Strathclyde Institute of Pharmaceutical and Biomedical Sciences

Development of Technologies for a *Toxoplasma gondii* Vaccine

DALIA AHMED AL-TAIE

Thesis presented in fulfilment of the requirement for the degree of

Doctor of Philosophy

2019

Declaration

Acknowledgments

This thesis would not have been possible without the help and support of many individuals. I would especially like to thank my supervisor Prof. Craig W Roberts for his expert guidance, invaluable advice and unfailing support. I particularly appreciate his meticulous attention to detail, his patience in explaining things in the simplest terms and for his promptness in providing feedback to drafts and responding to email queries. I would like to thank my second supervisor Dr. M. Wiese, I extend my continual appreciation for his keenness and support in all my studies. I have learnt much from him during the time I spent at laboratory. Thanks to the BPU staff for being always so friendly and helpful.

I would like to extend my thanks to the Ministry of Higher Education in Iraq and Baghdad University for awarding me this PhD scholarship and providing me with the financial support to study in the United Kingdom.

In a more personal level, I would like especially thank my mother, my sister and my brother who supported and encouraged me during this journey. I would like to dedicate this thesis to soul of my father (***I wish you could have been with me at this moment to share this with me)***

Conferences and Courses Attended

- Presenting a poster at Glasgow University entitled "Expression and Purification of Recombinant *Toxoplasma gondii* Vaccine Candidates (SAG1 and Cyc18) in *Leishmania tarentolae*" as a part of WSIG showcase in September 2017.

- Attending a workshop for bioinformatics at Glasgow University on 16 October 2017.

- Attend a workshop for Jalview training course at Dundee University on 23 November 2017.

- A poster presenting entitled "Expression and Purification of Recombinant *Toxoplasma gondii* Vaccine Candidates (SAG1 and Cyc18) in *Leishmania tarentola*e "at international conference DGP 18 BERLIN, the 28th ANNUAL Meeting of the German Society for Parasitology 21-24th of March 2018 at FREIE UNIVERSITAT, Berlin, Germany.

- Oral presentation at Toxo2018, held at Imperial College London on 1st and 2nd November 2018.

- Membership British Society for Parasitology 2016-2017.

- Personal license number: SAB/SCT-W15/85, date 30th October 2015.

Abbreviations

APC	Antigen presenting cell
Amp	Ampicillin
BAG1	Bradyzoite antigen 1
BSA	Bovine Serum Albumin
bp	Base pairs
CCR	Chemokine receptor
CD	Cluster of differentiation
CMI	Cell mediated immunity
CNS	Central nervous system
DC	Dendritic cell
ddH_2O	Double Distilled Water
DTT	1,4-dithiothreitol
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylene diamine tetraacetic acid
ER	Endoplasmic Reticulum
FCS	Foetal calf serum
GPI	Glycosylphosphatidylinositol
gDNA	Genomic DNA
HIV	Human immunodeficiency virus
HSP	Heat shock protein
HRP	Horse Radish Peroxidase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iNOS	inducible nitric oxide synthase
kDa	kilo-Dalton

LB	Luria-Bertani (broth)
LPS	Lipopolysaccharide
MΦ	Macrophage
MCP	Monocyte chemotactic protein
MHC	Major histocompatibility complex
Mic	Microneme
MyD88	Myeloid differentiation primary response gene 88
NISV	Non Ionic Surfactant Vesicles
NK	Natural killer
NO	Nitric oxide
ORF	Open Reading Frame
OT	Ocular toxoplasmosis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PV	Parasitophorous vacuole
PVDF	Polyvinylidene Fluoride
RFLP	Restriction fragment length polymorphisms
RNA	Ribonucleic acid
ROI	Reactive oxygen intermediates
ROP	Rhoptry
RPM	Revolutions per minute
SDS	PAGE Sodium dodecyl sulphate polyacrylamide gel electrophoresis
S.E.M	Standard error of the mean
SSU	Small ribosomal Subunit
STAT	Signal transducer and activator of transcription
TE	Toxoplasmic encephalitis
TGF	Tumor growth factor
TgHSP70	*T. gondii* heat shock protein 70
Th	T helper
TLA	*Toxoplasma* lysate antigen
TLR	Toll like receptor
TNF	Tumor necrosis factor
Treg	T regulatory

Contents

List of tables

List of Figures

Abstract

Studies were undertaken to test the utility of Surface Antigen 1 (SAG1), a surface glycoprotein and Cyclophilin 18 (TgCyc18) a chemokine mimic specific for CCR5 as a vaccine in a murine model of *T. gondii* infection. SAG1 has been shown to provide some protection against infection in previous studies. As Cyc18 induces, IL-12 when it binds to CCR5 it could act as an adjuvant to drive Th1 and $CD8^+$ T cell development. As SAG1 is known to undergo post-translational modification, initial experiments focused on development of a novel eukaryotic expression system. The system as envisaged would utilise heterologous expression of filamentous *L. mexicana* secreted acid phosphatases fused to the proteins of interest in *Leishmania tarentolae.* It was anticipated that these filaments would facilitate purification. Plasmids were constructed and transfected into *L. tarentolae*. Recombinant parasites were found to express and secrete the fusion protein into the media. The identities of proteins were confirmed by immunoblot, immunofluorescence, ELISA, and mass spectrometry. Levels of secreted proteins were too low for vaccination studies. DNA vaccination was used as an alternative approach. TgSAG1 and TgCyc18 genes were cloned into the eukaryotic expression vector (pVAX-1). pVAX-Cyc18 induced IL-12 in murine bone marrow-derived dendritic cells. The inclusion of TgCyc18 gene in the vaccine augmented Th1 cell activation in BALB/c mice as their splenocytes produced significantly greater levels of IFN-ɣ, TNF-α, IL-2 and IgG2a compared with mice vaccinated with SAG1 alone. These mice also had reduced weight loss compared with mice vaccinated with SAG1 alone. Addition of SAG4 and SAG4.2 genes to this vaccine cocktail conferred significant protection following oral challenge with *T. gondii* cysts as measured by the reduction of mortality rate and reduced body weight

loss. These results demonstrate that TgCyc18 has potential as adjuvant in multicomponent vaccines where a Th1 response is required.

Chapter 1

Introduction

Chapter 1: Introduction

1.1 History and Classification

Toxoplasma gondii (*T. gondii*) is a protozoan parasite belonging to the Apicomplexa phylum. It was discovered by Nicolle and Manceaux in 1908 when they were working on a small north African rodent called *Ctenodactylus gundi* (Nicolle and Manceaux 1908). *T. gondii* was so named due to its morphology (Toxo= meaning arc or bow, Plasma=meaning life) and gondii from the rodent in which it was discovered. The same parasite was described in a rabbit in Brazil by Splendore in 1908 and it was originally mistaken for *Leishmania* (Splendore, 1908). *T. gondii* was ultimately classified under the Apicomplexa phylum as shown in table 1.1 (reviewed, Dubey, 2009). *T. gondii* is an obligate, intracellular, ubiquitous protozoan parasite with the ability to infect all mammals and birds as intermediate hosts. The Felidae (especially domestic cats) family is considered the definitive host as the sexual reproduction cycle is only known to occur in this host (Weiss & Dubey, 2009). Since cats are known to be a source of the disease, they have been considered to be a health problem for both wildlife and humans (Montoya & Remington, 2008). Humans normally acquire infection with *T. gondii* by consumption of infected raw meat from intermediate hosts like sheep, goats and cattle, or by oral ingestion of food or water contaminated with oocysts from cat feces (Montoya & Remington, 2008). Environmentally resistant oocysts are responsible for spreading the infection to herbivores via contaminated food and water (Dubey, 2010). In addition to waterborne outbreaks, oocysts have been known to cause infections via aerosol exposures and to people working in the garden (Jones *et al.*, 2006). In addition, other routes of infection include congenital transmission and through tissue transplantation. Toxoplasmosis is considered as a zoonosis.

Table 1.1 classification of *T. gondii*

Kingdom	Protista
Phylum	Apicomplexa
Class	Sporozoa
Subclass	Coccidiasina
Order	Eucoccidiorida
Suborder	Eimeriorina
Family	Sarcocystidae
Subfamily	Toxoplasmatinae
Genus	*Toxoplasma*
Species	*gondii*

1.2 Population Structure of *T. gondii* Varies by Geographical Location

Genetic analyses are used to distinguish the genetic markers of *T. gondii* in different geographical countries. *T. gondii* was long thought to have three clonal lineages with less than 1% differences in their genetic composition (SU *et al*., 2010). These three clonal lineages were simultaneously named as type I, II and III also defined as type 1, 2 and 3, by the 2 groups of researchers that classified them by restriction length polymorphisms (Sibley *et al.*, 2009; Dardé *et al.*, 1992). Other genotypes (IV and V) were rarely distinguished and they were named as exotic or atypical because for their genetic polymorphisms that do not fit with the three clonal lineages and these strains include recombinant allele genotypes derived from types I, II, III (Su *et al.*, 2012). Type I strains have been associated with congenital and ocular toxoplasmosis (reviewed, Hakimi *et al.*, 2017). Thus, type I strains have the ability to cross blood- brain barrier, placenta and intestinal epithelium and that seemed to be related with its virulence (Barragan & Sibley, 2002). Furthermore, strains once referred to as atypical but now known to be common in some geographical regions including South America have been reported to be responsible for severe acute infections in patients in South America and Africa (Saeij *et*

al., 2005b). Type II strains are associated with *T. gondii* infection in North America and Europe (Saeij *et al.*, 2005b). For instance, type II of *T. gondii* is mostly dominant in UK followed by type III (Herrmann *et al.*, 2012). *T. gondii* type II strains are often considered to be the most common in prevalence (but this might be an artifact of geographical sampling bias) and account for 95% of congenital toxoplasmosis cases in France from 2002 through 2009 (Ajzenberg *et al.*, 2015). However, recently the Advisory Committee on the Microbiological Safety of Food reported that less information is available for *T. gondii* incidence for Scotland (Burrells *et al.*, 2016). Some polymorphisms in specific molecules between types of *T. gondii* are known to directly influence virulence as discussed in section 1.4.

Type I, II and III strains are relatively common in domestic and wild animals in North America and Europe (Mondragon *et al.*, 1998). Whereas, several diverse genotypes of *T. gondii* are associated with severe infections in agricultural animals in South America (Cañón-Franco *et al.*, 2013; Dubey *et al.*, 2012).

1.3 Incidence of Infection Varies by Geographical Location

The estimated incidence of *T. gondii* infection in humans is about 500 million individuals worldwide (Weiss & Dubey, 2009). Absolute incidences of infection vary in geographical areas and populations according to differences in the type of consumed food, environmental conditions and the dominance of animal species used in the food industry. Incidences in warm climates are greater than cold climates and incidences tend to be lower in mountainous countries (Barbosa *et al.*, 2009). Generally, the serological prevalence is about 25% of the world's human population (reviewed, Hakimi *et al.*, 2017). It has been shown that seroprevalence in USA is 10-20%; South and Central America:

50–80%; Eastern and Central Europe: 20–60%; Middle East: 30-50%; Southeast Asia: 20–60% and Africa: 20–55% (Pappas *et al.*, 2009). In healthy adults, toxoplasmosis produces a relatively mild infection, with elevated fever, enlarged lymph nodes, and muscle weakness (Petersen, 2007). Whereas, in immunocompromised adults, severe infections occur, typically from a reactivation of chronic infection, as seen in AIDS, organ transplant, or chemotherapy patients (Montoya & Liesenfeld, 2004).

