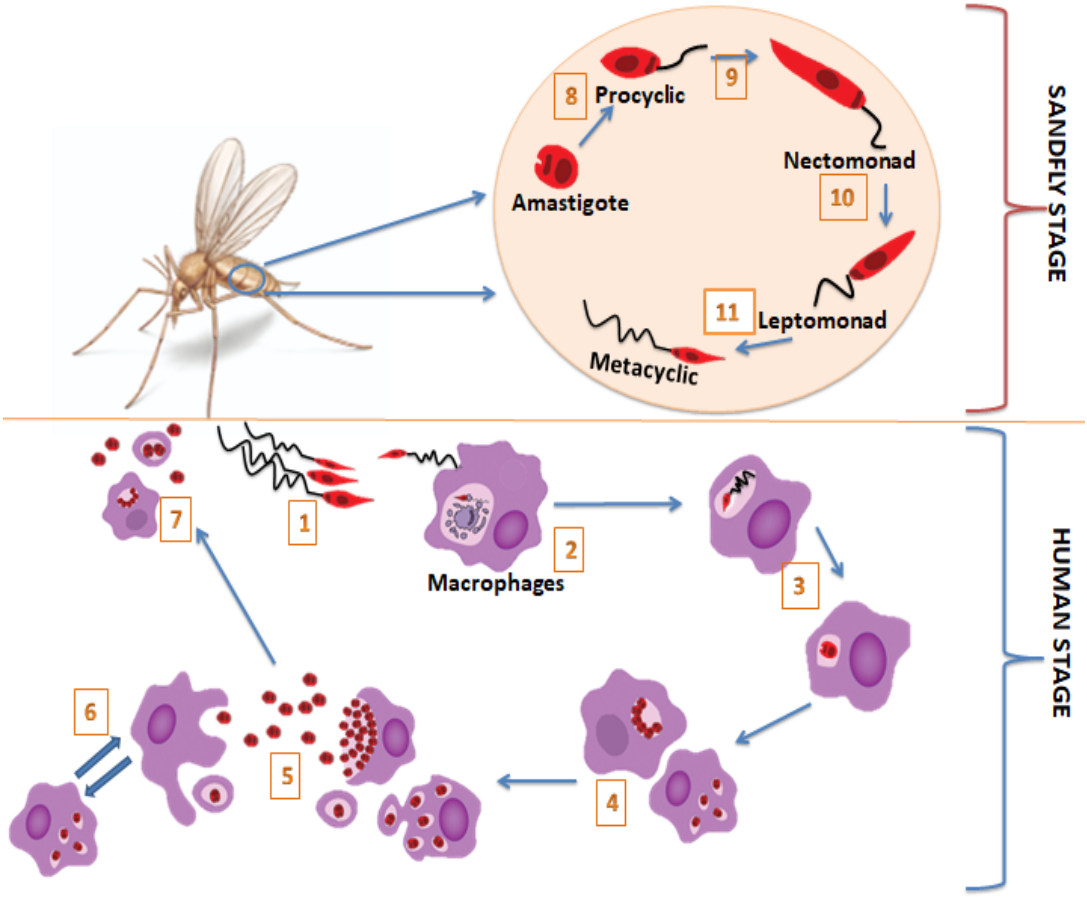


**Figure 1.2 The life cycle of *Leishmania.*** 1. Host infection by metacyclic promastigotes deposited in the skin when the vector feeds; 2. Macrophages infected with promastigotes; 3. Promastigote differentiation into amastigote; 4. Multiplication of amastigotes in individual macrophage; 5. Release of free amastigotes within host cell membranes; 6. Infection of new cells; 7. Ingestion of free parasites and infected cells by a sand fly when it takes a bloodmeal; 8. Amastigotes differentiate into procyclic promastigotes inside the sand fly's gut; 9. Differentiation to nectomonad promastigotes; 10. Differentiation to leptomonad promastigotes; 11. Differentiation to metacyclic promastigotes [adapted from (Serafim *et al.*, 2018)].

### *1.3 Immunity to* **Leishmania**

The host's immune system has a key role in controlling *Leishmania* infections and responses can be divided into early, innate immune responses and later adaptive immune responses. Innate immune responses against *Leishmania* involve neutrophils, macrophages, dendritic cells (DC), monocytes and natural killer cells (NK cells), whereas adaptive immune responses involve $CD4^+$ T cells, $CD8^+$ T cells and B cells. Neutrophils have various roles in host immunity against *Leishmania*. At the beginning of infection neutrophils move to the site of infection in response to the injury and inflammatory response induced by the sand fly's bite (Dos Santos Meira and Gedamu, 2019). Neutrophils use different killing mechanisms to control the parasite which include release of granules that contain microbicidal proteins, phagocytosis and the production of reactive oxygen species (ROS) and formation of neutrophil extracellular traps (NETs) (Regli *et al.*, 2020). Three NETosis activation pathways including suicidal NETosis, vital NETosis and caspase-dependent NETosis have been characterised (Li *et al.*, 2023). Studies have shown that neutrophil depletion boosted parasite burdens and size of lesion size in mice infected with *L. major* (Passelli *et al.*, 2021). Thus indicating that neutrophils are important in protection against *Leishmania.* However, *Leishmania* can escape NET killing activity via the presence of specific cell surface components, which can resist NET protease activity (Regli *et al.*, 2017). *Leishmania* promastigotes have a glycocalyx coat made up from lipophosphoglycan (LPG), a 63 kDa metalloproteinase (GP63) and proteophosphoglycan (PPG). LPG is a key molecule for immune detection and protecting the parasite from the host immune system (Franco *et al.*, 2012). It has been shown that *L. donovani* LPG enhances parasite viability in the presence of NETs (Quintela-Carvalho *et al.*, 2022; Regli *et al.*, 2017).

LPG prevents the attachment of complement molecules, or it can inactivate a functional complement complex at the promastigote surface (Yasmin *et al.*, 2022). Pattern recognition receptors (PRRs) recognise pathogen-associated molecular patterns (PAMPs) on the pathogen and initiate the anti-microbial defence mechanisms of the innate immune system. One type of PRRs are Toll-like receptors (TLRs) which are mainly expressed by phagocytes (Merida-de-Barros *et al.*, 2018; Sacramento *et al.*, 2017). Recognition of TLR-2 by LPG can trigger NO and ROS production by macrophages to help parasite elimination, but it may also help parasite persistence as it stimulates the expression of more TLR-2 on the surface of neutrophils in *L. major* infection (Jafarzadeh *et al.*, 2019). Increased LPG-THR-2 interaction can then boost parasite uptake by neutrophils. In this way neutrophils can help spread *Leishmania* infection. It has been suggested that these cells act as 'Trojan horses' (Figure 1.3) and that parasites infect macrophages without inducing any antiparasitic responses when the neutrophils are taken up by macrophages (Regli *et al.*, 2020).

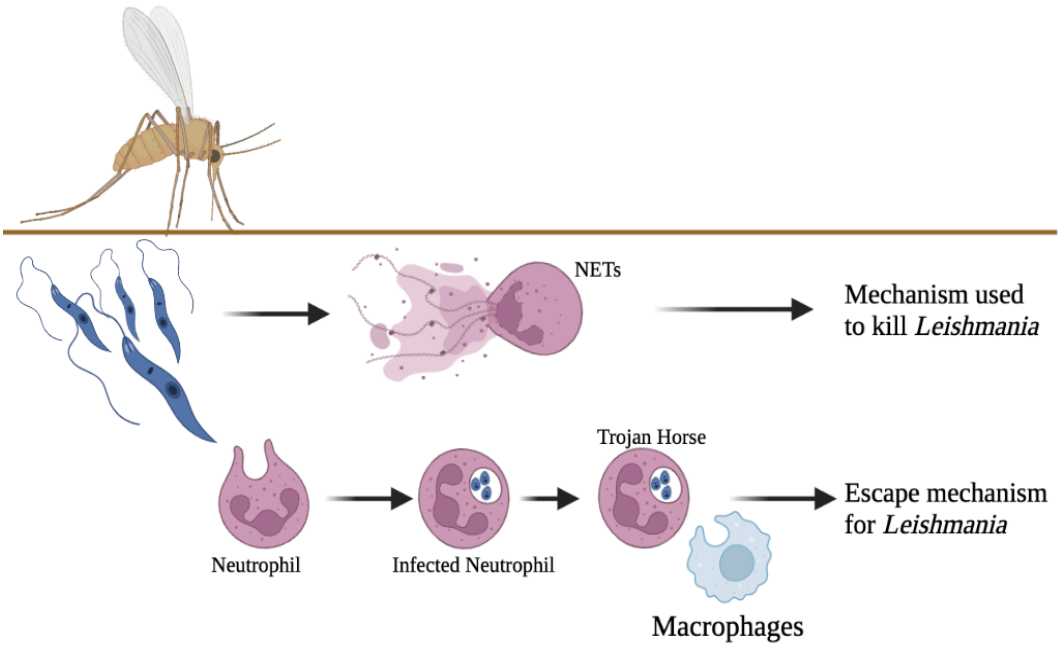


**Figure 1.3 Interaction of neutrophils with *Leishmania*.** Interaction of neutrophils with *Leishmania* promotes gathering of neutrophils. Neutrophil extracellular traps (NETs) can eliminate parasites. On the other hand amastigotes can be transferred silently by apoptotic neutrophils that act as a "Trojan Horse" [adapted from (Dos Santos Meira and Gedamu, 2019)].

Macrophages can be divided into two main groups according to their functions i.e. M1 and M2 cells. M1 cells display microbicidal properties whereas M2 cells are involved in inflammation and tissue repair. M1 macrophages produce IL-12 which can influence T-helper 1 (Th1) cells and they secrete tumour necrosis factor alpha (TNF-α) for polarisation of M1 macrophages. T cells and endothelial cells can then cause the induction of more M1 macrophages via their production of interferon gamma (IFN-γ) and/or TNF-α. In contrast, M2 macrophages produce IL-10, which is a negative regulator of IFN-γ and/or TNF-α (Goto and Mizobuchi, 2023). M1 macrophages can kill internalised pathogens by producing ROS such as superoxide, hydrogen peroxide and reactive nitrogen species (RNS) such as nitric oxide (NO), which is produced using

the enzyme inducible nitric oxide synthase (iNOS) (Loría-Cervera and Andrade-Narvaez, 2020). *Leishmania* promastigotes have developed various strategies for escaping these killing mechanisms (Figure 1.4). For example, LPG can shield promastigotes from damage by ROS. In addition, arginase secreted by *Leishmania* for producing crucial nutrients such as L-ornithine, is a substrate required by iNOS and this reduces the production of NO (Rossi and Fasel, 2018). Oxidative stress within macrophages can also be reduced by the presence of glycoprotein 63 (GP63), a surface metalloprotease with a molecular weight of 63 kDa. GP63 prevents the induction of NOX2 and iNOS in this way it protects promastigotes from macrophage killing. Another escape mechanism is via the production of superoxide dismutase (SOD), an enzyme that removes excess superoxide radicals ($O^{2-}$) by converting it to hydrogen peroxide and oxygen and is produced by *Leishmania* and host macrophages (Pawłowska *et al.*, 2023). *L. braziliensis* and *L. amazonensis* can enhance macrophage SOD expression, which breaks down superoxide and enhances parasite survival (Rossi and Fasel, 2018).

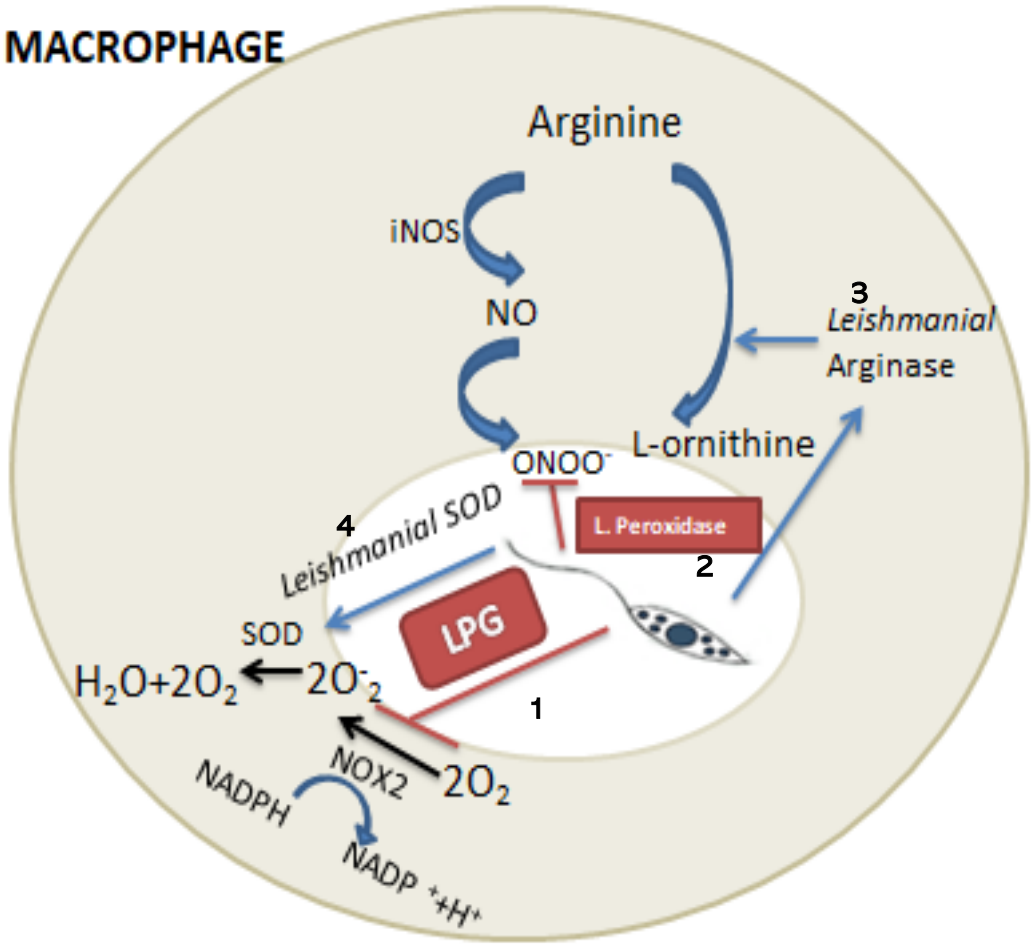


**Figure 1.4 Mechanisms used by *Leishmania* promastigotes to survive inside macrophages**. 1. *Leishmania* LPG coat hinders the NOX2 complex assembly at the phagosome membrane and the production of superoxide ($O_2^-$) is blocked. 2. *Leishmania* peroxidase secretion detoxify $ONOO^-$. 3. Arginase can be secreted by *Leishmania* and it promotes to convert arginine into ornithine and produce SOD homologues. 4. Arginase enhance the parasitoxic superoxide production [adapted from (Rossi and Fasel, 2018)].

NK cells are also involved in early innate responses to *Leishmania*. NK cells produce IFN-γ upon activation and stimulate macrophage anti-leishmanicidal activities and enhance the production of Th1 cells (Cavalcante *et al.*, 2022). Studies have shown that mice lacking NK cells secreted lower amounts of IFN-γ, higher amounts of IL-4 and had higher *L. major* burdens. NK cells may also enhance macrophage production of IL-12 and IL-18, which in turn may increase IFN-γ production and also cause DC cell activation. *L. donovani* and *L. mexicana* promastigotes can activate IFN-γ production by NK cells in the absence of IL-12 (Alizadeh *et al.*, 2023).

DC cells are considered as the main link between innate and adaptive immunity (Elmahallawy *et al.*, 2021). DCs and *Leishmania* interaction controls subsequent immune responses, using different cytokines and multiple signalling pathways (Margaroni *et al.*, 2022). DCs express both major histocompatibility complex (MHC) class I and class II molecules so they can present antigens to $CD4^+$ T cells and $CD8^+$ T cells (Figure 1.5). Antigen presentation occurs mostly in lymphoid tissues (Tiburcio *et al.*, 2019). After $CD4^+$ T and MHC-II interaction, $CD4^+$ T cells can differentiate into a number of different subsets including Th1, Th2, Th17, Th9 and T regulatory [Tregs] cells (Pennock *et al.*, 2013). Th1 cells secrete IFN-γ and this cytokine can act on macrophages and upregulate their production of RNS and ROS, inducing parasite elimination. Th2 cell produce IL-4, IL-5 and IL-13 and they can deactivate macrophages and increase parasite survival (Tiburcio *et al.*, 2019). Studies using mice infected with *L. major* proved that resistance to infection was associated with Th1 cells releasing IFN-γ, whereas susceptibility was related to Th2 cell production and the release of IL-10 and IL-4 (Castellano *et al.*, 2009). These T cells subsets cause polarisation of specific antibody responses in mice with Th1 and Th2 immune

responses being associated with IgG2a and IgG1 induction, respectively (Varotto-Boccazzi *et al.*, 2023). Th9 cells produce IL-9 and this cytokine induces the production of Th17 and Treg cells and upregulates the production of IL-10. Th17 cells produce IL-17, IL-21 and IL-22, which are responsible for pathogen eradication (Alizadeh *et al.*, 2023). DCs presenting antigens via the MHC-I pathway activate specific $CD8^+$ T cells. It has been showed that antigen-specific $CD8^+$ T cells have a significant role in immunity against *Leishmania* (Tiburcio *et al., 2019)*. Activated $CD8^+$ T cells turn into effector cells, which can release perforin, and granzymes, which can cause lysis of target cells and killing of infected host cells (Stäger and Rafati, 2012). Activated $CD8^+$ T cells produce IFN-γ, which stimulates macrophages to kill their intracellular *Leishmania* parasites (Novais and Scott, 2015). This explains why $CD8^+$ T cell have been shown to have a protective role against VL caused by infection with *L. infantum* or *L. donovani* in humans and murine models (Rossi and Fasel, 2018). In contrast, in *L. major* and *L. braziliensis* infection $CD8^+$ T cells which migrated into the infection site and secreted perforin and granzyme A were associated with the production of a skin ulcer and nodular lesion progression (Campos *et al.*, 2020).

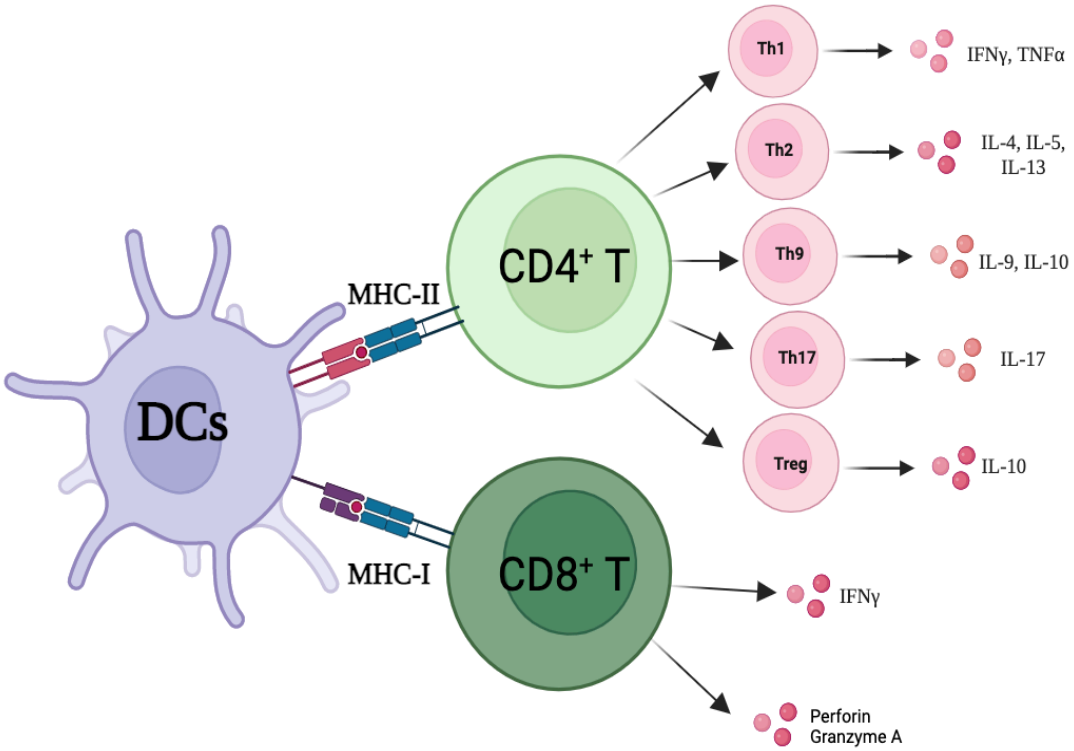


**Figure 1.5 The role of DCs in adaptive immune responses in *Leishmania* infection.** $CD8^+$ T cells recognise specific antigens presented via class I MHC and produce IFN-γ. $CD4^+$T cells recognise specific antigens presented via class II MHC and differentiate into different Th subtypes, which produce different cytokines [adapted from (Teufel *et al.*, 2020; Tiburcio *et al.*, 2019)].

B cells are important producers of pathogen specific antibodies and they can also act as antigen presenting cells (Nothelfer *et al.*, 2015). B cells are produced in the bone marrow, migrate to the spleen and other secondary lymphoid tissues where they mature and differentiate into immunocompetent B cells. B cell activation occurs in germinal centres of lymphoid organs via antigen recognition by the B cell receptor and a co-stimulatory signal provided by either helper T cells or the antigen itself. This results in B cell proliferation and the production of mature B cells, which differentiate into long-lived plasma cells or memory B cells.

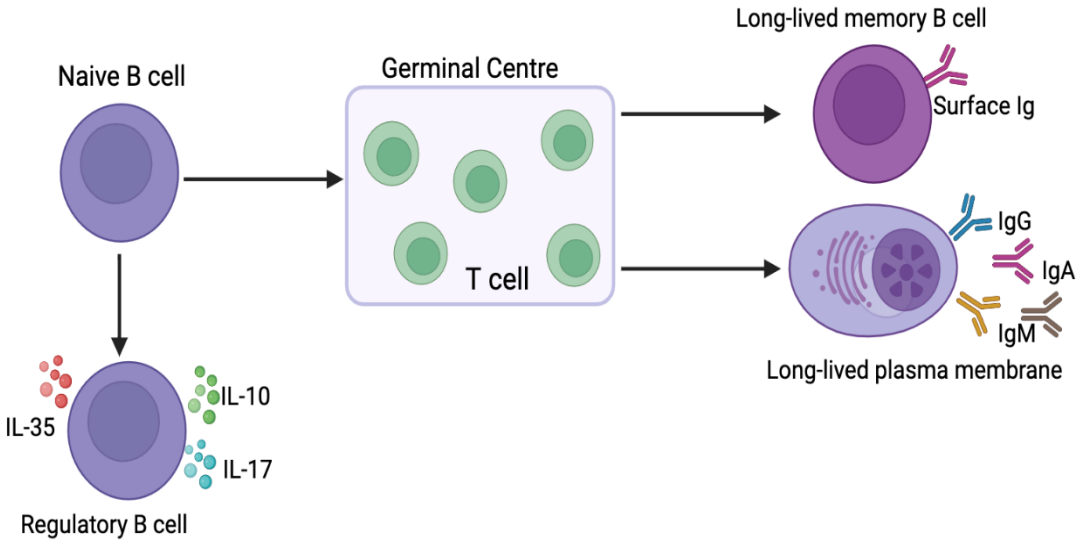


**Figure 1.6 The role of B cells in *Leishmania* infection.** *Leishmania* induces B cell differentiation and inhibits protective immune responses. In the germinal centre, to confer a protective immune response naive B cells differentiate into plasma cells, which produce an antibody of one specificity, or memory B cells, which provide a quick response following exposure. Regulatory B cell secrete immunosuppressive cytokines, thereby modulating a T cell response via the production of IL-10 and IL-17 [adapted from (Nothelfer *et al.*, 2015)].

B cells play a key role in both innate and adaptive immune response and studies have shown that regulatory B cells secrete different cytokines which have different roles, i.e., IL-10 is anti-inflammatory, IL-17 and IL-35 are immunoregulatory (Figure 1.6) (Conde *et al.*, 2022; Nothelfer *et al.*, 2015). B cell responses can be manipulated in distinct ways by *Leishmania,* depending on the infecting species (Silva-Barrios *et al.*, 2018). Studies have indicated a protective role for B cells, for example, mutants lacking B cells develop bigger cutaneous lesions and cannot control parasite growth in *L. major* infections (Firmino-Cruz *et al.*, 2020).

## *1.4 Treatment of leishmaniasis*

There is a limited number of drugs to treat leishmaniasis patients, but all of them have some drawback e.g. toxicity, high cost and/or a multidose treatment regimen (Table 1.2). Drug resistance is one of the main reasons that leishmaniasis reappears in drug treated patients and its induction is often related to using a long treatment protocol (Tom *et al.*, 2024). The high cost of drugs in endemic areas and the adverse effects associated with drug treatment often leads to poor patient compliance, which would favour the selection of drug resistant parasites in patients that do not complete the recommended treatment regimen (Baber *et al.*, 2024; Ghorbani and Farhoudi, 2018). Pentavalent antimonials such as sodium stibogluconate or meglumine antimoniate have been used since 1920's, but drug resistance in endemic parasites has limited their use, especially against VL (Ponte-Sucre *et al.*, 2017). Antimonials are not recommended for pregnant women (Ponte-Sucre *et al.*, 2017) and can cause adverse effects such as cardiac arrhythmia, nephrotoxicity and hepatotoxicity (Berger *et al.*, 2017). Amphotericin B (AmB), originally an antifungal drug, is also used to treat leishmaniasis (Thakur *et al.*, 2020). It is also available as lipid formulations which are

less toxic, but unfortunately more expensive and their high cost limits their use (Shirzadi, 2019). AmB is now available as an oral formulation, which has a lower toxicity than intravenous administered AmB as the formulation prevents AmB aggregation, but it has a poor shelf-life (Frézard *et al.*, 2022). Miltefosine, which has high antileishmanial efficiency but its teratogenicity problems mean that it cannot be used by pregnant women (Melcon-Fernandez *et al.*, 2023). Miltefosine was an anticancer agent, which was repurposed for antileishmanial therapy. The drug has a long *in vivo* half-life, which promotes the induction of drug resistance (Thakur *et al.*, 2020). Research in Ethiopia with CL patients showed that miltefosine had tolerable side-effects but it was not efficient in treating severe or protracted CL (van Henten *et al.*, 2021). Combination therapy may be one way to improve the therapeutic outcome for leishmaniasis as studies have shown that using two antileishmanial drugs reduces both the duration of therapy and the drug dose required, thereby reducing toxic side effects and overall treatment costs. For example, combination treatment with miltefosine and AmB is more effective than treatment with miltefosine alone (Chakravarty and Sundar, 2019).

**Table 1.2 Drugs used in the treatment of leishmaniasis.** The table shows the dose, disadvantages, side effects and the type of leishmaniasis treated by different drugs [adapted from (Ansari *et al.*, 2017; Chakravarty and Sundar, 2019; Sreedharan and Rao, 2023).

| Drug | Dose | Disadvantage | Efficiency | Clinical Forms | Mechanism |
|---|---|---|---|---|---|
| **Amphotericin B** | 1 mg/kg over 30 days | Toxicity | >95% | VL | Cell death with permeability increase |
| **Liposomal amphotericin B** | 20 mg/kg over 4 doses | Poor efficacy<br>High cost | 98% | VL | Killing parasite and infected host cell |
| **Sodium stibogluconate** | 20 mg $Sb^{v}$/kg day for 30 days | Toxicity<br>Resistance | 35-95% | CL,VL | Macromolecule biosynthesis inhibition in amastigotes |
| **Miltefosine** | 100 mg/day for 28 days | Low compliance<br>Teratogenicity | 94-97% | CL ,VL | Inhibition of cytochromec oxidase |
| **Pentamidine** | 4 mg/kg monthly for 12 months | Poor efficacy | 70-80% | CL,VL | Inhibition of transcription |

### *1.5 Vaccination*

Vaccines are an impactful invention against diseases that cause significant mortality and morbidity and they are the best way to control deadly diseases (Campagnani *et al.*, 2022). Vaccines have health, social and economic benefits for individuals and society and can provide herd immunity. Studies indicate that vaccines have prevented almost six million deaths/year from life-threatening vaccine-preventable diseases (Rodrigues and Plotkin, 2020). They are also a cost-effective way to control diseases as they prevent hospitalisation and can be used as part of a government's disease control programme (Tahamtan *et al.*, 2017). This has clearly been demonstrated in the COVID 19 pandemic where 19.9 million lives were saved by COVID 19 vaccines. A vaccine relies on using a pathogen's specific antigen or antigens to induce a protective immune response, which prevents clinical symptoms normally associated with infection with the pathogen (Watson *et al.*, 2022). In an ideal situation a vaccine induces a protective immune response that would block the ability of the pathogen to survive in the host. Vaccines can be divided into three categories based on the type of antigenic material used. First generation vaccines are inactivated or attenuated vaccines that use a weakened form of a pathogen, so it cannot cause disease (Tahamtan *et al.*, 2017). However, first generation vaccines may have safety concerns as the pathogen could revert back to an infective form and may cause toxic side effects (Jain and Jain, 2015). Second generation vaccines contain synthetic or recombinant proteins produced using bacterial or viral expression systems. Third generation vaccines encode specific gene sequence(s) of a pathogen that is (are) relevant for protection. DNA and RNA vaccines are examples of this group of vaccines (Tahamtan *et al.*, 2017). An ideal *Leishmania* vaccine should meet specific criteria: it should induce long-term immunity, be stable

even at tropical temperatures, be safe to use and be cost effective. Previous studies have shown that recombinant protein technology can help meet these criteria (Pollet *et al.*, 2021). Recombinant protein vaccines can be produced using different expression systems, which use different expression vectors, promotors, selection markers and purification methods. Several vaccine antigens have successfully been produced using this method. For example, a recombinant hepatitis B vaccine has been produced from yeast (Nascimento and Leite, 2012). Trumenba®, a vaccine against meningococcal serogroup B bacterium, has been produced using an *E. coli* expression platform by Pfizer (Pollet *et al.*, 2021). This technology has been used to produce vaccines against severe acute respiratory syndrome caused by coronavirus (SAR-CoV) e.g. the Soberana 2 vaccine contains the spike protein from SARS-CoV and uses a Chinese hamster ovary cell expression system. A recombinant spike protein vaccine produced by Sanofi and GlaxoSmithKline plc in clinical phase III trials is produced in insect cells (Pollet *et al.*, 2021; Toledo-Romaní *et al.*, 2023). Leishmaniasis is a potentially vaccine-preventable disease but there is no clinically approved available vaccine available at present (Kaye *et al.*, 2023). A number of different types of vaccines have been used for leishmaniasis and examples are shown in Table 1.3.

Dogs are one of the reservoir hosts for *L. infantum* and there are vaccines available to prevent canine leishmaniasis. Leishmune®, which was withdrawn from market because of lack of effectiveness was the first licensed vaccine in Brazil. Leish-Tec® was licensed in Brazil in 2007 and CaniLeish® and LetiFend® were licensed in Europe in 2016 against canine leishmaniasis (Velez and Gallego, 2020). Leish-Tec® was formulated with *L. donovani* recombinant A2 protein and it enhanced IFN-γ levels after vaccination. Studies showed that the IgG2/IgG1 ratio was higher than 1.0

showing that the vaccine induced a predominant Th1 response (Coelho and Christodoulides, 2023). CaniLeish® is a second generation vaccine and it contains *L. infantum* excretion-secretion proteins (LiESP) and studies showed specific IgG2 humoral response was elicited after vaccination. LetiFend® contains four recombinant *L. infantum* proteins (Morales-Yuste *et al.*, 2022) and has a 72% protection rate against canine leishmaniasis (Reguera *et al.*, 2016). Data from 61% of dog owners living in Europe showed that 40% preferred to use CaniLeish® and 39% preferred to use LetiFend® (Morales-Yuste *et al.*, 2022).

**Table 1.3 Overview of different types of *Leishmania* vaccine formulations tested.** First, second and third generation vaccine used against *Leishmania* [adapted from (Abdellahi *et al.*, 2022)].

| Candidate of vaccine | Type of disease/ species | Adjuvant | Animal model | Result / Limitation |
|---|---|---|---|---|
| **FIRST GENERATION** | | | | |
| Non-pathogenic *L. tarentolae* promastigotes | VL/*L. donovani* | | BALB/c | Protection/ 80-85% parasite reduction but IL-4 production |
| Attenuated $10^7$ promastigotes | VL/*L. chagasi* | | BALB/c | No protection |
| lpg2−mutant promastigotes | CL/*L. major* | | BALB/c | Protection/ Low IFN-γ |
| **SECOND GENERATION VACCINE** | | | | |
| gp36 | VL/*L. donovani* | Saponin | BALB/c | 68.1% liver parasite reduction/ high IgG2a, IgG2b, and IgG1antibodies |
| rLeish-111f | CL/*L. major* | rIL-12 | BALB/c | no detectable IL-4, mixed IgG1, and IgG2a response |
| rLACK | CL/*L. major* | rIL-12 | BALB/c | Not observed long term protection |
| rORFF | VL/*L. donovani* | | BALB/c | Low IgG2a/IgG1 ratio, |
| **THIRD GENERATION VACCINE** | | | | |
| NH36 | VL/*L. mexicana* | VR1012 | BALB/c | Increase in IFN-γ, but low antibody response |
| KMP-11 | VL/*L. donovani* | pCMV-LIC | Hamster | Mixed Th1/Th2 response |
| LACK | CL/*L. major* | MIDGE | BALB/c | High ratio of IgG2a/IgG1 |

## *1.6 Gamma Glutamylcysteine Synthetase (γGCS)*

The trypanothione biosynthesis pathway has an important role in oxidative stress and maintains the cellular redox balance in *Leishmania* (Figure 1.7). In the pathway trypanothione is reduced to form the products thioredoxin and tryparedoxin, which decrease peroxidase activity in *Leishmania.* Trypanothione decreases nitrogen and iron-containing products in a cell and this can protect the parasite from the ROS produced within the host macrophage. Trypanothione is only produced by *Leishmania* and is not made by the host macrophage, but the host macrophage can provide the substrate spermidine (Raj *et al.*, 2020).

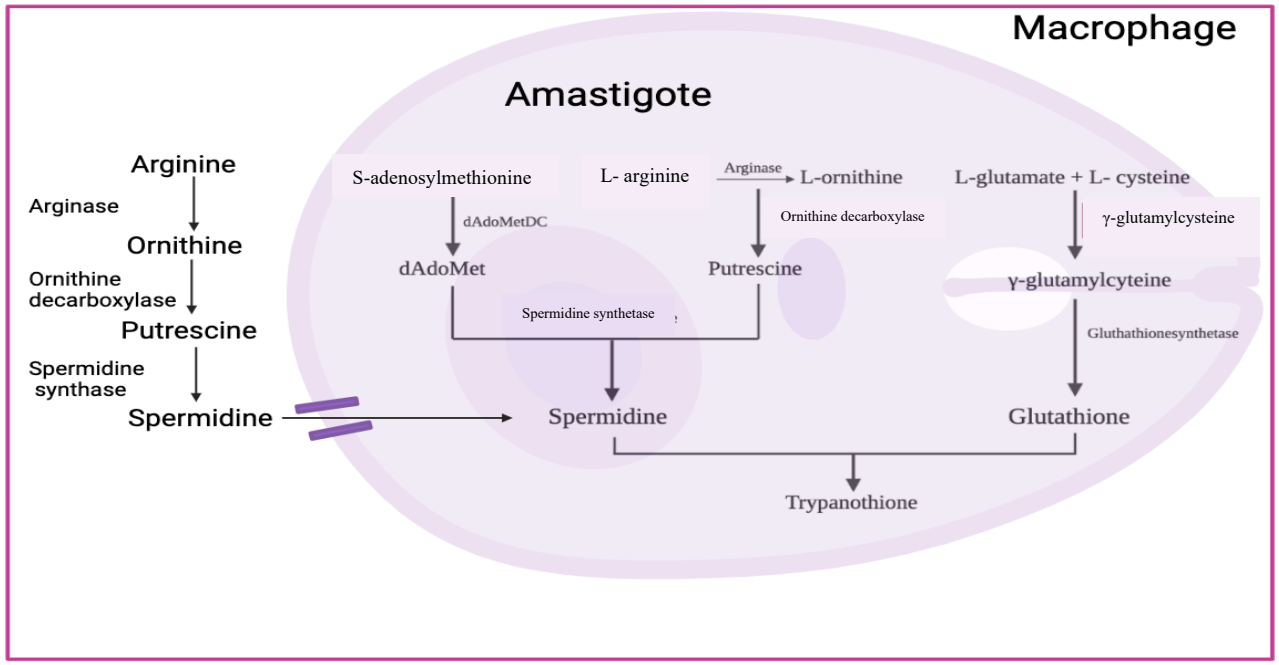


**Figure 1.7 The pathway used to synthesise trypanothione in *Leishmania*.** The pathway shows that spermidine and glutathione are used to make trypanothione [adapted from (Raj *et al.*, 2020)**].** Glutathione biosynthesis has two steps; the first step uses L-cysteine and L-glutamate and is catalysed by γGCS to produce γ-glutamylcysteine. In the second step, γ-glutamylcysteine is converted to glutathione using the enzyme glutathione synthetase. Spermidine is produced from dAdoMet and putrescine and this reaction is catalysed by ornithine decarboxylase and spermidine synthase (Agnihotri *et al.*, 2016).

Protective antigens used in a vaccine can be proteins that are released by a pathogen or intracellular proteins. Immunodominant Mtb antigen studies using *Mycobacterium tuberculosis* showed that *in vivo* antigen expression can control the functionality of T cells (Clemmensen *et al.*, 2021). γGCS is expressed in the parasite's cytoplasm and plays a crucial role in the survival of *Leishmania*. This was demonstrated by the fact that a γGCS null mutant could not be generated in *L. infantum* even if exogenous glutathione was added to the culture medium (Mukherjee *et al.* 2009). Studies in murine models have shown that γGCS is a vaccine candidate for leishmaniasis. Thus *L. donovani* γGCS used as a recombinant fusion protein vaccine (Ldγ GCS) or DNA vaccine protected against *L. donovani* infection in a BALB/c mouse model (Carter *et al.*, 2007; Henriquez *et al.*, 2010). Vaccination with *Ldγ GCS* protected mice against *L. major* or *L. mexicana* infection (Campbell *et al.*, 2012). In previous studies, pure full-length γGCS recombinant protein was not isolated from the soluble fraction of transfected *E. coli* and vaccination with the γGCS DNA or *Ldγ GCS* vaccine failed to induce sterile immunity.