1.4 Population Structure and Genetic Diversity of *T. gondii*

T. gondii differs according to the virulence and the genotype of the strain (Badr *et al*., 2016; Burrells *et al*., 2013). The three distinct clonal lineages of *T. gondii* strains differ genetically by 1% or less according to PCR-RFLP markers (restriction fragment length polymorphisms) single nucleotide polymorphisms (SNP) (Ferreira *et al.*, 2008; Khan *et al.*, 2005) and isoenzymes (Sibley *et al.*, 1992; Dardé *et al.*, 1992). Strains of type I genotype are highly virulent in mice, as lethal dose is a single viable parasite, regardless of the genetic background of the mouse host. Type I strains are differentiated by their high mortality with rapid death in mouse model, and level of acute virulence diseases in immunocompromised patients (Saeij *et al.*, 2005b; Barragan & Sibley, 2002). In contrast, type II and III strains are considered less virulent in lab models and have 50% lethal doses (LD50) of 10^2 to > 10^5 parasites and the effect of infection is dependent on the genotype of the host (Saeij *et al.*, 2005a). The distribution of SNPs revealed genetic crosses by sequencing of large numbers of cDNAs from the three clonal lineages that occurred in between highly similar parental strains (reviewed, Hakimi *et al.*, 2017). It is now recognised that the population structure of *T. gondii* is more complex than initially

believed as more samples from South America, Africa and Southeast Asia are analysed (Minot *et al.*, 2012; Lorenzi *et al.*, 2016).

1.5 Ultrastructure of *Toxoplasma gondii*

The ultrastructure of *T. gondii* has been extensively investigated by electron microscopy (Dubremetz & Ferguson, 2009). *T. gondii* possesses organelles like other eukaryotic cells: the nucleus is located at the posterior end of the parasite with the endoplasmic reticulum (ER) behind it. At the anterior end, there are rhoptries, dense granules and micronemes. The parasite has one single mitochondrion and an apicoplast. The three secretory organelles, rhoptries, dense granules and micronemes, and the proteins they secrete are essential for attachment, invasion and modification of the host cells. The proteins they produce are designated as ROP, GRA and MIC followed by a number (McFadden, 2011). The ultrastructure of *T. gondii* is shown in figure (1.1).

1.5.1. Rhoptries are located at the apical end of the parasite and they are the largest of the three secretory organelles. They are club-shaped and their size varies with the size of the life cycle stage (Hajj *et al*., 2006). They manufacture secretory proteins and enzymes and they possess a narrow apical duct and bulbous basal region. They synthesise pre-proteins, which are modified before secretion (Sadak *et al*., 1988). Dubremetz and Fergusson (2009) distinguished 30 rhoptry proteins, which are released during invasion. Some ROP proteins are associated with the parasitophorous vacuole membrane that aids the parasite to survive within host cells (Dubremetz & Ferguson, 2009). A number of ROP proteins are known to be immunomodulatory (see section 1.13).

1.5.2. Micronemes

Micronemes are membrane-bound organelles located at the anterior end of the parasite. To date few proteins have been directly identified in positioning or trafficking along microtubules (reviewed, Dubois & Soldati-Favre, 2019). However, there are 13 distinct proteins designated MIC 1-13 (Fritz *et al*., 2012). Microneme proteins have important roles in adhesion, host cell invasion, and critically participate in egress from infected cells (Soldati *et al.*, 2001).

1.5.3. Dense granules

Dense granules are known to contain at least 16 dense granules and 2 Nucleotides triphosphate hydrolases (NTPases) (designated NTPases I and II). These proteins were originally identified in the tachyzoites and are known to be secreted from the apical tip of the parasite. Secretion of many soluble proteins is through fusion with the plasma membrane and is the default pathway for proteins either associated with cell invasion or maintenance of the parasitophorous vacuole membrane (PVM), like GRA5 and GRA7 (Mercier *et al*., 2002).

1.5.4. Apicoplast

The apicoplast organelle is a secondary plastid originating from an endosymbiotic red algae-like organism (Wilson *et al.*, 1996). It is similar to a chloroplast in some respects, but lacks the ability to photosynthesise. *T. gondii* has an apicoplast, which is important for parasite survival (Waller *et al.*, 1998). The apicoplast has essential roles in metabolism of fatty acids for *T. gondii* survival through providing lipid substrates required for the final step of parasite division (Martins-Duarte *et al.*, 2016).

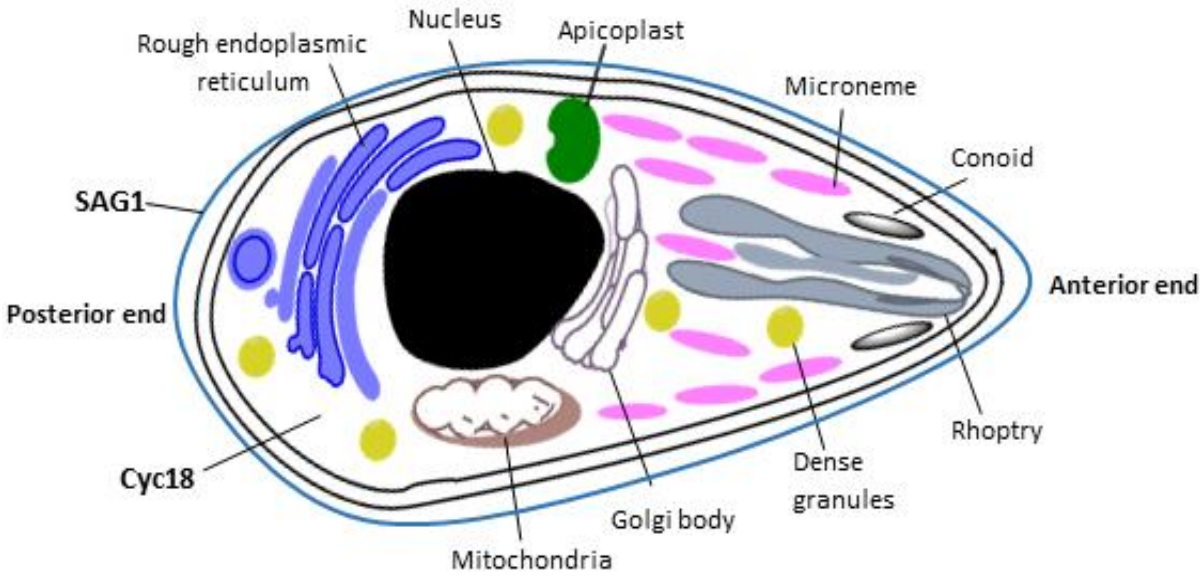


Figure 1. 1 Ultrastructure of *T. gondii*.

It demonstrates the anterior and the posterior ends as well as the distribution of the organelles in them. The SAG-related sequences (SRS) family proteins including SAG1. *T. gondii* contains many cytosolic proteins including Cyc18 (adapted from Ajioka *et al*., 2001).

1.6 Transmission of *T. gondii*

T. gondii can infect the host by many routes and by different life stages illustrated in figure (1.2). Ingestion of oocysts in contaminated water or in food is a common route of infection and has been associated with localised outbreaks of disease (Ferguson, 2009). Ingestion of tissue cysts from raw or undercooked meat from an infected intermediate host is another common means of infection in humans. If an intermediate host is pregnant when they were infected, or in some cases, carrying a chronic infection then vertical transmission of *T. gondii* can occur from infected mother to the fetus. Research suggests that tachyzoites can cross the placenta and disseminate into the foetal circulation to cause infection (Ajzenberg *et al.*, 2015).

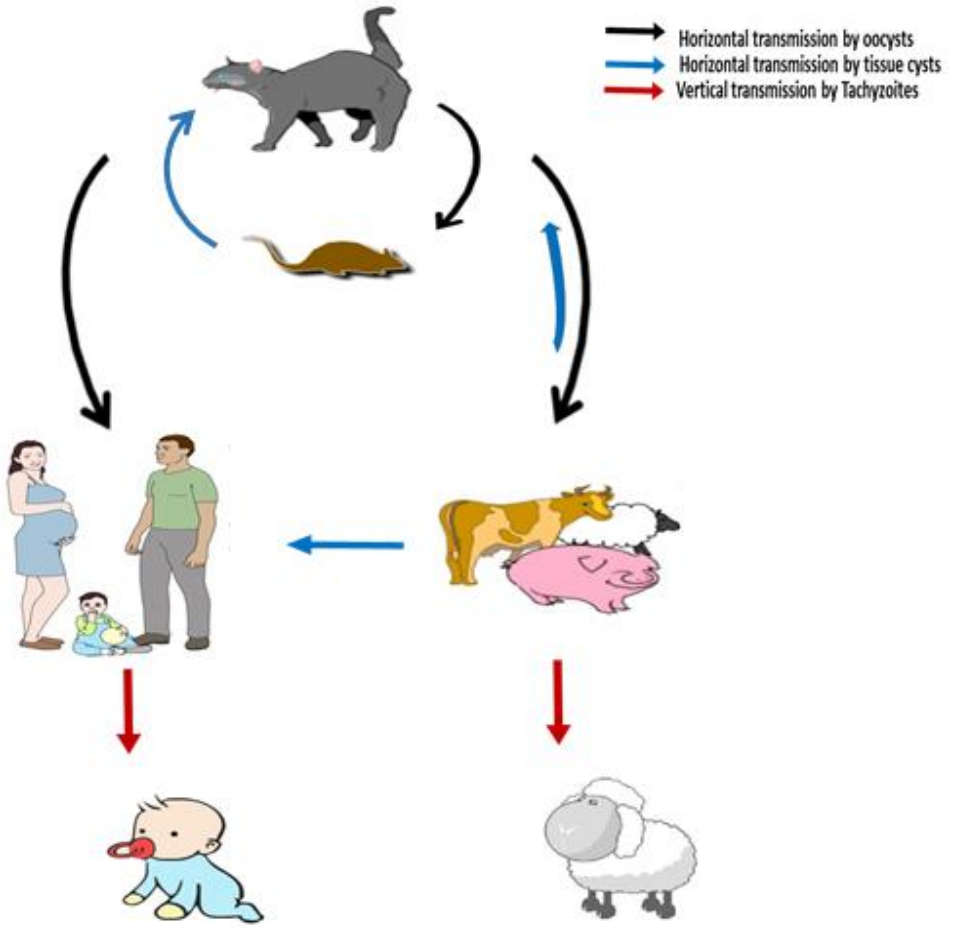


Figure 1. 2 Transmission of *T. gondii*

The cat is the final host and its feces includes the oocysts, which are transmitted horizontally (black and blue arrows) to the intermediate hosts. Most mammals, such as humans, cows, and sheep are considered intermediate hosts. Consumption of uncooked meat (contains tissue cyst) is another horizontal route of parasite transmission.There is a vertical transmission (see red arrows) from the infected mother to the fetus. During pregnancy the parasite may be vertically transmitted in all hosts.

1.7 Life Cycle of *T. gondii*

1.7.1 Extra-intestinal Phase

The extra-intestinal phase may also be called asexual phase and occurs in all intermediate hosts. After ingestion of oocysts or tissue cysts and exposure to the acidic gastric environment, sporozoites (from oocyst) or bradyzoites (from tissue cysts) are released. These infect the gut epithelial cells. After 12-18 hours these stages, namely, bradyzoites or sporozoites are converted to tachyzoites. This occurs within parasitophorous vacuoles (PV) (Hehl *et al.*, 2015; Dubey *et al.*, 1998). The proliferative tachyzoite form can infect almost any cell and divide within the host by a specialised process called endodyogeny, which was first described by Goldman *et al* (1958). Tachyzoites disseminate throughout the host via lymph and blood. Cells of the immune system such as macrophages and dendritic cells are infected by the parasite and have been reported to act as “Trojan horses” to help dissemination (Lambert *et al.*, 2009). Fast dividing tachyzoites in acute infection convert into slow dividing bradyzoites in the chronic phase. The conversion of tachyzoites is characterised by changes in gene expression and protein contents including differential expression of surface antigens (Lyons *et al.*, 2002).

Tissue cysts are found mostly in the brain, skeletal and cardiac muscles and these cysts may exist in the host tissues for the life of the immunocompetent host without causing overt illness (Hunter and Riechman, 2001). However, disease and reactivation of infection associated with tachyzoite multiplication can occur in the host after a decrease of immunity due to neoplasia, HIV infection or post-transplant immunosuppressive treatment.

1.7.2 Intra-epithelial Phase

The intra-epithelial phase refers to the sexual and asexual stages of *T. gondii* development that occurs exclusively in the intestinal cells of cats. Infection of cats is normally and most efficiently initiated after ingestion of tissue cysts, but can also be initiated by oocyst ingestion. Intestinal cells are infected by bradyzoites or sporozoites released from tissue cysts or oocysts, respectively, which then divide asexually by a process of schizogony to produce merozoites. Merozoites are released and infect new cells where they form macrogametes or microgametes in a process called gametogenesis (Hehl *et al*., 2015). The macrogamete is fertilised by a microgamete to form the zygote, which eventually forms the oocyst. Cats start to shed oocysts within 10 days after ingestion of bradyzoites. This time between infection with a bradyzoite and the demonstration of the oocyst in the feces is referred to as the prepatent period (Murata *et al.*, 2017).

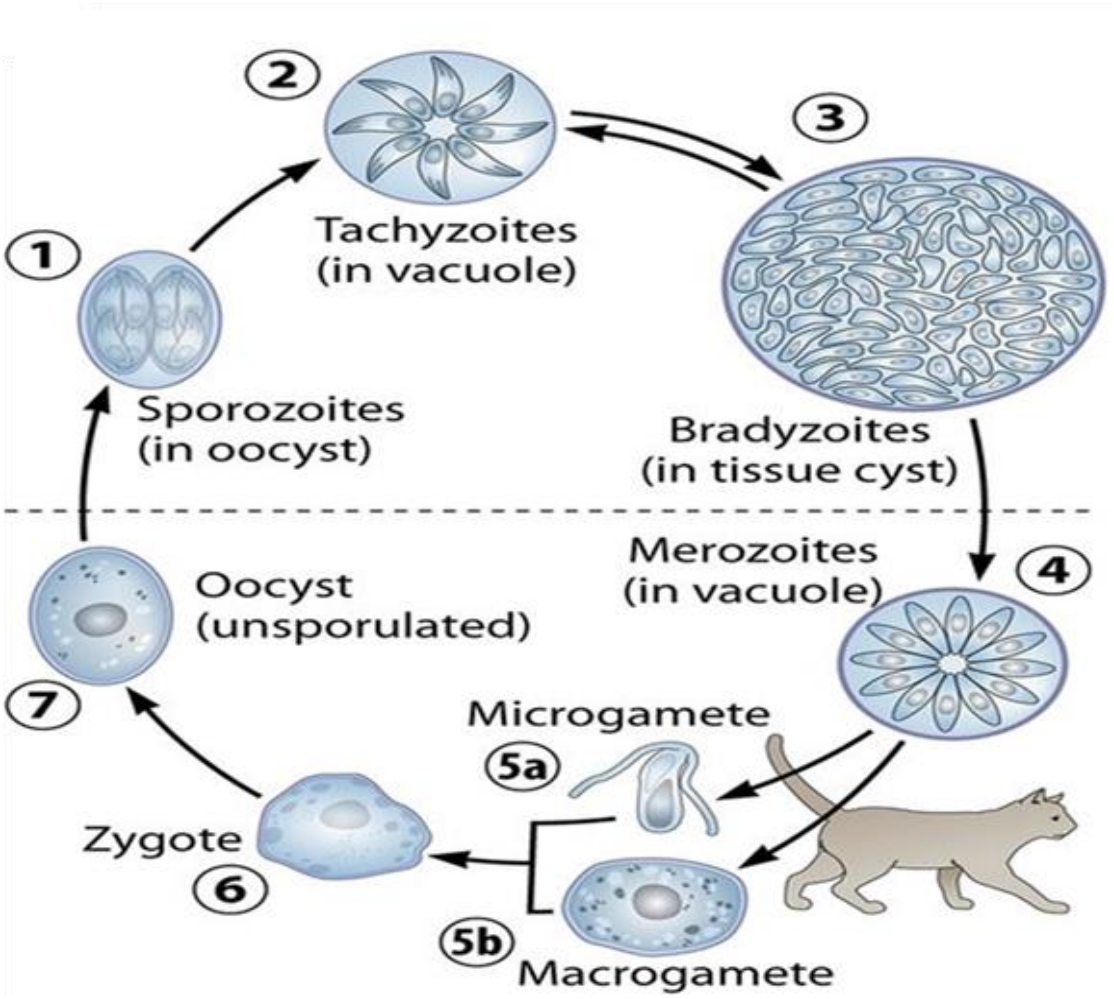


Figure 1. 3 Extra-intestinal and intra-epithelial phases of *T. gondii* life cycle

(1) Sporulated oocyst. Tachyzoites (2) are converted into bradyzoites (3) in the host cell cyst. Ingestion of the cyst by the cat will release the bradyzoites (4), which infect the epithelial cells of the cat giving rise to merozoites. Sexual division starts by differentiation into microgamete (5a) and macrogamete (5b) by gametogenesis. The microgamete fertilises the macrogamete to form the zygote (6). Subsequently this zygote develops into a non-sporulating oocyst (7), which is excreted in the feces of the cat (adapted from Morrissette, 2015).

1.8 Adult Acquired Toxoplasmosis

Acquired toxoplasmosis has a vast diversity in clinical symptoms, which are related to the *T. gondii* clonal lineage, the host immune status, the host genetic susceptibility, and the life- and cell cycle stage initiating the infection. Most infections are asymptomatic and are rarely diagnosed as toxoplasmosis and left untreated to become a chronic infection with no overt disease symptoms (Ajzenberg *et al*., 2016). Neuropsychiatric disease such as, schizophrenia, bipolar disease, memory loss and dementia symptoms have been associated in some studies with chronic *T. gondii* infection, but alternatively they could be a predisposing factor for the infection (Godwin, 2012; Guenter *et al.*, 2012). Depression of intracellular immunity can result in disease reactivation and overt clinical disease (see below). Nevertheless, highly virulent strains (exotic or recombinant strains) especially as observed in South America are known to cause severe toxoplasmosis even in immunocompetent patients (Montoya and Liesenfeld, 2004). Chronic infections with these exotic strains might cause ocular disease, toxoplasmic encephalitis, mental retardation or even blindness (Montoya and Liesenfeld, 2004).

1.8.1 Toxoplasmosis in the Immunocompromised Host

The immune status of the host plays an important role in the pathogenicity of *T. gondii* infection. Immunocompromised patients often develop atypical encephalitis, life-threatening pneumonitis, myocarditis, and sight-threatening and severe retinochoroiditis (Lihtech, 2005). The most common clinical symptoms in HIV patients are encephalitis, usually accompanied by multiple brain lesions. Toxoplasmic encephalitis normally develops within days to weeks following infection and often includes neurologic abnormalities like weakness and speech disturbances, headache, fever, confusion,

lethargy, and seizures (Montoya and Liesenfeld, 2004). Patients also may have cranial nerve abnormalities, visual defects, movement disorders, stroke, personality changes, and other neuropsychiatric abnormalities. *Toxoplasma* can also cause extracerebral toxoplasmosis, which affects both the eyes and the lungs. Patients with choriodoretinitis typically develop blurred vision, pain, or photophobia (Rabaud *et a*l., 1994).

1.9 Congenitally Acquired Toxoplasmosis

While this is generally said to only occur in humans if the person is infected for the first time, an increasing number of exceptions have been reported where vertical transmission has occurred in a chronically infected pregnant woman (Andrade *et al.*, 2010). In addition, vertical transmission from a chronic infection has been well documented in some strains of mice and at least one strain of sheep (Chessa *et al.*, 2014; Roberts & Alexander, 1992). During gestation when the mother becomes infected it is difficult to determine whether the fetus is infected or not and what is the severity of the disease. In humans, the likely consequences of infection for the fetus are more severe during the first three months of pregnancy resulting in a high risk of abortion, or where the fetus is carried to term, mental retardation and or encephalitis. Maternal infection during the final months of pregnancy is more likely to results in congenital transmission, but is likely to cause less severe congenital disease or even be asymptomatic at birth. Spontaneous abortion is less likely when maternal infection occurs in the third trimester (Waldorf and McAdams, 2013; Allain *et al*., 1998). Congenital toxoplasmosis occurs in humans and other species of animals especially sheep, goats and rodents (Weiss 2013; Beverley,1959). The symptoms of congenital toxoplasmosis were described by (Sabin, 1942) and include hydrocephalus, chorioretinitis and intracerebral calcification. In humans, samples collected from amniotic fluid, placenta tissue and umbellical cord blood of the aborted fetus can be used for

diagnosis. The estimated rate of congenital toxoplasmosis was 500–5000 infants each year in the United States (Montoya & Remington, 2008). Although, many infants seem to be healthy at birth, serious disease and long-term sequelae may become clear months or years later (Montoya & Remington, 2008). It is widely believed that essentially all congenitally infected individuals will develop eye disease later in life.

1.10 Ocular Toxoplasmosis

Ocular toxoplasmosis can be the result of adult or congenital infection. The first isolation of *T. gondii* from an eye was from a thirty-years old male (Jacobs *et al.*, 1954). Virtually all cases of ocular toxoplasmosis before 1950 were considered a result of congenital transmission (Perkins, 1961). In a study executed from (1948 – 1951) 99% of 103 congenitally infected children had eye lesions (Feldman, 1953). However, another study by Burnett *et al*., (1998) found that 20 of 95 patients had ocular toxoplasmosis from acute toxoplasmosis in 1995 related with a Canadian waterborne outbreak. Ocular toxoplasmosis is the most common cause of posterior uveitis in the USA (Weiss and Kim, 2014). The common findings are retinochoroiditis that might lead to blindness. In Brazil, virulent atypical strains are now also associated with severe ocular disease even when acquired post-natally (Khan *et al.*, 2006a; Vallochi *et al.*, 2005). Due to the low volume of ocular fluid, diagnosis of ocular toxoplasmosis and genotyping analyses have been challenging. Moreover, ocular fluid samples are usually collected when there is a typical and/or severe case to perform genotyping analyses (Subauste *et al*., 2011). Therefore, it now appears that *T. gondii* infection can result in ocular toxoplasmosis even when acquired post-natally, especially in South America.

1.11 Treatment

Acute toxoplasmosis is mostly diagnosed in pregnant women by serology and is generally treated immediately with Spiramycin. Immunocompetent, non-pregnant patients are generally not treated unless severe symptoms continue beyond a few weeks. Treatment of pregnant mothers in early stages of infection with Spiramycin can decrease the possibility of congenital toxoplasmosis up to 60% (De Paschale *et al.*, 2008). Maternal treatment started within three weeks of seroconversion had a minor influence on reduction of vertical transmission (Thiébaut, *et al*., 2007). Spiramycin helps to prevent transmission to the fetus because it concentrates in the placenta (Buyukbaba *et al*., 2012; Couvreur *et al*., 1988). However, Spiramycin does not cross the placenta effectively and therefore is not a treatment of an infected fetus. Although newborns affected by congenital infection might seem normal at first examination, serious sequelae, such as neurological impairment and blindness can occur subsequently (Varella *et al.*, 2003). Therefore, PCR (polymerase chain reaction) testing of amniotic fluid is recommended at least four weeks after maternal infection. If PCR results are negative, Spiramycin is continued for the rest of the pregnancy. If the PCR is positive, alternative treatment (combination of Pyrimethamine and Sulfadiazine) are necessary to reduce the severity of congenital toxoplasmosis. This has been shown to increase the percentage of asymptomatic infants at birth (Laboudi and Sadak, 2017; Hohlfeld *et a*l.,1989; Daffos *et al*., 1988). Pyrimethamine can suppress bone marrow function and result in leucopenia, subsequent anemia and thrombocytopenia. Both drugs are considered as teratogenic in animals and can cause reversible renal failure when used in large doses. Due to the prospective toxicity of these drugs, they are only used if fetal infection has been documented during pregnancy. Azithromycin has effectively treated *T. gondii* in animal

models and in humans with AIDS (Godofsky, 1994; Araujo *et al*., 1988). In women intolerant to pyrimethamine, trimethoprim-sulfamethoxazole may be used. However, the safety and efficacy of these drugs for treating in utero toxoplasmosis are unknown (Hermann *et al*., 2017; Derouin *et al*., 2000).