### *1.7 Aim of this study*

Leishmaniasis is a vector-borne, infectious disease caused by *Leishmania* parasites transmitted to people via the bite of a sand fly. Leishmaniasis as one of the neglected tropical diseases and affects the world's poorest populations in more than 90 countries. Leishmaniasis is endemic in Africa, the Americas, the Mediterranean region and Asia. 1.5 - 2 million new cases take place on a yearly basis across the globe and leishmaniasis gives rise to 70,000 deaths each year. 350 million people carry the risk of being infected with this detrimental disease. No vaccines are currently approved to protect people against leishmaniasis. More than one species of *Leishmania* can be found in some regions of Brazil, Sudan and Colombia (Babiker *et al.*, 2014, Ramírez *et al.*, 2016, Esteves *et al*., 2018).

At this point, γGCS stands out as a potential vaccine candidate. γGCS plays a crucial role in the survival of *Leishmania* as it catalyses the rate-limiting step in the generation of glutathione, a significant anti-oxidant. Previous studies have revealed that recombinant γGCS can provide protection against *L. donovani* and *L. major* infection in a murine model.

This study aims to first develop a combination vaccine made of bacterially expressed and purified recombinant *L. donovani, L. major* and *L. mexicana* full-length γGCS proteins. Second, evaluate the efficacy of a live vaccine composed of a mixture of *L. tarentolae* parasites expressing either γGCS from *L. major, L. mexicana* or *L. donovani,* with/without adjuvant PODS releasing IL-2, against *L. donovani* infection in a murine model.

### *1.8 Objectives*

In order to develop a recombinant vaccine utilising the corresponding *L. donovani, L. major* and *L. mexicana* full-length and pure γGCS proteins, the objectives are:

1.To generate an expression plasmid containing the gene sequence of *L. donovani, L. major* or *L. mexicana* γGCS using pET21a(+), including a sequence encoding for the T7-tag followed by a protease cleavage site at the 5'-end of the gene and a second, different protease cleavage site followed by a hexahistidine-tag at the 3'-end of the gene,

2.To use the expression plasmid for the *L. donovani, L. major* or *L. mexicana* γGCS fusion protein in *E. coli* and optimise the production of pure full-length fusion proteins.

To evaluate the efficacy of a live vaccine containing a mix of *L. tarentolae* expressing γGCS from *L. major, L. mexicana* or *L. donovani,* with/without adjuvant PODS releasing IL-2, against *L. donovani* infection, the objectives are:

1.To characterise the immune responses in mice following vaccination by the determination of nitrite production, IFN-γ and IL-10 cytokine production, and IgG1/IgG2a specific antibody response, *in vivo* imaging, and by an *in vitro* proliferation assay.

### *1.9 Hypothesis*

1.The pET21a(+) based expression system is able to allow the expression of full-length γGCS in *E. coli*.

2.Vaccination with a mixture of live *L. tarentolae* expressing γGCS from *L. major*, *L. mexicana* or *L. donovani* is able to protect mice against *L. donovani* infection.

# Chapter 2 Material and Methods

## *2.1 Materials*

### *2.1.1 Materials*

The source of the reagents used in studies is shown in Table 2.1.

**Table 2.1 Chemical materials used in experimental studies and their manufacturer.**

| Chemical | Manufacturer |
|---|---|
| Acetic Acid | Fisher Scientific UK Ltd, Loughborough, UK |
| Acrylamide | Bio-Rad Laboratories GmbH, München, Germany |
| Adenosine | Sigma Aldrich, Irvine, UK |
| Agar | Sigma Aldrich, Irvine, UK |
| Agarose | Fisher Scientific, Loughborough, UK |
| Ammonium Chloride | Sigma Aldrich, Irvine, UK |
| Antibiotics (Carbenicillin, tetracycline, chloramphenicol) | Sigma Aldrich, Irvine, UK<br>Carl Roth, Germany |
| Anti His-tag XP rabbit mAb HRP antibody | Cell Signalling Technology, UK |
| Anti-T7-Tag XP® Rabbit mAb HRP antibody | Thermo Scientific, Wilmington, USA |
| B-PER Bacterial Protein Extraction | Fisher Scientific, Loughborough, UK |
| Biotin | Sigma Aldrich, Irvine, UK |
| Bio-Rad protein assay reagent | Fisher Scientific UK Ltd, Loughborough, UK |
| Boric Acid | Severn Biotech, England, UK |
| Bromophenol blue stain | Sigma Aldrich, Irvine, UK |
| Bovine Serum Albumin | Sigma Aldrich, Irvine, UK |
| Calcium Chloride | Fisher Scientific UK Ltd, Loughborough, UK |
| Carboxypeptidase A | Sigma Aldrich, Irvine, UK |
| Colour prestained protein markers (20-250 kDa) | New England Biolabs, Hitchin, UK |
| Coomassie Brilliant Blue R Stain | Bio-Rad, Watford, UK |
| D-(+)-Glucose | Sigma Aldrich, Irvine, UK |
| DNA ladder 1 Kb | Promega, Southampton, UK |
| Deoxyribonucleotide Triphosphate Mix | BioLine, Nottingham, UK |
| Dimethyl Sulfoxide | Sigma Aldrich, Irvine, UK |
| Ethanol | Sigma Aldrich, Irvine, UK |
| Ethidium Bromide | Sigma Aldrich, Irvine, UK |
| Ethylenediamine Tetra-Acetic Acid | BDH Laboratory, Poole, England |
| Folic acid | Sigma Aldrich, Irvine, UK |
| GeneRuler 1 kb plus DNA ladder | Thermo Scientific, Wilmington USA |
| Glacial Acetic Acid | Sigma Aldrich, Irvine, UK |
| Glycerol | Sigma Aldrich, Irvine, UK |

**Table 2.1 continued**

| | |
|---|---|
| Glycine | Fisher Scientific, Loughborough, UK |
| Glucose | Sigma Aldrich, Irvine, UK |
| Guanosine | Sigma Aldrich, Irvine, UK |
| Glucosamine-HCl | Sigma Aldrich, Irvine, UK |
| Hemin | Alfa Aesar, Lancashire, UK |
| HEPES | Sigma Aldrich, Irvine, UK |
| Hydrogen Peroxide | Sigma Aldrich, Irvine, UK |
| Hydrochloric Acid | Sigma Aldrich, Irvine, UK |
| HisTrap Columns (0.1mL) | Thermo Scientific, Wilmington USA |
| HisPur™ Ni-NTA Spin Columns | Thermo Scientific, Wilmington USA |
| Horseradish peroxidase (HRP) conjugated goat antimouse IgG1 and IgG2a | Southern Biotechnology Associates Inc, Birmingham, USA |
| Imidazole | Sigma Aldrich, Irvine, UK |
| Isopropanol | Techmate Ltd, Milton Keyes, UK |
| Isopropyl-β-D-thiogalactopyranoside | Apollo, Manchester, UK |
| LB broth | Sigma Aldrich, Irvine, UK |
| LB agar | Sigma Aldrich, Irvine, UK |
| Lucigenin | Thermo Scientific, Wilmington USA |
| Luminol | Thermo Scientific, Wilmington USA |
| L- alanine | Sigma Aldrich, Irvine, UK |
| L- arginine | Sigma Aldrich, Irvine, UK |
| L- glutamine | Sigma Aldrich, Irvine, UK |
| L-methionine | Sigma Aldrich, Irvine, UK |
| L-phenylalanine | Sigma Aldrich, Irvine, UK |
| L-proline | Sigma Aldrich, Irvine, UK |
| L-serine | Sigma Aldrich, Irvine, UK |
| L-threonine | Sigma Aldrich, Irvine, UK |
| L-taurine | Sigma Aldrich, Irvine, UK |
| L-tyrosine | Sigma Aldrich, Irvine, UK |
| Lithium Chloride | Sigma Aldrich, Irvine, UK |
| Manganese II Chloride Tetrahydrate | Sigma Aldrich, Irvine, UK |
| 2-mercaptoethenol | Sigma Aldrich, Irvine, UK |
| Methanol | Sigma Aldrich, Irvine, UK |
| MEM, F-14 powder | Gibco BRL, Paisley, UK |
| MEM amino acids | Gibco BRL, Paisley, UK |
| MEM non-essential amino acids | Gibco BRL, Paisley, UK |
| MOPS | Fisher Scientific, Loughborough, UK |
| NucleoSpin Extract II Kit | Macherey & Nagel, Dueren, Germany |
| NucleoSpin Plasmid Miniprep Kit | Macherey & Nagel, Dueren, Germany |
| pBHXGCS plasmids | Biomatik, Ontario, Canada |

**Table 2.1 continued**

| | |
|---|---|
| Pierce™ Chromogenic Endotoxin Quant Kit | Thermo Scientific, Wilmington USA |
| Potassium Chloride | Fisher Scientific, Loughborough, UK |
| Protein assay dye reagent | Bio-Rad Laboratories GmbH, München, Germany |
| RPMI-1640 medium | Fisher Scientific Loughborough, UK |
| Rubidium chloride | Sigma Aldrich, Irvine, UK |
| Glycerol | Sigma Aldrich, Irvine, UK |
| Gel extraction kit | Macherey & Nagel, Dueren, Germany |
| Shrimp alkaline phosphatase | Roche Diagnostics, Mannheim, Germany |
| Sigma Fast ™ 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) tablets | Sigma Aldrich, Irvine, UK |
| Sodium Hydrogen Carbonate | Fisher Scientific Loughborough, UK |
| Sodium Acetate | Fisher Scientific, Loughborough, UK |
| Sodium chloride | Fisher Scientific, Loughborough, UK |
| Sodium dihydrogen orthophosphate 1-hydrate | BDH Laboratory, Poole, England |
| Sodium dodecyl sulphate | Sigma Aldrich, Irvine, UK |
| Sodium hydroxide | Sigma Aldrich, Irvine, UK |
| Sodium phosphate | Sigma Aldrich, Irvine, UK |
| SuperSignal™ West Pico PLUS chemiluminescent substrate | Thermo Scientific, Wilmington USA |
| Ponceau S solution | Sigma Aldrich, Irvine, UK |
| Potassium acetate | Sigma Aldrich, Irvine, UK |
| Potassium chloride | Sigma Aldrich, Irvine, UK |
| Potassium dihydrogen phosphate | Sigma Aldrich, Irvine, UK |
| Resazurin | Sigma Aldrich, Irvine, UK |
| Prestained protein markers (7-175 kDa) | New England Biolabs, Hitchin, UK |
| Protein assay dye reagent | Bio-Rad Laboratories, München, Germany |
| Plasmid DNA purification kit | Macherey & Nagel, Dueren, Germany |
| Nitrocellulose membrane | Fisher Scientific, Loughborough, UK |
| RPMI 1640 medium | Gibco BRL, Paisley, UK |
| N,N,N′,N′ -Tetramethylethylenediamine | Sigma Aldrich, Irvine, UK |
| T4 DNA Ligase kit | Roche Diagnostics, Mannheim, Germany |
| T7-Tag affinity purification kit | Merck Millipore, Darmstadt, Germany |
| Trizma | Sigma Aldrich, Irvine, UK |
| Tryptone | Sigma Aldrich, Irvine, UK |
| Tween-20 | Fisher Scientific, Loughborough, UK |

### *2.1.2 Animals, parasites and PODS*

BALB/c (20-25g) mice, female and male, were supplied from the University of Strathclyde colony. The following parasites were used in studies: wild type *L. donovani* (strain MHOM/ET/67:LV82), *L. tarentolae* wild type parasites, transgenic *Leishmania tarentolae* [strains *L. tarentolae L. don* γGCS, *L. tarentolae L. maj* γGCS, and *L. tarentolae L. mex* γGCS supplied by Muattaz Hussain (Hussain, 2017, Topuz Ata *et al.*, 2023).Two types of PODS supplied by Cell Guidance System (Cambridge, UK) were used in this study. PODS alone (PODS-Empty) or PODS that would release human interleukin-2 (PODS-IL-2). *In vivo* studies were carried out in accordance with local ethical approval and had United Kingdom Home Office approval (Licence number PPL: PF669CAE).

### *2.1.3 Plasmids*

pBHXLmexGCS, pBHXLmajGCS, pBHXLdonGCS were commercial plasmids that included part of the gene and the sequence encoding for T7 tag. pET21aLmexGCS6His(M), pET21aLmajGCS6His(M), pTHGFPLdonGCS were prepared by Muattaz Hussain (Hussain, 2017) and carry the full length γGCS sequence, however with a mutation in the beginning of the gene.

## *2.2 Molecular Biology Methods*

The following methods were used in Chapter 3.

### *2.2.1 Production of competent cells*

Competent *Escherichia coli* cells were prepared for studies as described before (Hanahan, 1983). A single *E. coli* colony, selected from LB agar plate (1% w/v tryptone, 0.5% w/v yeast extract, 1% w/v sodium chloride, 1.5% w/v agar in dd$H_2O$), was inoculated into 3 mL Luria-Bertani broth containing suitable antibiotics and the

culture was grown overnight at 37°C and 230 rpm in a shaking incubator. One hundred mL fresh LB broth and 500 µL of culture were mixed and the culture was grown until it had a 0.2 optical density at a wavelength of 600 nm (OD600). The culture was kept on ice for 15 minutes, split between two 50 mL sterile tubes and centrifuged at 4°C at 3500 × g for 15 minutes. The resulting cell pellets were resuspended and pooled using 8 mL cold sterile RF1 (100 mM rubidium chloride, 50 mM manganese (II) chloride tetrahydrate, 10 mM calcium chloride dihydrate, 30 mM potassium acetate, 15% w/v glycerol, adjusted to pH 5.8 with 1% v/v acetic acid) and incubated on ice for 90 minutes. The cell suspension was centrifuged again, and the pellet was resuspended with 8 mL sterile cold RF2 solution (10 mM rubidium chloride, 75 mM calcium chloride dihydrate, 10 mM 3-[N-morpholino] propane sulfonic acid, 15% w/v glycerol, adjusted to pH 6.8 with 1N sodium hydroxide) and kept on ice for 15 minutes. The resulting competent cells were aliquoted in 100 µL lots, snap-frozen in liquid nitrogen, and stored at -80°C until required.

### *2.2.2 Transformation of E. coli*

5 µL plasmid and 100 µL competent *E. coli* cells were incubated on ice for 1 hour. Then the mixture was incubated at 42°C for 90 seconds and was followed by 10 minutes ice incubation. Then 900 µL LB medium was mixed with the cells and placed in a shaking incubator at 37°C for 1 hour and then 100 µL and 200 µL of the cell suspension were plated on Luria-Bertani agar containing the appropriate selection antibiotic(s) using a sterile spreader. These plates were incubated at 37°C overnight, in an upside-down position.

### *2.2.3 Preparation of glycerol stocks*

Glycerol stocks of bacterial suspensions were prepared to cryopreserve relevant bacterial samples for future use. Aliquots of overnight bacterial cultures (500 µL) and 700 µL glycerol were added to a sterile cryotube and incubated on ice for 10 minutes. The samples were then stored at -80°C until required.

### *2.2.4 Plasmid DNA mini-preparation*

Five mL LB medium (1% w/v tryptone, 0.5% w/v yeast extract, 1% w/v sodium chloride in dd$H_2O$) containing the appropriate antibiotic was inoculated with a single colony from a plate spread after transformation of *E. coli* and incubated at 37°C overnight. One mL of each sample was transferred into a 1.5 mL tube and centrifuged at 4°C 11,000 × g for 30 seconds. The supernatant was discarded, and 'Plasmid DNA Purification' kit instructions were followed so that pure DNA (final volume 50 µL) was extracted from the sample. The plasmid DNA was stored at -20°C until required for studies.

### *2.2.5 Plasmid DNA midi-preparation*

Two hundred mL LB medium containing the appropriate antibiotics was inoculated from a single colony selected from an agar plate and incubated overnight at 37°C. The culture was centrifuged for 15 minutes 4,000 × g at 4°C. The 'Low-copy plasmid purification' method detailed in the 'Plasmid DNA Purification kit' instructions were followed so that pure plasmid DNA was extracted from the bacterial pellet. Six portions of 833 µL of eluted sample were distributed into six 1.5 mL tubes, mixed with 583 µL isopropanol at room temperature and the samples were centrifuged for 30 minutes at 15,000 × g and 4°C. The resulting supernatant was discarded, and the pellets were washed with 500 µL of 70% ethanol. The mixture was centrifuged using the same

conditions for 10 minutes. The resulting pellets were air-dried and 30 µL $ddH_2O$ was added to each tube. The DNA (total final volume 180 µL) was pooled and stored at -20°C until required.

### *2.2.6 Klenow protocol*

Fifty-five µL eluted DNA was recovered from a gel and 7 µL NEB buffer 2.1, 3 µL DNA polymerase large (Klenow) Fragment, 1.5 µL dNTPs and 3.5 µL $ddH_2O$ were carefully mixed with the sample and it was incubated at 25°C for 15 minutes. One µL 0.5 M EDTA was added to stopping the reaction and the sample was then incubated at 75°C for 20 minutes.

### *2.2.7 Shrimp alkaline phosphatase treatment*

Twenty-six µL DNA recovered from a gel were combined with 3 µL 10× SAP buffer and 1 µL SAP enzyme. Firstly, samples were incubated in a shaking incubator at 37°C for 2 hours and then incubated at 65°C for 30 minutes. The sample was used in a ligation process.

### *2.2.8 Ligation*

One and half µL T4 ligase buffer, 0.8 µL T4 ligase enzyme, one time vector, three-fold insert DNA were combined and completed up to 15 µL with $ddH_2O$. Samples were incubated at 13°C overnight in a thermocycler.

### *2.2.9 Restriction analysis*

One µL plasmid, 0.6 µL restriction enzyme and 1.5 µL of the appropriate 10× enzyme buffer was added to a 1.5 mL microcentrifuge tube, mixed and the volume made up to 15 µL with $ddH_2O$. The mixture was incubated at 37°C with shaking at 750 rpm for 3 hours. For fragment isolation, 60 µL plasmid, 10 µL of the appropriate 10× buffer

selected according to enzyme and 2.5 µL of each a restriction enzyme were mixed and in a 1.5 mL microfuge tube and the volume of the suspension made up to 100 µL with $ddH_2O$. The mixture was incubated for 3 hours at 37°C in a shaking incubator set at 750 rpm.

### *2.2.10 Agarose gel electrophoresis*

Agarose gels with 0.8% (w/v) agarose were prepared using 0.5×TBE (0.45 M Tris base, 0.45 M boric acid, 10 mM EDTA pH 8.0) and 0.5 µg/mL ethidium bromide. For analytical gels, 5 µL sample, 10 µL $ddH_2O$ and 1.5 µL loading dye were combined. Samples and 5 µL GeneRuler DNA ladder were loaded into the appropriate wells of the gel. DNA was separated using a VWR™ power source at 120 V for 45-60 minutes. For preparative gels, 95 µL sample mixed with 8 µL AP dye were loaded into the well of the gel and gels were run at 60 V for 15 minutes and followed by 100 V until fragments were sufficiently separated.

### *2.2.11 Primer sequences*

**Table 2.2 Oligonucleotide primers for sequencing of γGCS**

| Primer Name | Primer Sequence |
|---|---|
| pET21a_1.for: | 5'-ATGCGTCCGGCGTAGAGGAT-3' |
| LmajGCS_1.rev | 5'-TCGTAGCGAGACTTGAGGAT-3' |
| LmexGCS_1.rev: | 5'-TGCAGAGCGCTGCTACCCAT-3' |
| T7 reverse : | 5'- GCTAGTTATTGCTCAGCGG -3' |

## *2.3 Protein Expression and Purification*

The following methods were used in Chapter 4.

### *2.3.1 Optimisation of protein expression*

Small scale cultures were used to optimise the method used to produce recombinant protein. Firstly, 2 µL of the appropriate pET21a(+) *Leishmania* γGCS plasmid was used to transfect 100 µL Rosetta Blue *E. coli* cells, using the method detailed in section 2.2.1. The bacterial sample was incubated on ice for 20 minutes, followed by 2 minutes incubation at 42°C, then 2 minutes incubation on ice again. The bacterial sample was then incubated for 1 hour at 37°C with 250 mL LB medium. A sample of the transformed bacteria (100 µL or 200 µL) was plated out on LB agar containing carbenicillin (100 µg/mL), tetracycline (12.5 µg/mL) and chloramphenicol (34 µg/mL) and the plates were incubated overnight, upside down, at 37°C. All colonies were used to inoculate 50 mL LB broth containing the same antibiotics. This culture was incubated at 37°C until an $OD_{600}$ between 0.6-0.8. Recombinant protein expression was then induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG, 0.1 mM) and the 50 mL bacterial culture was split into four equal samples i.e. 12.5 mL/sample. The samples were then incubated overnight at different temperatures (i.e. 18°C, 25°C, 30°C or 37°C) on a shaking incubator (New Brunswick Scientific, Stevenage, UK) set at 230 rpm. The $OD_{600}$ value was measured over the course of the experiment and then each culture was pelleted by centrifugation at 4,000 × g at 4°C for 15 minutes in a Beckman Coulter High Speed Centrifuge (Avanti JXN2-6, London, UK). The resulting pellets were stored at -20°C until required. These studies were repeated on a larger scale, using 50 mL or 250 mL as the starter culture volume to seed a 1 L or 6 L volume of LB broth containing selection antibiotics and IPTG (0.1 mM). Pellets from uninduced and non-transformed *E. coli* were also prepared as experimental controls.

### *2.3.2 Lysis of bacterial pellets*

Pellets from bacterial cultures were lysed by resuspension in 1:1 or 1:4 ratio of B-PER™ bacterial protein extraction reagent/gram pellet. After various incubation times, the resulting suspension was pelleted by centrifugation at 18,000 × g at 4°C for 20 min in a Beckman Coulter High Speed Centrifuge (Avanti JXN2-6). The resulting pellet contained insoluble bacterial extract whilst the supernatant contained the soluble bacterial extract. The samples were stored at -20°C until required.

### *2.3.3 Recombinant protein purification*

A HisPur™ Ni-NTA spin purification kit was set up to collect hexahistidine-tagged soluble protein as indicated by the manufacturer's instructions. Briefly, the column had its end cut off and the fluid present was allowed to drain away. The column was washed by adding 1 mL binding buffer and then centrifuged at 700 × g at 4°C for 2 min. The fluid in the collection section was discarded and the soluble protein was added to the column until all the soluble protein had been loaded. This involved adding 1 mL soluble protein to the column, incubating on ice for 2 min and then centrifuging for 2 min as before. The flow through sample was collected and then the column was washed twice with 1 mL wash buffer using the process above and the wash samples were collected. The column was then incubated with 1 mL elution buffer for 2 min and the protein eluted off the column collected. The eluted sample was stored -20°C until required.

A T7•Tag® affinity purification kit was set up to collect T7-tagged soluble protein as indicated by the manufacturer's instructions. The T7•Tag antibody agarose provided with the kit was loaded into the column provided and equilibrated to room temperature. Wash buffer was added to the column and allowed to drain through the column under

gravity. The sample containing recombinant protein was added to the column and allowed to flow through the column and the resulting flow through sample collected. The column was washed with wash buffer and five microcentrifuge tubes containing 150 µL of neutralisation buffer prepared. The elution buffer was prepared as instructed, and 1 mL serially added to the column 5 times. The resulting elution samples were stored at -20°C until required.

### *2.3.4 Protein assay*

The amount of protein present in samples was determined using a Bio-Rad protein assay. Briefly, 10 µL of the sample or a protein standard (BSA 0.1-1 mg/mL) was added in to 96 well ELISA plate and then 200 µL of freshly diluted Bio-Rad protein assay reagent was added to each sample (1:5 diluted with distilled water). The absorbance of the samples was measured at $OD_{595}$ using a Softmax Molecular Device plate reader (Molecular Devices Corporation, Sunnyvale. USA). The amount of protein present in unknown samples was determined by linear regression using the standard curve produced for the protein standards. A correlation coefficient > 0.95 was obtained in all assays.

### *2.3.5 SDS polyacrylamide gel electrophoresis (SDS-PAGE)*

The molecular weight of proteins in soluble extracts from recombinant protein studies was determined using SDS polyacrylamide gel electrophoresis. An experimental sample was mixed with sample buffer (0.5 M Tris-HCl pH 6.8, 20% glycerol, 10% w/v sodium dodecyl sulphate solution, 5% v/v of 2-mercaptoethenol, 1% v/v bromophenol blue) and boiled at 100°C for 10 minutes. The resulting sample was then cooled on ice and 15 µL of the sample or 3 µL of a protein marker mix (NEB, Colour Prestained Protein Standard, Broad Range (10-250 kDa) was loaded to the appropriate

well of an SDS-PAGE gel. The gel consisted of a 12% w/v resolving gel [30% (w/v) acrylamide-bisacrylamide, 1.5 M Tris (pH 8.8), 0.4% (w/v) SDS, 10% ammonium persulfate, TEMED, $ddH_2O$] and 5% w/v stacking gel [30% (w/v) acrylamide-bisacrylamide, 0.5 M Tris (pH 6.8), 0.4% (w/v) SDS, 10% ammonium persulfate, TEMED, $ddH_2O$]. The proteins present in the sample were separated by gel electrophoresis using a XCell SureLock™ (Invitrogen™, Carlsbad, USA) set at 150 V, 30 mA for 90 minutes and running buffer (0.29% w/v Tris base, 1.44% w/v glycine, 0.1% w/v sodium dodecyl sulphate, pH 8). The gel was either stained with Coomassie dye to visualise the proteins present or used in western blot studies. In staining studies, the gel was stained for 1 hour with Coomassie blue dye (0.1% v/v Coomassie Brilliant Blue R-250, 50% v/v methanol and 10% v/v glacial acetic acid) and then incubated in destaining solution (40% v/v methanol and 10% v/v glacial acetic acid). The stained gels were photographed to record the results obtained.

### *2.3.6 Western blot studies*

Western blot studies were used to identify which separated proteins contained a hexahistidine-tag and/or T7-tag. The proteins separated by gel electrophoresis were transferred on to a nitrocellulose membrane using a Xcell SureLock™ E10001 (Invitrogen™, Carlsbad, CA92008) system. Blotting pads and filter papers were cut according to the gel size and soaked in transfer buffer (12 mM Tris base, 96 mM glycine (v/v), in 100 % methanol). Three blotting pads were placed at the bottom of the transfer cassette, filter paper was laid after then gel was placed and covered with nitrocellulose membrane. Another three blotting pads were placed on top and cassette was closed. The proteins were transferred to the nitrocellulose membrane by exposing to a voltage of 30 V for 1 hrs. Ponceau S solution was used to stain the proteins on the

membrane to show that transfer had been successful. The membrane was washed three times for 5 min with wash buffer (20 mM Tris base, 150 mM sodium chloride, 0.05% Tween-20 in water) to remove the Ponceau solution and then incubated for1 hour at room temperature with blocking buffer (PBST, 20 mM Tris, 150 mM NaCl, 0.05 %, Tween-20, 5% w/v non-fat dry milk in water). The membrane was then incubated as before for 1 hour with anti-His-tag XP rabbit mAb horseradish peroxidase (HRP) antibody conjugate (1:1000 dilution in blocking buffer). The membrane was washed three times with wash buffer.

Similar studies were completed for the T7-tag as described above, but in this case the nitrocellulose membrane was probed with an anti-T7-Tag XP® mAb HRP-conjugated antibody. After transfer of separated proteins on to the nitrocellulose membrane, the membrane was stained with Ponceau solution to ensure that the proteins had successfully transferred. The membrane was washed with wash buffer (37 mM Tris base, 2.7 mM sodium chloride, 10 mM disodium phosphate, and 1.8 mM monopotassium phosphate, 0.2% Tween-20 in water) to remove the Ponceau solution. An anti-T7-Tag XP® Rabbit mAb HRP conjugate (1:10,000 dilution in blocking buffer (37 mM Tris base, 2.7 mM potassium chloride, 10 mM disodium phosphate and 1.8 mM monopotassium phosphate, 0.2% Tween-20, 5% w/v non-fat dry milk in water) was used in studies. The membrane was washed three times with wash buffer.

After washing step for two membrane, the SuperSignal™ West Pico PLUS chemiluminescent substrate was used as the substrate as this allows detection of positive proteins present at low concentrations. Following incubation with the anti-His or anti-T7 conjugate the membrane was incubated with chemiluminescence substrate containing peroxide solution and luminol/enhancer solution (1 mL v/v mixture) for 2

min. If the conjugate attached to a separated protein, then the interaction results in light production and this causes a dark spot to appear on exposed X ray film. A piece of X ray film was cut to the size of the membrane, and it was exposed in a dark room to the membrane for 15 seconds - 10 minutes. A picture of the exposed X ray film was taken to record the results obtained.

## *2.4 Vaccine studies*

The following methods were used in Chapter 5.

### *2.4.1 Preparation of Soluble Antigen*

*Leishmania* promastigotes (*L. donovani*) were cultivated in SDM-79 medium and *L. tarentolae* promastigotes were cultivated in RPMI-1640 medium supplemented with hemin (7.5 μg/mL) and heat-inactivated foetal calf serum (FCS, 10% v/v) in 25 $cm^2$ tissue culture flasks. *Leishmania* promastigotes from *in vitro* cultures were pelleted by centrifugation at 3,000 × g for 5 minutes. The pellets were resuspended in 10 mL PBS and then centrifuged as before to remove any medium contaminating the parasites. Pellets from different cultures were pooled and resuspended in 5 mL PBS pH 7.4 and 1 mL aliquots were transferred into cryotubes. The parasites sample were then freeze-thawed in liquid nitrogen four times. The resulting suspensions were pooled and then pelleted as before. The amount of protein present/mL in the supernatant (soluble protein, SAG) was determined using the Bio-Rad assay as described in 2.3.4 and aliquots of the SAG protein were stored at -20°C until required.

### *2.4.2 Vaccination with transgenic parasites*

BALB/c mice (n = 5/group) were injected subcutaneously into the loose neck skin on day 0 and day 21 with 0.2 mL PBS pH 7.4 alone (control), PBS containing $2 \times 10^7$/mL

wild type *L. tarentolae* promastigotes, 2 × $10^7$/mL transgenic *L. tarentolae* promastigotes (expressing γGCS from *L. donovani* alone or a 1:1:1 mixture of parasites expressing γGCS from three pathogenic species, Figure 2.1).

Three plasmids for DNA fragment isolation were constructed and the fragments integrated into the ribosomal RNA gene locus of *L. tarentolae* including either of the gene sequences for *L. donovani, L. major*, or *L. mexicana γGCS.* These parasites were provided by Dr Muattaz Hussain (Topuz Ata *et al.*, 2023).

The first vaccine experiment had four experimental groups: control (group 1), *L. tarentolae* WT (group 2)*, L. tarentolae* promastigotes expressing *L. don* γGCS (group 3) and *L. tarentolae* promastigotes expressing *L. don* γGCS, *L. maj* γGCS, *L. mex* γGCS (group 4). The second vaccine experiment had 6 experimental groups: control (group 1), PODS-Empty (50 million/mouse, group 2), PODS-IL-2 alone (50 million/mouse, group 3), *L. tarentolae* WT (group 4)*, L. tarentolae* promastigotes expressing *L. don* γGCS (group 5) and *L. tarentolae* promastigotes expressing *L. don* γGCS PODS-IL-2 (50 million/mouse, group 6). Three weeks later the mice were infected by intravenous tail injection (no anaesthetic) with *L. donovani* amastigotes (0.2 mL RPMI-1640 supplemented with 100 μg/mL penicillin/streptomycin and 2 mM L-glutamine, 2 × $10^8$/mL), harvested from the spleen of an infected stock hamster. In the course of the experiment, blood samples were collected and centrifuged at 13,000 xg for 10 minutes at 4°C after being incubated overnight 4°C. The resulting serum sample collected by centrifugation was stored -20°C until required. Parasite burdens in all mice were assessed on day 14 following the method of Carter *et al.* (1988).

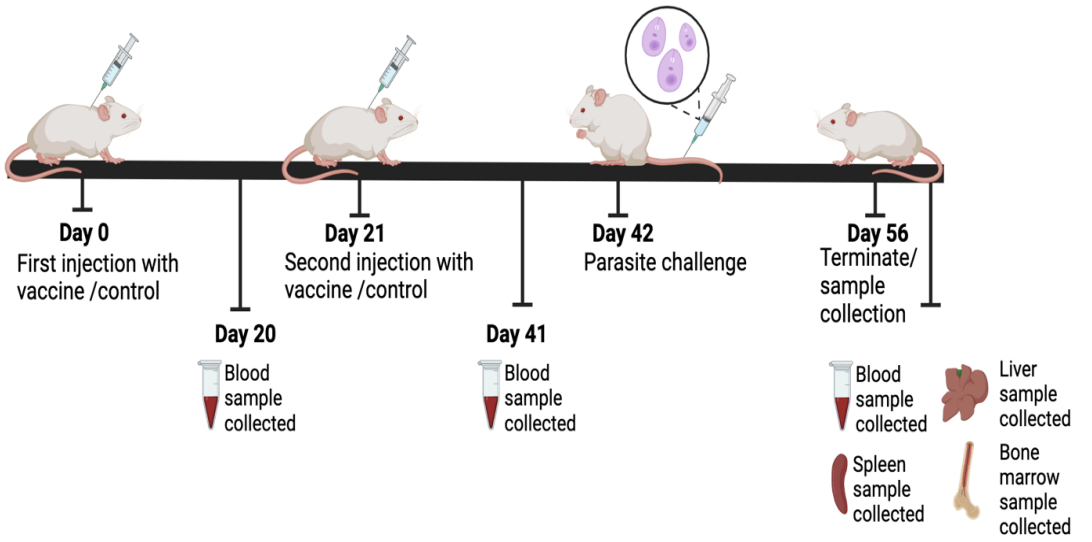


**Figure 2.1 Immunisation protocol followed for *L. donovani* experiment**. The BALB/c mice (n=5/treatment) were immunised with *Leishmania γGCS (from L. donovani, L. mexicana or L. major)* on day 0 and day 21. On day 42, 2 ×$10^7$ *L. donovani* amastigotes were injected by intravenous tail injection. Blood samples were collected over the course of the experiment to specify specific antibodies at different stages of the experiment. At the end of the experiment the animals were sacrificed and parasite numbers in the spleen, bone marrow and liver were determined to assess vaccine effectiveness.

### *2.4.3* In vivo *imaging*

The effect of vaccination or infection on neutrophil or macrophage recruitment was determined over the course of an experiment (Tseng Kung, 2012) using an *in vivo* imaging system (IVIS Spectrum, Perkin Elmer, UK). Mice were injected intraperitoneally with luminol solution (150 mg/kg, 10 mg/mL in saline) 3 hours after immunisation in neutrophil studies and 72 hours after treatment with lucigenin solution (10 mg/kg, 2.5 mg/mL in saline) for macrophage studies. Mice were imaged 5 min

after injection (medium binning, 2 min imaging) and the amount of bioluminescence (total flux, photon/sec) emitted in each region of interest (ROI) was determined using the Living Image software (4.8.0, Perkin Elmer, UK). The same sized region of interest was used for each mouse at each time point so that the area was the same in all studies. Data is expressed as the mean bioluminescent signal (BLI, photons/sec) ± SE in studies.

### *2.4.5* In vitro *proliferation assays*

Lymphocyte proliferation studies were completed with spleen samples (Carter *et al.*, 2007). Spleen samples, collected aseptically from control or vaccinated mice, were added to sterile Petri dishes and disrupted in approx. 5 mL complete RPMI 1640 (RMPI medium containing 10% v/v FCS, 100 µg/mL penicillin/streptomycin and 2 mM L-glutamine) using the end of a 2 mL syringe. The resulting cell suspension was transferred to a sterile universal tube, pelleted by centrifugation for 5 minutes at 3,000 × g at 4°C, and the pellet was resuspended in 3 mL Boyle's solution (0.17M Tris, 0.16M ammonium chloride, mixed 1:9 and a final pH of 7.2). The suspension was incubated for 5 minutes at 37°C and the cells were then pelleted by centrifugation for 5 minutes 3,000 × g. The cells were washed three times using medium. Cells ($5 \times 10^5$/well, 100 µL/well) were added to the appropriate wells of a 96 well tissue culture plate and incubated with 100 µL medium alone (un-stimulated controls), SAG (50 µg/mL), prepared from *L. donovani*, wild type *L. tarentolae* or transfected *L. tarentolae* (expressing *L. donovani* γGCS, *L. major* γGCS or *L. mexicana* γGCS) promastigotes or concanavalin A (10 µg/mL, positive control). Plates were incubated for 72 hours at 37°C in an atmosphere of 5% carbon dioxide. After 72 hours, the plates were stored at -20°C until cytokine or nitrite levels could be determined. In some

studies, the effect of treatment on splenocytes proliferation was assessed by adding 20 µL/well resazurin solution (0.125 mg/mL in dd$H_2O$) after seventy-two hours to all samples and the absorbance of the samples was determined at 570 nm wavelength using a Softmax Molecular Device (Molecular Devices Corporation, California. USA).

### *2.4.6 Cytokine determination*

An ELISA assay was used to determine the type of cytokines present in cell supernatants from lymphocyte proliferation assays (Carter *et al.*, 2007). Briefly, the appropriate wells of a 96 well ELISA plate were coated with 50 µL rat anti-mouse anti IL-10, IL-6 or IFN-γ antibody (2 µg/mL, diluted in PBS pH 9.0) and the plate was incubated overnight at 4°C. The plates were then washed three times in wash buffer (PBS pH 7.4 containing 0.05% v/v Tween-20) and then 150 µL blocking buffer **(**PBS pH 7.4 containing 10% v/v FCS) was added to the appropriate wells. The plate was incubated at 37°C for 1 hour. Plates were washed as before and then 30 µL of the cell supernatant or 30 µL of the appropriate cytokine standard (20 ng/mL, diluted with 10% v/v FCS in PBS pH 7.4, run in duplicate) was added to the appropriate wells of the ELISA plate. The plates were incubated at 37°C for 2 hours and then washed three times as before. After the washing step, 100 µL of the appropriate biotin anti-cytokine antibody (1 µg/ml, 10% v/v FCS in PBS pH 7.4) was added to the appropriate wells of the plate. The plates were then incubated for one hour and then washed three times as before. Streptavidin alkaline phosphate conjugate (100 µL, diluted 1:4,000 in 10% v/v FCS in PBS pH 7.4) was added to all the appropriate wells of the plate and the plate was incubated for 1 hour at 37°C. The plates were washed as before, then 100 µL substrate (p-nitrophenyl phosphate disodium salt hexahydrate, 1 mg/mL, diluted in

dd$H_2O$) were added to all the used wells and the plate was incubated in the dark until the colour developed in the wells. This occurred within 20-60 minutes. The absorbance of the samples was determined at 405 nm and a standard curve plotted for the standards run on each plate using the software Softmax Molecular Device (Molecular Devices Corporation, California. USA) provided on the ELISA reader (Spectramax 190). The correlation coefficient for the standards was always > 0.97 in assays. The software automatically calculated the amount of cytokine present (mg/mL) in the unknown samples run on the sample ELISA plate. The data is presented as the mean cytokine production (ng/mL ± SE) for each treatment.