1.12 Immune Response

Most of what we know about the immune response to *T. gondii* has been worked out from rodent models of infection. While many of the finding have been confirmed using human cells, differences have also been identified. The immune response is dependent on several factors: inoculum size, stage of life cycle causing the infection, route of infection, the parasite strain, as well as the immunological status and genetic susceptibility of the host species (Dutra *et al.*, 2013). *In vitro* studies have shown that different strains vary in their severity and immune responses they induce. It has been suggested that virulent strains might be more effective in triggering an immune response, tissue penetration and the ability to encyst compared with other strains (Kim, 2006; Robben *et al.*, 2004). There have also been a number of strain specific virulence factors identified that are known to influence the development of immunity at least in rodents.

Immune responses can be divided into innate and adaptive immune response and each has its role in the immunity. This will be explained in detail:

1.12.1 Innate Immune Response

1.12.1.1 Interaction Between *T. gondii* and Host Cells

The innate immune response controls *T. gondii* but does not provide long-term protection. *T. gondii* has a number of PAMPs (pathogen-associated molecular patterns), which include heat shock protein 70 (HSP70) and glycosylphosphatidylinositol anchors (GPIs) and profilin (Debierre-Grockiego *et al.*, 2007). Toll-like receptors (TLR) are proteins that play a role in the innate immune response. HSP70 and GPI-anchors interact with TLR2 and TLR4, respectively. While, profilin can ligate with TLR11 in mice and other mammals, but is not found in humans (Yarovinsky *et al.*, 2005). A signaling cascade is started with the production of cytokines (IL-12 and TNF-α) from antigen-presenting cells (dendritic cells, macrophages and neutrophils). Following infection with *T. gondii*, monocytes, neutrophils and dendritic cells (DCs) are recruited to the site of infections, and all of these cell types have been implicated in resistance to the parasite.

A. Neutrophils

Rapid infiltration of neutrophils to the site of infection has a significant role in the acute phase of *T. gondii* infection. The earliest recruited neutrophils have a phagocytic role (Bliss *et al.*, 2000). While some studies have suggested that neutrophils are also an the early source of IL-12 and TNF-α production in mice, there is evidence that monocytes are important for IL-12 production in humans (Ehmen & Lüder., 2019; Abdallah *et al.*, 2012). Consequently, neutrophils inhibit parasite proliferation in the early stages of infection (Bliss *et al.*, 2001). Approximately 85% of neutrophils exhibit intracellular storage of IL-12. Bliss *et al.* (2000) found that depletion of neutrophils during the first six days of infection leads to mortality in *T. gondii* infected mice, which was associated with decreased of IL-12 and IFN-ɣ production by splenocytes (Bliss *et al.*, 2000). Cytokines

produced by neutrophils are likely to influence the nature of downstream immune cell activation including macrophage activation and T cell subset differentiation.

B. Dendritic Cells (DC)

DCs are recognised as a source of IL-12 production during *T. gondii* infection (de Sousa *et al.*, 1997). DCs in murine spleen cells are positive for IL-12 after *ex vivo* stimulation with *T. gondii* tachyzoites. IL-12 is considered the most important inducer of IFN-ɣ synthesis during acute infection. DCs acquire parasite antigen by phagocytosis leading to upregulation of MHC expression and production of pro-inflammatory IL-12 cytokines (Aliberti *et al.*, 2003). IL-12 production from DCs has been known to be TLR-dependent resulting in signaling through MyD88 (Scanga *et al.*, 2002). Mice genetically deficient in MyD88, which is essential for most TLR signaling, are susceptible to avirulent *T. gondii* infection and exhibit an impaired IL-12 response (Scanga *et al.*, 2002).

Cytokine chemokine receptor 5 (CCR5) is upregulated on the surface of DCs after signaling from infected cells following IL-12 stimulation (Aliberti *et al.*, 2000). *T. gondii* cyclophilin-18 is recognised as the principal molecule derived from the parasite that triggers IL-12 production through binding host CCR5 (Aliberti *et al.*, 2003). Cyclophilin-18 from a viable parasite can influence the DC's function. Therefore, DCs are able to distinguish between live and dead tachyzoites (Aliberti *et al.*, 2003). Dendritic cells are also known to play significant roles in disseminating the parasite within the host as they are capable of migration and have been postulated to transport tachyzoites into tissues including the brain (Lambert *et al.*, 2009).

C. Macrophages and Monocytes

Macrophages can also produce IL-12 after activation by tachyzoites or tachyzoite-derived moieties (Gazzinelli *et al.*, 1996). The different *T. gondii* genotypes affect macrophages differently. Type I tachyzoites induce IL-12 production through MyD88-independent

mechanisms, while type II tachyzoites do so by both MyD88-dependent and -independent mechanisms (Robben *et al.*, 2004). Macrophages and monocytes like dendritic cells have been postulated to have a pathogenic role through facilitating dissemination of parasites during acute toxoplasmosis and they have been found in the brain and lymph nodes of infected mice during chronic infection (Suzuki *et al.*, 2005). Macrophages are capable of phagocytosis of tachyzoites and production of nitric oxide (NO) and reactive oxygen, which can mediate killing of *T. gondii* and inhibit parasite replication (Masek and Hunter, 2013).

IFN-ɣ-induced monocyte-derived macrophages (MDM) express indoleamine 2,3-dioxygenase (IDO), which degrades tryptophan leading to impair *T. gondii* growth (MacKenzie *et al.*, 2008; Gupta *et al.*, 1994; Pfefferkorn, 1984). Murakami *et al.* (2012) indicated that IDO-deficient mice have an increased lung tryptophan concentration compared to wild type after infection and this results in increased *T. gondii* levels (Murakami *et al.*, 2012). Furthermore, IFN-ɣ was shown to inhibit *T. gondii* replication in rat enterocytes by iron depletion (Dimier & Bout, 1998). Although, IFN-ɣ-activated human monocytes were shown to downregulate transferrin receptor expression to limit the growth of other microbes (Byrd & Horwitz, 1989).

D. Natural Killer Cells (NK)

NK cells are considered the third most abundant lymphocyte population and have an important role in IFN-ɣ production during acute toxoplasmosis (Backström *et al.*, 2004). NK cells become activated by IL-12, which is the main cytokine released by neutrophils, dendritic cells and macrophages in response to *T. gondii* infection. Other cytokines including TNF-α, IL-1b and IL-15 have a minor role compared with IL-12 activating NK cells (Carson *et al.*, 1995). SCID mice (severe combined immune deficiency mutant mice), which are susceptible to *T. gondii* infection in terms of mortality can be made more

susceptible by neutralisation of IL-12 or IFN-ɣ, indicating a protective role for NK cell activation and production of IFN-ɣ (Gazzinelli *et al.*, 1994). NK cells have direct cytotoxic effects on infected cells and are responsible for elimination or reduction of tachyzoites in acute infection via IFN-ɣ (Denkers *et al.*, 1996). NK cells also help the development of cytotoxic $CD8^+$ T cell and expansion of Th1 $CD4^+$ T cells and suppression of Th2 responses through IFN-ɣ.

1.12.2 Adaptive Immune Response

Long-term protection is dependent on the adaptive response*. T. gondii* proteins are cleaved in the proteasome then transferred to the endoplasmic reticulum (ER) then to Golgi apparatus through TAP (transporter associated antigen processing). *T. gondii* proteins are thus processed and presented on the surface of the cells in the context of major histocompatibility complex class I (MHCI). *T. gondii* proteins are also processed through the exogenous pathway and are thus displayed in the context of class II (MHC II) (York & Rock, 1996). After *T. gondii* infection, macrophages and NK cells are activated to produce cytokines, like IL-12 that would direct the cell mediated response of $CD4^+$ and $CD8^+$ T cells (Goldszmid *et al.*, 2007). The importance of the adaptive immune responses for resistance to *T. gondii* is exemplified by the increased susceptibility of mice experimentally depleted of $CD4^+$ T cells or $CD8^+$ T cells or SCID and nude mice that are congenitally deficient in these cells (Lütjen *et al.*, 2006).

1.12.2.1. T cells

Initial studies in the nude mouse established a crucial role for T cells in mediating protection against overwhelming fatal toxoplasmosis (Lindberg & Frenkel, 1977). In recent years, a number of studies has widely considered the role of the T cell subsets

and how they interact to mediate protection. $CD4^+$ and $CD8^+$ T cells are differentiated from naive helper T cells (Th0 cells) after the infection has occurred. T subsets can eliminate *T. gondii* or *T. gondii*-infected cells by a number of ways. $CD4^+$ T cells are capable of releasing IFN-ɣ, which can activate various intracellular killing mechanism as previously discussed. $CD8^+$ T cell can recognise and kill infected cells through the *T. gondii* peptide displayed on MHC class 1.

A. $CD4^+$ T Lymphocytes

Processing of the parasite proteins and their presentation on the surface of the APC (antigens processing cells) is critical for the activation of $CD4^+$ T cells (Debierre-Grockiego *et al.*, 2007). $CD4^+$ T cells are critical for *T. gondii* resistance in the mouse, the lack of $CD4^+$ T cells is associated with increased susceptibility during the chronic stage of infection (Hwang *et al.*, 2016). $CD4^+$ and $CD8^+$ T lymphocytes both control chronic infection by production of IFN-ɣ as treating infected mice with anti-$CD4^+$ and anti-$CD8^+$ monoclonal antibodies results in disease exacerbation (Gazzinelli *et al.*, 1992). Treated mice showed increased pathology and mortality (Gazzinelli *et al.*, 1992). $CD4^+$ T cells are also necessary to promote B cell responses as mice deficient in $CD4^+$ T cells display lower parasite-specific antibody titers (Ware *et al.*, 2012; Johnson & Sayles, 2002). Naive $CD4^+$ T cells may differentiate into one of several T cells, including Th1, Th2, Th17, TFH, and Treg, as defined by their pattern of cytokine production and function (reviewed, Zhu, Yamane & Paul, 2010).

A) Th1 and Th2 cells

Th1 and Th2 are differentiated from naive $CD4^+$ T cells after they are stimulated by APC cells to become effector and/or memory cells (Mosmann *et al.*, 1986). Th1 cell differentiation is favoured during *T. gondii* infection as IL-12 preferentially expands this

subset (Magram *et al.*, 1996). It has been shown that $CD4^+$ T cells can be made to differentiate into Th1 cells *in vivo* and *in vitro* by incubation with an IL-4 neutralising antibody and IL-12 (Le Gros *et al.*, 1990). Interleukin 4 (IL-4) is a cytokine that promotes differentiation of naive $CD4^+$ T cells into Th2 cells. Upon stimulation by IL-4, Th2 cells consequently yield additional IL-4 as a positive feedback (Van Gisbergen *et al.*, 2005). The cell that primarily produces IL-4, has not been recognised but previous studies concluded that basophils and mast cells could be the effector cell (Van Gisbergen *et al.*, 2005). Activated Th2 cells, subsequently produce IL-4, IL-5 and IL-13 cytokines, which can downregulate Th1-mediated killing mechanisms, but conversely protect against cytokine shock (Sokol & Medzhitov, 2010). A protective role for Th1 cells against *T. gondii* infection was implied by studies that selectively adoptively transferred T cell subsets or selectively depleted $CD4^+$ T cells and/or IFN-ɣ (Gazzinelli *et al.*, 1992). This was further reinforced with later studies of mice genetically deficient in IFN-ɣ or the IFN-ɣR (Yap & Sher, 1999). However, when mice deficient in IL-4 and IL-10 became available, it also became clear that Th2 cells (and Treg cells) were also important in limiting inflammation and mortality during *T. gondii* infection (Roberts *et al.*, 1996).

B) Th17, Treg and TFH Cells

Th17 cells were identified as the third major effector population of $CD4^+$ T cell. They were named this due to their production of IL-17A and IL-17F, although they also produce IL-21, and IL-22, which are not produced by Th1 or Th2 (Korn *et al.*, 2007; Harrington *et al.*, 2005; Cua *et al.*, 2003). The role of Th17 cells during *T. gondii* infection is controversial, as they have been shown to be required for control of *T. gondii* infection in mice. IL-17A-deficient mice were highly susceptible to *T. gondii* infection during the acute phase and

decreased the survival rate of infected mice compared with wild type mice against *T. gondii* infection (Moroda *et al.*, 2017).