### *2.4.7 Nitrite determination*

Nitrite levels in cell supernatants from lymphocyte proliferation assays were determined (Carter *et al.*, 2005). The appropriate sample (50 µL cell supernatant or nitrite standard, serially diluted from 100 µM in PBS pH 7.4) was added to the appropriate wells of a 96 well ELISA plate. Then 50 µL Griess reagent (1:1 mix, 2% w/v aqueous sulphanilamide, 5% v/v aqueous orthophosphoric acid: 0.2% w/v aqueous naphthalene diamide hydrogen chloride) was added to the appropriate wells of the plate and the plate was incubated for 5 minutes at room temperature. The absorbance of each sample at 540 nm was determined on a plate reader. Nitrite levels (µM) present in each sample was calculated using linear regression using the standard curve plotted from standards run on the same plate. The correlation coefficient for the standards was always > 0.95. Data are shown as the mean nitrite level/treatment ± SE.

### *2.4.8 Specific antibody response*

Specific IgG1 and IgG2a antibody titres were determined using an ELISA (Carter *et al.*, 2007). Briefly, a 96 well micro titre plate was coated with 100 µL *Leishmania*

γGCS soluble antigen (*L. tarentolae L. don*, *L. tarentolae L. maj*, *L. tarentolae L. mex*, or *L. donovani,* 0.1 μg/mL in PBS, pH 9) and incubated overnight at 4°C. The plates were washed three times with wash buffer (PBS pH 7.4 with 0.05% v/v Tween-20) and then blocked by adding blocking buffer (150 μL PBS pH 7.4 containing 10% v/v FCS) to the appropriate wells and incubating samples at 37˚C for 1 hour. Plates were washed three times with wash buffer as before and then 100 μL of a serum sample collected from mice over the course of experiment (serially diluted from 1:100 dilution in blocking buffer) was added to appropriate wells of a plate. The plates were and then incubated for 1 hour at 37˚C. The plates were again washed three times with wash buffer and then 100 μL horseradish peroxidase (HRP) conjugated goat anti-mouse IgG1 or IgG2a (1:4,000 in 10% v/v FCS in PBS pH 7.4) were added to the appropriate wells and the plate was incubated for 1 hour at 37°C. The plates were again washed three times with wash buffer and then 100 μL of the substrate (25 mL with sodium acetate buffer pH 5.5 containing 250 μL tetramethylbenzidine [6 mg/mL dimethyl sulfoxide] and 7 μL of hydrogen peroxide were added to the appropriate wells and the plate was incubated at room temperature. After 20 minutes, the reaction was stopped by adding 50 μL 10% v/v aqueous sulphuric acid to each well and the absorbance of each sample was determined at 405 nm using an ELISA plate reader (Spectramax 190). The mean endpoint and ± SE were then calculated for each treatment.

### *2.4.9 Statistical analysis of data*

Specific antibody, cytokine determination, nitrate assay and proliferation assay results and bioluminescence (BLI, photon/sec) data collected with Living Image® software (Perkin Elmer, United Kingdom) were analysed to determine any significant differences between treatments. Data involving two treatments were analysed using a

Mann Whitney U test using an online tool (https://www.statskingdom.com/170median_mann_whitney.html). Data involving more than two groups were analysed using a Kruskal-Wallis test using an online tool (https://www.statskingdom.com/kruskal-wallis-calculator.html). A p value of $< 0.05$ was considered significant.

# Chapter 3 Generation of *Leishmania* γGCS expression constructs

## *3.1 Introduction*

Recombinant DNA technology involves modifying genetic material to obtain a desired characteristic or product (Figure 3.1). Recombinant protein studies require production of an expression vector containing the gene sequence for a specific protein, which can then be introduced into cells to produce the recombinant protein (Khan *et al.*, 2016). Plasmid vectors contain regulatory elements, a promotor region or regions, endonuclease cleavage sites, which allow insertion of DNA sequences, antibiotic selection marker sequence or sequences, and a region coding for a tag to allow rapid purification of the expressed protein (Celie *et al.*, 2016). The origin of replication (ori) is required for survival and production of plasmids within the host cell (Kazi *et al.*, 2022). Antibiotic resistance genes allow easy identification of successful transfection as only modified cells will grow in the presence of the antibiotic or antibiotics that are inactivated by the products of the drug resistance genes on the plasmid (Kazi *et al.*, 2022). An appropriate transcription promotor is critical for high gene expression as transcription starts at the promotor region and determines the direction of translation for the recombinant protein. Promotors can be of prokaryotic, eukaryotic or viral origin (Wang *et al.*, 2009). Plasmid vectors can be obtained from a commercial source or custom designed from a template vector provided by a company or a collaborator. Custom vectors come with maps to show the various regions within the DNA sequence and the vector can be specific for a particular application or for transfection of particular host cells (Khan *et al.*, 2016).

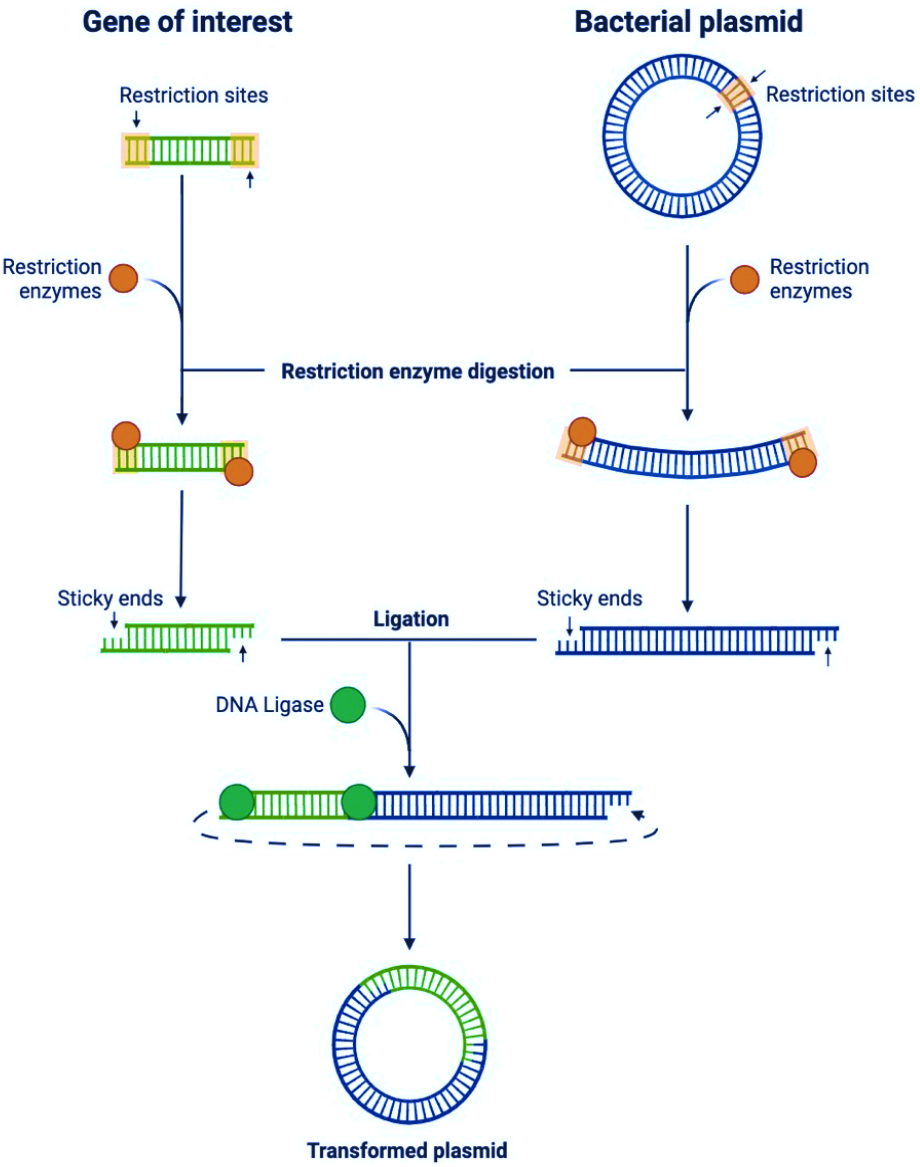


**Figure 3.1 Procedure for desired gene sequence insertion into an expression vector.** The gene of interest and plasmid are cleaved with restriction enzymes to give two linear DNA sequences. The two sequences are then ligated using DNA ligase to give a complete circular plasmid, which can be inserted into cells for expression.

The pET expression system is one of the most frequently used systems used to express recombinant proteins. There are a number of advantages to using this expression system. For example, the pET vector has one of the strongest promotors (Rosano *et al.*, 2019) and uses IPTG induction to induce protein expression. The presence of a T7-tag in the vector sequence is known to reduce the formation of inclusion bodies by expressed proteins (Muttar and Panfchal, 2022). The T7 promotor in the pET expression system is derived from gene*10* of the T7 bacteriophage. Target genes transcribed under the control of the T7 promotor are not used by *E. coli* RNA polymerase but are controlled by the lac promotor in *E. coli* strains (Kaur *et al.*, 2018).

In this study, the pET21a(+) expression vector was used to produce recombinant proteins. This expression vector allowed the insertion of the N-terminal T7-tag and C-terminal His-tag coding sequence with different restriction enzyme sites. Two types of plasmids were made in this project, plasmids that allowed the expression of *L. donovani, L. major* and *L. mexicana* γGCS protein with a T7-tag and a His-tag, and plasmids that allowed expression of the γGCS proteins with just a His-tag. It had been suggested that a T7-tag may boost fusion protein expression (Kimple and Sondek, 2004), so having the two constructs allowed this hypothesis to be studied in this project. The two tags were used to allow separation of the full-length fusion protein from the other soluble proteins produced by transfected *E. coli*.

Protease cleavage site encoding sequences were inserted next to the tag coding sequences so that it would be possible to remove the tags from the recombinant fusion protein and thus, allow purification of full-length γGCS recombinant protein. This was

essential as recombinant protein vaccines for clinical use should not contain any exogenous amino acid sequences (Schneier *et al.*, 2020) (Figure 3.2).

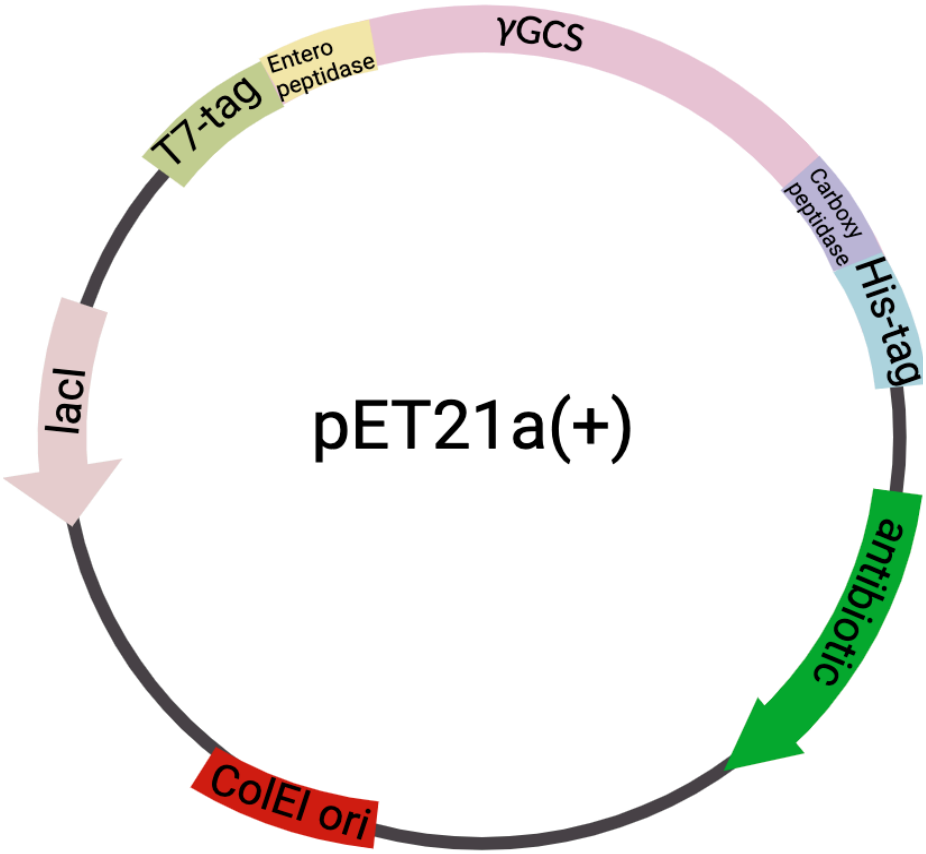


**Figure 3.2 Schematic of pet21a plasmid for expression of γGCS.** The protein has the amino acid sequence for γGCS from *L. major*, *L. mexicana* or *L. donovani,* which is flanked by a His-tag at the C-terminus and a T7-tag at the N-terminus.

Clone manager 9.2 software was used to design the gene constructs used. This tool provides cloning simulation to show the product sizes of gene fragments after cleavage of plasmids with restriction enzymes. Production of *L. donovani* γGCS recombinant protein required more cloning steps than for the other two *Leishmania* species as previous constructs (Hussain, 2017) had mutations at the start of the *L. donovani* γGCS

gene sequence. In this study initial cloning used commercially synthesised gene sequences (i.e. pBHXLdonGCS, pBHXLmajGCS, pBHXLmexGCS (Appendix 1)) and plasmids prepared by a previous researcher in the laboratory (pET21aLdonGCS6His(M), pET21aLmajGCS6His(M), pET21aLmexGCS6His(M), and pTHGFPLdonGCS (Hussain, 2017)). The synthesised constructs contain a codon-optimised part of the γGCS genes and a T7-tag coding sequence at the start of each gene. Relevant DNA sequences were obtained from existing plasmids using restriction enzyme cleavage and fragment ligation to produce a new plasmid. Successful integration of a DNA sequence in the correct orientation into a plasmid was then checked by restriction analysis and final confirmation of the correct plasmid constructs was obtained by having the plasmids sequenced by a commercial supplier.

The aims of this chapter were to:

1. Clone the genes for *L. donovani, L. major, L. mexicana* γGCS into a pET-21a (+) expression vector.
2. Obtain expression plasmids with N-terminal T7-tag and C-terminal His-tag sequence and generate expression plasmids that only had a C-terminal His-tag sequence.
3. Verify that final γGCS expression plasmids had the correct DNA sequence.

## *3.2 Result*

### *3.2.1 Cloning of* L. mexicana *and* L. major *γGCS into pET21a (+) expression vector*

Cloning of *L. major* and *L. mexicana* γGCS required the pBHX vectors, pET21aLmajGCS6His(M), and pET21aLmexGCS6His(M) plasmids that were produced by Muattaz Hussain (Hussain, 2017).

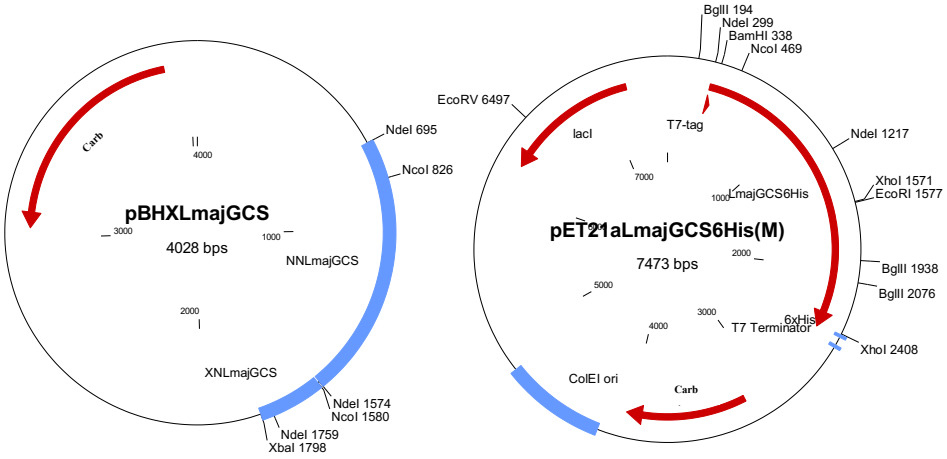


**Figure 3.3 Plasmid maps for pBHXLmajGCS and pET21aLmajGCS6His(M).**

Carb, Carbenicillin resistance gene; lacI, lac operon; ColE1 ori, replication origin. NNLmajGCS and XNLmajGCS region were synthesized by company. LmajGCS6His that has incorrect gene sequence was generated by Muattaz.

pBHXLmajGCS (Figure 3.3) was cleaved with NdeI to generate 165 bp, 879 bp, and 2,964 bp DNA fragments. The 879 bp fragment was isolated from an agarose gel using a NucleoSpin Extract II Kit (Figure 3.6). pET21aLmajGCS6His(M) (Figure 3.3) was cleaved with NdeI. This resulted in a 918 bp and 6,555 bp fragments from which the 6,555 bp fragment was isolated using the NucleoSpin Extract II Kit (Figure 3.6). The 879 bp and 6,555 bp were ligated to generate pET21asLmajGCS6His (Figure 3.5), which was used to transform competent *E. coli* cells. The cells were then plated on

agar containing carbenicillin. Six colonies were individually cultured in 5 mL LB broth containing the same antibiotic. Plasmids were isolated from the resulting transfected bacteria using a NucleoSpin Plasmid Miniprep kit (section 2.2.4). If the two DNA fragments had ligated in the correct orientation then cleavage with NcoI and XhoI (Table 3.1, column 5) would produce DNA fragments with the predicted sizes shown in Table 3.1 (column 6) and Figure 3.7.

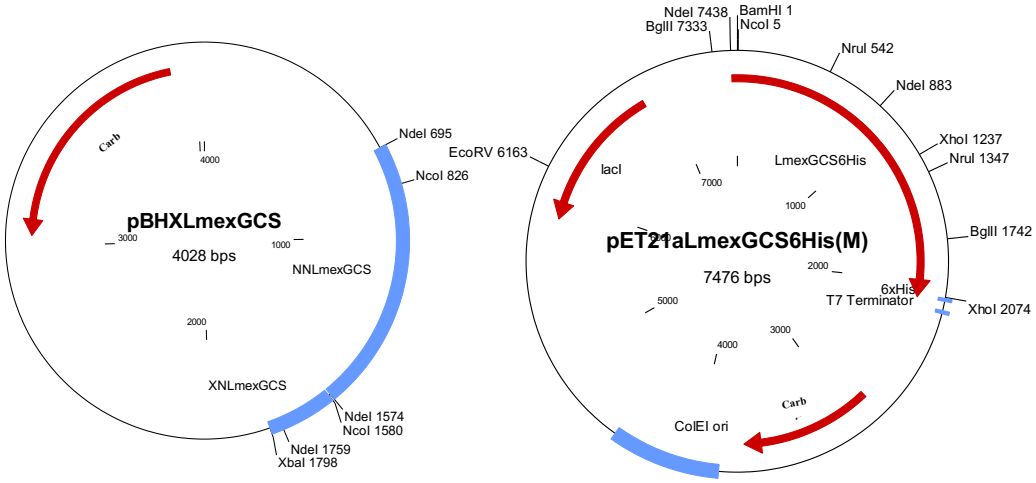


**Figure 3.4 Plasmid maps for pBHXLmexGCS and pET21aLmexGCS6His(M).** Carb, Carbenicillin resistance gene; lacI, lac operon; ColE1 ori, replication origin. NNLmexGCS and XNLmexGCS regions were synthesized by the company. LmexGCS6His that has incorrect gene sequence was generated by Muattaz.

The pBHXLmexGCS plasmid (Figure 3.4) plasmid was cleaved with NdeI and produced products with sizes of 165 bp, 879 bp, and 2,964 bp. The 879 bp fragment was isolated from an agarose gel with a NucleoSpin Extract II Kit (Figure 3.6). The pET21aLmexGCS6His(M) plasmid (Figure 3.4) was also cleaved with NdeI and gave products with sizes of 918 bp and 6,555 bp (Figure 3.6). The 879 bp and 6,555 bp

fragments were ligated to generate pET21asLmexGCS6His plasmid (Figure 3.5). This plasmid was used to transform competent *E. coli*, and the bacteria were plated on agar containing carbenicillin. Selected colonies were grown in medium containing the same antibiotic and a NucleoSpin Plasmid Miniprep was used to isolate pET21asLmexGCS6His. If the two DNA fragments had ligated in the correct orientation the restriction analysis with NcoI and XhoI (Table 3.1, column 5) would produce DNA fragments with the predicted sizes shown in Table 3.1 (column 6) and Figure 3.7.

**Table 3.1 Details of plasmids, restriction enzymes, and the predicted size of restriction digest products**

| Column 1 | Column 2 | Column 3 | Column 4 | Column 5 | Column 6 |
|---|---|---|---|---|---|
| Plasmid Name | Restriction Enzyme | Size of isolated fragment | Plasmid after ligation | Restriction Enzymes of Miniprep with | Expected fragment size |
| pET21aLmajGCS6His(M) | NdeI | 6,555 bp | pET21asLmajGCS6His | NcoI and XhoI | 837 bp, 1,102 bp, 5,495 bp |
| pBHXLmajGCS | | 879 bp | | | |
| pET21aLmexGCS6His(M) | NdeI | 6,555 bp | pET21asLmexGCS6His | NcoI and XhoI | 837 bp, 1,102 bp, 5,495 bp |
| pBHXLmexGCS | | 879 bp | | | |

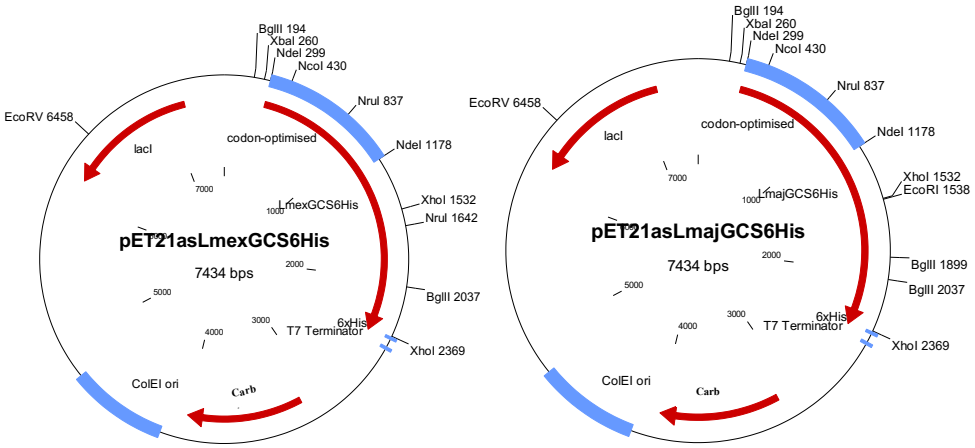


**Figure 3.5 The pET21asLmajGCS6His and pET21asLmexGCS6His plasmid maps.** Carb, Carbenicillin resistance gene; lacI, lac operon; ColE1 ori, replication origin. LmajGCS6His and LmexGCS6His were generated with codon optimised regions.

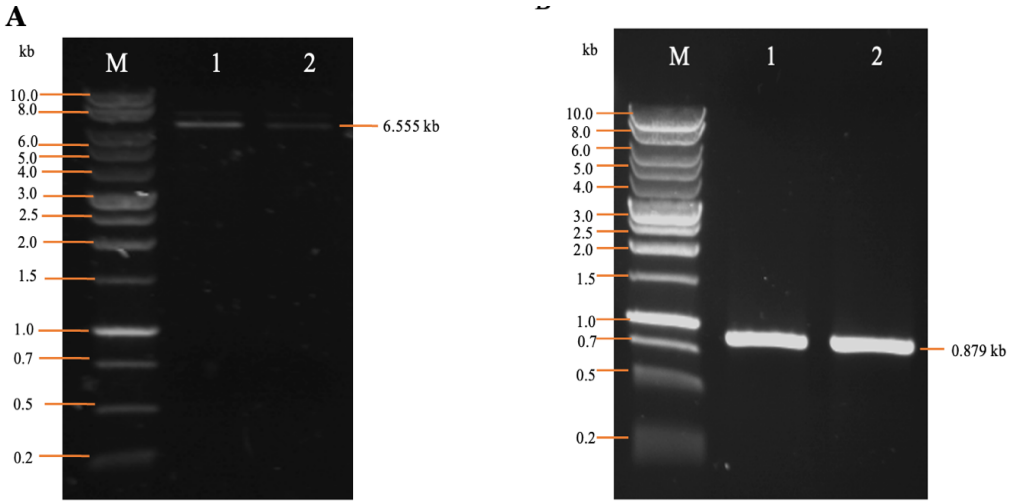


**Figure 3.6 Agarose gels showing isolated DNA fragments produced after digestion of plasmids with NdeI.** (A) DNA-fragments derived from pET21a parent plasmids. M, DNA size marker; lane 1, 6,555 bp NdeI fragment from pET21aLmexGCS6His(M); lane 2, 6,555 bp NdeI fragment from pET21aLmajGCS6His(M). (B) DNA-fragments derived from pBHX plasmids. M, DNA size marker; lane 1, 879 bp NdeI fragment from pBHXLmexGCS; lane 2, 879 bp NdeI fragment from pBHXLmajGCS.

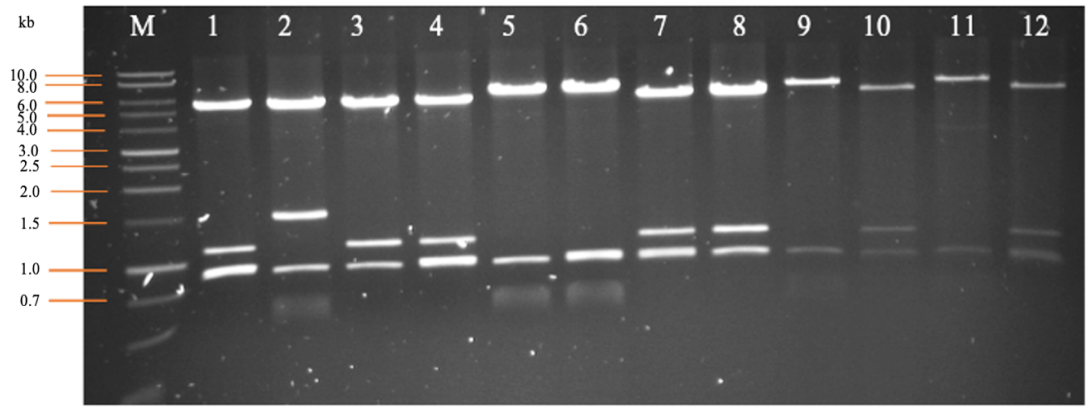


**Figure 3.7 Agarose gel showing DNA products produced after cleavage of pET21asLmajGCS6His and pET21asLmexGCS6His with NcoI and XhoI.** M, DNA size marker; lanes 1-6, pET21asLmajGCS6His; lanes 7-12, pET21asLmexGCS6His. The expected band sizes are 837 bp, 1,102 bp, 5,495 bp.

Colonies 1, 3 and 4 from the pET21asLmajGCS6His transformation were identified as putative positive clones as restriction analysis of the isolated plasmids with NcoI and XhoI produced 837 bp, 1,102 bp, 5,495 bp fragments. Plasmid DNA from colony 3 was isolated using a NucleoSpin Plasmid Midiprep kit and a sample was used in restriction analysis (Table 3.2, column 2). Table 3.2 (column 3) shows the resultant fragments of the analysed plasmid in the correct orientation. Hence, colony 3 was a positive colony as it contained a plasmid producing bands of the expected sizes in restriction analyses (Figure 3.8).

Colonies 7, 8, 10 and 12 of the pET21aLmexGCS6His plasmid gave 837 bp, 1,102 bp, 5,495 bp fragments upon cleavage with NcoI and XhoI, and were thus identified as putative positive colonies. Colony 8 gave bands of the expected sizes (Table 3.2, column 1, Figure 3.5). This plasmid was further analysed with different restriction

enzyme combinations (Table 3.2, column 2) and it gave products with the expected size (Table 3.2, column 3), indicating that the plasmid had incorporated DNA sequences in the correct orientation (Figure 3.8).

**Table 3.2 Details of plasmids, restriction enzymes, and the predicted size of restriction digest products.** One of plasmid DNAs of colonies carrying the correct orientation were cleaved. Bacteria from selected colonies were used to produce more plasmid DNA in a midiprep and subjected to restriction analysis.

| Column 1 | Column 2 | Column 3 |
|---|---|---|
| Plasmid name | Restriction analysis of Midiprep | Expected Fragment Size |
| pET21asLmajGCS6His (colony 3) | XbaI | 7,434 bp |
| | BglII | 138 bp, 1,705 bp, 5,591 bp |
| | EcoRV and EcoRI | 2,512 bp, 4,922 bp |
| pET21asLmexGCS6His (colony 8) | NcoI, | 7,434 bp |
| | XhoI and EcoRI | 837 bp, 6,537 bp |
| | BglII | 1,843 bp, 5,591 bp |
| | | |

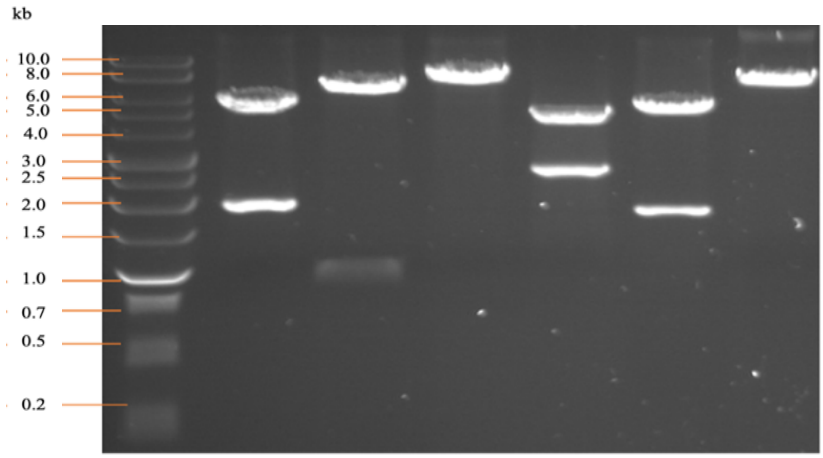


**Figure 3.8 Restriction analysis of pET21asLmajGCS6His and pET21asLmexGCS6His on an agarose gel.** Miniprep control was completed with restriction enzymes and one colony that had the correct fragment sizes was chosen. Midiprep was performed with the chosen colony and extracted DNA was checked with restriction enzymes. M, DNA size marker; midiprep of colony 3 for pET21asLmexGCS6His; lane 1, BglII; lane 2, BglII, lane 3, NcoI; midiprep of colony 8 for pET21asLmajGCS6His; lane 4, EcoRV and EcoRI lane 5, BglII; lane 6, XbaI.

pET21asLmajGCS6His and pET21asLmexGCS6His generated had the correct γGCS gene sequence, in the correct orientation and a His-tag fused to the C-terminus. Therefore the next step involved adding the T7-tag to the N-terminus of the γGCS gene sequence. The T7-tag may allow to enhance protein production (Köppl *et al.*, 2022).

pBHXLmajGCS was commercially generated by gene synthesis. It was cleaved with NcoI and XbaI to produce 218 bp, 754 bp and 3,056 bp DNA fragments. The 218 bp fragment was isolated from an agarose gel (Table 3.3, column 3)(Figure 3.10). pET21asLmajGCS6His was cleaved with NcoI and XbaI to give products with a size

of 170 bp and 7,264 bp. The 7,264 bp fragment was isolated from an agarose gel with a NucleoSpin Extract II Kit (Figure 3.10). This fragment was dephosphorylated with SAP (section 2.2.7) to prevent vector self-ligation. The 218 bp fragment from pBHXLmajGCS and the 7,264 bp product from pET21asLmajGCS6His were ligated and used to transfect competent *E. coli* cells to generate pET21aT7LmajGCS6His (Figure 3.10). Selected colonies from transformed bacteria were used to produce plasmid DNA, and a sample was then treated with the restriction enzymes NheI and XhoI (Table 3.3, column 5). Positive colonies which had the correct DNA sequence in the correct orientation would produce products with the predicted sizes shown in Table 3.3, column 6; Figure 3.11).

Commercially obtained pBHXLmexGCS was cleaved with NcoI and XbaI to generate products with a size of 218 bp, 754 bp, 3,056 bp. The 218 bp fragment (Table 3.3, column 3) was isolated from an agarose gel (Figure 3.10). pET21asLmexGCS6His was cleaved with NcoI and XbaI to create 170 bp and 7,264 bp fragments. The 7,264 bp fragment was isolated from an agarose gel using a NucleoSpin Extract II Kit (Table 3.3, column 3) (Figure 3.10). The 7,264 bp fragment from pET21asLmexGCS6His was treated with SAP, ligated to the 218 bp fragment from pBHXLmexGCS, and then used to transform competent *E. coli* to generate pET21aT7LmexGCS6His (Figure 3.9). This plasmid was isolated from selected putative positive colonies using a NucleoSpin Plasmid Miniprep kit and a sample was cleaved with NcoI and XhoI (Table 3.3, column 5). This should produce products of the size shown in column 6, Table 3.3 (Figure 3.11) if the DNA fragments had been inserted in the correct orientation.

**Table 3.3 Details of plasmids, restriction enzymes, and expected DNA fragments.** pBHXGCS and pET21aGCS6His were used to generate constructs that had a T7-tag. NcoI and XbaI (column 2) of donor plasmids (column 1) was shown with size of fragments (column 3). Isolated DNA fragments were ligated to generate final plasmids. Ligation sample was transformed into *E. coli*. Plasmid DNA was isolated from selected colonies were cleaved with control enzyme combination after miniprep (column 5).

| Column 1 | Column 2 | Column 3 | Column 4 | Column 5 | Column 6 |
|---|---|---|---|---|---|
| Donor Plasmids | Restriction Enzyme | Size of Isolated Fragment | Plasmids after ligation | Restriction analysis of Midiprep with | Expected Fragment Size |
| pBHXLmajGCS | NcoI and XbaI | 218 bp | pET21aT7LmajGCS6His | NheI and XhoI | 837 bp, 1,275 bp, 5,370 bp |
| pET21asLmajGCS6His | | 7,264 bp | | | |
| pBHXLmexGCS | NcoI and XbaI | 218 bp | pET21aT7LmexGCS6His | NcoI and XhoI | 837 bp, 1,235 bp, 5,370 bp |
| pET21asLmexGCS6His | | 7,264 bp | | | |

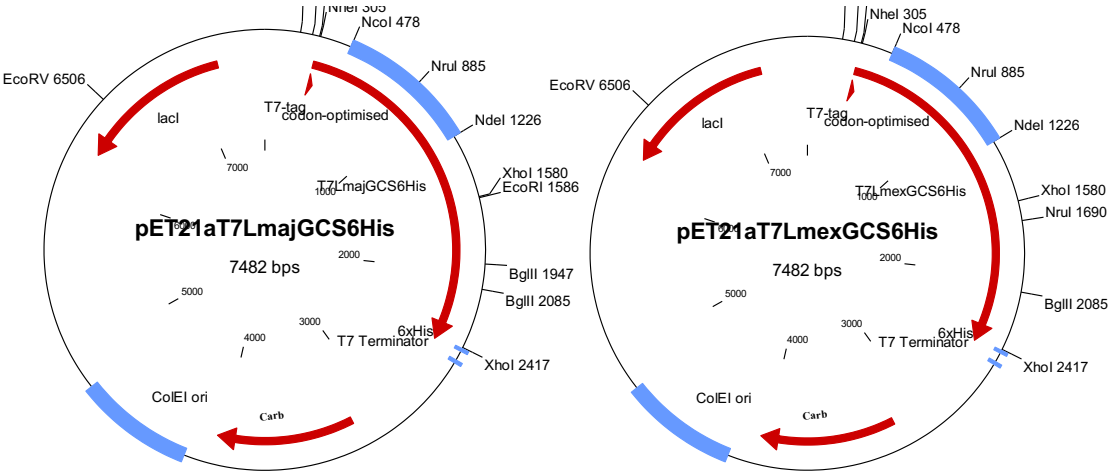


**Figure 3.9 Plasmid maps of pET21aT7LmajGCS6His and pET21aT7LmexGCS6His.** Carb, Carbenicillin resistance gene; lacI, lac operon; ColE1 ori, replication origin. LmajGCS6His and LmexGCS6His were generated with codon optimised regions.

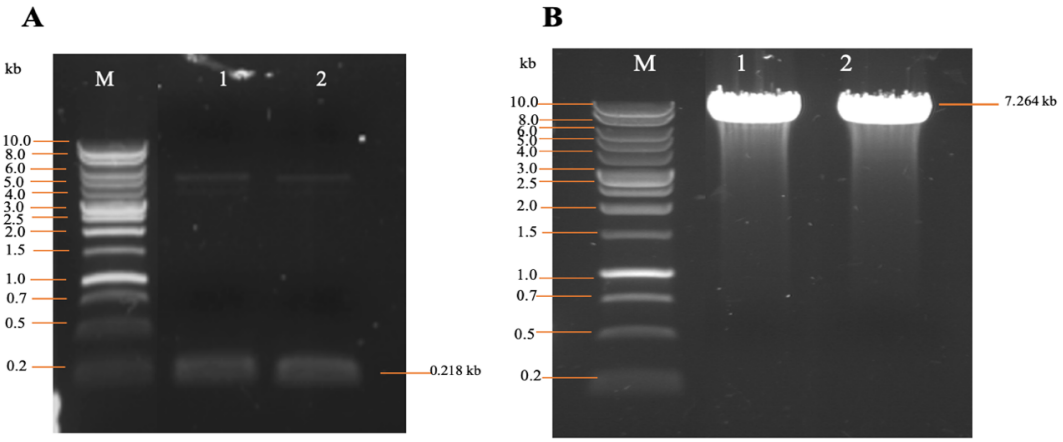


**Figure 3.10 Analysis of isolated DNA-fragments on agarose gels.** DNA-fragments derived from pBHXGCS, pET21asLmajGCS6His, and pET21asLmexGCS6His. (A) M, DNA size marker; lane 1, 218 bp fragment NcoI and XbaI pBHXLmajGCS fragment; lane 2, 218 bp fragment NcoI and XbaI pBHXLmexGCS fragment. (B) M, DNA size marker; lane 1, 7,264 bp NcoI and XbaI pET21asLmajGCS6His fragment; lane 2, 7,264 bp NcoI and XbaI pET21asLmajGCS6His fragment.

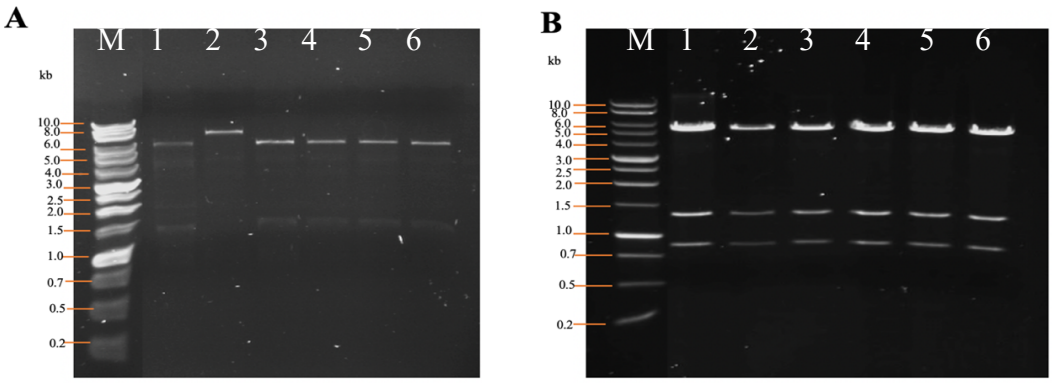


**Figure 3.11 Restriction analysis of pET21aT7LmajGCS6His and pET21aT7LmexGCS6His using agarose gels**. A DNA miniprep isolation was performed with single colonies for each plasmid. (A) M, DNA size marker; lane 1-6, miniprep DNA from colonies 1-6, pET21aT7LmajGCS6His NheI and XhoI result in 837 bp, 1,275 bp, 5,370 bp. (B) M, DNA size marker; lane 1-6, miniprep DNA from colonies 1-6, pET21aT7LmexGCS6His NcoI and XhoI result in 837 bp, 1,235 bp, 5,370 bp.

After miniprep control, possible positive colonies for pET21aT7LmajGCS6His, pET21aT7LmexGCS6His were selected. One positive colony for pET21aT7LmajGCS6His (Colony 3) and one for pET21aT7LmexGCS6His (Colony 1) was used to produce a higher amount of plasmid. A sample of each plasmid was cleaved with different restriction enzyme combinations (column 2, Table 3.4) to confirm that the DNA fragments had inserted in the correct orientation (Figure 3.12).

**Table 3.4 Details of plasmids, restriction enzymes, and expected DNA fragments.** Putative positive colonies were detected after miniprep control and midiprep was performed with one colony from each plasmid to obtain high amount of plasmid (column 1). Plasmids were digested with three different enzyme combinations (column 2) to prove that the DNA segments had integrated in the correct orientation in the plasmids. The size of the fragments detected after cleavage (column 3) confirmed correct integration.

| Column 1 | Column 2 | Column 3 |
|---|---|---|
| Plasmid | Restriction analysis of Midiprep | Expected fragment size |
| pET21aT7LmajGCS6His (Colony 3 ) | NdeI | 927 bp, 6,555 bp |
| | NcoI and EcoRV | 1,454 bp, 6,028 bp |
| | NheI and EcoRI | 1,281 bp, 6,201 bp |
| pET21aT7LmexGCS6His (Colony 1) | XhoI | 837 bp, 6,645 bp |
| | NheI and XhoI | 837 bp, 1,275 bp, 5,370 bp |
| | BgIII | 1,891 bp, 5,591 bp |

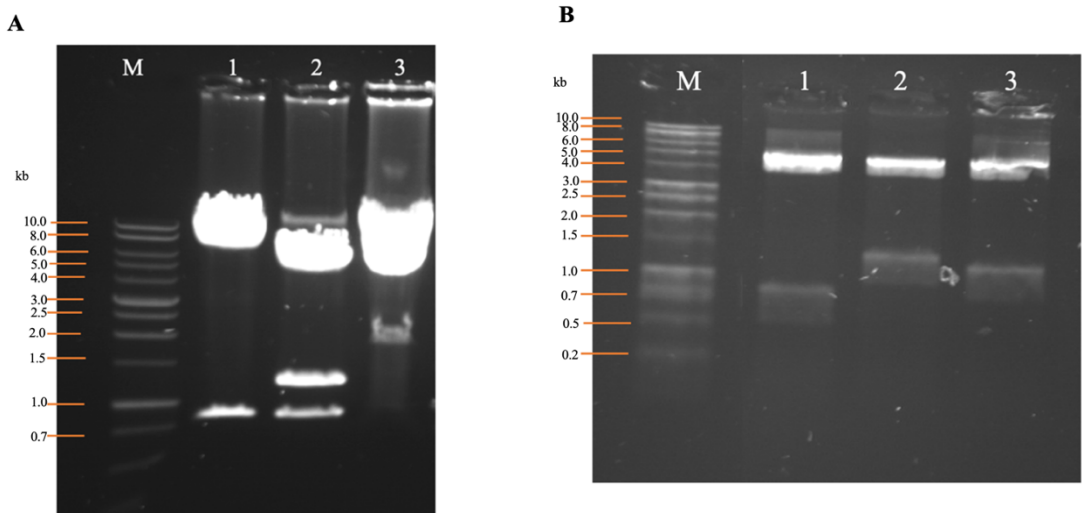


**Figure 3.12 Restriction analysis with pET21aT7LmexGCS6His and pET21aT7LmajGCS6His plasmids. (**A) M, DNA size marker; lane 1, XhoI; lane 2, NheI and XhoI; lane 3, BglII. (B) M, DNA size marker; lane 1, NdeI; lane 2, NcoI and EcoRV; lane 3, NheI and EcoRI.

### *3.2.2 Cloning of* Leishmania donovani *γGCS into the pET-21a (+) expression vector*

Cloning of *L. donovani* γGCS into pET-21a (+) required different steps from the other two *Leishmania* species. The first step is ligation of pET-21a (+) and pBHXLdonGCS carrying part of the γGCS gene sequence. These plasmids were cleaved with NdeI and the 5,443 bp fragment of pET21a (+) isolated from an agarose gel was treated with SAP (section 2.2.7, Table 3.5 column 1). The 879 bp fragment was isolated from an agarose gel for pBHXLdonGCS using a NucleoSpin Extact II kit (Table 3.5 column 1). The isolated fragments were ligated, used to transform competent *E. coli* cells and positive colonies carrying pET21aNLdonGCS (Table 3.5, column 2) were isolated. Plasmid DNA was isolated, and two putative positive colonies were cleaved with XbaI and HindIII to generate 982 bp, 5,340 bp fragments (Table 3.5 column 3, 4). One colony did not give DNA fragments of the expected sizes whereas the second one did

(Figure 3.14, lane 2). A Midiprep was performed to give a higher amount of plasmid from the positive colony carrying pET21aNLdonGCS plasmid and a sample was cleaved with the enzymes shown in column 5, Table 3.5 to see if it would give products of the predicted size if the plasmid had incorporated DNA in the correct orientation (column 6, Table 3.5, Figure 3.15).

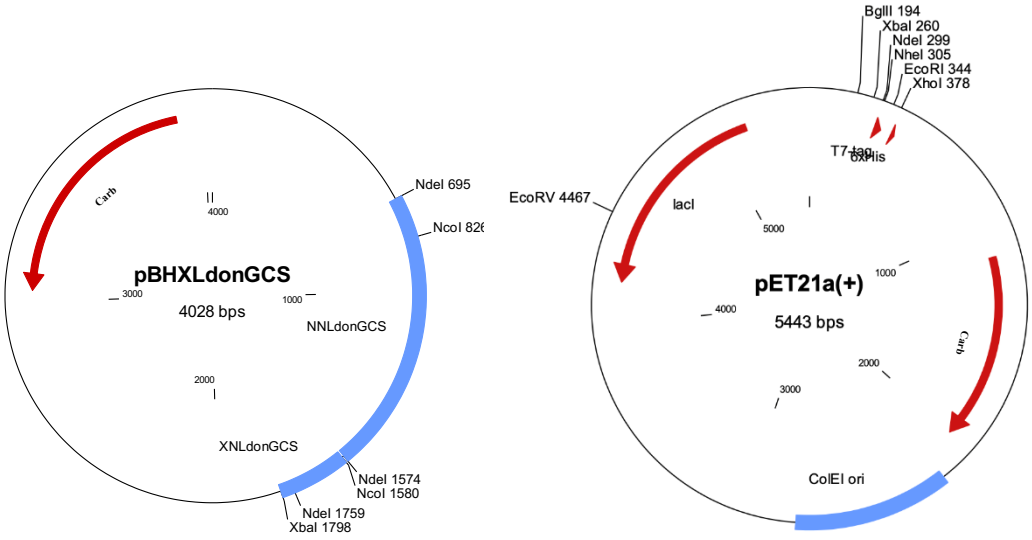


**Figure 3.13 Plasmid maps of pBHXLdonGCS and pET21a(+) plasmids.** Carb, Carbenicillin resistance gene; lacI, lac operon; ColE1 ori, replication origin. NNLdonGCS and XNLdonGCS regions were synthesised by the company.

**Table 3.5 Shows generation of pET21aNLdonGCS plasmid and control of the colonies carrying the plasmid.** pBHXLdonGCS was cleaved with NdeI to generate an 879 bp fragment, pET21a(+) was linearised with NdeI (column 1) and both fragments were ligated to form pET21aNLdonGCS. The ligation was transformed into *E. coli* and plasmids isolated from putative positive colonies were checked with XbaI and HindIII (column 3). A colony carrying a plasmid resulting in the predicted fragment sizes (column 4) was used to performed a DNA midiprep. A high amount of pET21aNLdonGCS DNA was obtained and checked by restriction enzyme analysis (column 5).

| Column 1 | Column 2 | Column 3 | Column 4 | Column 5 | Column 6 |
|---|---|---|---|---|---|
| Donor Plasmids/ Isolated Fragment Size | Plasmid after Ligation | Restriction analysis of miniprep with | Expected Fragment Sizes | Restriction analysis of midiprep with | Expected Fragment Sizes |
| pBHXLdonGCS 879 bp | pET21aNLdonGCS | XbaI and HindIII | 982 bp 5,340 bp | XbaI | 6,322 bp |
| pET21a (+) 5,443 bp | | | | NcoI and EcoRV | 1,406 bp, 4,916 bp |
| | | | | NdeI | 879 bp, 5,443 bp |

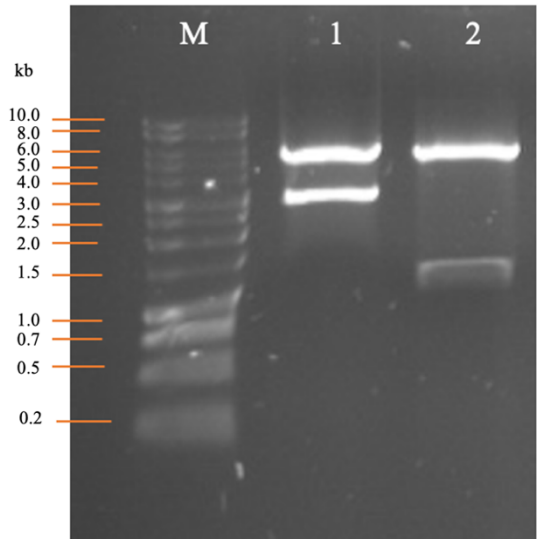


**Figure 3.14 Agarose gel of XbaI and HindIII restriction analysis of plasmids from two colonies after miniprep.** Expected fragment sizes are 982 bp and 5,340 bp. M, DNA size marker; lane 1, plasmid of pET21aNLdonGCS negative colony; lane 2, plasmid of pET21aNLdonGCS positive colony.

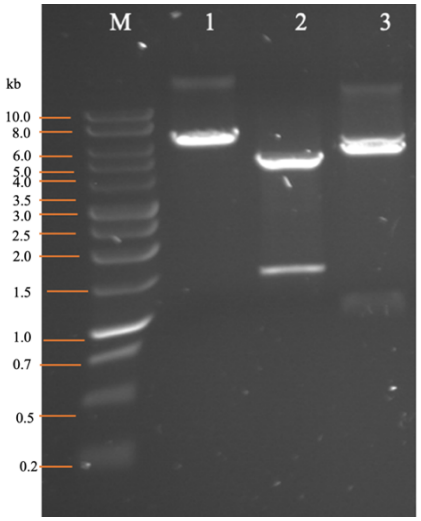


**Figure 3.15 Agarose gel of pET21aNLdonGCS restriction analysis.** M, DNA size marker; lane 1, XbaI; lane 2, NcoI and EcoRV; lane 3, NdeI.

Generating pET21aLdonGCS6His required pET21aNLdonGCS and pTHGFPLdonGCS prepared by Muattaz Hussain (Hussain, 2017).

pTHGFPLdonGCS was cleaved with NotI to generate 1,221 bp and 8,615 bp fragments (Figure 3.11). The 8,615 bp fragment was isolated, filled using the Klenow process (section 2.2.6) (Table 3.6, column 4) and cleaved with NcoI to generate 130 bp, 1,935 bp, 2,703 bp, and 3,847 bp fragments (column 5, Table 3.6). The 1,935 bp fragment was isolated using an agarose gel (Figure 3.12).

pET21aNLdonGCS was cleaved with XhoI to generate a 6,322 bp product (Figure 3.11) and its ends were filled using the Klenow process (section 2.2.6) (column 4, Table 3.6). The resulting fragment was cleaved with NcoI to generate 827 bp, 5,495 bp fragments (column 5, Table 3.6) and the latter was isolated from an agarose gel (Figure 3.12). The 1,935 bp fragment from pTHGFPLdonGCS and the 5,495 bp fragment from pET21aNLdonGCS were ligated to obtain pET21aLdonGCS6His (column 7, table 3.6).

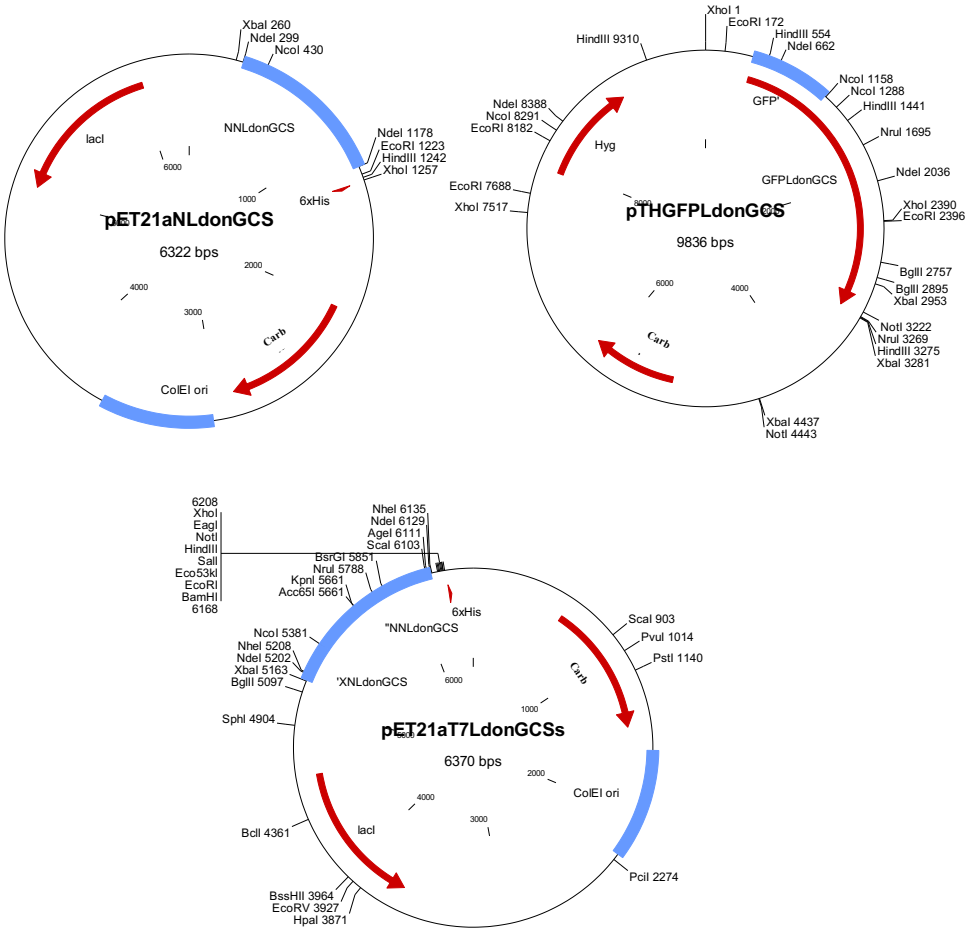


**Figure 3.16 Plasmid maps for pET21aNLdonGCS, pTHGFPLdonGCS and pET21aT7LdonGCSs plasmid maps.** Carb, Carbenicillin resistance gene; lacI, lac operon; ColE1 ori, replication origin. NNLdonGCS and XNLdonGCS regions were synthesised by the company.

**Table 3.6 Details of plasmids, restriction enzymes, and the predicted size of restriction digest products to generate pET21aLdonGCS6His.** Donor plasmids (column 1) were cleaved with the restriction enzymes shown in column 2 to produce fragments of the size shown in column 3.The desired fragments were filled-in using the Klenow process (column 4) and cleaved with NcoI to produce the fragment of the size shown in column 6. These fragments were ligated to produce pET21aLdonGCS6His (column 7).

| Column 1 | Column 2 | Column 3 | Column 4 | Column 5 | Column 6 | Column 7 |
|---|---|---|---|---|---|---|
| Donor Plasmids | Restriction Enzyme | Expected Size of Fragments | | Restriction Enzyme | Expected Fragment Size | Plasmid after ligation |
| pTHGFPLdonGCS | NotI | 1,221 bp<br>8,615 bp | 8,615 kb filled- in with Klenow process | NcoI | 1,935 bp | pET21aLdonGCS6His |
| pET21aNLdonGCS | XhoI | 6,322 bp | 6,322 kb filled in with Klenow process | NcoI | 5,495 bp | |

The generation of pET21aT7LdonGCS6His required an additional cloning step. pET21aT7LdonGCSs (Figure 3.16) was generated with pET21aNLdonGCS and pBHXLdonGCS.

pET21aNLdonGCS was cleaved 1 to produce fragments of 6,125 bp and 170 bp. pBHXLdonGCS was cleaved with NcoI and XbaI to produce products with a size of 218 bp, 754 bp and 3,056 bp.

The 6,125 bp fragment from pET21aNLdonGCS and the 218 bp fragment from pBHXLdonGCS were isolated, ligated and transformed into to competent *E. coli* cells to create pET21aT7LdonGCSs (Table 3.7) (Figure 3.16). Positive colonies were used to set up 5 mL bacterial cultures and plasmids were isolated from the colonies using a miniprep kit (section 2.2.4). One of the clones carrying the correct plasmid was selected to produce higher amounts of plasmid DNA using a midiprep kit (section 2.2.4). The identity of pET21aT7LdonGCSs was confirmed via restriction enzyme analysis.

**Table 3.7 Details of plasmids, restriction enzymes, and the predicted size of restriction digest products to generate pET21aT7LdonGCSs.** pTHGFPLdonGCS and pET21aNLdonGCS were cleaved with NcoI and XbaI, 128 bp and 6,125 bp fragments were isolated and ligated generating the pET21aT7LdonGCSs construct.

| Donor Plasmid | Restriction Enzymes | Expected size of fragments | Plasmid |
|---|---|---|---|
| pTHGFPLdonGCS | NcoI and XbaI | 218 kb | pET21aT7LdonGCSs |
| pET21aNLdonGCS | NcoI and XbaI | 6,125 kb | |

The final construct pET21aT7LdonGCS6His was obtained by ligation of fragments of pET21aT7LdonGCSs and pTHGFPLdonGCS (Hussain, 2017).

The pTHGFPLdonGCS plasmid prepared by Muattaz Hussain (Hussain, 2017) was cleaved with NotI to generate 1,221 bp and 8,615 bp DNA fragments (Table 3.8, column 3) (Figure 3.17). The 8,615 bp fragment was filled-in using the Klenow method (section 2.2.6) and cleaved with NcoI to generate 130 bp, 1,935 bp, 2,703 bp,

and 3,847 bp fragments (Table 3.8 column 4). The 1,935 bp fragment was then isolated from an agarose gel (Figure 3.12) (Table 3.8 column 6).

pET21aT7LdonGCSs was linearised with XhoI, the 6,370 bp fragment (Table 3.8 column 4) was isolated from an agarose gel and filled-in using the Klenow method (section 2.2.6). The 6,370 bp fragment was cleaved with NcoI to generate 827 bp and 5,543 bp fragments and the 5,543 bp fragment isolated from an agarose gel (Figure 3.18, Table 3.8 column 6).

The 1,935 bp pTHGFPLdonGCS and 5,543 bp pET21aT7LdonGCSs fragments were ligated to produce pET21aT7LdonGCS6His and this was used to transfect competent *E. coli* cells.

**Table 3.8 Details of plasmids, restriction enzymes, and the predicted size of restriction digest products to generate pET21aT7LdonGCS.** pET21aT7LdonGCSs was cleaved with XhoI that created 6,370 bp and pTHGFPLdonGCS was cleaved with NotI that created 1,221 bp and 8,615 bp fragments (column 3). 6,370 bp linearised pET21aT7LdonGCSs and 8,615 bp pTHGFPLdonGCS were filled in with Klenow process (column 4), then they were cleaved with NcoI (column 5). After ligation of the 5,543 bp and 1,935 bp fragments pET21aT7LdonGCS6His was obtained (column 7).

| Column 1 | Column 2 | Column 3 | Column 4 | Column 5 | Column 6 | Column 7 |
|---|---|---|---|---|---|---|
| Donor Plasmids | Restriction Enzyme | Expected size of fragments | | Restriction Enzyme | Expected size of fragments | Ligation 5,543 bp + 1,935 bp |
| pET21aT7LdonGCSs | XhoI | 6,370 bp | 6,370 bp fragment filled-in with Klenow | NcoI | 5,543 bp, 827 bp | pET21aT7LdonGCS6His |
| pTHGFPLdonGCS | NotI | 1,221 bp, 8,615 bp | 8,615 bp fragment filled-in with Klenow | NcoI | 1,935 bp, 5,495 bp | |

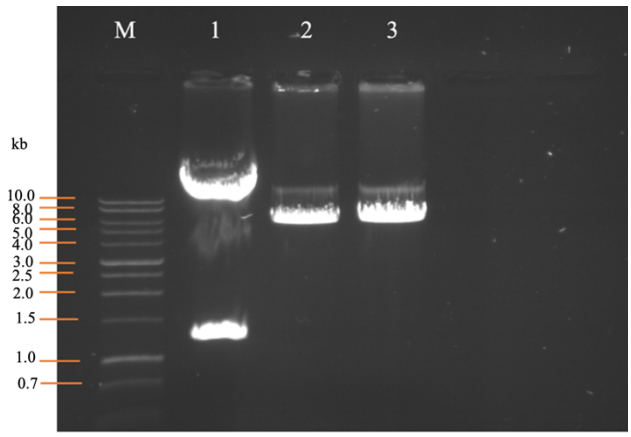


**Figure 3.17 Agarose gel of desired fragments generated with restriction enzymes.** M; DNA size marker; lane 1, 1,221 bp and 8,615 bp fragments from pTHGFPLdonGCS using NotI; lane 2, 6,322 bp linear pET21aNLdonGCS plasmid with XhoI; lane 3, 6,370 bp linear plasmid pET21aT7LdonGCSs with XhoI.

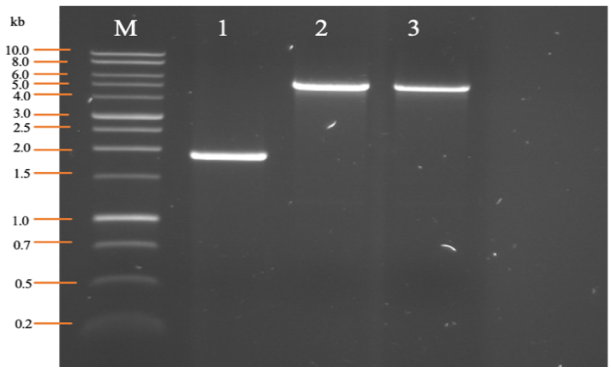


**Figure 3.18 Agarose gel of isolated DNA-fragments of pTHGFPLdonGCS, pET21aNLdonGCS and pET21aT7LdonGCSs.** M, DNA size marker; lane 1, 1,935 bp fragment from pTHGFPLdonGCS; lane 2, 5,495 bp fragment from pET21aNLdonGCS; lane 3, 5,543 bp fragment from pET21aT7LdonGCSs. The 1,935 bp fragment from pTHGFPLdonGCS and 5,495 bp fragment from pET21aNLdonGCS were ligated to generate pET21aLdonGCS6His. The 1,935 bp fragment from pTHGFPLdonGCS and 5,543 bp fragment from pET21aT7LdonGCSs were ligated to obtain pET21aT7LdonGCS.

Colonies carrying pET21aLdonGCS6His or pET21aT7LdonGCS6His (Figure 19) were used to produce 5 mL bacterial broth. Samples of bacteria were used to isolate plasmid using a miniprep kit and digested with XhoI (pET21aLdonGCS6His) or NheI and XhoI (pET21aT7LdonGCS6His) to determine if the DNA fragments had inserted in the correct orientation (Table 3.9, Figure 3.20). A larger quantity of DNA was isolated from bacterial colonies that gave the expected result using a midiprep kit ( section 2.2.5, Table 3.10 , Figure 3.21).

**Table 3.9 Size of fragments produced by cleaving pET21aLdonGCS6His and pET21aT7LdonGCS6His with different restriction enzymes.**

| Plasmid Names | Restriction Enzyme | Expected size of fragments |
|---|---|---|
| pET21aLdonGCS6His | XhoI | 837 bp, 6,594 bp |
| pET21aT7LdonGCS6His | NheI and XhoI | 837 bp, 1,275 bp, 5,370 bp |

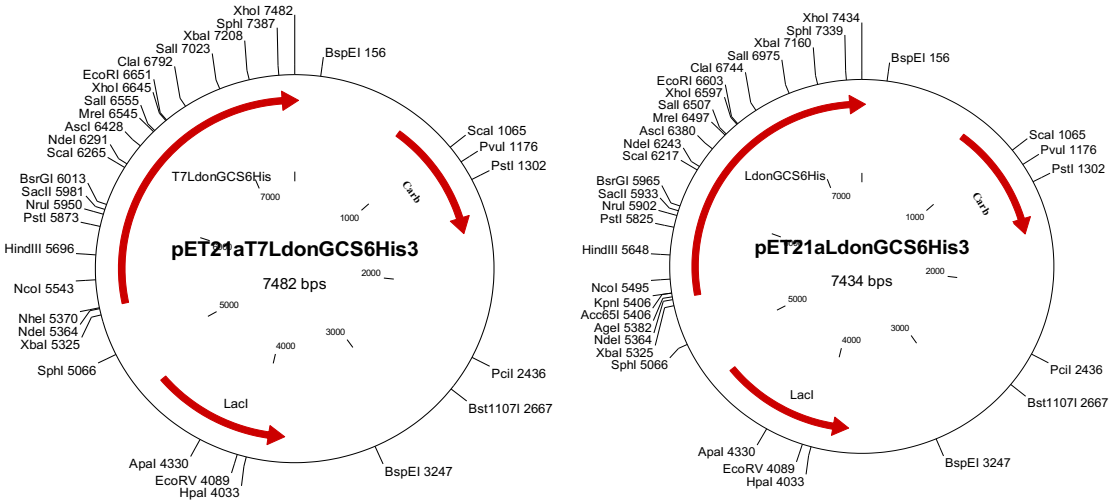


**Figure 3.19 Plasmid maps of pET21aLdonGCS6His and pET21aT7LdonGCS6His.**

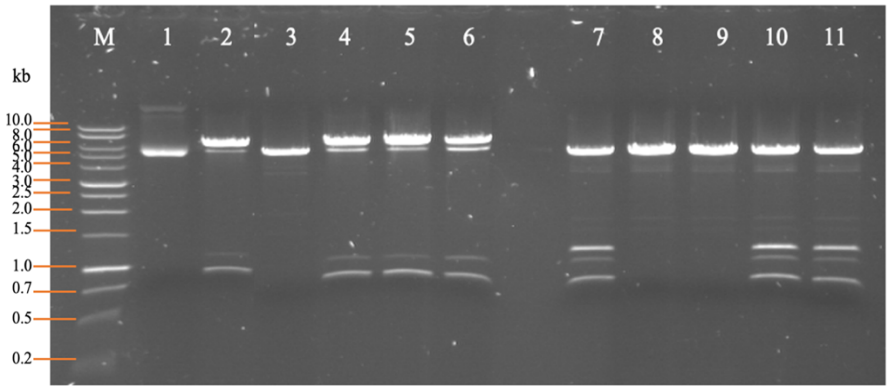


**Figure 3.20 Restriction analysis of pET21aLdonGCS6His and pET21aT7LdonGCS6His plasmid DNAs.** M, DNA size marker; lane 1-6, pET21aLdonGCS6His miniprep colonies XhoI result in 837 bp, 6,594 bp; lane 7-8-9-10-11, pET21aT7LdonGCS6His miniprep colonies NheI and XhoI result in 837 bp, 1,275 bp, 5,370 bp.

**Table 3.10 Restriction analysis of midiprep DNAs.** pET21aLdonGCS6His and pET21aT7LdonGCS6His were cleaved with different restriction enzymes.

| Plasmid Names | Restriction Enzyme | Expected size of fragments |
|---|---|---|
| pET21aLdonGCS6His | XhoI | 837 bp, 6,597 bp |
| | EcoRI and EcoRV | 2514 bp, 4,920 bp |
| | HindIII | 7,434 bp |
| | XbaI | 1,835 bp, 5,599 bp |
| pET21aT7LdonGCS6His | NheI | 7,434 bp |
| | EcoRI and EcoRV | 2,560 bp, 4,922 bp |
| | HindIII | 7,434 bp |
| | NheI and XhoI | 837 bp, 1,275 bp, 5,370 bp |

Restriction analyses indicated that *L. donovani, L. major, L. mexicana* γGCS gene sequences were successfully cloned into pET-21a (+) expression vector with either one (His-tag) or two tags (His-tag and T7-tag). Therefore, samples of the pET21aLdonGCS6His, pET21aT7LdonGCS6His, pET21aLmajGCS6His, pET21aT7LmajGCS6His, pET21aLmexGCS6His and pET21aT7LmexGCS6His plasmids were sent for sequencing by a commercial company.

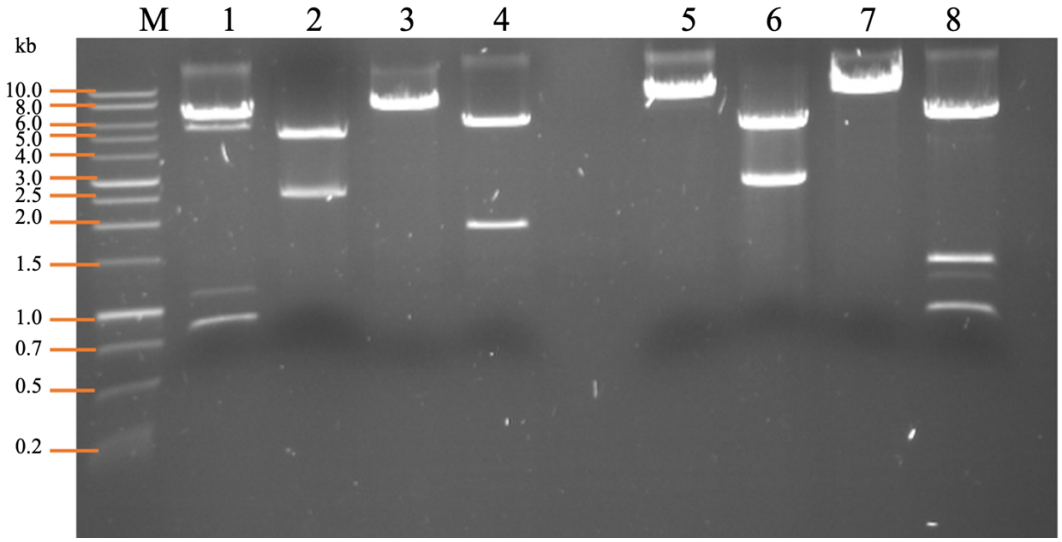


**Figure 3.21 Restriction analysis of pET21aLdonGCS6His and pET21aT7LdonGCS6His.** M, DNA size marker; lanes 1-4, pET21aLdonGCS6His; lane 1, XhoI; lane 2, EcoRI and EcoRV; lane 3, HindIII; lane 4, XbaI; lanes 5-8, pET21aT7LdonGCS6His; lane 5, NheI; lane 6, EcoRI and EcoRV; lane 7, HindIII; lane 8, NheI and XhoI.

### *3.3 Discussion*

γGCS genes from *L. donovani, L. major* and *L. mexicana* were successfully cloned into the pET21a(+) expression vector and fused with the sequence coding for an N-terminal T7-tag and a C-terminal His-tag. This process took nearly a year to finish as each plasmid construction required a large number of steps and often incorrect plasmids were obtained. Therefore, individual cloning steps had to repeated more than once. Thus when the plasmid DNA, isolated from the antibiotic selected colonies, were subjected to the restriction digest analyses the wrong fragment sizes were produced. The ligation process was often modified, for example by using different ligation ratios to obtain positive colonies carrying the desired plasmids.

Previous plasmids including the related gene sequences obtained from Muattaz Hussain (Hussain, 2017) had mutations in the start of the genes. Sequencing analysis revealed that the amino acid sequences at beginning of the γGCS proteins were not correct. Therefore, a partial γGCS gene sequence was synthesised by a commercial supplier. This was provided in a plasmid and allowed to incorporate the end sequence of the appropriate *Leishmania* species γGCS, and manipulate the initial DNA sequence of the gene to facilitate expression in *E. coli.* Codon optimisation is a crucial step in heterologous gene expression that can have an effect on the level of protein expression (Constant *et al.,* 2023). Codon optimisation of the full-length gene may enhance protein expression; however, synthesis of the full-length γGCS, which is a relatively large gene, would have come at a high-cost. pET21aLdonGCS6His, pET21aT7LdonGCS6His, pET21aT7LmajGCS6His, pET21asLmexGCS6His and pET21aT7LmexGCS6His were confirmed by commercial sequencing of the plasmids (Appendix 1). These plasmids were then used in expression studies detailed in Chapter 4.

# Chapter 4 Expression and purification of recombinant *Leishmania* γGCS protein.

### *4.1 Introduction*

Vaccines are an impactful way of preventing disease and reducing mortality and morbidity caused by harmful pathogens; vaccines have prevented almost six million deaths/year (Orenstein and Ahmed, 2017). They are a cost-effective way to control diseases as they prevent hospitalisation and can be used as part of a government's disease control programme (Pollard and Bijker, 2021). Vaccines use an antigen from the relevant pathogen, which can induce a protective immune response in the immunised individual. The problem for vaccine studies is identifying which specific antigen or collection of antigens can induce a protective immune response.

Vaccines can be classified into three different types. First generation vaccines are inactivated or attenuated vaccines that use a weakened form of a pathogen, so it cannot cause disease (Acosta-Coley *et al.*, 2022; Tahamtan *et al.*, 2017). However, first generation vaccines have safety concerns as the pathogen could revert back to a harmful form (Jain and Jain, 2015). Second generation vaccines are genetically engineered pathogen-specific proteins or synthetic or recombinant proteins produced using bacterial or viral expression systems. Third generation vaccines contain specific gene sequence(s) of a pathogen that is (are) relevant for protection. DNA and RNA vaccines are examples of this group of vaccines and they have been useful in helping combat the COVID19 pandemic (Tahamtan *et al.*, 2017).

Recombinant vaccines have many advantages over the other types of vaccines. For example, they can induce long-term immunity, are cost effective to produce and are more stable than other types e.g. RNA vaccines (Pollet *et al.*, 2021). Recombinant protein vaccines can be produced using expression systems, which use different expression vectors, promotors, selection markers and purification methods

(Nascimento and Leite, 2012). There are many expression systems such as bacteria, yeast, insect or mammalian cells. The one selected is likely to depend on the pathogen protein produced as high-quality recombinant protein is required for clinical use. Each system has particular advantages, for example, *E. coli* bacteria can grow quickly and give a high recombinant protein yield, whereas yeast can allow post-translational modifications of expressed proteins and grow in cost effective medium (Tripathi and Shrivastava, 2019).

Several vaccine antigens have successfully been produced using recombinant protein technology. For example, a hepatitis B vaccine has been produced from yeast (Nascimento and Leite, 2012). This vaccine has been used in 189 countries around the world by the end of 2019. Children younger than 5 years of age showed low prevalence of chronic hepatitis B infection with less than 1% in 2019 (Pattyn *et al.*, 2021). Trumenba®, a vaccine used to protect against *Neisseria meningitidis*, is produced using an *E. coli* expression platform by Pfizer (Pollet *et al.*, 2021). It was approved for use by the US Food and Drug Administration in 2014 (Sulis *et al.*, 2022). Trumenba® vaccination induced a 4-fold increase in antibody responses against four *N. meningitidis* strains (Bagwe *et al.*, 2020). Recombinant technology was also used to produce vaccines against severe acute respiratory syndrome coronavirus (SARS-CoV). For example, the Soberana 2 vaccine, which contains the spike protein from SARS-CoV, used a Chinese hamster ovary cell expression system to produce the recombinant protein (Hotez and Bottazzi, 2022). These studies show how effective recombinant protein vaccines can be in controlling diseases.

Leishmaniasis is a vector borne disease that affects nearly 14 million people around the world (Raj *et al.*, 2020). Although studies indicate that leishmaniasis can

potentially be a vaccine-preventable disease, there is currently no clinically approved available vaccine (Mann *et al.*, 2021). This is not because of a lack of research, but rather a difficulty in translating experimental research into a viable clinical product.

Various recombinant protein candidates have been developed for leishmaniasis, and these include gp63, fucose mannose ligand (FML), cysteine proteinase (CP), promastigote surface antigen 2 (PSA-2) and *Leishmania* homolog of receptors for activated kinase (LACK) (Nagill and Kaur, 2011). The *L. donovani* p45 (rLD45) protein, which is a methionine aminopeptidase produced using an *E. coli* C41 (DE3) expression system, induced significant protection in a hamster model against VL (Gupta *et al.*, 2012; Jain and Jain, 2015). Vaccination with recombinant gp63, produced using *S. typhimurium,* protected mice against *L. major* or *L. donovani* infection, and protection was associated with the ability of splenocytes from vaccinated mice to produce higher levels of IFN-γ (Abdellahi *et al.*, 2022). Another study showed that BALB/c mice immunised with recombinant hypothetical amastigote-specific *L. infantum* protein (LiHyp1), produced in *E. coli*, given with saponin adjuvant, also protected against *L. amazonensis* infection. Protection was associated with enhanced Th1 immune responses as immunised mice had higher IFN-γ and IL-12 responses compared to controls in spleen cell culture (Jesus *et al.*, 2023). The aim of this study was to produce recombinant *Leishmania* γGCS, using *E. coli*. *E. coli* is an ideal vaccine expression system as the bacterium grows quickly, allowing large amounts of recombinant protein to be produced, it has low cost medium requirements and it has a genome that is easy to manipulate. However, there are some disadvantages with this expression system. For example, it cannot carry out the post-translational modifications that may be required for some mammalian proteins.

Moreover, expressed proteins may be incorporated into inclusion bodies rather than being expressed as soluble proteins, and some proteins are only produced in low quantities (Lozano Terol *et al.*, 2021).