Treg cells are also known as suppressor T cells. Treg can modulate the immune system and prevent autoimmune disease by down regulation of the proliferation of effector T cells, largely due to their production of IL-10 (DiPaolo *et al.*, 2007). TGFβ, is essential for differentiation of Treg from naive $CD4^+$ cells and for Treg maintenance (reviewed, Chen, 2011). Treg cells would appear to be important in limiting excessive inflammation and thus have a beneficial role in murine models of infection (Fonseca *et al.*, 2012).

TFH have a helper function for antibody production. TFH cells are responsible for induction and control of class-switching immunoglobulin production and plasma cell differentiation via IL-21 production (Reinhardt *et al.*, 2009). Recently, a beneficial role for TFH cells during murine toxoplasmosis has been suggested as IL-21 would appear to prevent $CD8^+$ T cell dysfunction (Moretto et al., 2017).

B. $CD8^+$ T Lymphocytes

$CD8^+$ T cells provide strong protective immunity during adaptive immune response and have the ability of killing the infected cells via release of IFN-ɣ cytokines (Yap & Sher, 1999). IL-12 and IL-2 favour the expansion of cytolytic $CD8^+$ T cells. IL-2 is produced from $CD4^+$ T and is necessary to prime the protective response of $CD8^+$ T cell. Neutralisation of IL-2 in mice results in diminished $CD8^+$ T cell responses and decreased protection (Denkers *et al.*, 1996). $CD4^+$ T cells are necessary for the maintenance of $CD8^+$ T cell during the chronic stage of infection, but are not essential during the acute infection (Lütjen *et al.*, 2006). $CD8^+$ T cells have been shown to have cytolytic activity in mice and humans during chronic infection and are necessary to prevent disease reactivation (Ochiaia *et al.*, 2016). Mice deficient in $CD8^+$ T cells show increased susceptibility to

toxoplasmosis, succumbing approximately 50 days post-infection. Adoptive transfer of $CD8^+$ T cells from chronically infected or mice vaccinated with an attenuated strain of *T. gondii* display resistance (Gigley *et al.*, 2009; Denkers *et al.*, 1997; Parker *et al.*, 1991). *in vivo,* depletion studies of $CD8^+$ T cells and $CD4^+$ T cells result in reactivation of the infection and severe disease, but depletion of $CD4^+$T cells alone had limited impact (Gazzinelli *et al.*, 1992). Some of the earliest studies on the $CD8^+$ T cell responses identified MHC1/CD8 epitopes from the Surface Antigen 1 (SAG-1) protein (Khan *et al.*, 1988).

1.12.3. Controlling *T. gondii* Infection

IFN-ɣ has effector mechanisms to induce anti-toxoplasma activity in host cells, these mechanisms include: Nitric oxide (NO) production, tryptophan starvation, generation of reactive oxygen intermediates (ROI), iron deprivation, immunity-related GTPases (IRG) and P65 guanylate-binding proteins (Gbp). Following IFN-ɣ binding to its receptor (IFN-ɣ -R) at the cell surface there is initiation of a cascade signaling, involving JAK family of tyrosine kinases and STAT (signal transducer and activator of transcription) family factors (Halonen *et al.*, 2006). Consequently, IFN-ɣ activates genes that control the immune response, expression of pro-inflammatory cytokines and MHC-mediated pathway. Invasion of host cells by *T. gondii* induces phosphorylation of the signal transducer and activator of transcription STAT. STAT1 is a main pathway and is used by *T. gondii* to support its survival by utilising reduction of IFN-ɣ production from $CD4^+$ and $CD8^+$ T cells (Gavrilescu *et al.*, 2004). Furthermore, *T. gondii* activates STAT3 and STAT6 and thus the production IL-6 and IL-10 (Ong *et al.*, 2010; Saeij *et al.*, 2007).

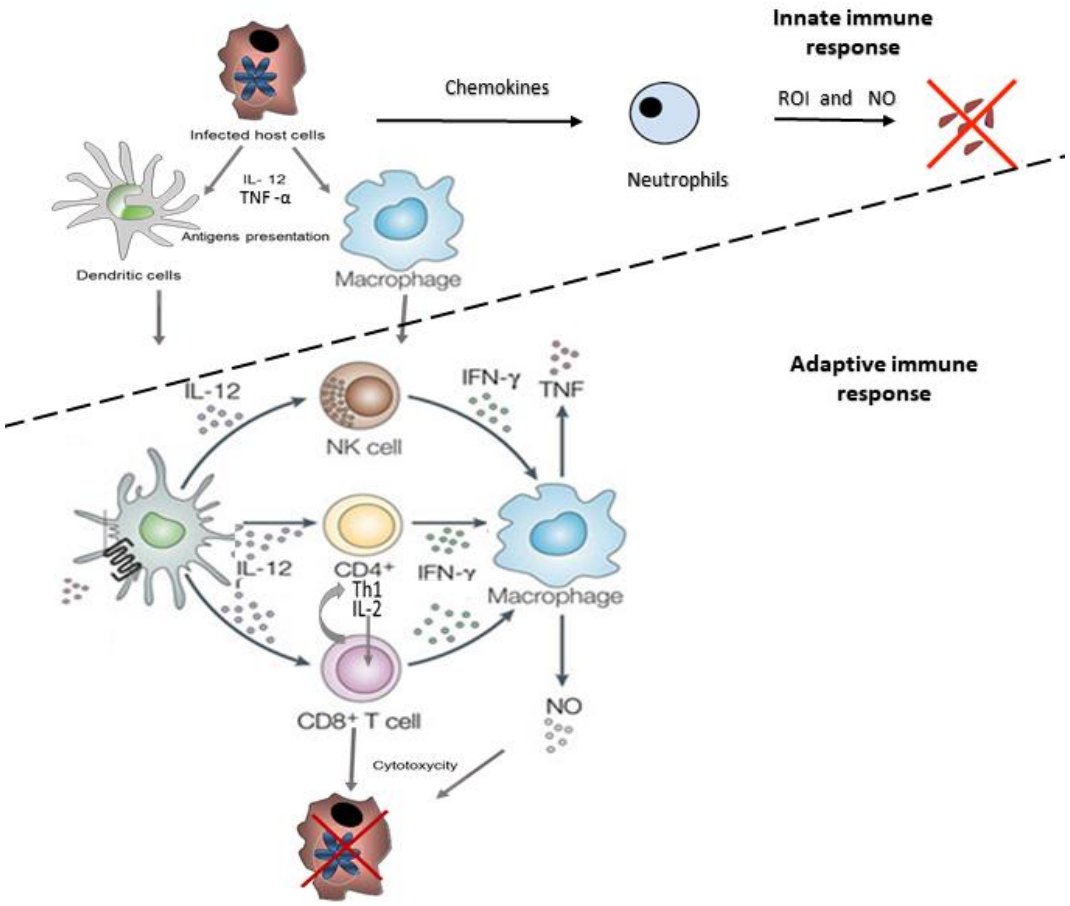


Figure 1.4 Innate and adaptive immune response against *T. gondii* infection.

T. gondii induces dendritic cell activation and IL-12 through the interaction of PAMPs with TLRs and through *T. gondii* cyclophilin 18 binding CCR5. IL-12 has a critical role, as an essential mediator, to induce NK cells to secrete IFN-ɣ that has cytotoxic effect on infected cells. IL-12 also has an important role in activation of $CD4^+$ and $CD8^+$ T cells. Macrophages have a significant role in the elimination of the parasite and are activated by IFN-ɣ to induce NO production that can directly kill *T. gondii* (Lewis and Wilson, 2005).

1.12.4. Humoral Immunity

The intracellular localisation of *T. gondii* suggested that the vast majority of the control of the parasite relies on the cell-mediated immunity. Nonetheless, production of antibodies assists cell-mediated immunity to control *T. gondii* infection. An important role for B cells in resistance against *T. gondii* was demonstrated using B cell-deficient (muMT) mice. Mutant mice died within 3-4 weeks, after infection with 10 cysts of the ME49 strain of *T. gondii*. muMT mice had more parasites in their brains and lungs than control mice (Kang *et al.*, 2000). It has been found that monoclonal and polyclonal antibodies specific to SAG1 of *T. gondii* inhibit infection of human fibroblasts and murine enterocytes (Mineo *et al.*, 1993). Other reports have been published on the protective effect of monoclonal antibodies that recognise SAG1 or dense granule proteins. These antibodies inhibit tachyzoite invasion of host cells *in vitro* and *in vivo* (Cha *et al.*, 2001). *In vitro*, specific murine anti-*T. gondii* antibodies have the ability to bind extracellular tachyzoites and kill them through the classical complement pathway (Kaneko *et al.*, 2004; Suzuki *et al.*, 1987). Activation of the alternative complement pathway occurred in non-immune human serum, with deposition of equivalent amounts of C3, which bound covalently to parasites to mediate killing (Fuhrman & Joiner, 1989). The classical complement pathway was activated by binding of natural specific IgM antibodies to the tachyzoites, leading to lysis. IgM antibodies have an important role to block tachyzoite cell invasion during early acute *T. gondii* infection and that was identified by using mice deficient in IgM (Couper *et al.*, 2005). Mutant mice displayed significantly increased tachyzoite dissemination to the liver, lung, and spleen compared to wild type mice. IgA has the ability to interfere with *T. gondii* tachyzoites to prevent adhesion to the mucosal surface and thereby preventing parasite invasion of host cells (Zorgi *et al.*, 2011). Furthermore, a study that successfully induced

high titers of IgA following intranasal vaccination of mice found that this was associated with the development of reduced numbers of cysts and implies a protective role for this antibody type (Igarashi *et al.*, 2008).

1.13 Immunomodulation by *T. gondii*

The virulence of *T. gondii* strains is related to a number of excreted/secreted effector molecules including rhoptry and dense granule proteins, which have been demonstrated to be key in determining virulence in mice and likely also account for the different clinical prognoses observed when humans are infected with different lineages (Taylor *et al.*, 2006).

It has been shown that ROP5 and ROP18 are *T. gondii* virulence factors during mouse infection (Niedelman *et al.*, 2012; Behnke *et al.*, 2015; Shwab *et al.*, 2016). Most strains of *T. gondii* from South America have alleles of ROP18 and ROP5 that are related to type 1 (Niedelman *et al.*, 2012). A genetic cross between the type 1 strain from South America and the type 2 (ME49) strain from North America confirmed the role of ROP18 and ROP5 in mediating virulence during acute toxoplasmosis, and this was further extended by using CRISPR-Cas9 to delete these genes from strains of several additional South American lineages (Behnke *et al.*, 2015).

T. gondii ROP16 activates STAT3 and STAT6, which results in suppression of the immune response through control of pro-inflammatory mediators and production of anti-inflammatory mediators such as IL-6 and IL-10 (Ong *et al.*, 2010; Saeij *et al.*, 2007). Paradoxically, *T. gondii* engineered to lack ROP16 fail to induce Arg1 and thus are more virulent as they have access to increased amounts of cellular arginine that they require for multiplication.

A number of dense proteins (GRA proteins) are known to be immunomodulatory and are sometimes referred to as virulence factors. For example, GRA15, is a tachyzoite virulence factor that has a distinct role in parasite survival in mice (Hehl *et al.*, 2015).

IFN-ɣ acts at the frontline of defense against *T. gondii* and is produced by $CD4^+$ and $CD8^+$ T cells, resulting from phosphorylation of STAT1-dependent pathway. *T. gondii* has evolved a secretory protein named inhibitor of STAT transcription (TgIST) as it is able to inhibit STAT1 and thus ablate the activity of the IFN-ɣ-dependent signaling pathway (Olias *et al.*, 2016). TgIST plays a major role early during acute infection by protecting the invading tachyzoites within host cells by blocking potent parasite killing (Olias *et al.*, 2016). However, there are other STAT1-dependent pathways that overcome this block and that are required to control toxoplasmosis (Gavrilescu *et al.*, 2004).