Studies have shown that growing *E. coli* at high temperatures and/or using high concentrations of an inducer favour inclusion body formation (Bhatwa *et al.*, 2021). Specific bacterial strains have been developed to overcome these problems e.g. the *E. coli* RosettaBlue strain. RosettaBlue™ host strains are NovaBlue derivatives that combine high transformation efficiency and recA endA lacIq mutations with enhanced expression of eukaryotic proteins that contain codons rarely used in *E. coli*. These strains supply tRNAs for AGG, AGA, AUA, CUA, CCC, and GGA on a chloramphenicol-resistant plasmid. It has been shown to give significantly better protein expression than the traditionally used *E. coli* BL21 (DE3) strain (Tegel *et al.*, 2010). Glucose is the main source of energy in bacteria, but when it is not available lactose is used as a second energy source. This switch in using a different energy source is facilitated by the *Lac* operon in *E. coli*, which contains the gene sequences for *Lac* Z, *Lac* Y and *Lac* A. The products of these genes allow the bacterium to metabolise lactose (Velazco *et al.*, 2021). The expression vector can have a T7 promotor. This promotor contains the recognition sequence for a T7 RNA polymerase and has an adjacent LacO site: a site containing a lac operator sequence. The inhibitor of the lac operon, LacI, binds to the LacO site, and prevents the binding of T7 RNA polymerase to the T7 promotor. Isopropyl β-d-1-thiogalactopyranoside (IPTG), which is used to induce expression of recombinant proteins in *E. coli* is an analogue of lactose and can induce the expression of the *Lac* operon (Khani and Bagheri, 2020). Its binding to LacI stops Lac I binding to LacO and switches on expression of the recombinant

protein (Shilling *et al.*, 2020). BL21 (DE3) contains the lambda DE3 prophage that carries the gene for T7 RNA polymerase under control of a lacUV5 promotor, allowing expression of the T7 RNA polymerase to be induced with IPTG and can direct high-level expression of cloned genes under the control of the T7 promotor (Figure 4.1).

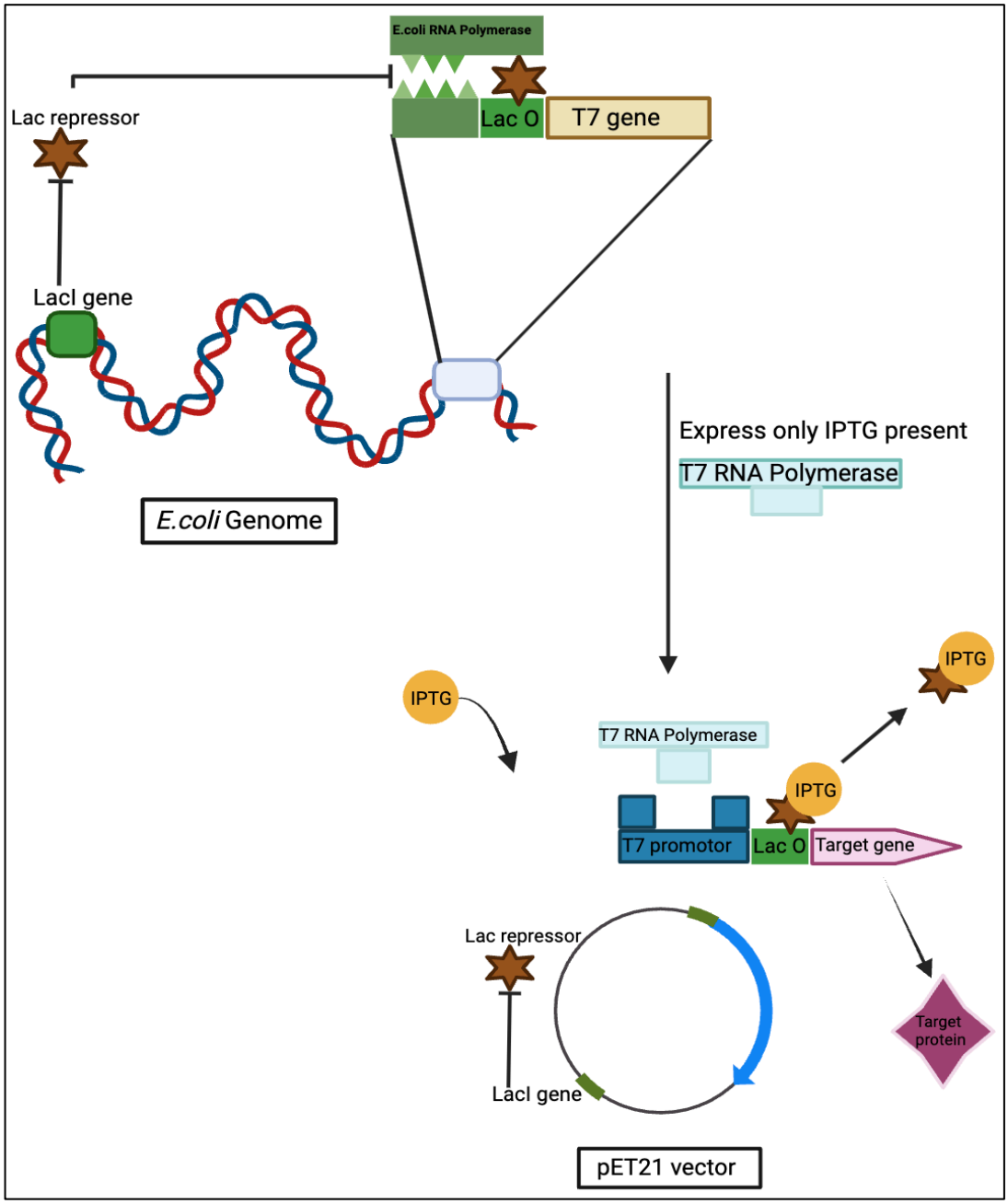


**Figure 4.1 Summary of induction mechanism with IPTG.** Lac repressors bind Lac O and gene transcription is prevented. When IPTG is present, Lac repressor is removed, and gene transcription is initiated by the T7 RNA polymerase. This then drives expression of the vaccine protein [adapted from (Novagen; pET system manual, 11$^{th}$ edition)].

A recombinant protein can be engineered so that it has additional components in the protein that facilitate its manufacture. Thus recombinant proteins often have 'tags' incorporated into their amino acid sequence to monitor protein production and for purification from soluble/insoluble protein collected from transfected bacteria (Mahmoodi *et al.*, 2019). For example, a T7-tag or His-tag (Pao *et al.*, 2022) can be detected using a specific antibody and used in immunoblot studies to confirm recombinant protein expression. It is possible to demonstrate that full-length recombinant proteins are produced by engineering different tags onto the N-terminus and C-terminus of a recombinant protein. This is one of the reasons why two tags were engineered into our recombinant protein gene sequence in this project. A his-tag is often used to isolate recombinant proteins using immobilised metal affinity chromatography (IMAC) using $Zn^{2+}$, $Ni^{2+}$, $Cu^{2+}$, $Co^{2+}$, $Fe^{3+}$ or $Ca^{2+}$ ions, which bind to histidine. The his-tag normally consists of 6 histidine residues, which are not normally present in bacterial proteins. Proteins that have a poly-histidine tag bind strongly to a metal ion attached to a matrix, other proteins are washed away, leaving the recombinant protein attached to the matrix. The most common metal ion used is $Ni^{2+}$ and it is often bound to a resin prepared using nitrilotriacetic acid (NTA) (Kimple *et al.*, 2013). The process uses bind-wash-elute steps that allow the purification of the His-tagged recombinant protein. However, tags may interfere with the structure and function of a protein (Zhao *et al.*, 2013). Therefore, it is important that any tags are removed from the final recombinant vaccine protein. It is possible to remove tags by engineering the cleavage recognition sequence of a protease between the tag and the recombinant protein itself.

In this project new plasmids constructs containing the gene sequence of gamma glutamyl cysteine synthetase from *L. donovani*, *L. major* or *L. mexicana* were cloned into the pET-21a expression plasmid (Chapter 3). The plasmids were used to transform the RosettaBlue strain of *E. coli* as previous studies had shown that this strain was the best for γGCS protein production (Doro, 2014). Previous expression studies using different plasmid constructs had shown that 18°C was the optimal temperature for protein production after IPTG induction (Doro, 2014). Nevertheless, it was deemed possible that the new constructs may have a different temperature requirement, so the effect of temperature on protein production was investigated in this study. As part of the manufacture method, bacteria need to be lysed to release their expressed protein and this is often achieved using sonication, which may cause protein degradation or damage by heat generation (Danaeifar, 2022). Because of these reasons, and the availability of new commercial reagents, in this study cell lysis was carried out using B-PER extraction reagent, which allows quick lysis without denaturating proteins. It also allows the extracted protein to be used directly in downstream processing (Wahab, 2024)

DNA sequences coding for protease recognition and cleavage sites were introduced at each end of the γGCS gene so that the His and T7 tags could be removed from the recombinant fusion protein using the corresponding proteases. Thus, enteropeptidase would be used to remove the T7 N-terminal tag and carboxypeptidase A would be used to remove the C-terminal His-tag. Previous studies using different constructs for γGCS recombinant protein had found most of the expressed recombinant protein was produced in the insoluble fraction in inclusion bodies in *E. coli*, rather than being expressed as soluble protein. The insoluble protein was treated with urea and then

dialysed to release soluble γGCS recombinant protein. Unfortunately, this process resulted in the production of truncated forms of soluble γGCS (Doro, 2014). It was hoped that the new constructs would allow the production of full-length recombinant proteins in the soluble fraction extracted from transfected bacteria.

Therefore, the aims of these studies were to:

1. Identify the optimal conditions for *Leishmania* γGCS fusion protein expression in RosettaBlue *E. coli.*
2. Determine the effect of a T7-tag on *Leishmania* γGCS fusion protein expression in RosettaBlue *E. coli.*
3. Identify the optimal conditions for *Leishmania* γGCS fusion protein purification from RosettaBlue *E. coli.*
4. Identify the optimal conditions for cleaving the His- and T7-tag from the *Leishmania* γGCS fusion protein so that pure full-length *Leishmania* γGCS was obtained.

## *4.2 Results*

### *4.2.1 Expression of recombinant* Leishmania *γGCS protein of* L. donovani, L. mexicana and L. major *using the pET-21a expression plasmid in RosettaBlue cells.*

The presence of numerous colonies on the agar containing antibiotics showed that *E. coli* Rosetta cells were successfully transformed with the expression plasmids containing the gene sequence of γGCS from *L donovani, L. major* or *L. mexicana* (Figure 4.2).

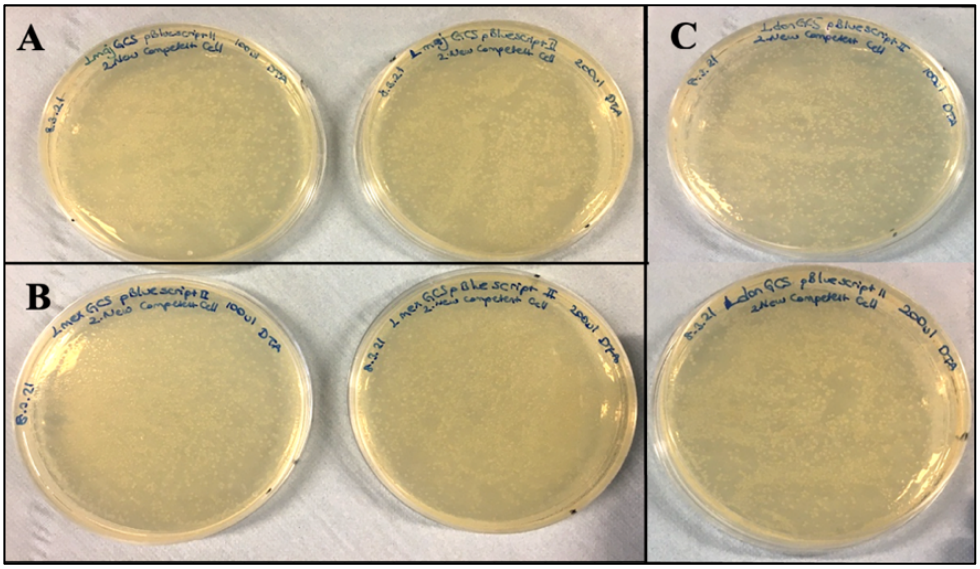


**Figure 4.2 The appearance of the culture plates for *E. coli* transfected with different plasmids. 100 µL Rosetta *E. coli* cells were transformed with 2 µL pET21a(+) plasmids that contained the *Leishmania* γGCS gene sequence**. Samples were incubated for 20 minutes on ice, then for 2 minutes 42°C, then for 2 minutes on ice. Then, 250 µL LB broth was added to the transformed bacteria and cultured for 1 hour at 37°C shaking. After transformation 125 µL of the transformed bacteria were spread onto a sterile LB-agar plate containing carbenicillin, tetracycline and chloramphenicol antibiotics. The plates were incubated overnight at 37°C, in an 'upside down' position. The plates were photographed to show the presence of colonies. (A) *L. major* γGCS plate; (B) *L. mexicana* γGCS plate; (C) *L. donovani* γGCS plate.

### *4.2.2 Expression studies showed that 18°C was the optimal incubation temperature for growing transformed bacteria after induction with IPTG.*

All the transformed bacterial colonies on a plate were used to inoculate broth containing the same antibiotics as the LB agar. This, as well as good aseptic technique, ensured that only transfected bacteria grew in cultures. Experiments were carried out to identify the effect of temperature on bacterial growth after recombinant protein expression induction, as temperature can affect bacterial growth and protein aggregation into inclusion bodies (de Groot Ventura, 2006). In these studies, only expression of *L. mexicana* γGCS fusion protein was determined. Bacteria were transfected with pET21asLmexGCS6His plasmid (Figure 4.3), grown until the culture had an absorbance of 0.6-0.8 $OD_{600}$ and IPTG was added to start protein expression. The bacterial culture was then split into four flasks and grown overnight at 18°C, 25°C, 30°C or 37°C. The bacteria present in each flask were then pelleted and the pellet was treated with B-reagent to lyse the bacterial cells to release the soluble proteins of the bacteria. The presence of different molecular weight proteins in the soluble fraction was then determined by SDS-PAGE gel electrophoresis followed by Coomassie blue staining to visualise the proteins present. The *L. mexicana* γGCS fusion protein should have a MW of 79.6 kDa. Inspection of proteins present in each sample indicated that the best protein expression occurred when bacteria were cultured at 18°C after induction (Figure 4.3). Therefore, this temperature was used in subsequent expression studies for all three recombinant *Leishmania* proteins.

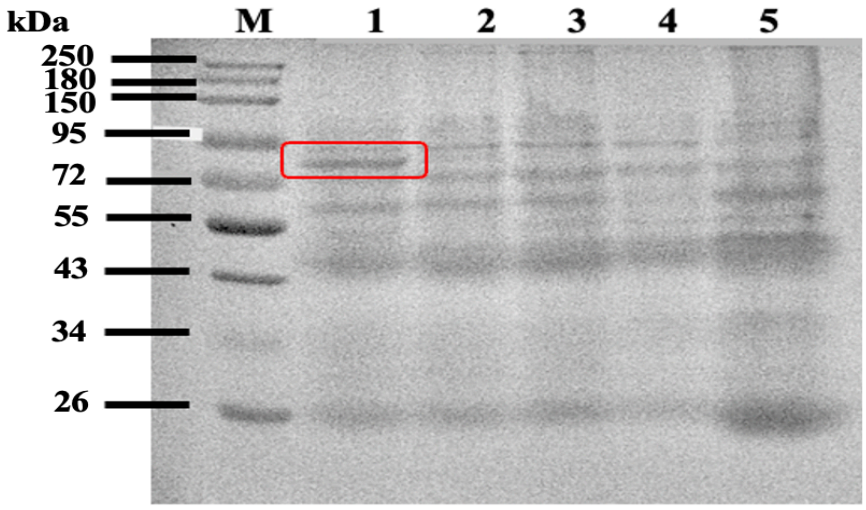


**Figure 4.3 Image of SDS-PAGE gel to show the effect of incubation temperature on protein expression in *E. coli*.** *E. coli* RosettaBlue bacteria were transformed with the pET21asLmexGCS6His plasmid and the bacteria were then plated out on LB agar plates containing selective antibiotics and the plates were incubated overnight at 37°C. The resulting colonies were washed off the plates and added to 50 mL LB broth containing the same antibiotics and incubated at 37°C until they reached an absorbance of 0.6-0.8 $OD_{600}$ and the culture was split into five equal volumes. Then expression was started by adding 0.1 mM IPTG to all four of them and the samples were incubated overnight at 18˚C, 25˚C, 30˚C, 37˚C and 37˚C (uninduced). The bacteria present in each sample were pelleted and soluble and insoluble protein collected. The soluble protein was then mixed with sample buffer, boiled and cooled, added to the appropriate lane of an SDS-PAGE gel. The proteins were separated by gel electrophoresis and visualised using Coomassie blue staining. Lanes: M, Marker; lane 1, 18˚C; lane 2, 25˚C; lane 3, 30˚C; lane 4, 37˚C; lane 5, 37˚C (uninduced).

### *4.2.3 Incorporation of a T7-tag into the γGCS gene sequence did not improve recombinant γGCS protein production.*

Bacteria were transfected with the pET21aT7LdonGCS6His plasmid, pET21aLdonGCS6His plasmid, pET21asLmexGCS6His plasmid or the pET21aT7LmexGCS6His plasmid. This experiment was carried out to determine whether incorporating a T7-tag gene sequence into the gene sequence for γGCS improved recombinant protein expression as affinity tags such as T7 have been shown to increase fusion protein expression (Kimple *et al.*, 2013). This time the culture volume was increased to 1L, using a 50 mL bacterial starter culture volume. The resulting bacteria in each 1 L volume cultures were pelleted by centrifugation and were treated with B-per reagent so that soluble proteins in the cytosol of the bacteria were released. A sample of the soluble protein was then run on an SDS-PAGE gel to separate individual proteins and the gel was used to transfer the separated proteins on to a nitrocellulose membrane. The nitrocellulose membrane was probed with an anti-6His antibody to show which proteins had a histidine tag attached. The results showed that a band equivalent to the full-length γGCS recombinant protein was present for all four bacterial lysates i.e. *Ld*γGCS6His, T7*Ld*γGCS6His, *Lm*γGCS6His and T7*Lm*γGCS6His (Figure 4.4) and this protein had a histidine tag. Unfortunately, all four bacterial lysates also had lower molecular weight proteins that contained a histidine tag, indicating degradation of the recombinant protein. Therefore, the recombinant protein was broken down during expression and/or during processing. The amount of protein present in the soluble bacterial extract was determined so that the potential yield/litre bacterial broth could be calculated (Table 4.1). Similar amounts of recombinant protein were produced by bacteria transformed with plasmids with or

without the T7-tag for both *Ld*γGCS and *Lm*γGCS. Therefore, the presence of a T7 tag did not appear to improve the amount of fusion protein expressed. The results indicated that further purification methods would be required to separate the full-length protein from the lower molecular weight contaminants

**Table 4.1 The total amount of soluble protein (mg) produced from RosettaBlue *E. coli* bacteria transformed with pET21aT7LdonGCS6His, pET21asLdonGCS6His, pET21asLmexGCS6His or pET21aT7LmexGCS6His.**

| Plasmid used to transfect bacteria | Culture volume | Protein concentration in Rosetta (DE3) cells soluble extract |
|---|---|---|
| pET21asLdonGCS6His | 1 L | 1,784 mg/mL |
| pET21aT7LdonGCS6His | 1 L | 1,375 mg/mL |
| pET21asLmexGCS6His | 1 L | 1,631 mg/mL |
| pET21aT7LmexGCS6His | 1 L | 1,789 mg/mL |

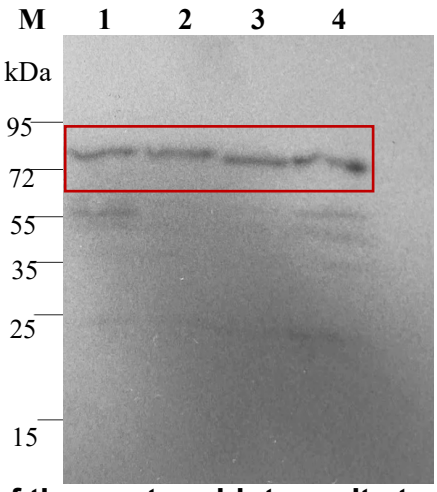


**Figure 4.4 Image of the western blot results to confirm the production of recombinant LdγGCS6His, T7LdγGCS6His, LmγGCS6His and T7LmγGCS6His by transfected *E. coli*.** RosettaBlue *E. coli* were transfected with pET21aLdonGCS6His, pET21aT7LdonGCS6His, pET21asLmexGCS6His, pET21aT7LmexGCS6His. The colonies produced were used to inoculate 50 mL starter cultures containing selective antibiotics. The resulting bacteria from the overnight cultures were added to 1 L LB broth, containing the same concentration of antibiotics, and cultured as before. Once the culture reached the required absorbance 0.1 mM IPTG was added and cultures were incubated at 18°C overnight. The bacteria present were then pelleted, and soluble protein extracted. The proteins present in a sample of the soluble protein were separated by SDS-PAGE gel electrophoresis and transferred onto a nitrocellulose membrane. The presence of 6His-tagged protein was determined by probing with an anti-His-tag antibody and using chemiluminnescence. Images of the membrane were captured on to X ray film, which was photographed to show the results. M, Marker; lane 1, LdγGCS6His; lane 2, T7LdγGCS6His; lane 3, LmγGCS6His; lane 4, T7Lmγ-GCS6His. Full-length protein bands are clearly seen.

The experiment was repeated and this time an uninduced control sample was added to show that production of the fusion protein did not occur in untransformed bacteria and that 18°C was a suitable temperature for production of fusion proteins from all three species. RosettaBlue *E. coli* were transfected with pET21a(+) vector alone, pET21aT7LdonGCS6His, pET21aT7LmajGCS6His or pET21aT7LmexGCS6His (Figure 4.5). The colonies produced were used to inoculate a 50 mL starter culture containing antibiotics at 37°C. The resulting bacteria from the overnight cultures were added to 1 L LB broth, containing the same antibiotics. Colonies grown on agar plates were used for LB medium cultures containing all three antibiotics. Recombinant protein production was induced in transfected bacteria by adding 0.1 mM IPTG when the desired OD value was reached, and cultures were incubated overnight at 18°C. As expected, the negative control bacterial sample did not produce a protein that had a 6His-tag as none of the proteins present in this sample were recognised by the anti-His antibody in Western blotting studies. Uninduced samples also showed that recombinant protein expression did not occur if IPTG was not added to the culture.

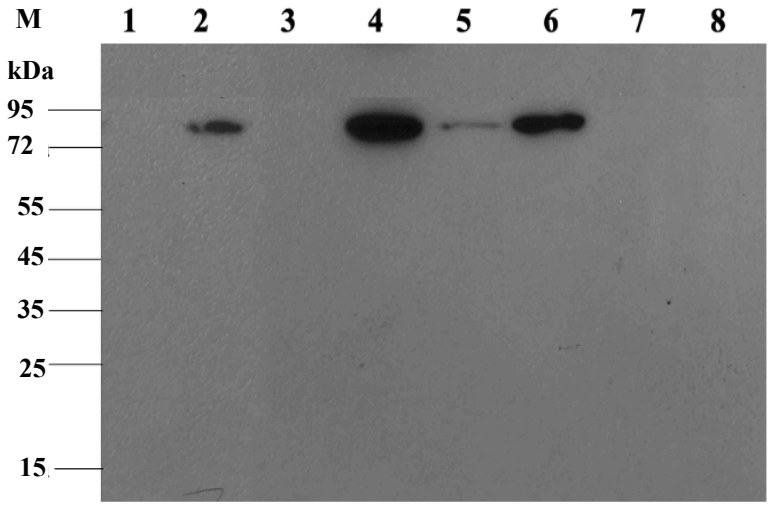


**Figure 4.5 Western blot for T7LdonGCS6His, T7LmajGCS6His T7LmexGCS6His induced cultures and uninduced cultures, and RosettaBlue *E. coli* cells without vector, pET21a(+) vector alone (control/induced).** Transfected bacteria were either induced with IPTG when they reached the required OD value or left uninduced. The proteins present in a soluble extract of each culture were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The presence of his-tagged protein was determined by incubating the membrane with a mouse anti-His-tag antibody, followed by incubation with an anti-mouse HRP conjugate. The membrane was then incubated with a substrate that produced a chemiluminescent signal in the presence of bound HRP. Images of the membrane were captured on to X-ray film. M, Marker; lane 1, T7LdγGCS6His total protein uninduced; lane 2, T7LdγGCS6His total protein induced; lane 3, T7LmjγGCS6His total protein uninduced; lane 4, T7LmjGCS6His total protein induced; lane 5, T7LmγGCS6His total protein uniduced; lane 6, T7LmγGCS6His total protein induced; lane 7, RosettaBlue *E. coli*; lane 8, pET21a(+).

### *4.2.4 Effect of using HisPur™ Ni-NTA Spin Purification and T7•Tag® Affinity Purification on production of recombinant γGCS from* **L. donovani, L. mexicana** *or* **L. major**

The above studies had indicated that the γGCS fusion protein had been produced. Therefore, the next step was to purify the fusion protein using the two affinity tags as this would allow pure full-length γGCS fusion protein to be isolated from the extracted bacterial proteins. Expressions were completed as before using 18°C as the culture temperature and 0.1 mM IPTG to induce recombinant protein expression. The bacterial culture was then pelleted and B-per reagent added to lyse the bacteria. The cell lysate was centrifuged and the resulting soluble bacterial extract was used in purification studies. T7LdγGCS6His, T7LmjγGCS6His and T7LmγGCS6His protein was purified using a HisPur™ Ni-NTA spin purification kit. Purified protein samples were collected during processing and used in gel electrophoresis (Figure 4.6) and Western blot studies (Figure 4.7) so that the efficiency of the purification process could be monitored.

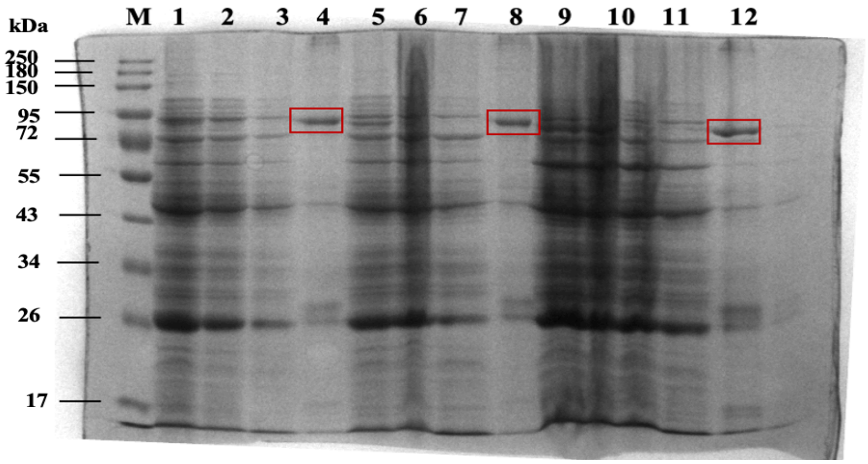


**Figure 4.6 SDS-PAGE gel to show the effectiveness of the HisPur™ Ni-NTA spin purification process on recombinant protein isolation**. *E. coli* was transformed with the pET21aT7LdonγGCS6His, pET21aT7LmajγGCS6His or pET21aT7LmexγGCS6His and incubated at 37°C until cultures reached OD values of between 0.6-0.8. Recombinant protein expression was then induced by adding 0.1 mM IPTG, and the bacterial cultures were incubated overnight at 18°C. The bacteria from the resulting cultures were pelleted by centrifugation and the soluble protein extracted from bacterial cells using B-per reagent. The proteins present in the soluble protein extract samples were separated by SDS-PAGE gel electrophoresis. Lane M, marker; lane 1, T7LdγGCS6His total protein; lane 2, T7LdγGCS6His flow through; lane 3, T7LdγGCS6His wash with 25 mM imidazole wash buffer; lane 4, T7LdγGCS6His elution with 250 mM imidazole buffer; lane 5, T7LmjγGCS6His total protein; lane 6, T7Lmjγ-GCS6His flow through; lane 7, T7LmjγGCS6His wash with 25 mM imidazole wash buffer; lane 8, T7LmjγGCS6His elution with 250 mM elution buffer; lane 9, T7LmγGCS6His total protein; lane 10, T7LmγGCS6His flow through; lane 11, T7LmγGCS6His wash with 25 mM imidazole wash buffer; lane 12, T7LmγGCS6His elution with 250 mM imidazole buffer.

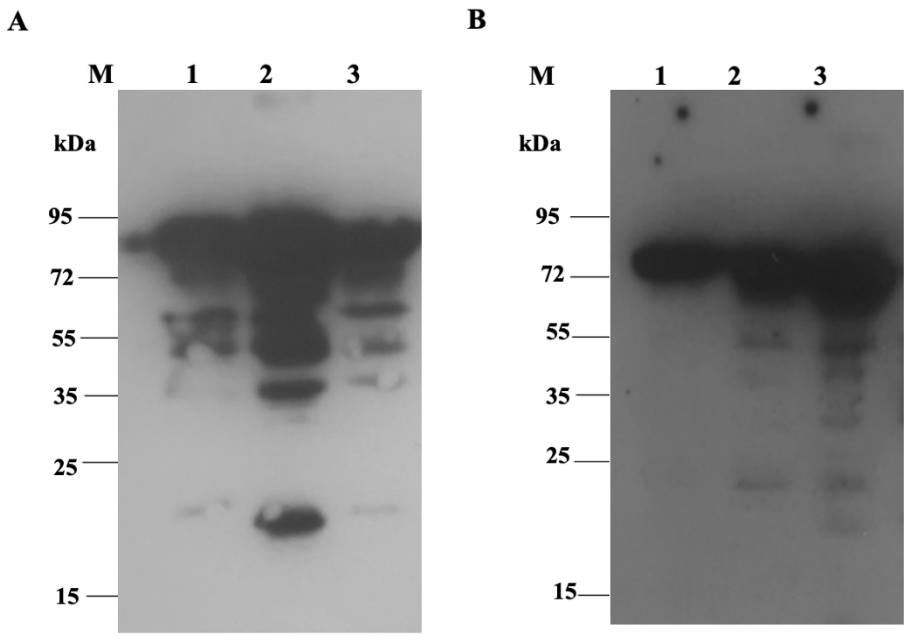


**Figure 4.7 Western blot to show the effectiveness of the HisPur™ Ni-NTA spin purification process in recombinant protein isolation.** T7LdγGCS6His, T7LmjγGCS6His and T7LmγGCS6His recombinant protein were purified from the soluble bacterial extract using a HisPur™ Ni-NTA spin column. The proteins present in eluted samples were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was then used in Western blot studies to identify the presence of His-tagged proteins. M, marker; lane 1, eluted recombinant T7LdγGCS6His from HisPur™ Ni-NTA spin purification column; lane 2, eluted recombinant T7LmjγGCS6His from HisPur™ Ni-NTA spin purification column; lane 3, eluted recombinant T7LmγGCS6His from HisPur™ Ni-NTA spin purification column. (A) detection by His-tag antibody, (B) detection by T7-tag antibody.

Western blot results showed that the isolated protein still had undesired lower molecular weight proteins that contained a 6His-tag (Figure 4.7A) and a T7-tag (Figure 4.7B) in the purified sample. A two-stage isolation process was then used to isolate pure full-length recombinant γGCS fusion protein as this should remove the contaminating lower molecular weight proteins. Therefore, soluble protein purified using a HisPur™ Ni-NTA spin purification kit was used with a T7•Tag® affinity purification kit. The eluted sample from the T7•Tag® affinity purification column had very little protein present as determined by protein assay and SDS-PAGE (data not shown). Therefore, the T7•Tag® affinity kit was used as the first purification method and the HisPur™ Ni-NTA spin purification kit was used second, as this may improve protein isolation by loading more protein on the T7•Tag® affinity kit. However, only a small amount of protein was detected in the eluate and very little protein of the correct molecular weight for the fusion protein was present (Figure 4.8). However, there were fewer lower molecular weight proteins in the eluted sample. It was decided to abandon studies using the T7•Tag® affinity kit and concentrate on comparing different purification methods using the 6His-tag purification kit.

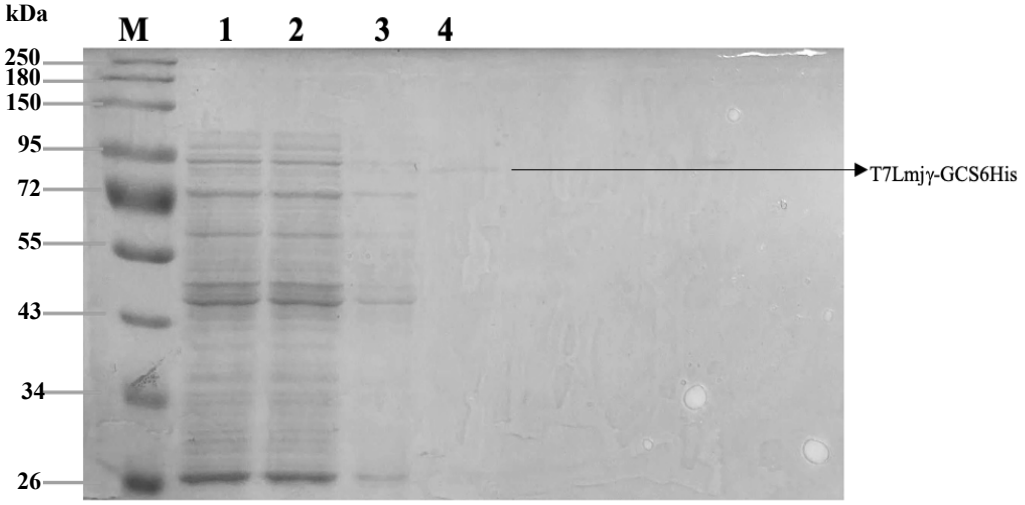


**Figure 4.8 SDS-PAGE gel showing protein present in T7Lmjγ-GCS6His sample during protein purification using the T7•Tag® affinity purification method.** *E. coli* was transformed with pET21aT7LmajγGCS6His and incubated at 37°C until an OD value of between 0.6-0.8 was reached. Then the culture was induced by adding 0.1 mM IPTG and incubated overnight at 18°C. The bacteria present in the culture were pelleted, incubated overnight with B-per reagent, and the soluble extract isolated. The recombinant protein present in the soluble extract was purified using the T7•Tag® affinity purification kit. Samples collected during recombinant protein isolation were analysed using SDS-PAGE. M, marker; lane 1, T7LmjγGCS6His total protein sample; lane 2, T7LmjγGCS6His flow through sample; lane 3, T7LmjγGCS6His wash sample; lane 4, eluted protein of T7LmjγGCS6His sample.

### *4.2.5 Comparison of HisPur™ Ni-NTA Spin column and HisTrap HP column using the ÄKTA protein purification system*

Studies were carried out to determine whether the HisPur™ Ni-NTA Spin columns were better than the ÄKTA protein purification system at isolating γGCS fusion protein from the soluble protein extracted from transfected bacteria. pET21aT7LmajGCS6His was used to transform RosettaBlue *E. coli* cells. All the colonies from an agar plate were used to produce a starter culture, which was used to inoculate two 1 L LB broth cultures. Soluble protein was extracted from each 1 L broth and used in two different purification methods i.e. HisPur™ Ni-NTA spin column and an ÄKTA protein purification system.

Purification using a HisPur™ Ni-NTA spin column was performed according to the manufacturer's instructions. 75 mM imidazole wash buffer was used to remove unwanted proteins and 250 mM imidazole was used to elute bound protein. A HisTrap HP column was used with the ÄKTA protein purification system to purify recombinant protein. The machine was cleaned with distilled water. A 1 mL HisTrap HP column attached. The column was washed with 3 mL distilled water and then buffer (0.05 M Tris, 0.2 M NaCl pH 7.4 containing 40 mM imidazole) using 0.3 mL/min flow rate. The bacterial total soluble protein from a 1 L broth was loaded onto the column, and then 6His-tagged protein was eluted with elution buffer (50 mM Tris, 0.2 M sodium chloride pH 7.4 containing 500 mM imidazole). The recombinant protein present in the soluble protein from the other 1 L broth was isolated as before using a HisPur™ Ni-NTA spin column. Figure 4.9 shows the 6His-tagged protein present in the eluted protein from each method. The HisPur™ Ni-NTA spin column method produced a higher concentration of the 6His-tagged protein, but more undesired protein bands

were obtained. Because of this reason, ÄKTA protein purification system was abandoned and it was decided to improve the HisPur™ Ni-NTA spin purification method by optimising the washing steps before elution by altering the imidazole concentration used.

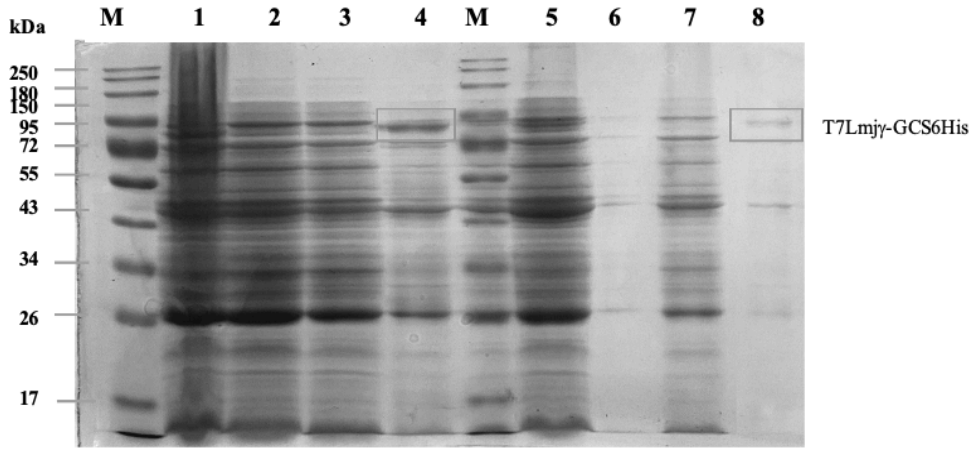


**Figure 4.9 SDS-PAGE gel image comparing the protein profile for T7LmjγGCS6His recombinant protein purified using a HisPur™ Ni-NTA Spin purification kit and ÄKTA protein purification system**. *E. coli* was transformed with pET21aT7LmajγGCS6His and incubated at 37°C until the OD value reached between 0.6-0.8. And then they were induced with 0.1 mM IPTG, incubated overnight at 18°C. Cell pellets were collected by centrifugation and incubated overnight with B-reagent. After incubation, homogenates were centrifuged to collect total protein lysate. T7LmjγGCS6His recombinant protein was purified using a HisPur™ Ni-NTA spin column as directed by the manufacturer. The column was washed with 75 mM imidazole wash buffer to remove unbound protein, and the recombinant protein eluted with 200 mM imidazole elution buffer. T7LmjγGCS6His recombinant protein was purified with a HisTrap HP His tagged protein purification column (1 mL) using an ÄKTA system. M, marker; lane 1, T7LmjγGCS6His total protein; lane 2, Flow through sample; lane 3, 25 mM imidazole wash buffer; lane 4, 250 mM imidazole elution buffer obtained from HisPur™ Ni-NTA spin column. M, marker; lane 5, T7LmjγGCS6His total protein sample; lane 6, flow through sample; lane 7, wash sample; lane 8, elution sample obtained from HisTrap HP His tagged protein column.

### *4.2.6 Optimisation of HisPur™ Ni-NTA Spin column purification*

It was possible that altering the imidazole concentration in the wash buffer could eliminate lower molecular weight contamination of the eluted protein (Bhat *et al.*, 2018). Initially 25 mM, 75 mM, and 150 mM imidazole were sequentially used in the wash buffer and the molecular weight of the proteins eluted from the column after each wash step and after final elution using 250 mM and 500 mM imidazole was analysed by gel electrophoresis (Figure 4.10A). The results showed that using washing steps with wash buffer containing 25 mM and 75 mM imidazole did not eliminate the presence of undesired bands without eluting the full-length γGCS fusion protein. Subsequent studies using 35 mM, 40 mM, 45 mM, 50 mM, 55 mM, 60 mM, 65 mM, 70 mM imidazole wash buffer concentrations were carried out to define an optimal imidazole wash concentration. Using imidazole at 45 mM was the best concentration to remove unwanted proteins without eluting the *L. major* γGCS fusion protein (Figure 4.10B). A higher concentration of full-length fusion protein was obtained with HisPur™ Ni-NTA Spin column after optimisation. Therefore, a larger batch of soluble protein was processed using a HisPur™ Ni-NTA Spin column washed three times with wash buffer containing 45 mM imidazole (Figure 4.11, lanes 3, 4 and 5) and elution buffer containing 250 mM imidazole, but this time the column was incubated with the elution buffer for 30 minutes with rotation at 4°C. A sample of the eluted protein was then analysed using SDS-PAGE gel electrophoresis followed by Western blot analysis to determine the effect of these changes on the amount of *L. major* γGCS fusion protein isolated. Unfortunately, both full-length and truncated 6His-tagged fusion protein was present in the eluted sample (Figure 4.11).

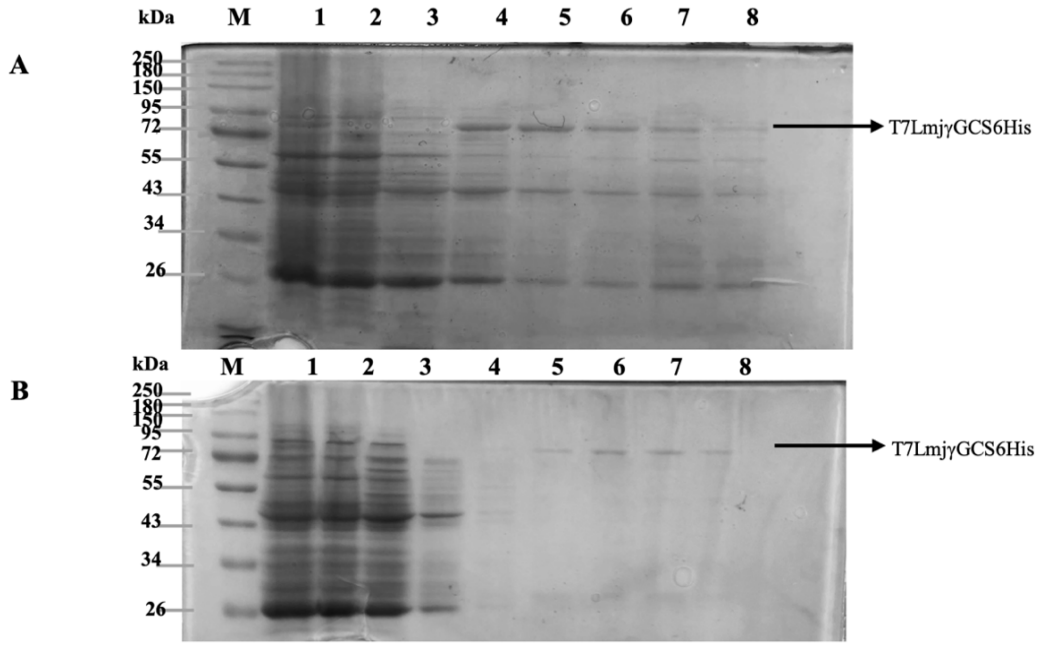


**Figure 4.10 The effect of optimisation studies on the recovery of T7LmjγGCS6His uisng a HisPur™ Ni-NTA spin column kit.** *E. coli* was transfected with pET21aT7*Lmajor*γGCS6His and soluble protein extracted from induced bacterial cultures. T7LmjγGCS6His recombinant protein purification was performed with HisPur™ Ni-NTA spin column with additional wash and elution steps. The proteins present in different samples collected during purification were analysed using SDS-PAGE. (A) M, marker; lane 1, T7LmjγGCS6His total protein lane 2, flow through; lane 3, 25 mM wash buffer; lane 4, 75 mM wash buffer; lane 5, 150 mM wash buffer; lane 6, 250 mM elution buffer; lane 7, 500 mM elution buffer. (B) M, marker; lane 1, T7LmjγGCS6His total protein; lane 2, flow through; lane 3, wash buffer; lane 4, 40 mM wash buffer; lane 5, 45 mM wash buffer; lane 6, 50 mM wash buffer; lane 7, 55mM wash buffer; lane 8, 60 mM wash buffer; lane 9, 65 mM wash buffer; lane 10, 70 mM wash buffer; lane 11, 250 mM elution buffer; lane 12, 500 mM elution buffer.

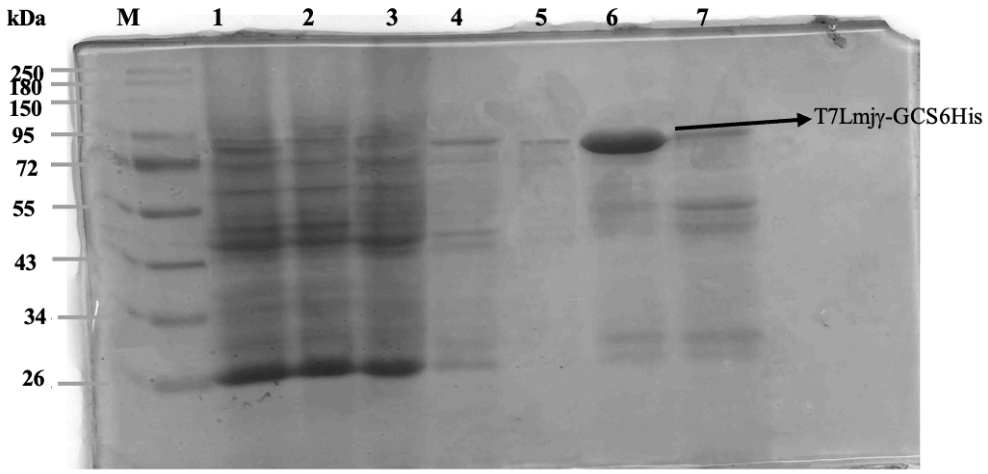


**Figure 4.11 The effect of changing the washing and elution method on the recovery of T7LmjγGCS6His using a HisPur™ Ni-NTA spin column kit.** *E. coli* was transfected with the pET21aT7*Lmajor* γGCS6His and soluble protein extracted from induced cultures. T7LmjγGCS6His recombinant protein was purified using HisPur™ Ni-NTA spin column, with additional wash and elution steps to remove lower molecular weight contaminants. The proteins present in different samples collected during purification were analysed using SDS-PAGE (A) M, marker; lane 1, T7LmjγGCS6His total protein; lane 2, flow through; lane 3, 45 mM wash buffer; lane 4, 45 mM wash buffer; lane 5, 45 mM wash buffer; lane 6, 250 mM elution buffer; lane 7, 250 mM elution buffer.

### *4.2.7 Tag Removal by Enzymatic Cleavage*

Purification of pure recombinant γGCS protein from the expressed fusion protein requires the 6His- and T7-tag to be cleaved from the protein. This can be achieved by using carboxypeptidase A to remove the 6His-tag and enteropeptidase to remove the T7-tag. However, optimisation of these cleavage processes is difficult and could affect target protein structure and function (Nguyen *et al.*, 2019). Cleavage of the 6His-tag was attempted using different enzyme concentrations and treatment times using a 25°C incubation temperature. SDS-PAGE gel and Western blot analyses of cleaved protein samples showed that the 6His-tag was not completely removed from the fusion protein (Figure 4.12 and Figure 4.13). Unfortunately, there was insufficient time for further studies.

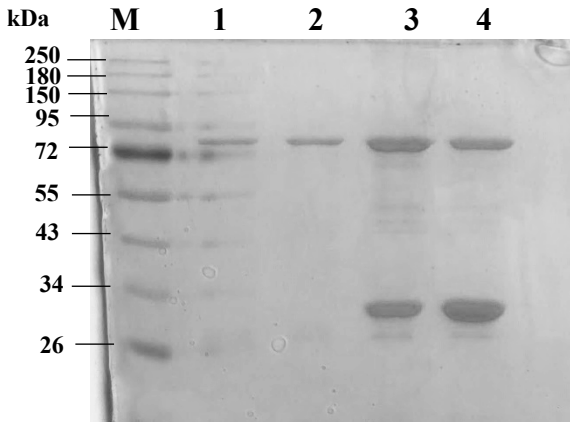


**Figure 4.12 Removal of the His-tag from the *L. major* γGCS fusion protein with Carboxypeptidase A.** Different incubation conditions were used to remove the 6His-tag from *L. major* γGCS fusion protein purifed using a HisPur™ Ni-NTA spin column. The proteins present in samples were separated by SDS-PAGE. M, marker; lane 1, *L. major* γGCS fusion protein; lane 2, *L. major* γGCS fusion protein without enyzme treatment for 30 minutes at 25˚C; lane 3, *L. major* γGCS fusion protein 0.5 µL carboxypeptidase A (>0.375 units/µL) 30 minutes 25˚C treatment sample; lane 4, T7LmjγGCS6His fusion protein 1.5 µL carboxypeptidase A 30 minutes 25˚C treatment sample.

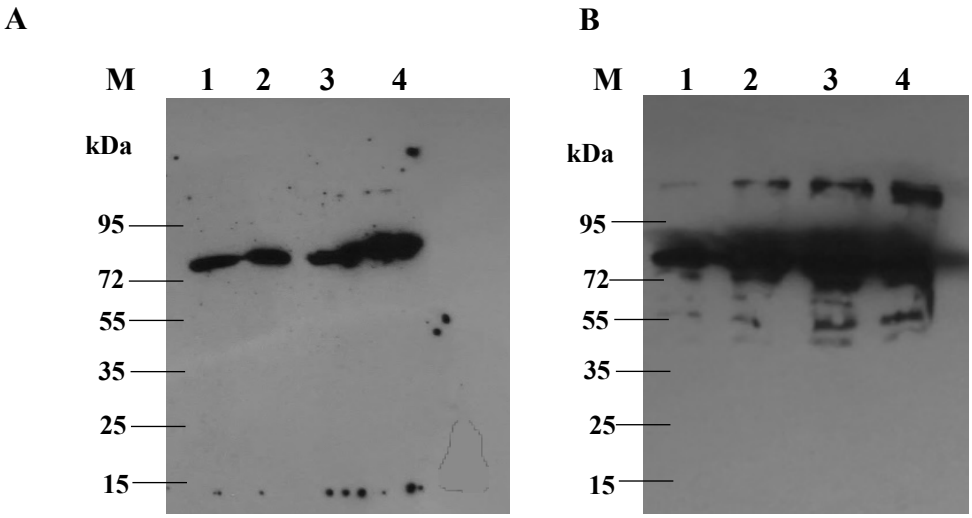


**Figure 4.13 Different carboxypeptidase A treatments to remove of the His-tag from the *L. major* γGCS fusion protein.** Different incubation conditions were used to remove the 6His-tag from *L. major* γGCS fusion protein purifed using a HisPur™ Ni-NTA spin column. The proteins present in samples were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was then used in Western blot studies to identify the presence of His-tagged and T7-tagged proteins. (A) T7 antibody treatment. (B) His-tag antibody treatment. M, marker; lane 1, *L. major* γGCS fusion protein; lane 2, *L. major* γGCS fusion protein for 30 minutes at 25˚C without enzyme treatment; lane 3, *L. major* γGCS fusion protein 0.5 µL carboxypeptidase A (>0.375 units/µL) for 30 minutes at 25˚C; lane 4, *L. major* γGCS fusion protein 1.5 µL carboxypeptidase A for 30 minutes at 25˚C.

## *4.3 Discussion*

The results of these studies showed that the optimal temperature for γGCS fusion protein expression after induction was 18˚C for the new γGCS constructs. This was the temperature used in previous studies for the previous γGCS constructs (Henriquez *et al.*, 2010, Campbell *et al.*, 2012). The same temperature has also been used in other studies expressing *Leishmania* proteins. For example, *L. donovani* hypothetical protein (LdHyP) was expressed using a pET28a expression vector system and *E. coli* with an expression temperature of 18˚C. The expression was confirmed using Western blotting with an anti-His primary antibody (Yadav *et al.*, 2021). And the same temperature was also used for a *Leishmania* disulphide isomerase (PDI) protein expressed using a pET15b vector and *E. coli* BL21 (DE3) cells. A yield of 600 mg/L recombinant protein was obtained and its identity confirmed via SDS-PAGE and Western blot analysis (Ali *et al.*, 2016).

In this study, the fusion protein was expressed in the soluble fraction and truncated and full-length γGCS fusion proteins were present in eluted samples. Formation of the truncated forms could be caused by proteolysis during purification, incorrect translation, or because high levels of recombinant protein expression occurred (Jennings *et al.*, 2016; Singh *et al.*, 2015). In this study, while protein was obtained between 0.3 and 0.5 mg/L of bacterial culture after HisPur™ Ni-NTA spin column purification, further purification using a T7•Tag® affinity kit to eliminate truncated forms of protein decreased the yield to between 0.3 and 2.4 µg/L. This seems a very low amount of protein but variable protein yields are reported for other recombinantly expressed proteins from *Leishmania*. For example, *L. chagasi* rLci2B and rLci1A heat shock proteins gave a total protein yield of 10.5 mg and 18 mg with RSETB and pBK-

CMV vectors, respectively from 1 L transfected *E. coli* strain *BL21(DE*3) culture (De Souza *et al.*, 2012). The *L. major* leucyl-aminopeptidase gene codes for a 62 kDa protein, was codon-optimised and cloned into the pET15b vector for expression in *E. coli* Lemo21(DE3). Recombinant leucyl-aminopeptidase was purified in a single step by immobilized metal ion affinity chromatography purification gave a yield of 2.5 mg/L (Aguado *et al.*, 2021).

The IPTG concentration used for induction of protein expression can influence inclusion body formation. Therefore, manipulating the IPTG levels can avoid or decrease inclusion body production (Rizkia *et al.*, 2015). We used an IPTG concentration of 0.1 mM in this study, as this was the optimal concentration identified in previous studies (Doro, 2014), although different plasmid constructs were used. This concentration has also been utilised in various *Leishmania* protein expression studies. For example, *Leishmania infantum* GDP-mannose pyrophosphorylase (GDP-MP) protein reached 12.8 mg/mL after purification with Size Exclusion Chromatography and was expressed in *E. coli* BL21(DE3) using 0.1 mM IPTG (Mao *et al.*, 2022). Another example is recombinant mannosyl transferase/phosphorylase (MTPs) protein. It was induced using the same IPTG concentration, expressed in *E. coli* BL21(DE3) and purified between 12 and 60 mg/mL (Sernee *et al.*, 2019). The presence of truncated contaminants is not a major problem as a purification step should allow isolation of the full-length protein. However, an earlier study suggested that γGCS is an unstable protein and easily broken down to smaller subunits (Agnihotri *et al*., 2016). One way to eliminate lower MW contaminants is to use membrane filtration (Tripathi and Shrivastava, 2019). Unfortunately, this method could not be assessed in this study due to time constraints.

Fusion tags can help protein folding and improve functional activity (Ki and Pack, 2020). In this study we hoped that the T7-tag would also help to improve recombinant protein production, but its presence had no obvious effect as similar amounts of protein were produced using *E. coli* culture for both *Ld*γGCS6His and *Lm*γGCS6His. Previous studies have shown that using different tag systems e.g. maltose-binding protein (MBP) and glutathione S-transferase (GST) can help increase target protein expression (Ki and Pack, 2020). Therefore, in future studies testing these tags could be investigated. Another thing that can be tested is changing the broth used to culture *E. coli*. Although LB is often used to grow bacteria, switching to richer broths may increase bacterial growth and using autoinduction media may give higher bacterial densities (Rosano *et al.*, 2019). *E. coli* expression systems allow to express protein in high yield. Expression is controlled by various factors such as, size of the heterologous protein, codon bias or toxicity (Patil *et al.,* 2022). In our study, performing codon optimisation of part of the gene might have affected protein expression. Codon optimisation of the full-length gene could potentially enhance the protein expression efficiency.

To improve recombinant protein expression in *E. coli* a different promotor, different strains of *E. coli* or different culture media could be used (Pouresmaeil and Azizi-Dargahlou, 2023). Alternatively, a different expression system could be tested. Yeast may be a good choice because they grow more rapidly than *E. coli* (Pollet *et al.*, 2021). Recombinant *L. donovani* nucleoside hydrolase protein could be expressed in *Pichia pastoris* in a stable, soluble form (Hudspeth *et al.*, 2016). Another factor that can affect protein expression is the molecular size of the recombinant protein. Studies have shown that large molecular weight (> 60 kDa) soluble proteins are produced in lower

concentrations in *E. coli* (Francis Page, 2010). In this project, γGCS fusion proteins are above 70 kDa, making them large recombinant proteins. *E. coli* can also have a high background of histidine-rich proteins, and this may result in relatively low recovery for recombinant proteins (Gomari *et al.*, 2020). In this study this was not the case as untransformed bacteria did not produce detectable amounts of 6His-tagged protein.

The dual purification system used in this study was unsuccessful and this was mainly due to purification via the T7-tag. This system has been used successfully for other proteins, for example, recombinant murine IL-33 and IL-1β were purified using T7 and His affinity purification (Hayakawa *et al.*, 2007). This study was the first time a T7-tag has been used for a γGCS fusion protein. Using a T7•Tag® affinity method can be problematic as a purification method as it can be affected by low pH, limited matrix reusability and protein folding can affect tag accessibility (Kimple *et al.*, 2013). Previous studies using *Rhizobium leguminosarum* TonB cloned into pET17b vector, and engineered with a T7-tag, showed that folding at the C-terminus resulted in poor binding to the T7 affinity column (Baxter, 2017). Therefore, it is possible that a similar problem occurred for our recombinant protein and that the T7-tag is not suitable for the *Leishmania* γGCS fusion proteins.

We tried two purification methods in this study, spin column purification and HisTrap HP column purification using the ÄKTA system. Time constraints did not allow optimisation using the ÄKTA system, but our initial studies indicated that more fusion protein was isolated using the HisPur™ Ni-NTA spin purification system. Poor resolution using the ÄKTA system may occur if a high sample volume is applied to

the system or if the sample used is too viscous (Ó'Fágáin *et al.*, 2017; Stanton, 2004). The HisPur™ Ni-NTA spin purification was optimised by testing different wash buffer concentrations, washing times and elution conditions. Similar to other studies (Spriestersbach *et al.*, 2015), the results of this study showed that increasing the imidazole concentration in the wash buffer helped remove contaminants.

In conclusion, constructs with two tags and codon-optimised gene region were transfected into *E. coli* RosettaBlue strain to express the protein at 18°C. It was possible to isolate the full and partial length recombinant γGCS from *L. donovani*, *L. major* and *L. mexicana* from the soluble protein extracted. However, using a dual-tag purification did not boost γGCS fusion protein purification. The study also indicated that a T7-tag is not suitable for the use with *Leishmania* γGCS fusion proteins. Obtaining a high amount of purified γGCS fusion protein without a T7 and His tag failed. Therefore, it was not possible to use the recombinant protein in the planned vaccine studies.

# Chapter 5 Vaccination with transgenic *L. tarentolae* parasites expressing γGCS protected BALB/c mice against *L. donovani* infection.

## *5.1 Introduction*

Live vaccines have helped prevent epidemic infectious diseases in the world, for example, rubella, yellow fever, and tuberculosis (Kamei, 2023). Live vaccines consist of an infective agent that expresses the antigens or antigens known to be important in inducing protective immunity against a pathogenic species, but it is modified so that it cannot induce the harmful effects associated with the specific pathogen (Pollard and Bijker, 2021). Vaccines candidates are likely to be more successful if they induce protective cellular and humoral responses (Manohar *et al.*, 2022). They have several advantages compared to killed vaccines e.g. they induce an immune response at the site of the normal infection, favour antigen presentation, they induce the same type of immunological responses as a pathogenic species, and they produce long-term immunity (Clem, 2011). However, live vaccines do have some limitations, for example, the immune responses generated are not as robust as the pathogenic organism and they require expensive transportation and storage costs as they often have poor thermal stability (Li *et al.*, 2023). Additionally, pathogen strains may mutate over time so that a new vaccine must be developed with the mutated protective antigen or antigens for disease control (Pandey *et al.*, 2020). Live vaccines have already been used in *Leishmania* studies. Centrin1 is an essential protein in the development of amastigotes and deletion of centrin1 in *L. donovani* amastigotes blocked parasite growth, thus stopping the parasite from developing within the host but not from infecting the host at the promastigote stage (Moreira *et al.*, 2023). A centrin gene deleted *L. major* live vaccine induced the same level of immunity as the pathogenic parasite but did not cause disease in an animal model. This live vaccine has reached the final stage of preclinical development (Kaye and Matlashewski *and et al.*, 2023).

Another approach is to use a related non-pathogenic species that expresses an antigen or antigens from the related pathogenic species used that is known to induce protective immunity i.e. using a transgenic live vaccine. For example, *L. tarentolae* promastigotes ($2 \times 10^7$/mouse) expressing lipophosphoglycan (LPG) from *L. infantum*, a virulence factor in *L. infantum* were used to vaccinate BALB/c mice against a homologous challenge. Vaccinated animals had enhanced Th-1 responses based on splenocytes. Vaccinated animals produced higher levels of IFN-γ and lower levels of IL-10 compared to infected controls. The vaccine reduced parasite burden significantly compared to control infected mice (Pirdel and Farajnia, 2017).

In another study *L. tarentolae* expressing *L. donovani* specific A2 protein were used as a live vaccine against *L. infantum*. Mice were immunised with either $2 \times 10^8$ stationary-phase *L. tarentolae* promastigotes given by intravenous injection or $5 \times 10^6$ stationary-phase promastigotes given by intraperitoneal injection. Vaccinated animals and controls were then infected with $10^7$ *L. infantum* promastigotes and parasite burdens and immune responses monitored. Antigen stimulated splenocytes from mice given the vaccine by the intraperitoneal route produced significantly more IFN-γ ($p < 0.001$) and lower amounts of IL-5 ($p < 0.02$ ) compared to controls, and vaccinated animals produced significantly lower amounts of specific IgG1 compared to controls. These mice also had significantly lower spleen and liver parasite burdens compared to controls, indicating that protection was associated with enhanced Th1 responses and lower Th2 responses (Mizbani *et al.*, 2009).

Previous studies using murine models have shown that γGCS as a recombinant protein vaccine or DNA vaccine from pathogenic *Leishmania* can protect against infection but

neither induced sterile immunity (Carter *et al.*, 2007; Henriquez *et al.*, 2010). This may be related to the inability to induce a strong protective immune response. Using *L. tarentolae* expressing γGCS as a live vaccine may be one way to enhance the immune response induced by γGCS, and using an adjuvant may boost immune responses even further (Zhao *et al.*, 2023). There are numerous types of adjuvants for vaccines and these include saponin, cytosine phosphoguanine (CpG) and cytokines (Ratnapriya *et al.*, 2019). Adjuvants have been used in *Leishmania* studies, for example, CpG was used to boost immune responses against *L. tarentolae* two proteins from *L. major* i.e. homolog receptor activated C kinase (LACK) and kinetoplastid membrane protein-11 (KMP11). Joint treatment with CpG and transfected parasites was significantly more effective at reducing *L. major* parasite burdens compared to vaccination with transfected parasites alone ($p < 0.05$). This protection was associated with a higher IgG2a/IgG1 specific antibody compared to the vaccine alone group ($p < 0.05$). Antigen stimulated spleen cells from the joint group produced significantly higher amounts of IFN-γ compared to other groups ($p < 0.05$), indicated that protection was associated with an enhanced Th1 response (Salari *et al.*, 2020). The polyhedrin delivery system (PODS®) is proprietary system owned by Cell Guidance System. It consist of polyhedrin protein produced by the silkworm virus (*Bombyx mori cypovirus*) and has been used to deliver proteins such as cytokines and growth hormones *in vitro*. PODS are 200 nm-5 μm in size, temperature-stable and non-brittle, and they release their active protein slowly (Wendler *et al.*, 2021). IL-2 has an essential role between innate and adaptive immune response (Bendickova and Fric, 2020) as it promotes T cell proliferation and B cell activation making it an ideal cytokine to act as an adjuvant (Zhou, 2022). However, IL-2 can also have adverse effects as it can promote T

regulatory cell production, which can suppress protective immunity (Overwijk *et al.*, 2021). PODS expressing IL-2 were assessed in this study for their ability to act as an adjuvant for a live *Leishmania* vaccine, i.e. *L. tarentolae* expressing γGCS. Studies have shown that innate immune responses are important in eliciting specific immune responses (Connors *et al.*, 2023). Therefore, it is important to understand the type of innate immune responses induced by vaccination. *In vivo* imaging using different chemicals can be used to monitor the local innate immune responses (Tseng and Kung, 2013). Luminol (5-amino-2,3-dihydro-1,4-phthalazine-dione) reacts with myeloperoxidase (MPO) produced by neutrophils, resulting in the production of a chemiluminescent product that can be detected by IVIS imaging (Bedouhène *et al.*, 2017). Lucigenin (bis-N-methylacridinium nitrate) interacts with phagocyte NADPH oxidase (phox) to produce a chemiluminescent product that can also be detected by IVIS imaging (Tseng Kung, 2012). In this study, luminol and lucigenin were used to monitor neutrophil and macrophage recruitment throughout the course of the study, to determine if the vaccines had the ability to enhance recruitment, which may improve antigen presentation in vaccinated mice.

*L. tarentolae* cell lines expressing *L. don* γGCS, *L. maj* γGCS and *L. mex* γGCS used as a live vaccine protected against *L. major* infection ($p < 0.001$ compared to control) and enhanced the production of IFN-γ from splenocytes stimulated specific antigen (Topuz Ata *et al.*, 2023). Therefore, the aim of this study was to determine if *L. tarentolae* expressing *L. don* γGCS, *L. maj* γGCS and *L. mex* γGCS could protect against *L. donovani* infection. In addition, it was determined if IL-2 releasing PODS could act as an adjuvant and enhance protective immune responses induced by the live *L. tarentolae* vaccine.

## *5.2 Results*

### *5.2.1 Immunisation with* **L. tarentolae** *promastigotes, expressing γGCS from pathogenic* **Leishmania,** *protected against* **L. donovani** *infection in a murine model.*

Studies were carried out to determine if immunisation with *L. tarentolae* promastigotes expressing γGCS from a pathogenic *Leishmania* species could protect against *L. donovani* infection. BALB/c mice were immunised on day 0 and 21 with $2 \times 10^7$ *L. tarentolae* promastigotes (*L. tarentolae* wild type WT group, *L. tarentolae* promastigotes expressing *L. don* γGCS alone, *L.t L don* γGCS *group* or 1:1:1 ratio of *L. tarentolae* expressing *L. don* γGCS, *L. maj* γGCS and *L. mex* γGCS, triple vaccine group). On day 42 all the mice (immunised and controls) were infected with *L. donovani* amastigotes (Figure 5.1). Controls were given PBS alone at immunisation or injected with WT *L. tarentolae* parasites to show that any protection was related to the production of γGCS from a pathogenic species of *Leishmania*. Immunisation with *L. tarentolae* was associated with significantly higher level of neutrophil recruitment based on chemiluminescence values after injection of luminol at both priming and boosting compared to PBS treated controls ($p < 0.05$). However, there was no significant difference in the enhanced neutrophil recruitment for mice injected with WT *L. tarentolae* or transgenic *L. tarentolae* (Figure 5.2A). Neutrophil recruitment was also determined after infection with *L. donovani* amastigotes on day 42 of the study. Infected mice immunised with the triple vaccine ($p< 0.01$) or *L.t L.don* γGCS parasites alone ($p < 0.05$) had significantly higher neutrophil levels based on chemiluminescence values compared to non-immunised infected controls (Figure 5.2A). In a second experiment, similar levels of neutrophil recruitment results were present for the WT and transgenic *L. tarentolae* vaccine groups (Appendix 3.1).

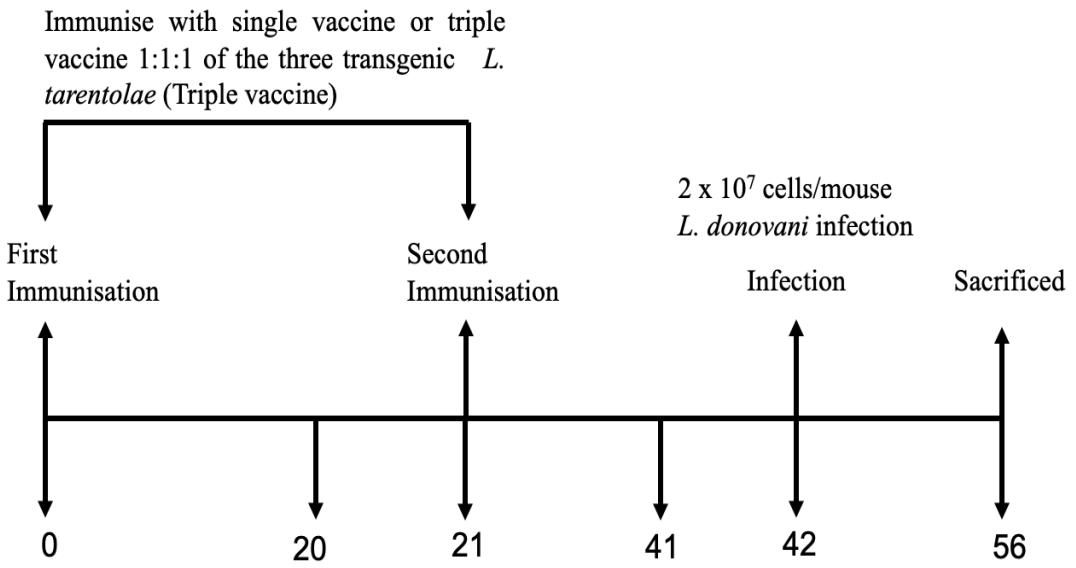


**Figure 5.1 Vaccination schedule used in experiments.** BALB/c mice (n = 5/treatment) were immunised on days 0 and 21 with 2 × $10^7$ *L. tarentolae* parasites (WT, *L. tarentolae* transfected with *L. don* γGCS gene sequence or a 1:1:1 mixture of *L. tarentolae L don* γGCS, *L maj* γGCS and *L mex* γGCS (triple vaccine). Blood samples were collected on day 20 and 41 and 56. Control and vaccinated mice were infected on day 42 with *L. donovani* amastigotes. Three weeks post infection, mice were sacrificed and spleen, liver and bone marrow parasite burdens were determined.

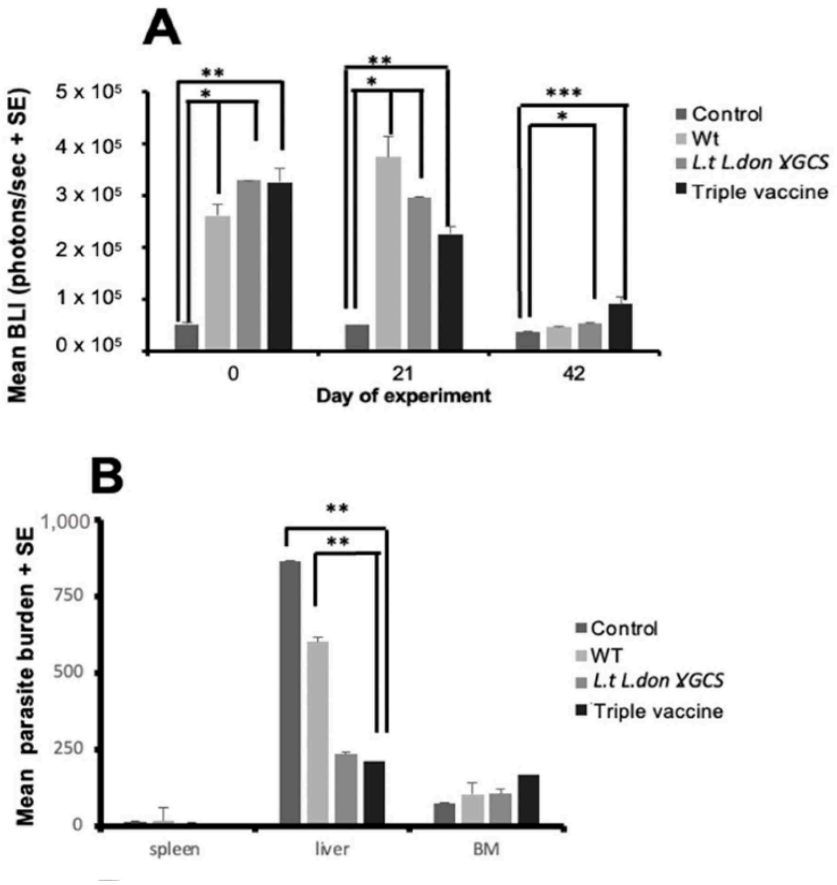


**Figure 5.2 The effect of vaccination on neutrophil recruitment (A) and parasite burdens (B).** BALB/c mice were injected on days 0 and 21 with PBS alone (control), $2 \times 10^7$ WT *L. tarentolae* (W*T),* $2 \times 10^7$ *L. tarentolae* expressing GCS from *L. donovani (L.t L.don γGCS*) or a 1:1:1 mixture of *L. tarentolae* expressing GCS from *L. donovani, L. major or L. mexicana* (triple vaccine). On day 42 all the mice were infected with $2 \times 10^7$ *L. donovani* amastigotes and spleen, liver and bone marrow parasite burdens in all the animals were determined 21 days later, on day 56 of the experiment. The effect of immunisation and infection on neutrophil recruitment was determined by measuring the amount of chemiluminescence emitted by mice (mean BLI, measured as photons/sec). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Blood samples collected over the course of the study were used to determine specific IgG1 and IgG2 antibody responses. Immunisation with WT, *L.t L don* γGCS or the triple vaccine significantly increased specific IgG1 and IgG2a responses at priming and boosting compared to PBS controls ($p < 0.05$, Figure 5.3). A higher specific IgG2a / IgG1 ratio is often associated with a protective immune response (Rostamian *et al.*, 2017). On day 42 post-infection the mean IgG2a / IgG1 for the different groups were infected control 1.35, WT *L. tarentolae* 1.64, *L.t L don γGCS* 1.18, triple vaccine 0.115.

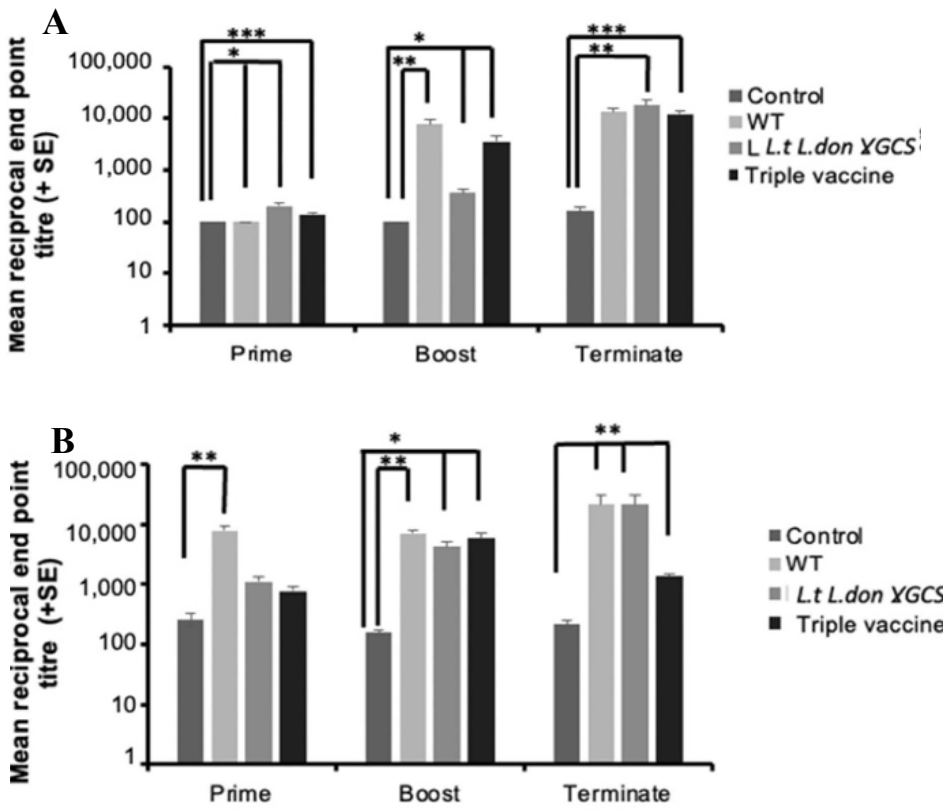


**Figure 5.3 The effect of vaccination on the specific antibody response of control and vaccinated mice.** Blood samples were collected from mice on day 21, 42 and 56 to determine the specific antibody responses**.** Specific IgG1 (A) and IgG2a (B) antibody titres were determined using an ELISA assay. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Cytokine production by splenocytes from in vitro proliferation assay can be used to indicate the type of immune response present in mice (Albert *et al*., 2019). Splenocytes from control and vaccinated mice produced similar amounts of IFN-γ in response to specific antigen stimulation. However, cells from mice immunised with the triple vaccine produced significantly higher level of IFN-γ compared to cells from controls ($p < 0.01$, Figure 5.4). Similar levels of IL-10 and nitrite were produced by antigen stimulated splenocytes from all groups of mice (Appendix 3.2). This result was not repeated in a second experiment, where similar amounts of IL-10, nitrite and IFN-γ were produced by antigen stimulated splenocytes from all groups of mice (Appendix 3.3).