T. gondii cyclophilin 18 (TgCyp18) a cytosolic protein appears to induce IL-12 production by interacting directly with CCR5, and can be inhibited by cyclosporine A (Yarovinsky *et al.*, 2004). It has been shown that TgCyp18 mutant proteins have reduced interactions with CCR5 and reduced IL-12 induction (Yarovinsky *et al.*, 2004). TgCyp18 appears to act as a structural mimic of CCR5-binding ligands, with no sequence similarity with the host CCR5 ligands, CCL3, CCL4, CCL5 and CCL8 (Ibrahim *et al.*, 2014).

Overall, *T. gondii* has evolved multiple molecules that interact with the host immune response. Most of these would seem to subvert the immune response and promote parasite establishment and survival to the detriment of the host and can therefore be thought of as virulence factors. However, other mechanisms induce immune events that limit parasite growth. These have likely evolved to facilitate host survival for long enough to allow the parasite to disseminate and encyst and thus allow the intermediate host to function as a means of perpetuating the life cycle.

1.14 Vaccination

T. gondii is a global pathogen posing a great risk of infection to humans. Thus, all humans deserve vaccination against this pathogen. Unfortunately, up to now there is no available vaccine for humans. Vaccinations against toxoplasmosis could have medical and economic benefits because of the inability of current treatments to kill the bradyzoites in cyst stages during chronic infection. Consequently, medications neither stop nor eradicate infection and therefore a vaccine would signify the best way of avoiding infection. Many vaccine candidates have been studied for control of *T. gondii* while no established method for assessing these vaccines is available. Researchers have mainly used mice as animal models for immunological studies and vaccine designs (Reese *et al.*, 2016). Previous studies reviewed the pathogenicity, immunogenicity and characterisation of *T. gondii* antigens expressed in all infective stages of the parasite to find the best vaccine candidates against toxoplasmosis (Rezaei *et al.*, 2019; Zhang *et al.*, 2015; Jongert *et al.*, 2009).

1.14.1 Killed Vaccines

Killed vaccines were investigated early in 1956 and include whole tachyzoite lysate, soluble fractions of the cysts or soluble fractions of the tachyzoite lysate (Cutchins & Warren, 1956). These vaccines required adjuvants such as lipid vesicles, Freund's Complete Adjuvant (FCA) and Freund's Incomplete Adjuvant (FIA) to increase their immunological effects (Krahenbuhl *et al.*, 1972). Limited protection was achieved by these vaccines in terms of reduced mortality after challenge infection or decreased cyst burden.

1.14.2 Live Attenuated Vaccines

Live attenuated vaccines of *T. gondii* are used to mimic natural infection that have a potential role in promoting long effective immunity. TS-4 is a chemically mutagenised version of the RH strain, selected for temperature sensitivity and has been used experimentally to give a strong protection of mice against lethal challenge with RH and reduce congenital toxoplasmosis (McLeod *et al.*, 1988). However, the TS-4 vaccine failed to give full protection and prevent tissue cyst formation against challenge with the avirulent strain (McLeod *et al.*, 1988).

Veterinary vaccines, in theory could be administered to cats to reduce oocyst shedding or be administered to livestock to prevent their infection or to prevent congenital transmission or abortion. The S48 strain of *T. gondii* (Toxovax), which was isolated from an aborted ovine foetus in 1988 is not able to form tissue cysts or oocysts and has been used to reduce abortion in sheep (Wilkins *et al.*, 1988). S48 was used to immunise lambs and vaccinated sheep showed a significant reduction of tissue cysts in various tissues and reduced incidence of abortion following challenge (Katzer *et al.*, 2014). Similar effects were found in pigs (Burrells *et al.*, 2015). T-263 live strain of *T. gondii* attenuated with Gamma irradiation was used and 84% of vaccinated cats reduced the number of oocysts shed against challenge infection but this vaccine has never been commercialised (Frenkel *et al.*, 1991).

A number of experimental vaccines exist and have been used in mice with varying degrees of success. Depletion of RPS13 a ribosomal protein in the *T. gondii* parasite has resulted in a much attenuated RH strain (ΔRPS13) that can provide protection to mice against subsequent challenge with wild type parasites (Hutson *et al.*, 2010). A *T. gondii* uracil auxotroph mutant was found to be completely avirulent in mice, including IFN-ɣ KO

mice. This mutant strain could induce complete protection against lethal infection with *T. gondii* RH strain (Fox & Bzik, 2015). Mice vaccinated with ultraviolet-attenuated *T. gondii* and co-administered with pidotimod drug had higher levels of IgG antibody, IL-2, IFN-ɣ and TNF-α and extended survival time with lower parasite burden compared with infected controls against RH tachyzoites infection (Zhao *et al.*, 2013). Due to safety concerns, the use of live attenuated vaccines in humans are still limited and it is doubtful if a live attenuated vaccine for *T. gondii* would pass the required regulatory steps or be accepted by the public.

1.14.3 Protein Vaccines

Previous trials have examined a large number of *T. gondii* proteins for their vaccine potential and to evaluate resultant protective immunity against acute and chronic toxoplasmosis (Roberts *et al.*, 2004). Examples of vaccine studies using recombinant proteins in murine studies are presented below.

One of the most studied and arguably one of the most promising vaccine candidates is SAG1. Vaccination with SAG1 has been shown to have potential when formulated with the adjuvant Quil A which protects 90% of mice against challenge infection (Khan *et al.*, 1991). Due to this protection, a number of studies have subsequently used SAG1 as part of a multi-component vaccine. For example, a subunit vaccine consisting of recombinant SAG1 (rSAG1) and recombinant GRA2 (rGRA2) proteins. This vaccine increased survival time of mice, which was associated with higher IFN-ɣ production, but lower IL-10 production (Allahyari et al., 2016). Other multi-component vaccine preparations have used *T. gondii* rhoptry and dense granule proteins. One such study used the recombinant ROP2, GRA5 and GRA7 in a vaccine study. Mice vaccinated with these recombinant proteins associated with cholera toxin had partial protection against tissue cyst formation

when challenged orally with tissue cysts from *T. gondii* (Igarashi *et al.*, 2008). Actin protein, which has an important role in the invasion of host cells by *T. gondii* has been used as an experimental vaccine to elicit the immune responses in BALB/c mice after nasal immunisation. This vaccine increased actin specific secretory IgA levels in nasal, vaginal and intestinal washes as well as the serum IgG2a, IFN-ɣ and IL-2. Vaccinated mice survived longer when challenged with a lethal infection with the virulent RH strain (Yin *et al.*, 2013). Another experimental vaccine used a combination of calcium-dependent protein kinase 6 (TgCDPK6) and ROP18 proteins adjuvanted with PLG (poly L-lactide-co-glycolide). This vaccine was able to increase specific antibodies and induce high levels of IFN-ɣ and IL-2. Vaccinated mice survived longer after challenge with *T. gondii* RH strain than control groups and developed fewer cysts in their brains when challenged with the cyst forming PRU stain of *T. gondii* (Zhang *et al.*, 2016).

1.14.4 Vaccines Making Use of Live Vectors

T. gondii proteins have also been cloned into viral vectors or even other protozoans and used for vaccination studies. A vaccine trial used the Pseudorabies virus (PRV) engineered to express *T. gondii* proteins SAG1 and MIC3. BALB/c mice immunised with both viruses induced specific IgG and significant levels of IFN-ɣ and IL-2 production. Vaccinated mice had increased resistance against a lethal challenge with *T. gondii* RH strain (Nie *et al.*, 2011). Another study using SAG1 expressing Adenovirus to vaccinate C57BL/6 mice was able to provide protection as measured by reduced cyst numbers in the brain (Mendes et al., 2013). A novel study also used *Eimeria tenella* as a vector for SAG1. This vaccine could induce protective immunity against *T. gondii* infections in chickens by eliciting TgSAG1-specific humoral and cellular immune responses. Vaccinated mice were found to have SAG1-specific Th1-dominated immune responses

and prolonged survival time compared with non-immunised mice after challenge with *T. gondii* (Tang *et al.*, 2016).

1.14.5 DNA Vaccines

The potential of plasmid DNA vaccines has been explored for prevention of toxoplasmosis in mice. DNA vaccines have a number of attractive features (i) They are easily constructed without needing to handle a virulent pathogen (ii) DNA vaccines induce cytotoxic $CD8^+$ T cells (iii) They are inexpensive relative to other vaccine technologies (iv) The proteins expressed following vaccination are post-translationally modified (reviewed Liu, 2011). A number of *T. gondii* genes have been cloned and tested in murine models of toxoplasmosis. For example, SAG1 was used as DNA vaccine candidate to enhance the immune response to protect adult BALB/c mice against *T. gondii* infection. Vaccination was able to increase splenocytes' IFN-ɣ production, prolong survival and decrease cyst burden. However, vaccination did not reduce the incidence of congenital disease (Couper *et al.*, 2003). Another study examined *T. gondii* glutathione reductase (TgGR) as a DNA vaccine. Mice immunised with pTgGR showed significantly raised titers of IgG1 and IgG2a, IgA and IgM as well as an increased production of IFN- ɣ, IL-4, IL-17 and TGF-β1 cytokines compared with the control groups. Following challenge with virulent *T. gondii* (RH strain) vaccinated mice had significantly longer survival times (Hassan *et al.*, 2014).

Studies have used multiple DNA vaccine candidates simultaneously with varying degrees of success in a number of murine models. Immunisation of BALB/c mice with a DNA cocktail containing SAG1 and ROP2 genes was found to induce a Th1-type response with the predominance of IgG2a. Vaccinated mice had an increased survival against challenge infection with the RH strain of *T. gondii* (Fachado *et al.*, 2003). GRA1 and

SAG1 have been demonstrated to prolong the survival rate of BALB/c mice following challenged with tachyzoites of the virulent *T. gondii* (RH strain). This protection was associated with high levels of IgG, IFN-ɣ and IL-2 and stimulated NK cell-killing activity (Wu *et al.*, 2012).

DNA vaccines have been used experimentally with some adjuvants to increase their effectiveness and modify the immune response. For example, inclusion of the granulocyte-macrophage colony-stimulating factor (GM-CSF) with a plasmid encoding *T. gondii* SAG1 and GRA4 antigens reduced mortality of susceptible C57BL/6 mice upon oral challenge with cysts. This DNA cocktail provided higher survival of newborns from immunised outbred mice exposed to infection during gestation with a specific humoral and cellular Th1 response (Mévélec *et al.*, 2005). It has been found that mice vaccinated with a multi-epitope DNA vaccine (comprising epitopes from SAG1, GRA2, GRA7 and ROP16) showed higher levels of IgG2a, significant IFN-ɣ production and had longer survival times against the acute infection of *T. gondii* compared with control groups. This multi-epitope DNA vaccine could be enhanced by adding a genetic adjuvant pEGFP-RANTES (pRANTES) which augmented the humoral and cellular immune responses (Cao *et al.*, 2015). Most recently, the *T. gondii* toxofilin gene was used as a DNA vaccine combined with a mixture of individual adjuvants, alum or monophosphoryl lipid A (MPL) and this resulted in induction of a strong humoral and cellular immune response in vaccinated mice. These mice had longer survival times and less cyst numbers following challenge with *T. gondii* infection compared with the control groups (Song *et al.*, 2017).

1.15 *Toxoplasma gondii* Surface Antigen 1 (SAG1) Protein

Surface antigen 1 (SAG1) is considered the major surface antigen in *T. gondii* (previously named p30) and comprises more than 5% of the total amount of protein of the tachyzoite stage of *T. gondii* (Lekutis *et al.*, 2001). SAG1 is a member of a large superfamily of surface proteins referred to as SAG-related sequences (SRS) (He *et al.*, 2002). *T. gondii* surface proteins are important for tachyzoite development as they facilitate adhesion to host cells and thus invasion of the host cells. SAG1 is considered a main immunodominant antigen (Tang *et al.*, 2016). SAG1 exists as a monomer of 250 amino acids with an approximate molecular weight of 30 kDa. It can form homodimer structures, which are attached to the parasite surface by glycosylphosphatidylinositol (GPI) anchors (He *et al.*, 2002). The protein consists of two domains. The N-terminal domain is designated domain 1 (D1), which consists of 130 amino acids. The C-terminal domain is designated domain 2 (D2) and is around 120 amino acids long. Two disulfide bonds are formed to stabilise each domain as shown in figure (1.5) (He *et al.*, 2002).

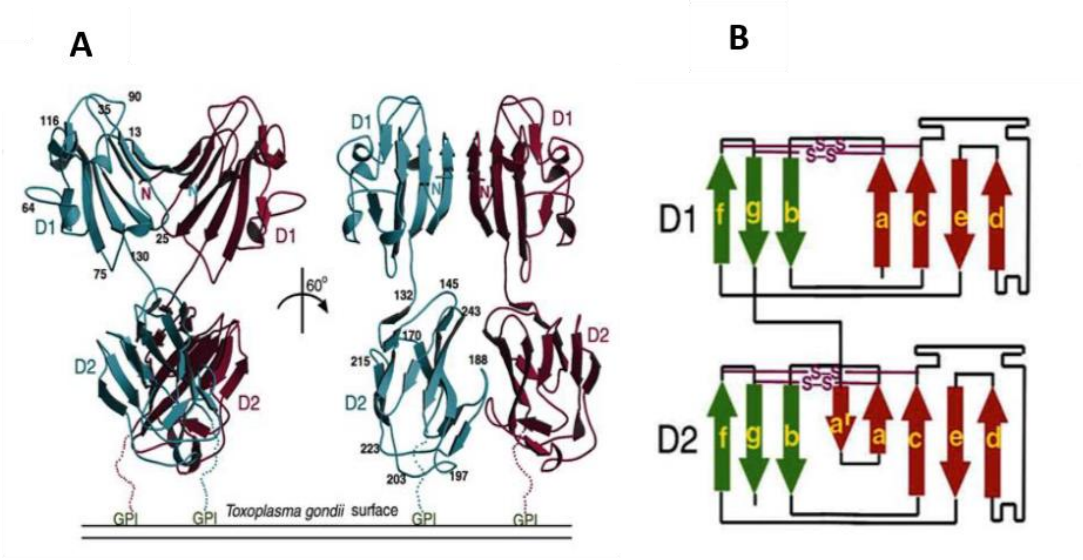


Figure 1. 5 Three-dimensional structure of the SAG1 protein.

(A) SAG1 dimer one monomer is pink and the other is cyan; C-terminus was extended with a dotted line to show the location of the GPI linkage with the membrane. **(B)** Secondary structure diagram of the SAG1 monomer. SAG1 D1 domain (C-terminal) binds to D2 domain via two disulfide bonds (adapted from He et al., 2002).

Previous studies have focused on SAG1 for vaccination because it has a critical importance for the immune response in the initial stage of infection involving both the humoral and cellular immune response (Abdizadeh *et al.*, 2015; Lekutis *et al.*, 2001). Vaccination of mice with SAG1 has provided encouraging results with significant protection as measured by a reduction of the number of cysts in vaccinated animals compared with control animals (Lekutis *et al.*, 2001). Therefore, preparation of SAG1 antigens from the parasite has immunogenic importance for vaccination studies. SAG1 was purified from tachyzoites or produced as a recombinant protein in *E. coli* or in a yeast (Wang & Yin, 2014). Moreover, an experimental DNA vaccine developed in a plasmid encoding SAG1 has been shown to provide mice with significant protection against mortality following challenge with the highly virulent RH strain of *T. gondii* (Angus *et al.*, 2000). Table 1.2 summarises various vaccine preparations using SAG1 protein or DNA and the protection they afforded with or without various adjuvants in animal models.

Table 1. 2 Experimental vaccine studies using SAG1 protein or DNA

Antigenic vaccine	**Adjuvant used**	**Animal model**	**Challenge strain of *T. gondii***	**Results**	**Reference**
Recombinant P1tPASAG-1 expressed in *E. coli*	Not applicable	C3H/HeN BALB/c mice	RH-strain	80-100% protection against challenge infection in cyst numbers	(Nielsen *et al.*, 1999)
DNA SAG1 vaccine	Freund's complete	C57BL/6 Mice Rat	RH, Me49 VEG	Reduced the mortality of immunised mice, increase IFN-ɣ, IL-2 production and decreased IL-4 production	(Angus *et al*. 2000)
Recombinant SAG1 protein expressed in *P. pastoris*	SBAS1	guinea pig	C56 (mildly virulent)	66 - 86% reduction in maternofetal transmission	(Haumont *et al.*, 2000)
P1tPASAG-1 DNA vaccine	Not applicable	BALB/c mice	Beverly type-2 strain	Enhanced Th-1 immune, increased splenocyte IFN-ɣ production and decreased brain cyst burden	(Couper *et al.*, 2003)

SAG1 protein expressed in *E. coli*	Not applicable	BALB/c mice	Me49 strain	50% reduction in maternofetal transmission	(Letscher-bru *et al.*, 2003)
SAG1 peptides	Not applicable	C3H/HeN mice	RH-strain	Gave a high protection against lethal challenge	(Siachoque *et al.*, 2006)
Recombinant SAG1 protein expressed in tobacco leaves	Incomplete Freund's adjuvant (IFA)	C3H/HeN mice	Non- Lethal dose of ME49 cysts	High level of IFN-ɣ 30%reduction of brain cysts	(Laguia-Becher *et al.*, 2010)
Recombinant SAG1 protein expressed in adenovirus (AdSAG1)	Not applicable	C57BL/6 mice	ME49 strain	Heterologous vaccine decreased the numbers of brain cyst compared with wild type viral vaccine	(Mendes *et al.*, 2013)
Recombinant pVAX1-SAG1 DNA vaccine	Not applicable	Not applicable	Not applicable	High level of pVAX1SAG1 expression vector in CHO cells as a suitable system for vaccine development	(Abdizadeh *et al.*, 2015)
Recombinant Et-TgSAG1 protein (SAG1engineered in *E. tenella*)	Not applicable	BALB/c mice	RH-strain	Th1- dominant immune response and prolong the survival rate	(Tang *et al.*, 2016)

1.16 *Toxoplasma gondii* Cyclophilin 18 (Cyc18)

T. gondii cyclophilin 18 (TgCyc18) has a direct immunogenic effect on cells that express the cysteine-cysteine chemokine receptor 5 (CCR5) including macrophages and dendritic cells. TgCyc18 is a chemokine mimic that interacts with CCR5, which is expressed on the surface of macrophages, dendritic cells and spleen cells (Ibrahim *et al.*, 2014). Upon binding CCR5 activates the dendritic cells to induce IL-12 production. Earlier, Albarti *et al* (2003) stated that IL-12 production was promoted by the activity of recombinant TgCyc18 compared to non-recombinant TgCyc18 in the soluble tachyzoite extract (Aliberti *et al.*, 2003). These results corresponded to the high affinity of recombinant TgCyp18 to trigger cell signalling of DCs through CCR5. TgCyc18 is a 18.5 kDa protein and is highly similar to cyclophilin TgCyc20 with 70% amino acid identity in their N-terminal regions (High *et al.*, 1994). However, both *T. gondii* cyclophilins have different intracellular distributions and functions related to differences in the amino acid sequences (High *et al*. 1994). TgCyc18 and TgCyc20 are similar to the human cyclophilin (hCyc18) protein in the core region, but TgCyc20 differs from hCyc18 by the insertion of seven amino acids (Ibrahim *et al.*, 2014). TgCyc18 can recruit cells including macrophages and lymphocytes to the site of infection through its chemotactic ability for CCR5-binding ligands (Ibrahim *et al.*, 2010). IL-12 produced by dendritic cells following CCR5 ligation by TgCyc18 has the ability to stimulate NK cells to produce IFN-ɣ and to promote Th1 T cell differentiation, which have each been demonstrated to be important for host immunity during acute toxoplasmosis. TgCyc18 has also been shown to induce the production of nitric oxide (NO), IL-6 and low levels of TNF-α which have also been demonstrated to mediate protection against *T. gondii* infection (Ibrahim *et al.*, 2009; Aliberti *et al.*, 2016).

Consequently, in this study the hypothesis is to test whether TgCyc18 is a suitable adjuvant for inclusion in a vaccine for toxoplasmosis.

1.17 Protein Expression Systems

There is a large number of systems for the expression of recombinant heterologous proteins. Each of these have their advantages and disadvantages and are thus suited to different applications. *Escherichia coli* is the most commonly used host for protein expression and as a standard prokaryotic expression system it can produce high protein yields but is characterised by the absence of post-translational modifications (PTMs) and sometimes prone to improper protein folding (Dortay & Mueller-Roeber, 2010). Eukaryotic protein expression systems generally can deliver PTMs and produce soluble protein, but at a significantly lower protein yield than the prokaryotic systems (Jenkins *et al.*, 2009). There are various protein expression systems in yeast using different species and these have been successful in medical and industrial settings, giving high yield of recombinant proteins but with high cost (Çelik & Çalık, 2012). Other eukaryotic expression systems include insects cells, protozoans and plants (Demain & Vaishnav, 2009). Table 1.3 shows the differences between a prokaryote (*E. coli*) and eukaryote (*L. tarentolae*) as protein expression system.

Table 1. 3 Comparison of characteristic features involved in expression system selection. Adapted from (Demain & Vaishnav, 2009).

Characteristic Feature	***E. coli***	***L. tarentolae***
Protein expression	Fast	High level
Protein yield	High	variable
Post-translational modifications	No	Glycosylation
Protein size	Mostly small size	Flexible
Protein folding	Proteins produced as inclusion bodies are inactive; require refolding	proper protein folding
Cost	Inexpensive	fair
Yield [mg/L]	200–300	0.4
High cell density	Toxic if oxygen not controlled	fair
Secretion protein	Strong secretion with no involvement of intracellular inclusion bodies	Easy manipulated

1.18 *Leishmania tarentolae* as a Protein Expression System

Ideally, an expression system for heterologous proteins should have both high protein yield and the appropriate PTMs. *L. tarentolae* is a suitable eukaryotic expression system for production of different proteins carrying PTMs with a protein yield of approximately 30 mg/L of culture (Breitling *et al.*, 2002). *L. tarentolae* is nonpathogenic to humans and lacks 250 genes for virulence coding sequences found in human pathogenic *Leishmania* species. Notably, it is deficient in major virulence and visceralisation factor A2 (Mizbani *et al.*, 2011). *Leishmania* are rich in glycoproteins both as modified secretory proteins and membrane bound proteins (Solá & Griebenow, 2011). Glycosylation is a mechanism for both *in vivo* and *in vitro* stability of proteins (Solá and Griebenow, 2011). Additionally, glycosylation provides protection for the modified proteins against enzymatic degradation, which is sometimes an issue in *E. coli*. Examples of proteins successfully expressed in *L. tarentolae* are shown in table 1.4.

Table 1. 4 Previous studies which have used *L. tarentolae* as protein expression system and their biological application.

Protein name	Type of protein expressed	Medical application	Reference
Convertase 4 (PC4)	Secreted soluble enzyme	PC4 precursors for reproductive pathway	(Basak *et al.*, 2008)
Human Laminin (LM)-332	glycoprotein	essential for epithelial basement membranes and cell adhesion in tissue engineering	(Phan *et al.*, 2009)
ASMT(N-acetyl serotonin methyl transferase)	Soluble recombinant protein	ASMT enzyme responsible for melatonin synthesis	(Ben-Abdallah *et al.*, 2011)
Human C-reactive protein (CRP)	pentameric protein with phosphocholine insoluble binding site	CRP is produced by the liver in response to inflammation	(Dortay *et al.*, 2011)
Human erythropoietin (EPO)	recombinant darbepoetin alfa protein (glycogen protein)	treating anemic patients with chronic disease	(Kianmehr *et al.*, 2016)

1.19 Mechanism of Protein Expression in *Leishmania tarentolae*

Leishmania tarentolae is a trypanosomatid protozoan parasite of the gecko and has been established as a new eukaryotic expression system for the production of recombinant proteins (Niimi, 2012). Trypanosomatidae are rich in glycoproteins with a glycosylation similar to that in mammals and higher vertebrates. Thus, one of the main advantages of the *L. tarentolae* expression system is the posttranslational modification of target proteins. *L. tarentolae* has a unique feature in their cellular transcription and translation systems. Numerous genes in *L. tarentolae* are arranged in repeated tandem arrays, and the mRNA is transcribed as a polycistronic precursor, with one individual pre-mRNA containing multiple tandemly organised open reading frames (Breitling *et al.*, 2002). Pre-mRNAs are post-transcriptionally modified in the nucleus by trans-splicing and polyadenylation, giving rise to mature mRNAs (Teixeira, 1998). Trans-splicing adds a capped 39 nucleotide leader sequence necessary for transport, stability and translation of the mRNA. Figure 1.6 shows how the polycistronic gene expression works for a construct integrated into the rRNA gene locus.