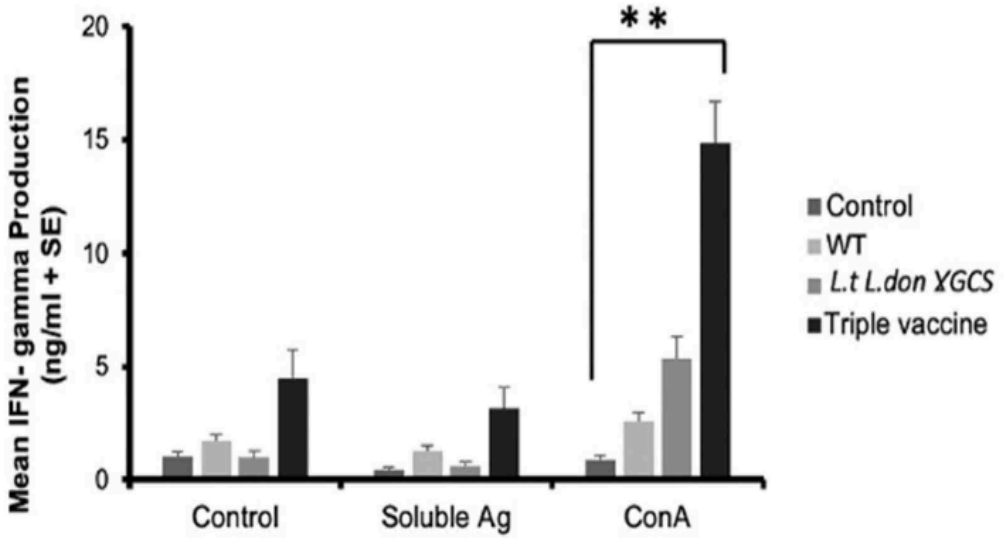


**Figure 5.4 Mean IFN-γ production was determined by antigen- and ConA-stimulated splenocytes**. Splenocytes cultures were prepared from mice at the end of experiment (5 ×$10^5$/mL) and incubated with medium alone (control), ConA (10 µg/ml) or *L. donovani* soluble antigen (50 µg/mL) for 72 hr. ** $p < 0.01$

The data from these studies indicated that immunisation with transgenic parasites expressing γGCS from a pathogenic species protected mice against *L. donovani* infection. However, protection was not associated with either a clear Th1 or a Th2 phenotype based on cytokine or antibody production. It is possible that using adjuvant could enhance the protection induced by immunising with transgenic *L. tarentolae* therefore the feasibility of using PODS expressing human IL-2 as an adjuvant was assessed.

### *5.2.2 PODS expressing IL-2 can cause proliferation of murine lymphocyte*

*In vitro* release studies showed, as expected, that IL-2 releasing PODS did indeed produce human IL-2 (Figure 5.5), with most of the cytokine being released within 24 hours. *In vitro* studies showed that PODS were not toxic to murine cells, and that human IL-2 released from PODS could stimulate the proliferation of murine splenocytes (Figure 5.6). This is not unexpected as the receptor of IL-2 is similar on human and murine lymphocytes (Abbas, 2020). A 72 hour incubation time was used in studies so that splenocytes had longer to proliferate.

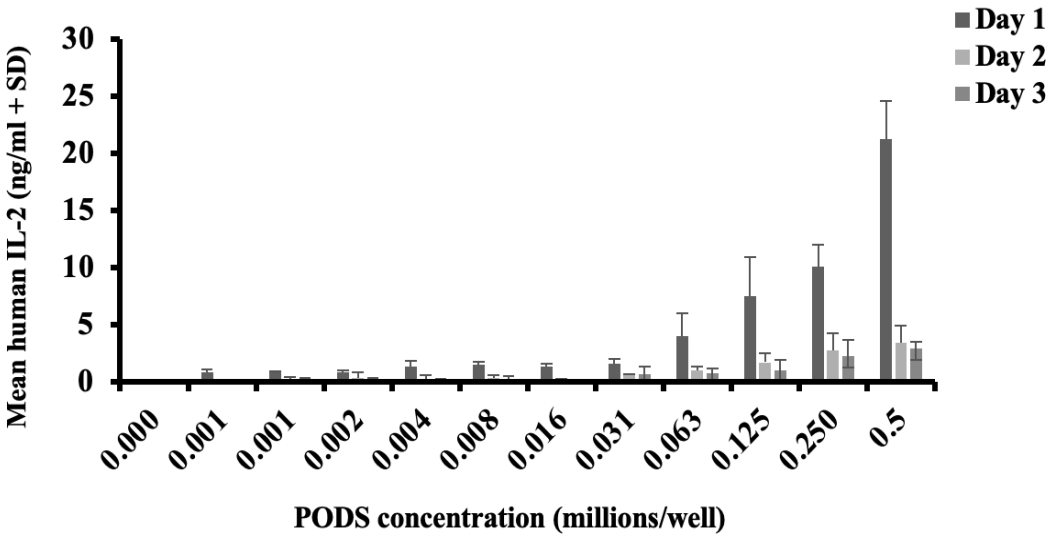


**Figure 5.5 The release of human IL-2 from PODS expressing IL-2.** Different concentrations of PODS releasing IL-2 were incubated in RPMI-1640 medium for 24, 48 or 72 hours in 96 well plates. The amount of human IL-2 present in the supernatant was determined by ELISA.

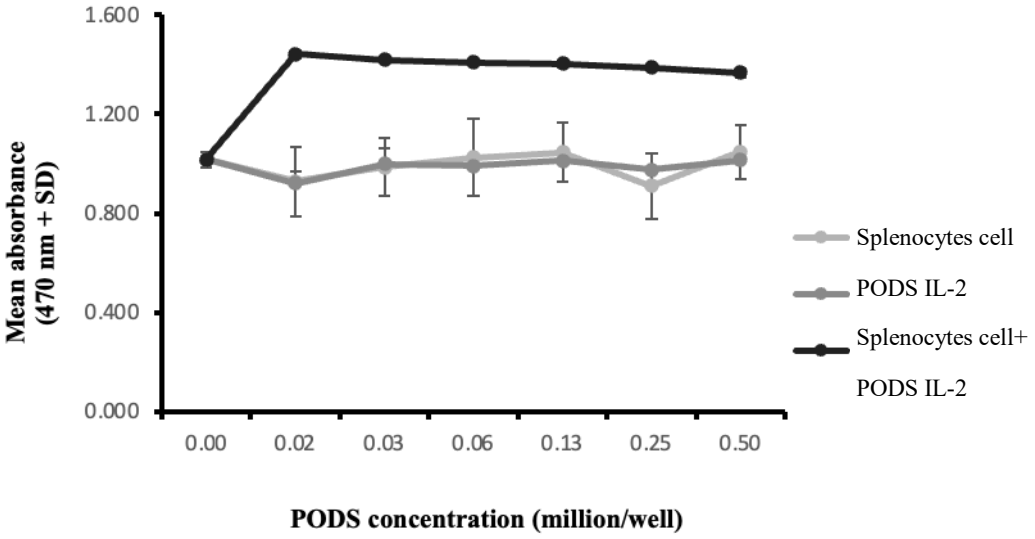


**Figure 5.6 The effect of incubation with IL-2 expressing PODS on the proliferation of murine splenocytes.** Splenocytes cells (5 × $10^5$/well, n= 3/treatment) were incubated with different concentrations of IL-2 releasing PODS (PODS IL2) for 72 hours. At 48 hours 20 µL resazurin solution (0.125 mg/mL) was added to each sample. The absorbance of each sample was determined at 570 nm. The absorbance of POD IL2 control is not at zero as complete medium containing resazurin solution does not give a zero value.

### *5.2.3 Immunisation with* L. tarentolae *expressing* L. donovani *γGCS and IL-2 producing PODS can protect against* L. donovani *infection*

Studies were carried out to determine if IL-2 producing PODS could act as an adjuvant for the transgenic *L. tarentolae* vaccine. Mice were immunised using the regimen shown in Figure 5.1. Immunisation with *L.t L.don* γGCS *L. tarentolae* with or without IL-2 producing PODS induced significantly higher levels of neutrophils at priming, boosting and infection ($p < 0.05$) compared to controls. However, there is no significant difference in the enhanced levels for mice given transgenic parasite alone and transgenic parasites and IL-2 producing PODS (Figure 5.7A). There was no significant difference in macrophage recruitment for all groups at priming and boosting. However, mice immunised with *L.t L.don* γGCS *L. tarentolae* and IL-2 producing PODS had significantly higher levels of macrophages present following infection compared to control mice ($p < 0.01$). Mice immunised with transgenic parasites alone had significantly higher levels of macrophages present at 4 minutes after lucigenin treatment ($p < 0.05$) but not 2 minutes after treatment (Figure 5.7B). However, there was no significant difference in macrophage recruitment for these two groups. Therefore, the overall results indicate that co-administration with IL-2 producing PODS did not enhance neutrophil or macrophage recruitment for mice immunised with transgenic parasites. Figure 5.8 shows an example of the images obtained in neutrophil and macrophage studies.

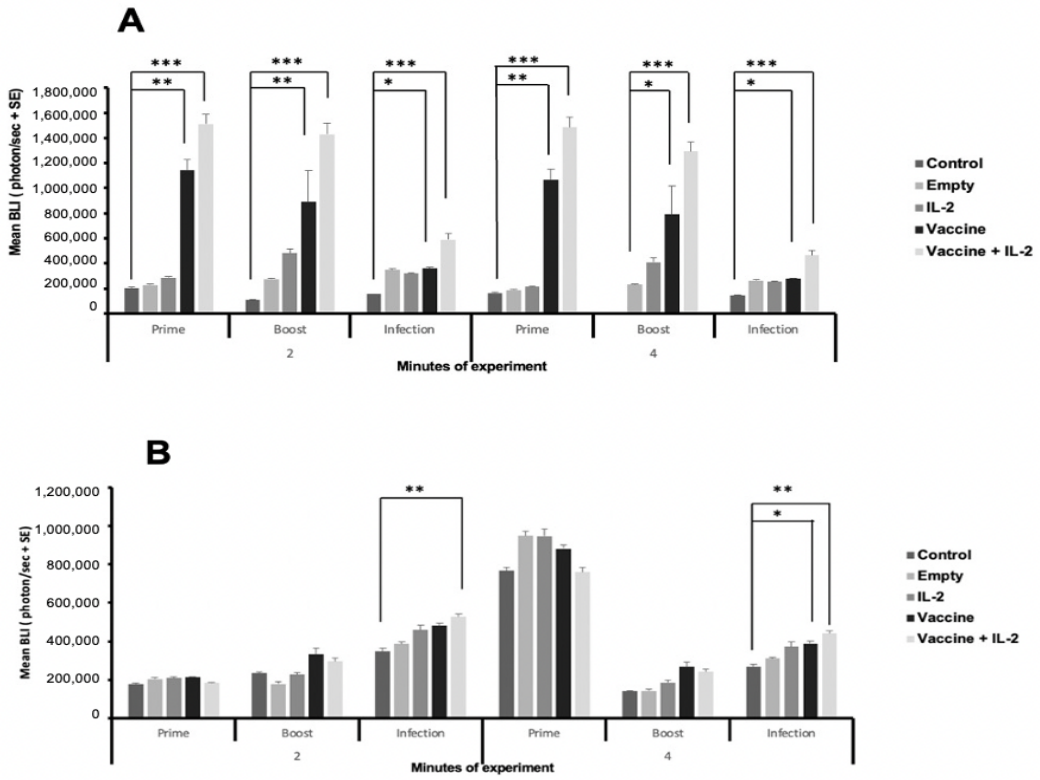


**Figure 5.7 The effect of vaccination on neutrophil and macrophage influx.** Mice (n = 5/treatment) were immunised on days 0 and 21. On day 42, the mice were infected by intravenous injection with $2 \times 10^7$ *L. donovani* amastigotes. Three hours after infection with *L. donovani* each mouse was injected with luminol solution (150 mg/mL, saline) and the effect on neutrophil recruitment was determined (A). The same mice were was injected with lucigenin solution (10 mg/kg, saline) and macrophage recruitment (B) assessed. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

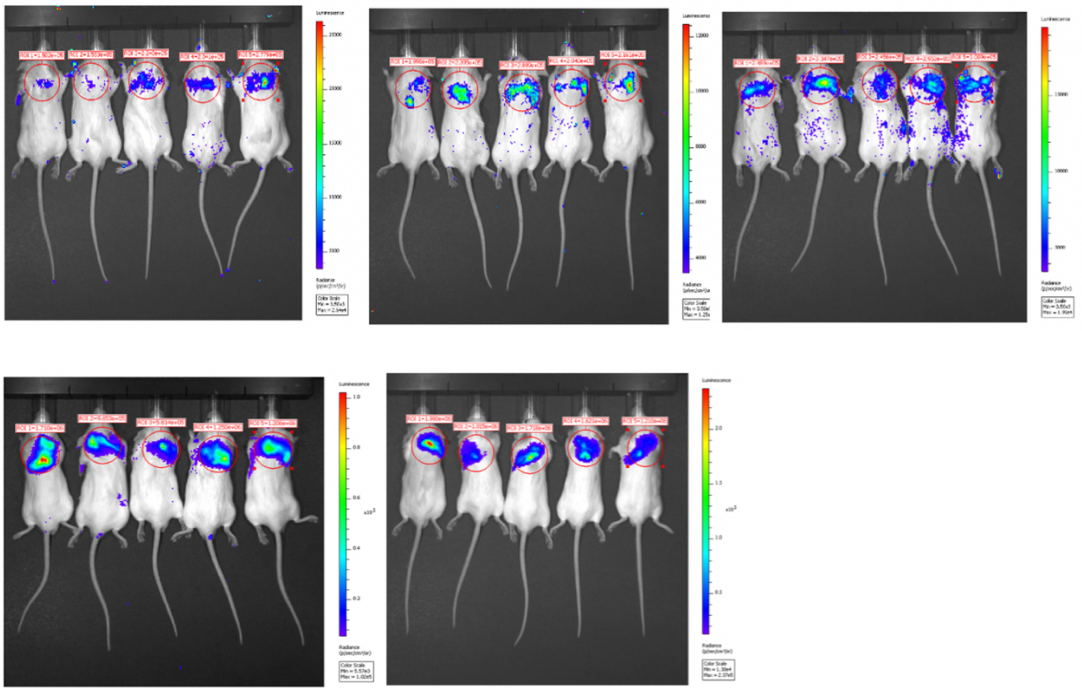

**Figure 5.8 An example of the type of images obtained in IVIS neutrophil imaging studies for *L. donovani* experiments. In** studies the same size region of interest was used for every reading in an experiment so that the same sized area was used to collect data. BALB/c mice (n = 5/treatment) were immunised on days 0 and 21 with PBS alone (control, A), PODS-Empty (50 million/mouse, B), PODS-IL-2 (50 million/mouse, C), $1 \times 10^7$ *L.t L.don* gGCS promastigotes alone (vaccine, D) or mixed with PODS-IL-2 (50 million/mouse, vaccine, E). The amount of bioluminescence emitted from the injection site (mean bioluminescence, total flux, p/s) was determined 3 hours after the first immunisation, mice were given 0.2 ml luminol solution (150 mg/mL, PBS) by intraperitoneal injection and imaged for 2 mins starting from 5 minutes after injection.

Determination of parasite burdens in the spleen, liver and bone marrow showed that vaccination with transgenic parasites, with or without IL-2 producing PODS caused a significant reduction in liver parasite burdens compared to control values ($p < 0.01$, Figure 5.9, Appendix 3.4B). Cotreatment with IL-2 producing PODS did not enhance the level of protection induced by vaccination with *L.t L.don* γGCS parasites.

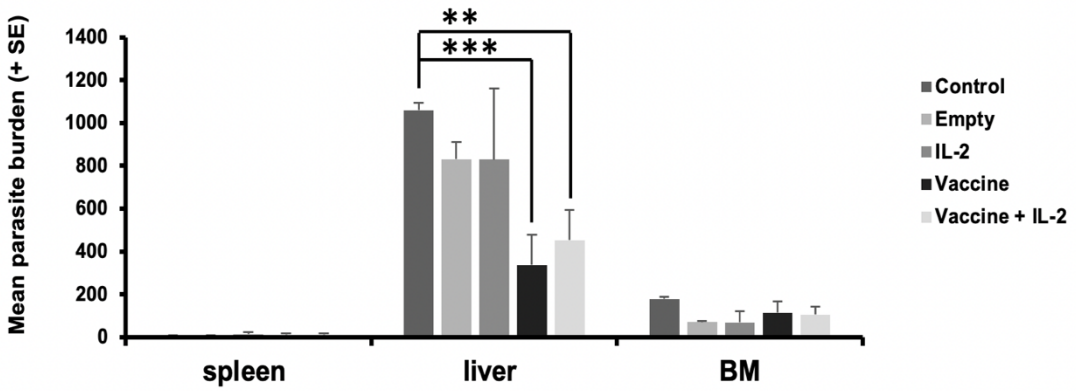


**Figure 5.9 The effect of vaccination on parasite burdens.** Spleen, liver and bone marrow parasite burdens were determined on day 21 post-infection in control and vaccinated mice. ** $p < 0.01$, *** $p < 0.001$.

Blood samples were collected from mice on days 21, 42 and 56 of the experiment to determine specific IgG1 and IgG2a antibody responses. Mice vaccinated with *L.t L.don* γGCS transgenic parasites and IL-2 releasing PODS had significantly higher specific IgG2a antibody titres at priming compared to controls ($p < 0.05$) but significantly higher IgG1 titres ($p < 0.05$) at boosting and at the end of the experiment. Mice vaccinated with *L.t L.don* γGCS transgenic parasites alone had significantly higher IgG1 levels at boosting and significantly higher IgG2a levels at the end of the experiment compared to controls ($p < 0.05$). The IgG2a/IgG1 ratios were infected control, 0.02, PODS-Empty, 0.10, PODS-IL 2, 0.01, vaccine, 0.71, vaccine and PODS IL-2, 0.25 at the end of the experiment, indicating that vaccination with the transgenic parasites, with or without IL-2 producing PODS, induced specific IgG1 as the main antibody (Figure 5.10).

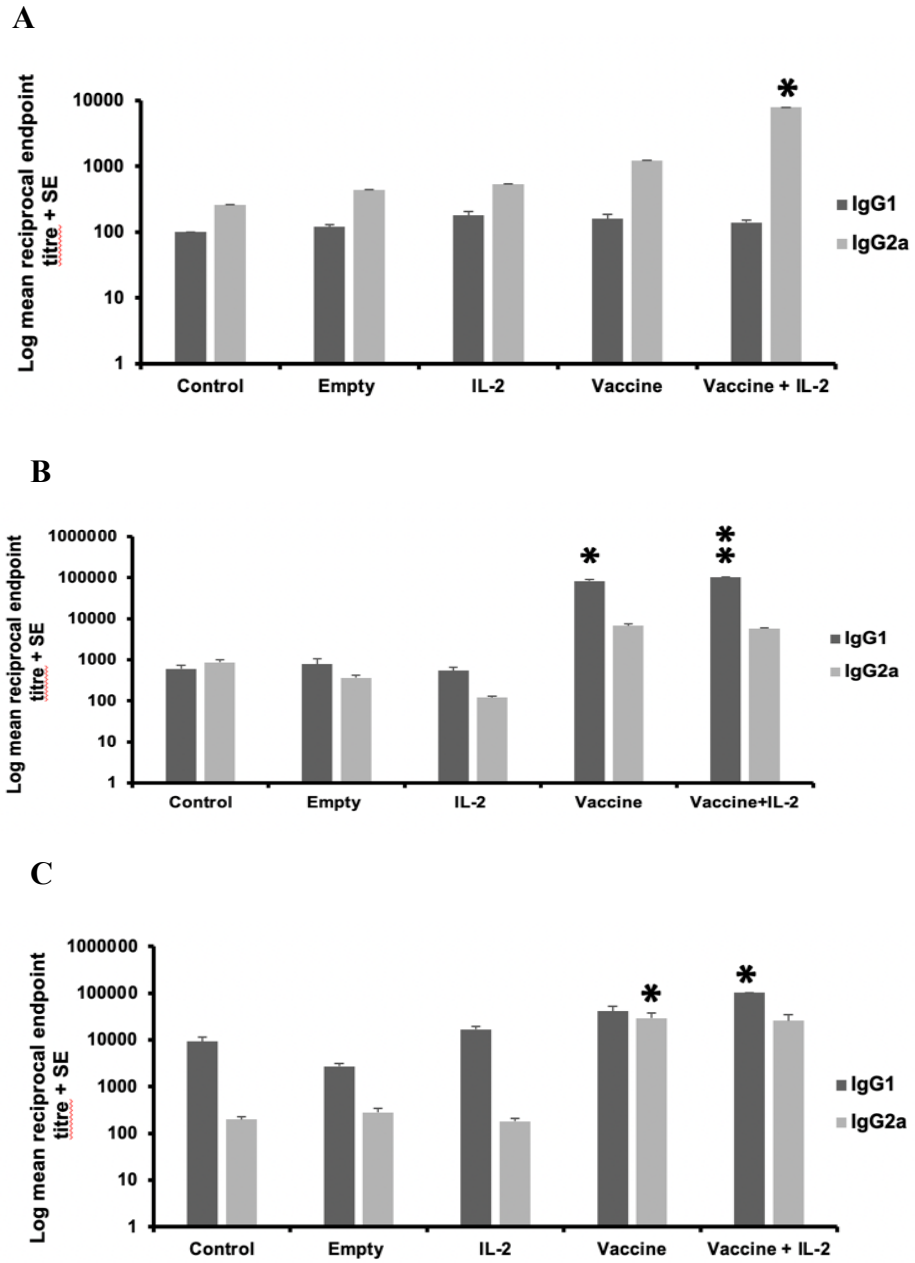


**Figure 5.10 Specific antibody determination outcome during vaccination.** On day (A) 21, 42 (B), and 56 (C) mice blood sample were collected from mice and used in ELISA assays to detect serum specific IgG1 and IgG2a specific antibody titres. * $p < 0.05$, ** $p < 0.01$.

The production of cytokines and nitrite by *in vitro* stimulated spleen cells was assessed to compare the phenotype of cells present in vaccinated and control animals at the end of the experiment. Spleen cells from infected control mice produced significantly lower levels of IL-10 ($p < 0.05$) and considerably higher levels of IFN-γ ($p < 0.05$) compared to cells from mice in the other groups (Fig 5.11). There was no clear difference in nitrite and IFN-γ production by splenocytes from mice in control and vaccinated groups in a repeat experiment, however cells from control mice and mice immunised with empty PODS produced significantly more IL-10 compared to the other groups ($p < 0.05$, Appendix 3.6).

Overall, these studies showed that immunisation with the *L. tarentolae* transgenic parasites protected mice against *L. donovani* infection and that co-treatment with PODS producing IL-2 did not enhance the level of protection obtained. Protection was not associated with a predominant Th1 response but rather a mixed Th1/Th2 response.

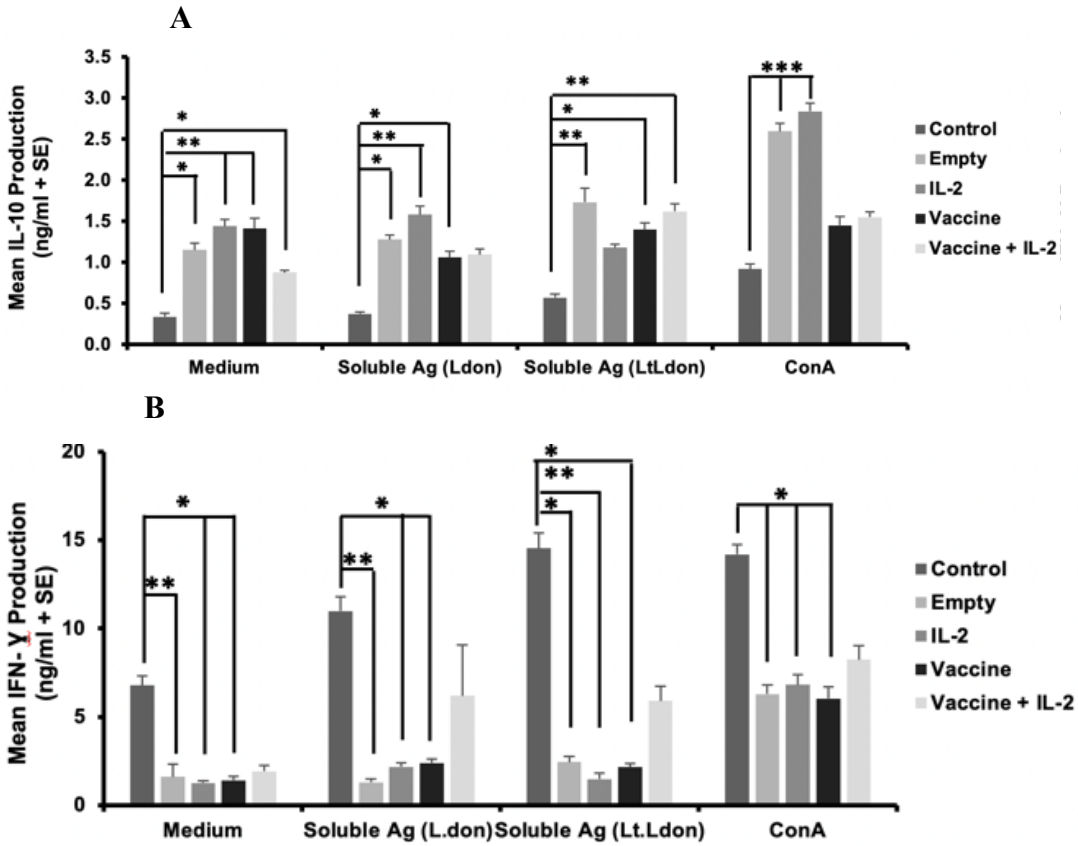


**Figure 5.11 IL-10 and IFN-γ production by splenocytes from control and vaccinated mice.** Splenocytes obtained from mice on day 21 post-infection were incubated with medium alone (control), ConA (10 μg/mL) or *L. donovani* soluble antigen (50 μg/mL) for 72 hr. The amount of IFN-γ and IL-10 present in supernatants were determined using an ELISA assay. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

## *5.3 Discussion*

The results of this study showed that vaccination with *L. tarentolae* expressing homologous and heterologous γGCS protected mice against *L.donovani* infection. A similar level of protection was induced by the triple vaccine (*L.don, L.maj* and *L.mex*) and the single *LtLdonγGCS* vaccine. This protective effect was related to a mixed Th1/Th2 response based on antibody response and cytokine production in immunised mice. This may not be surprising as studies in humans have shown that visceral leishmaniasis is associated with a mixed Th1/Th2 response (Balodi *et al.*, 2022). Immune response was identified as Th1/Th2 response, protective Th1 and disease promoting Th2 response against *Leishmania* infection (Goto and Mizobuchi, 2023). Animal model studies showed that a strong Th1 response with low level of Th2 was associated with healing, so Th1 is associated with a protective response against infection. APCs release IL-12, which trigger proliferation of Th1 cells inducing IFN-γ production. Macrophages were activated by IFN-γ and activation was associated with production of NO, which lead to parasite clearance (Divenuto *et al.*, 2023).

The results of this study showed that immunisation with *LtLdonγGCS* or the triple vaccine reduced liver but not splenic *L. donovani* parasites burdens. A similar study using a *L. tarentolae*-KMP11-NTGP96-GFP live vaccine significantly reduced both splenic and liver parasite burden in mice infected with *L. infantum*. Mice vaccinated with a *L. tarentolae*-KMP11-NTGP96-GFP showed enhanced IFN-γ/IL-4 and IgG2a/IgG1 ratios, indicating that protection was associated with a predominant Th1 response (Nasiri *et al.*, 2016). The difference in results compared to this study may be because different parasite proteins were used as vaccines or because of the use of

different pathogenic *Leishmania* species. Vaccination with *L. tarentolae* expressing γGCS from *L. donovani*, *L. major*, and *L. mexicana* protected against *L. major* infection. Protection was associated with a predominant Th1 response according to cytokines production from in vitro proliferation assays and specific IgG1 and IgG2a antibody (Topuz Ata *et al.*, 2023).

In this study, sterile immunity was not achieved but this may not be required as the presence of a few *Leishmania* parasites may be required to maintain immunity (Saljoughian *et al.*, 2014). However, the level of protection induced by transgenic *L. tarentolae* may be improved by expressing multiple proteins from pathogenic *Leishmania* species (Goto and Mizobuchi, 2023). After immunisation, a subcutaneous nodule occurs at the site of vaccination which would be an adverse effect if it occurred in vaccinated patients. One way to avoid this would be to administer a vaccine by the intramuscular rather than subcutaneous route (Collinson *et al.*, 2022). However, in this study vaccination was not associated with nodule formation.

In this study, the subcutaneous injection was given into the skin at the back of the neck instead of into the rump. This change was made on advice of our manager of the Biomedical Procedures Unit (BPU) as it would be more humane to the mice. This could have affected the local immune response. However, similar IgG1 and IgG2a responses of the BALB/c mice were detected after intramuscular injection (Campbell *et al.,* 2012).

Vaccination with *L. tarentolae* or *L. tarentolae* transfected with γGCS from *L. donovani or L. donovani, L. major*, *L. mexicana* enhanced specific IgG1 and IgG2a antibody response against a soluble *L. donovani* antigen, indicating that *L. tarentolae*

has some proteins that are also expressed in *L. donovani.* This study showed that immunisation with a triple live vaccine protected against *L. donovani* in mice. This is the first time, this type of vaccine has been used *in vivo* against *L. donovani* infection. Further study is required to determine if this vaccine can protect against *L. mexicana.*

Previous studies have shown that using an appropriate adjuvant can boost immune responses and selection of the correct adjuvant can favour Th1 polarisation (Varotto-Boccazzi *et al.*, 2022). In this study, PODS producing human IL-2 were assessed for their ability to boost protection induced by transgenic *L. tarentolae*. Unfortunately, co-treatment did not enhance the protection induced by transgenic *L. tarentolae* alone and there was no evidence that the IL-2 producing boosted Th1 or Th2 response. There could be various reasons for this, for example, perhaps a low amount of IL-2 was released *in vivo* from PODS or that PODS were quickly cleared by phagocytic cells at the site of injection. *In vitro* studies have shown that PODS are efficiently taken up by macrophages (Wendler *et al.*, 2021) and that macrophage uptake reduces the amount of cytokine released by PODS (Jones *et al.*, 2023). Treatment with IL-15 releasing PODS (total dose $4 \times 10^7$ PODS) reduced tumour growth in a metastatic renal cell carcinoma mouse model. However, the dosage of PODS caused adverse side-effects in treated mice (Jones *et al.*, 2023). In this study mice were injected with $5 \times 10^7$ PODS and no adverse side-effect was observed. In addition, vaccination with empty PODS did not enhance neutrophil or macrophage influx to the site of injection, indicating that PODS alone do not induce an inflammatory response. Therefore, the cytokine released from PODS may have an important effect on the results obtained.

Overall, studies have shown that genetically modified live vaccines are preferable against intracellular parasites (Pandey *et al.*, 2020). This study proved that γGCS is a valid antileishmanial vaccine. Transgenic parasite expressing γGCS from *L. donovani* can protect against *L. donovani* infection. This study showed that IL-2 PODS did not enhance a vaccine protective effect against *L. donovani* infection in a murine model.

# Chapter 6 Future Work and Conclusions

Leishmaniasis is a protozoan parasitic infectious disease caused by *Leishmania* species. At present, there are limited numbers of drugs available that have side effects and required repeated administration. Therefore, a clinical vaccine is required to protect people against infection (Van der Ende Schallig, 2023). Previous studies in our laboratory have demonstrated that intramuscular immunisation with plasmid carrying the *L. donovani* γGCS gene sequence against *L. donovani* infection (Carter *et al.*, 2007). Recombinant γGCS protein immunisation gave significant protection against *L. donovani* (Henriquez et al., 2010), *L. major* and *L. mexicana* (Campbell *et al., 2012)*. However, they did not give sterile immunity against *Leishmania* infection. This study was carried out to determine generating different recombinant γGCS constructions produce full-length, pure fusion protein for a vaccine candidate. Additionally, vaccination with recombinant γGCS produced, and whether vaccination using *L. tarentolae* expressing γGCS from pathogenic species would provide protection against *L. donovani* infection.

The γGCS sequences of three pathogenic *Leishmania* species i.e. *L. donovani, L. major* and *L. mexicana* with a C terminal His-tag and N terminal T7-tag or just a C terminal His-tag, were cloned into the pET-21a(+) expression vector. Sequencing studies showed that the γGCS gene sequences for all three *Leishmania* species were in the correct orientation except pET21asLmajGCS6His. Recombinant γGCS proteins for *L. donovani*, *L. major* and *L. mexicana* were expressed in *E. coli* and the results indicated that all three proteins were expressed in the soluble fraction. Constructs having two tags were used to determine of effect of N-terminal T7-tag on expression as studies had indicated that this tag could improve protein expression (Du *et al.*, 2022).

However, results of SDS-PAGE gel electrophoresis showed that the presence of a T7-tag did not increase the amount of protein compared to bacteria expressing γGCS with just a C-terminal His-tag. In future studies engineering different tags into the expression vector may improve protein expression (Malhotra, 2009). For example, maltose-binding protein is one of the most frequently used tags to boost solubility of the expressed protein. Studies showed that fusion proteins with MBP tag more highly soluble protein expressed (Bernier *et al.*, 2018).

The recombinant γGCS was purified from the soluble extract obtained from transfected bacteria using different purification methods i.e. using a HisPur™ Ni-NTA Spin column kit, a T7•Tag® affinity purification kit and a HisTrap HP column using the ÄKTA protein purification system. However, despite changing the conditions used experiments always resulted in the production of truncated protein as well as a full-length protein. The T7•Tag® affinity purification kit gave the poorest results which could indicate that either little full-length protein was present in the soluble protein or that the accessibility of the T7-tag within the folded protein prevented the tag from binding to the affinity column (Mills *et al.*, 2006). Purifying the protein using denaturing conditions e.g. extracting the protein using urea (Nekoufar *et al.*, 2020) could make the T7-tag accessible for affinity purification (Spriestersbach *et al.*, 2015).Therefore, this method should be tested in future studies.

One of the aims of this study was to produce full-length γGCS, which is a relatively large protein. However, it may be better to identify T or B cells epitopes in the protein and express these truncated peptides or use a commercial supplier to provide these peptides for testing. A vaccine combining a suitable γGCS peptide or peptides with

other smaller molecular weight *Leishmania* vaccine candidate protein might be provide protection. For example, vaccination with LACK1 and KMP11 antigens engineered into *L. tarentolae* protected against *L. major* in a murine model eliciting a Th1 response (Salari *et al.*, 2021).

Purification using the HisPur™ Ni-NTA spin column kit allowed isolation of full-length and truncated forms of γGCS from the soluble protein extracted from transfected *E. coli*. Improving the purification method with additional washes or modify imidazole concentration even further could allow the isolation of much more pure full-length γGCS.

Tags are exploited to facilitate expression and purification of desired recombinant protein but they have the potential to interfere with the function of the expressed protein and in vaccines, any foreign elements must be removed (Arnau *et al.*, 2006). Therefore, it is essential to engineer a way to remove any tag from genetic construct. Tags can be removed using chemical treatment. For this purpose proper methionine residues must be used. However, chemical methods may lead to amino acid modification in the target protein or protein denaturation. Enzymatic cleavage is more specific and can be achieved in mild conditions (Arnau *et al.*, 2006). In this study carboxypeptidase A was used to remove the 6His-tag and enteropeptidase to remove the T7-tag from the recombinant γGCS fusion protein. The optimisation of Carboxypeptidase A cleavage conditions can be problematic. However, carboxypeptidase A does remove a tag without leaving any residue behind C-terminal (Waugh, 2011). In thus study, different concentrations of carboxypeptidase A and various time periods were tried at 25°C, but total removal of the carboxypeptidase A

was not achieved despite using extended incubation times. Previous studies have shown that incubation at room temperature for 2 hours removed the His-tag from proteins (Austin *et al.*, 2011). In future studies using different incubation temperatures could improve the cleavage ability of the enzyme and could provide more protein without the C-terminal tag. However, it would be possible to remove uncleaved protein from purified γGCS by using a HisPur™ Ni-NTA spin column kit.

In this study, *L. tarentolae* parasites expressing *L. donovani, L. major* and *L.mexicana* γGCS were tested as a live vaccine against *L. donovani* infection. Protection of vaccine associated with mixed Th1/Th2 response but did not give sterile immunity. However, this may be overcome if a higher vaccine dose was used or by altering the number of doses given, for example, using 3 instead of 2 doses (Pirdel and Farajnia, 2017). Alternatively, γGCS as a protein or DNA could be used at prime or boost with the live *L. tarentolae* vaccine. This approach has been used in other studies, for example, *L. tarentolae* expressing cysteine proteinases and saliva protein, PpSP15 DNA were used as a vaccine. Immune response and parasite burden assessment showed that combination of cysteine proteinases and saliva protein boosted protective immune response against cutaneous leishmaniasis in mice (Zahedifard *et al.*, 2014). In this study, the triple live vaccine protected against *L. donovani* infection and showed this type of vaccine could protect against two species of *Leishmania i.e. L. donovani and L. major* (Topuz Ata *et al.*, 2023).

An adjuvant can enhance the immune response to a vaccine (Zhao *et al.*, 2023) and in this study PODS adjuvant releasing IL-2 cytokine were tested for their ability to improve the protective effect of the transgenic *L. tarentolae* vaccine used in this study.

Unfortunately, it was not effective. Using different adjuvants or using PODS, which express different cytokines could increase the efficacy of the *L. tarentolae* vaccine. Immunisation with recombinant *L. tarentolae* co-expressing PpSP15 and PsSP9 salivary protein with the adjuvant CpG resulted in significantly lower parasite burdens in the lymph nodes compared to controls in *L. major* infected mice (Lajevardi *et al.*, 2022). In another study, gardiquimod (a toll-like receptor-7 agonist) was evaluated as an adjuvant with the heat-killed antigen of *L.donovani.* Vaccination with the heat-killed antigen and gardiquimod induced significantly higher specific IgG2a levels and lower specific IgG levels compared to controls, and lower parasite burdens compared to controls against *L. donovani* infection (Goyal *et al.*, 2021). Cytokines are key regulators of immune response and IL-2 cytokine can enhance antiparasitic immunity (Osero *et al.*, 2020). Therefore, other cytokines that could be considered are IL-2, IL-15 and IFN-γ. Treatment with PODS expressing these cytokines were reported to be protective in a mouse model of renal cell carcinoma (Jones *et al.*, 2023). Murine forms of these cytokines would have to be engineered into PODS but the company only produces products that express human cytokines currently.

In summary, this project provided further evidence that γGCS is a promising vaccine candidate for *Leishmania*.

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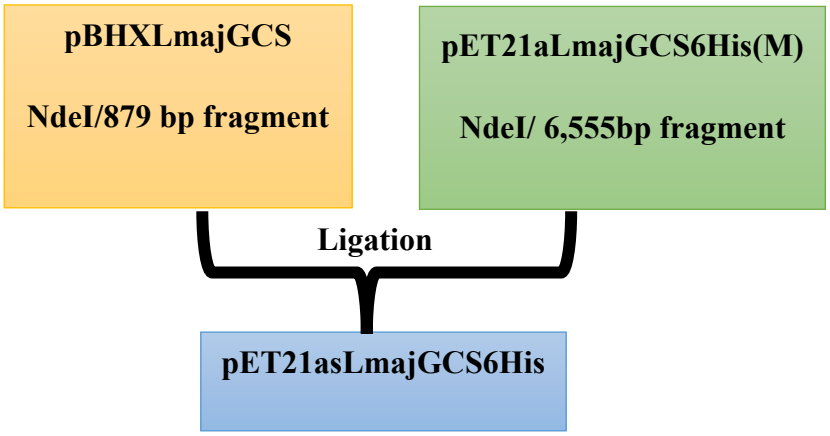
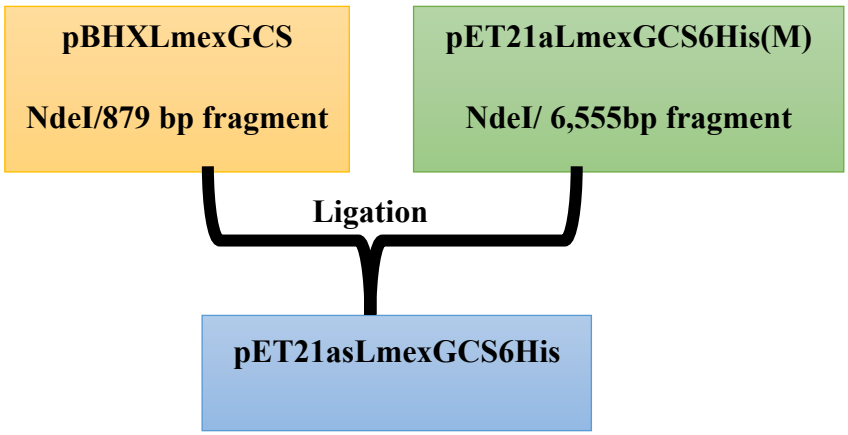
# APPENDIX

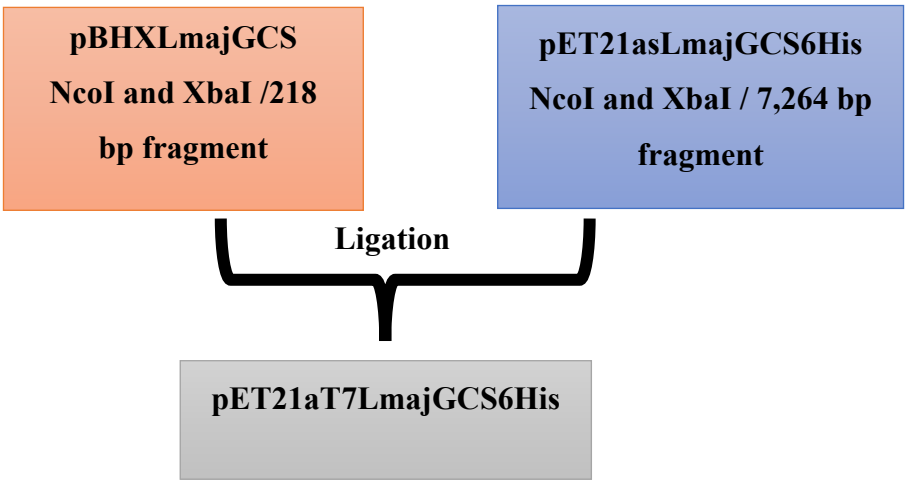
## *Appendix 1*



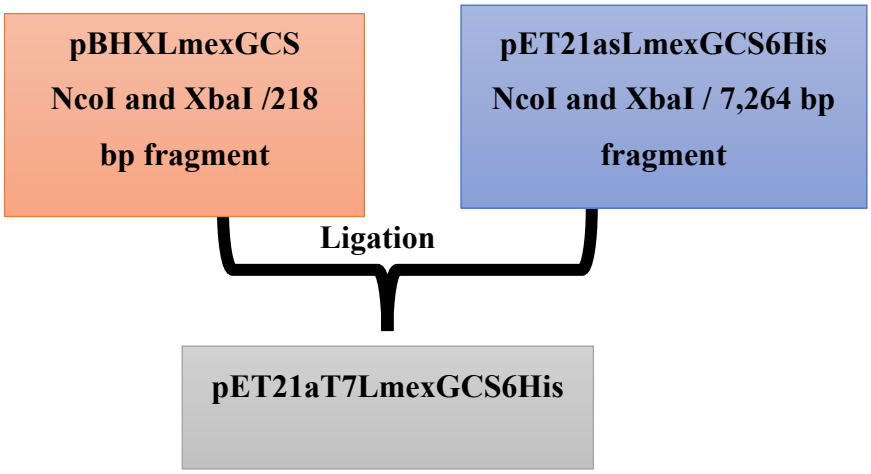

**Appendix 1.1 Flow chart of generation pET21asLmajGCS6His.** pBHXLmajGCS was cleaved with NdeI to generate 165 bp, 879 bp, and 2,964 bp DNA fragments. 879 bp fragment was isolated. pET21aLmajGCS6His(M) was cleaved with NdeI and 918 bp and 6,555 bp fragments were obtained. The 6,555 bp fragment was isolated and the 879 bp and 6,555 bp were ligated to generate pET21asLmajGCS6His.



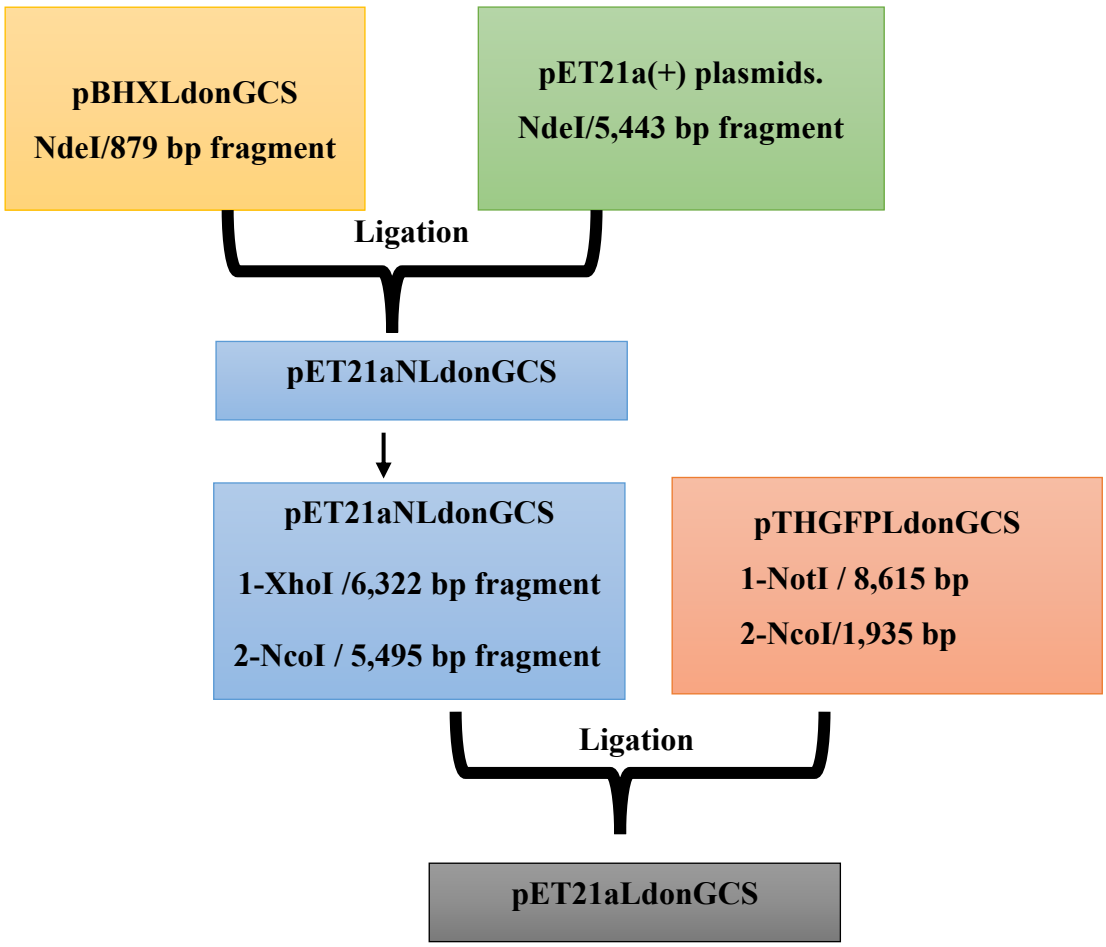

**Appendix 1.2 Flow chart of generation pET21asLmexGCS6His.** The pBHXLmexGCS plasmid was cleaved with NdeI 165 bp, 879 bp, and 2,964 bp were obtained. The 879 bp fragment was isolated. The pET21aLmexGCS6His(M) plasmid was also cleaved with NdeI and gave products with sizes of 918 bp and 6,555 bp The 879 bp and 6,555 bp fragments were ligated to generate pET21asLmexGCS6His plasmid.



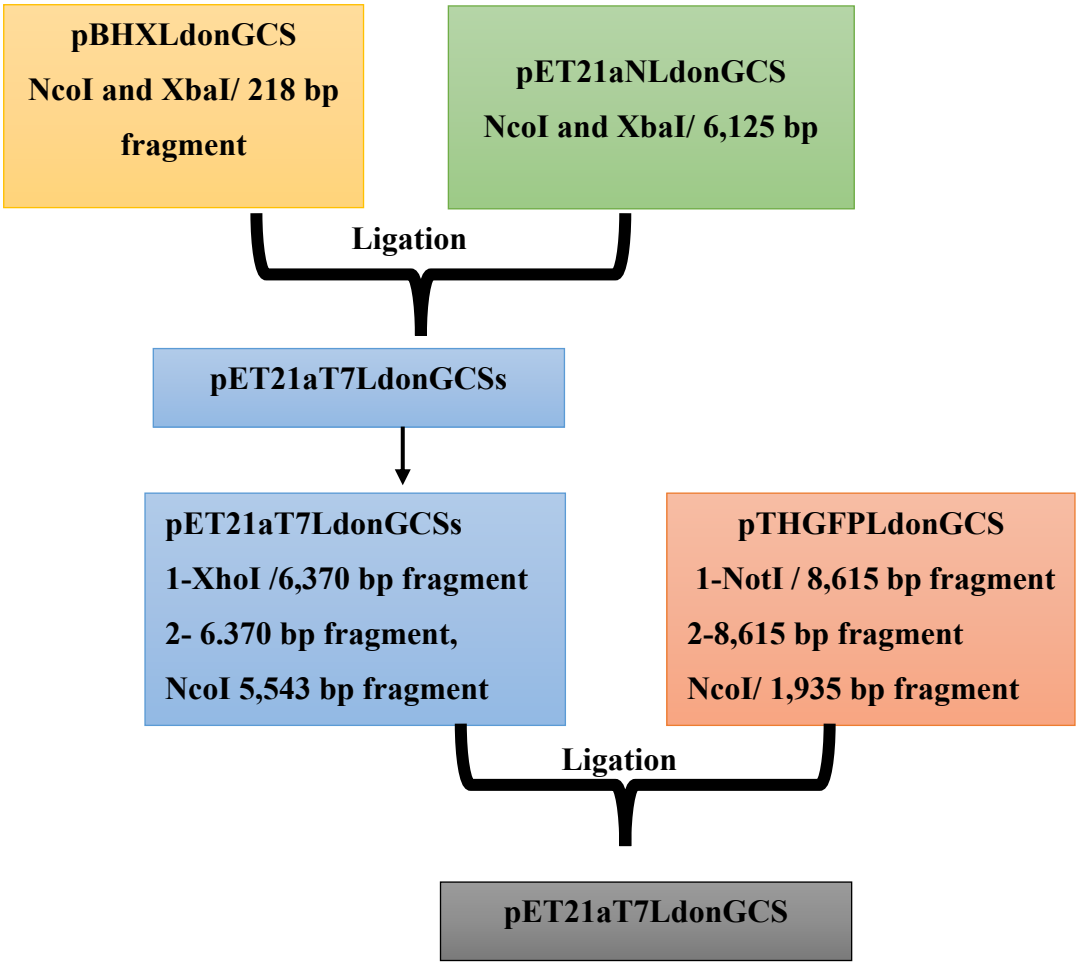

**Appendix 1.3 Flow chart of generation pET21aT7LmajGCS6His.** pBHXLmajGCS was cleaved with NcoI and XbaI to produce 218 bp, 754 bp and 3,056 bp DNA fragments. The 218 bp fragment was isolated. pET21asLmajGCS6His was cleaved with NcoI and XbaI to give products with a size of 170 bp and 7,264 bp. The 7,264 bp fragment was isolated The 218 bp fragment from pBHXLmajGCS and the 7,264 bp product from pET21asLmajGCS6His were ligated to generate pET21aT7LmajGCS6His.




**Appendix 1.4 Flow chart of generation pET21aT7LmexGCS6His.** pBHXLmexGCS was cleaved with NcoI and XbaI to generate products with a size of 218 bp, 754 bp, 3,056 bp. The 218 bp fragment was isolated from an agarose gel pET21asLmexGCS6His was cleaved with NcoI and XbaI to create 170 bp and 7,264 bp fragments and the 7,264 bp fragment was isolated.The 7,264 bp fragment from pET21asLmexGCS6His and the 218 bp fragment from pBHXLmexGCS were ligated.




**Appendix 1.5 Flow chart of generation pET21aLdonGCS.** pET21a (+) were cleaved with NdeI and the 5,443 bp fragment and the 879 bp fragment was isolated from pBHXLdonGCS. The isolated fragments were ligated to obtain pET21aNLdonGCS. pTHGFPLdonGCS was cleaved with NotI to generate 1,221 bp and 8,615 bp fragments. The 8,615 bp fragment was isolated, filled using the Klenow process and cleaved with NcoI to generate 130 bp, 1,935 bp, 2,703 bp, and 3,847 bp fragments. The 1,935 bp fragment was isolated. pET21aNLdonGCS was cleaved with XhoI to generate a 6,322 bp product and its ends were filled using the Klenow process The resulting fragment was cleaved with NcoI to generate 827 bp, 5,495 bp fragments. 5,495 bp fragment was isolated. The 1,935 bp fragment from pTHGFPLdonGCS and the 5,495 bp fragment from pET21aNLdonGCS were ligated to obtain pET21aLdonGCS6His.




**Appendix 1.5 Flow chart of generation pET21aT7LdonGCS.** The 6,125 bp fragment from pET21aNLdonGCS and the 218 bp fragment from pBHXLdonGCS were isolated, ligated to create pET21aT7LdonGCSs. The pTHGFPLdonGCS plasmid prepared by Muattaz Hussain was cleaved with NotI to generate 1,221 bp and 8,615 bp DNA fragments. The 8,615 bp fragment was filled-in using the Klenow and cleaved with NcoI to obtain 130 bp, 1,935 bp, 2,703 bp, and 3,847 bp fragments .The 1,935 bp fragment was then isolated. pET21aT7LdonGCSs was linearised with XhoI, the 6,370 bp fragment and filled-in using the Klenow method. The 6,370 bp fragment was cleaved with NcoI to generate 827 bp and 5,543 bp fragments and the 5,543 bp fragment isolated from an agarose gel. The 1,935 bp fragment from the pTHGFPLdonGCS and the 5,543 bp fragment from pET21aT7LdonGCSs were ligated to generate pET21aT7LdonGCS.

Plasmid DNA sequences:

## pBHXLdonGCS

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## pBHXLmexGCS

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## pET21aLdonGCS6His

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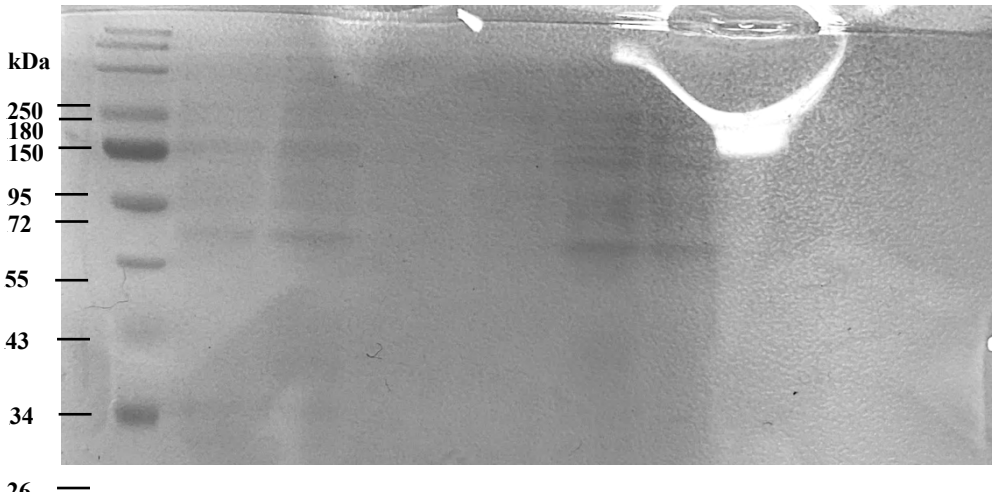
**Yellow, T7-tag**

**Pink, Enteropeptidase**

**Green, Carboxypeptidase A**

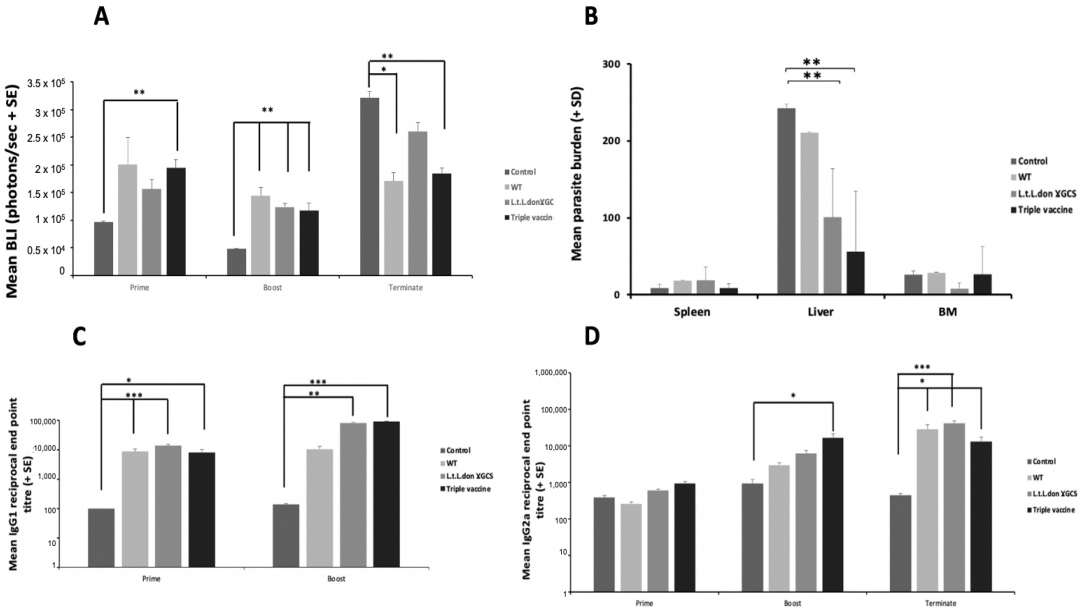
**Blue, His-tag**

## *Appendix 2*

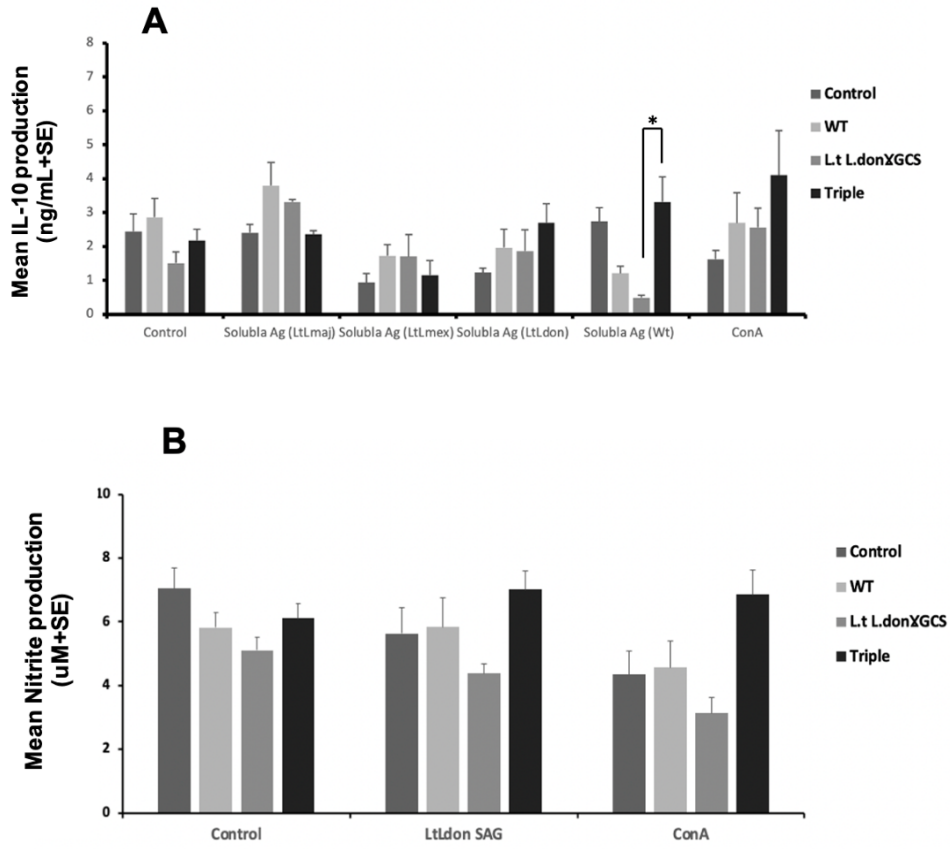



**Appendix 2.1** SDS-PAGE gel showing T7Ldγ-GCS6His, T7Lmγ-GCS6His protein purification using insoluble sample with the HisPur™ Ni-NTA spin purification. *E. coli* was transformed with the pET21aT7LdonγGCS6His, pET21aT7LmexγGCS6His and incubated at 37°C until the OD values reached between 0.6-0.8. Then they were induced with 0.1mM IPTG, incubated overnight 18°C. Pellets were collected with centrifugation and incubated overnight with B-per reagent. After incubation, insoluble sample was resuspend with extraction buffer (0.15 M tris. 0.2 M KCl, pH 7.4 containing 6 M urea, 20 ml/L culture) for 1 hour 30°C and centrifuged 12,000 × g at 4 °C to recover solubilised protein. Supernatant was diluted with 1:1 with extraction buffer supernatant. A HisPur™ Ni-NTA spin purification kit was used with 25 mM imidazole wash buffer and 250 mM imidazole elution buffer. Protein samples were separated by SDS-PAGE gel electrophoresis. Lanes M, Marker; lane 1, T7LdγGCS6His total protein; lane 2, T7LdγGCS6His flow through; lane 3, T7LdγGCS6His wash with 25 mM imidazole wash buffer; lane 4, T7LdγGCS6His elution with 250 mM imidazole buffer; lane 5, T7LmγGCS6His total protein; lane 6, T7LmγGCS6His flow through; lane 7, T7LmγGCS6His wash with 25 mM imidazole wash buffer; lane 8, T7LmγGCS6His elution with 250 mM elution buffer.

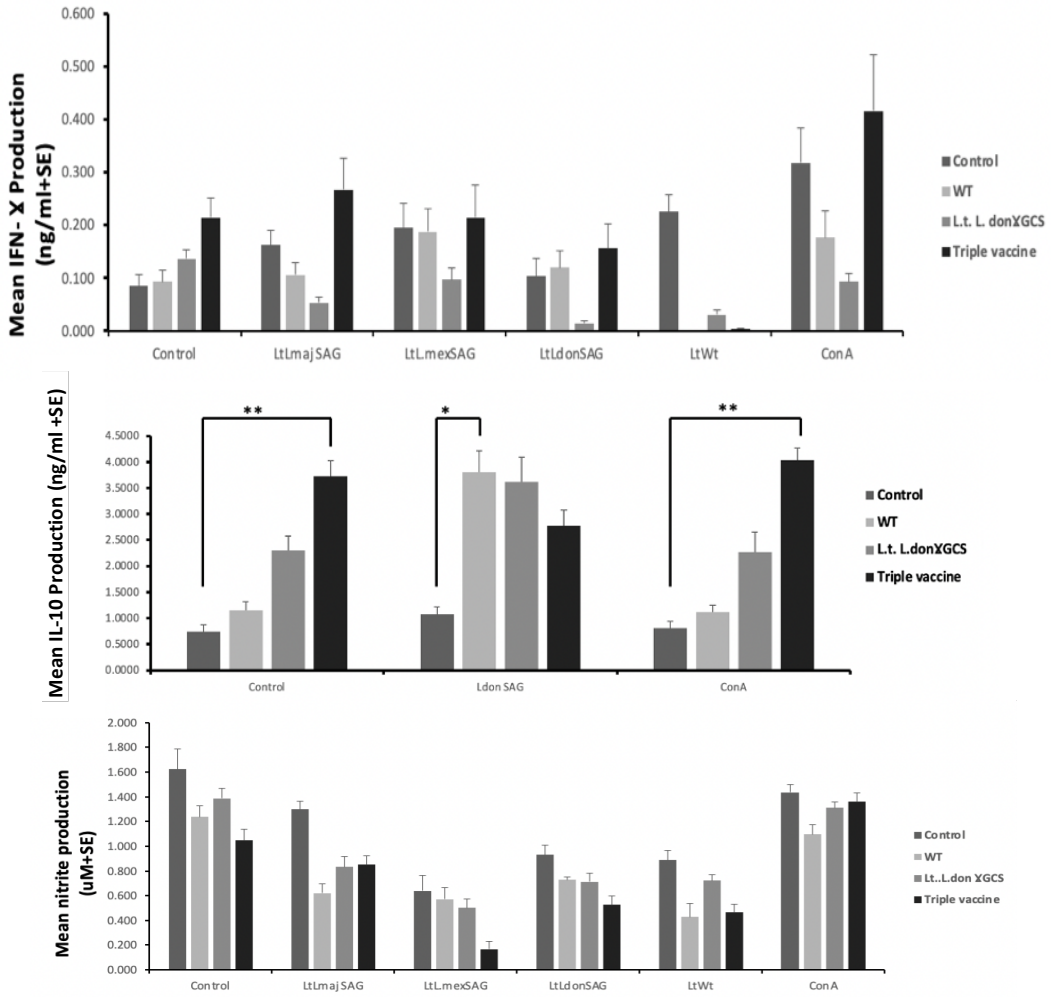
## *Appendix 3*



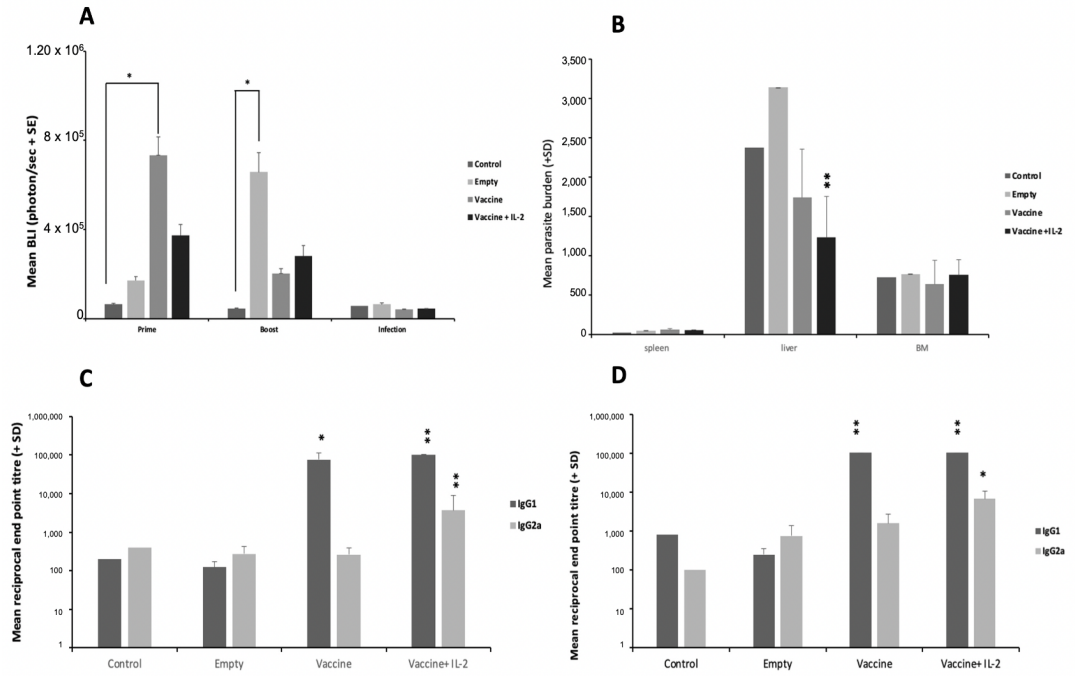

**Appendix 3.1** The effect of vaccination with different vaccines on neutrophil influx, antibody responses and parasite burdens of mice infected with *L. donovani*. Mice (n = 5/treatment) were immunised on days 0 and 21 with PBS alone (control), $1 \times 10^7$ *L. tarentolae* promastigotes (WT), $1 \times 10^7$ *L.t L.don* γGCS promastigotes (*L.t L.don* γGCS or a 1:1:1 mixture of *L.t L.don* gGCS, *L.t L.maj* gGCS, *L.t L.mex* gGCS transgenic parasites ($1 \times 10^7$, triple vaccine). The effect of vaccination on neutrophil recruitment 3 hours post-treatment, after injecting mice with luminol solution (150 mg/kg) and determining the amount of bioluminescence (mean bioluminescence, total flux, p/s) present at the injection site (A). Mice were infected on day 42 with $2 \times 10^7$ *L. donovani* amastigotes and parasite burdens were determined on day 56 (B). Specific *L. donovani* IgG1 (C) and IgG2a (D) antibody titres were determined over the course of the study. **P* < 0.05, ***P* < 0.001, ****P*< 0.0001 compared to control**.**



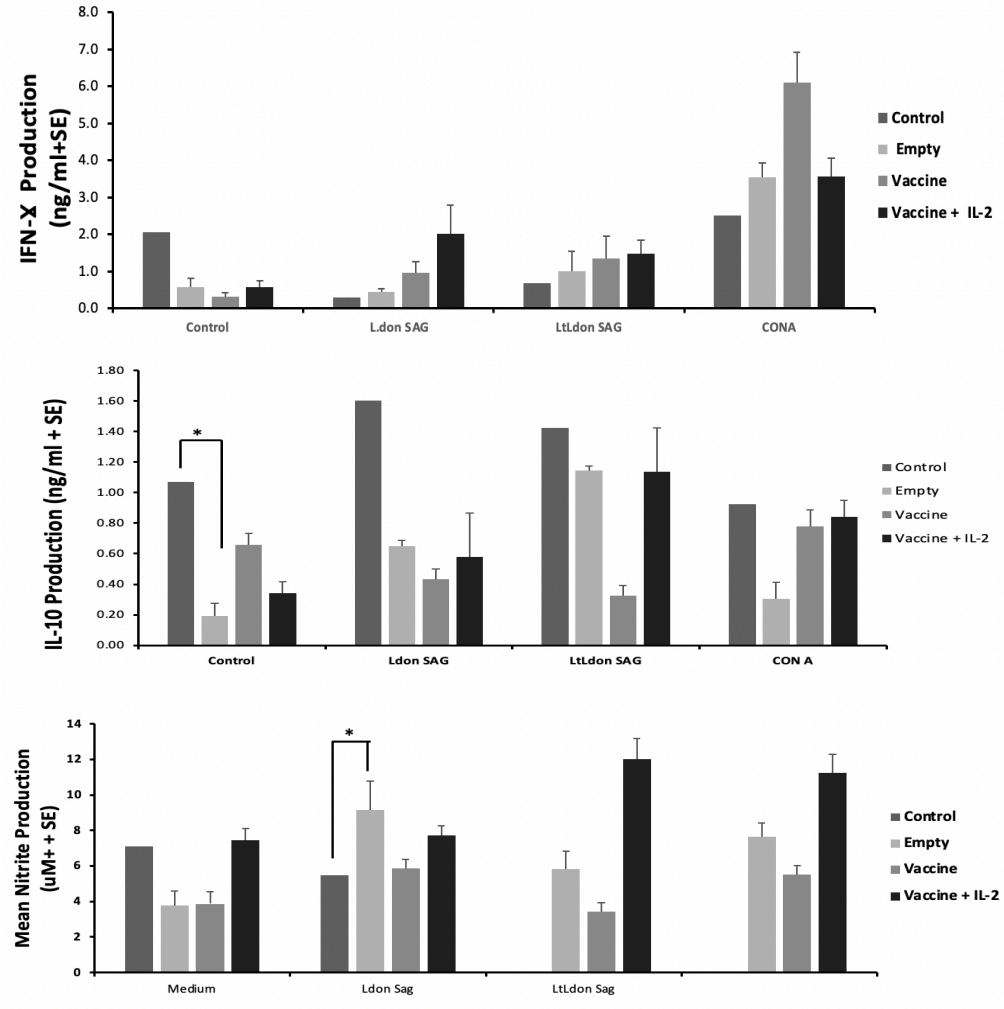

**Appendix 3.2** The effect of vaccination on IL-10 and nitrite production by splenocytes from control or vaccinated mice infected with *L. donovani*. Mice (n = 5/treatment) were immunised on days 0 and 21 with PBS alone (control) or $1 \times 10^7$ *L. tarentolae* promastigotes (WT), *L.t L.don* gGCS transgenic parasites (*Lt L.don* γGCS) or a 1:1:1 mixture of *L.t L.don* γGCS, *L.t L.maj* γGCS, *L.t L.mex* γGCS transgenic parasites ($1 \times 10^7$, triple vaccine). On day 42 mice were infected with $2 \times 10^7$ *L. donovani* amastigotes and on day 56 the study was terminated. Splenocytes ($5 \times 10^5$/mL) from each mouse were incubated with medium alone (controls), ConA (5 μg/mL) or *L. donovani* soluble antigen (50 μg/mL) for 72 h and the amount of IL-10 present in cell supernatants determined by ELISA (A); the amount of nitrite was determined using a Griess assay (B). *$P < 0.05$ for the groups compared.




**Appendix 3.3** IFN-g production by antigen and ConA stimulated splenocytes (A), IL-10 (B) and nitrite production (C) by splenocytes from control or vaccinated mice infected with *L. donovani*. Mice (n = 5/treatment) were immunised on days 0 and 21 with PBS alone (control) or $1 \times 10^7$ *L. tarentolae* promastigotes (WT) *L.t L.don* γGCS or a 1:1:1 mixture of *L.t L.don* γGCS, *L.t L.maj* gGCS, *L.t L.mex* γGCS transgenic parasites ($1 \times 10^7$, triple vaccine). On day 42, the mice were infected by intravenous injection with $2 \times 10^7$ *L. donovani* amastigotes and sacrificed on day 56. Splenocytes ($5 \times 10^5$/mL) from each mouse were incubated with medium alone (controls), ConA (5 μg/mL) or soluble antigen (50 μg/mL) for 72 hr and cytokines amount of present in cell supernatants determined by ELISA and the amount of nitrite was determined using a Griess assay. *$P < 0.05$, **$P < 0.001$ compared to control**.**




**Appendix 3.4** The effect of vaccination with different vaccines on parasite burdens and the immune responses of *L. donovani* infected mice. BALB/c mice (n = 5/treatment) were immunised on days 0 and 21 with PBS alone (control), PODS-Empty (50 million/mouse), $1 \times 10^7$ *L.t L.don* γGCS promastigotes alone (vaccine) or $1 \times 10^7$ *L.t L.don* γGCS promastigotes mixed with PODS-IL-2 (50 million/mouse, vaccine + IL-2). The effect of vaccination on neutrophil recruitment 3 hours post-treatment, after injecting mice with luminol solution (150 mg/kg) and determining the amount of bioluminescence (Mean BLI, total flux, p/s) present at the injection site (A). Mice were infected on day 42 with $2 \times 10^7$ *L. donovani* amastigotes and parasite burdens were determined on day 56 (B). The specific *L. donovani* antibody of mice on day 42 (C) and day 56 (D) are shown. **P* < 0.05, ***P* < 0.001 compared to control.




**Appendix 3.5** The effect of vaccination on the amount of IFN-g, IL-10 and nitrite produced by spleen cells from *L. donovani* infected mice. BALB/c mice (n = 5/treatment) were immunised on days 0 and 21 with PBS alone (control), PODS-Empty (50 million/mouse), $1 \times 10^7$ *L.t L.don* γGCS promastigotes alone (vaccine) or $1 \times 10^7$ *L.t L.don* γGCS promastigotes mixed with PODS-IL-2 (50 million/mouse, vaccine + IL-2). On day 42, the mice were infected by intravenous injection with $2 \times 10^7$ *L. donovani* amastigotes and sacrificed on day 56. Splenocytes ($5 \times 10^5$/mL) from each mouse were incubated with medium alone (controls), ConA (5 μg/mL) or soluble antigen (50 μg/mL) for 72 hr. The amount of amount of IFN-g (A) and IL-10 (B) produced by cells was determined by ELISA and the amount of nitrite (C) produced by cells was determined using a Griess assay. *$P < 0.05$ for groups indicated.

## *Scientific Activities*

### Poster

- Development of a vaccine against leishmaniasis. Derya Topuz Ata, Dr K. Chris Carter, Dr Martin Wiese. British Society for Immunology Congress December 2022, Liverpool

### Oral presentations

- Development of a vaccine against leishmaniasis. Derya Topuz Ata, Dr K. Chris Carter, Dr Martin Wiese. British Society for Immunology Summer School, September 2022, Coventry
- Development of a vaccine against leishmaniasis. Derya Topuz Ata, Muattaz Hussain, Michael Jones, Jonathan Best, Martin Wiese, K. Christine Carter · 17th International Conference on European Immunology April 2023, Roma.

### Publication

- Immunisation with Transgenic *L. tarentolae* Expressing Gamma Glutamyl Cysteine Synthetase from Pathogenic Leishmania Species Protected against *L. major* and *L. donovani* Infection in a Murine Model. Derya Topuz Ata, Muattaz Hussain, Michael Jones, Jonathan Best, Martin Wiese, K. Christine Carter. Microorganisms, 2023-05, DOI: 10.3390/microorganisms11051322.