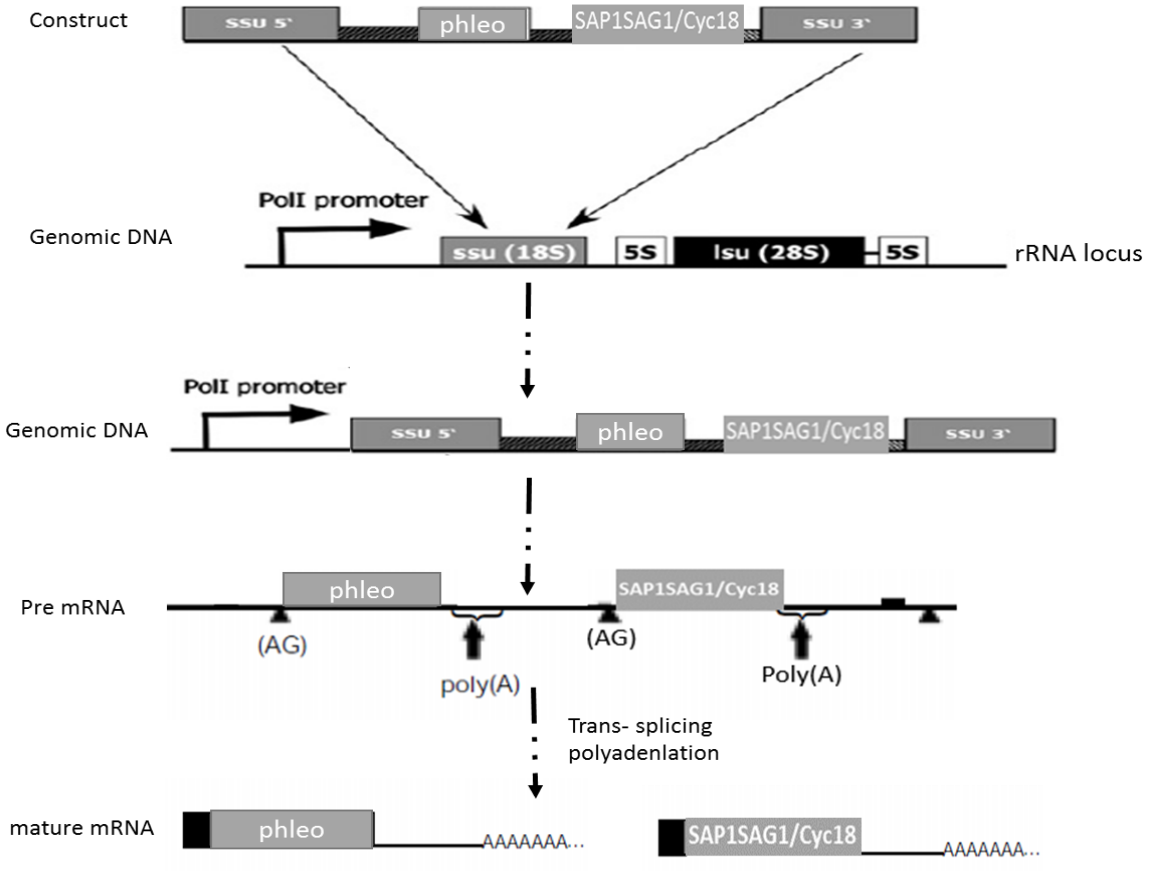


Figure 1. 6 Schematic illustrating mRNA synthesis for a construct integrated into the rRNA locus in *L. tarentolae:*

Gene cluster organised in a repeated tandem arrangement (Phleo and SAP1SAG1/Cyc18); RNA polymerase I transcription forms pre mRNA of all genes integrated into the small subunit rRNA(ssu) gene locus; the polycistronic mRNA is processed by adding the methylated 39-nucleotide splice leader at the splice addition site (AG) (arrow heads) and polyadenylation (arrows). Mature mRNA is composed of 5'and 3'UTR and a poly A tail.

1.20 Aims of the study

Despite a large number of experimental vaccine studies, there is still no vaccine available for prevention of *T. gondii* infection in humans. The studies described in this thesis have the aim of providing insight into how a vaccine might be constructed and produced. It can be divided into 2 aims:

Aim 1

To determine the viability of a novel protein expression system. Specifically, the studies will attempt to develop a novel filamentous protein expression system using *L. tarentolae* to express proteins of interest fused to *L. mexicana* secreted acid phosphatase (SAP). As *L. mexicana* secreted acid phosphatase (SAP) is secreted and self-assembles into filaments the proteins of interest should be easy to obtain from culture and purify. *L. tarentolae* is an attractive eukaryotic protein expression system for its ability to include post-translational modifications. As proof of principle and to test this system, 2 proteins from *T. gondii* have been chosen, SAG1 a promising vaccine candidate and cyclophilin 18 a chemokine mimic with the ability to bind and activate CCR5 and elicit IL-12 production.

Aim 2

To determine the ability of *T. gondii* cyclophilin 18 (TgCyc18) to work as an adjuvant in a vaccine for *T. gondii* infection. Specifically, studies will determine if TgCyc18 can increase the magnitude or alter quality of the immune response in mice vaccinated with SAG1 and if these mice have increased protection against *T. gondii* infection. It was originally envisaged that this would be achieved through the use of proteins expressed in Aim 1. However, as protein yields were low in this system this aim was achieved through the use of DNA vaccination.

Chapter 2

Materials and Methods

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Laboratory equipment	Source
<u>Centrifuges</u>	
Centrifuge 5424	Eppendorf, Hamburg, Germany
Centrifuge 5415R	Eppendorf, Hamburg, Germany
HERMLE Z 400 K	Hermle Labortechnik, Wehingen, Germany
Heraeus Multifuge 3SR+ Centrifuge	Heraeus, Hanau, Germany
<u>Microscopes</u>	
Axiovert 25 Inverted Microscope	Carl Zeiss, Jena, Germany
Axiostar transmission-light Microscope	Carl Zeiss, Jena, Germany
Eclipse TE2000S (Epifluorescent)	Nikon Instruments, Derby, UK
CFI Plan Fluor DLL-100x objective lens	Nikon Instruments, Derby, UK
Hamamatsu Orca-285 Camera	Hamamatsu, Garden City, UK
<u>Electrophoresis equipment</u>	
Agarose gel tanks	Biostep GmbH, Germany
SDS-PAGE tanks	Biometra, Göttingen, Germany
Power source 300W	VWR International, Lutterworth, UK
<u>Immunoblotting equipment</u>	
Fastblot B33 / B34	Biometra, Göttingen, Germany
Power Source 300W	VWR International, Lutterworth, UK
<u>Heat block</u>	
Thermomixer comfort	Eppendorf, Hamburg, Germany
<u>Photometer</u>	
BioPhotometer 6131	Eppendorf, Hamburg, Germany

Pharmacia LKB Ultrospec III	Pharmacia, Milton Keynes, UK

pH Meter

Digital-pH-Meter HI221	Hanna Instruments, Leighton Buzzard, UK

Shaking incubators

Innova 4230/4400	New Brunswick Scientific, Edison, NJ, USA
Thermomixer comfort	Eppendorf, Hamburg, Germany

Shaking water baths

GFL 1083	GFL, Burgwedel, Germany
mwg LAUDA M3	Heidolph Electro, Kehlheim, Germany

Sonicator

Branson Sonifier 250	Branson, Danbury, CT, USA

Thermocyclers

Gene Amp PCR System 9700 PE Applied	Biosystems, Weiterstadt, Germany
Surecycler 8800	Agilent Technology, Edinburgh, UK

Transfector

Nucleofector II	Amaxa Biosystems, Gaithersburg, MD, USA

Vortex

IKA-VIBRO-FIX VF2IKA	Labortechnik, Staufen, Germany

UV transilluminators

VWR Genosmart	VWR International, Lutterworth, UK
High Performance UV	UVP Cambridge, UK

Safety Cabinet

Thermo SAFE 2020, Class II	Fischer Scientific, Loughborough, UK

2.1.2 Chemicals

Acetic Acid	Carl Roth, Karlsruhe, Germany
Acrylamide 30% (w/v)	VWR, Lutterworth, UK
Adenosine triphosphate (ATP)	Roche Diagnostics, Sussex, UK
Agar-Agar	Techmate Ltd, Milton Keyes, UK
Agarose (Molecular biology grade)	Techmate Ltd, Milton Keyes, UK
Ammonium persulfate (APS)	Sigma-Aldrich, Steinheim, Germany
Bovine serum albumin (BSA)	Sigma-Aldrich, Steinheim, Germany
Bromophenol blue	Techmate Ltd, Milton Keynes, UK
Carbenicillin	Techmate Ltd, Milton Keyes, UK
Chloroform	Techmate Ltd, Milton Keyes, UK
Coomassie Brilliant Blue R250	Carl Roth, Karlsruhe, Germany
DABCO® (1,4-Diazabicyclo [2,2,2] octane)	Techmate Ltd, Milton Keyes, UK
DMSO	Roche Diagnostics, Mannheim, Germany
dNTP mix	BioLine, London, UK
EDTA disodium dihydrate	Sigma-Aldrich, Steinheim, Germany
Ethanol	VWR Chemicals, Lutterworth, UK
Ethidium Bromide	Sigma-Aldrich, Gillingham, UK
Fetal Calf Serum (FCS Gold) PAN	PAN Biotech, Aidenbach, Germany
Glucose	Techmate Ltd, Milton Keyes, UK
Glycerol	Techmate Ltd, Milton Keyes, UK
Hemin	Sigma-Aldrich, Steinheim, Germany
HEPES	Techmate Ltd, Milton Keynes, UK

Imidazole	Techmate Ltd, Milton Keynes, UK
IPTG	GerbuBiochemicals, Gaiberg, Germany
Isopropanol	Techmate Ltd, Milton Keynes, UK
Kanamycin sulfate	VWR, Lutterworth, UK
Methanol	Carl Roth, Karlsruhe, Germany
Milk powder	Carl Roth, Karlsruhe, Germany
Tryptone (Peptone)	Techmate Ltd, Milton Keynes, UK
Mowiol® 4-88	Sigma-Aldrich, Steinheim, Germany
p-formaldehyde	VWR, Lutterworth, UK
p-nitrophenylphosphate tablets 20 mg	Sigma-Aldrich, Steinheim, Germany
Penstrep (Penicillin 1,000U/ml,Streptomycin 10 mg/ml)	Life Technologies, Carlsbad, USA
Puromycin	Merck Biosciences, Schwalbach, Germany
Phleomycin	Merck Biosciences, Schwalbach, Germany
Potassium chloride	Sigma-Aldrich, Gillingham, UK
Potassium dihydrogen phosphate	Merck Biosciences, Schwalbach, Germany
Sodium dodecyl sulfate (SDS)	Fischer Scientific, Loughborough, UK
SDM79 Medium	Generon, Maidenhead, UK
Sodium Acetate	Merck Biosciences, Schwalbach, Germany

Sodium Chloride	Sigma-Aldrich, Gillingham, UK
Sodium Hydroxide	Techmate Ltd, Milton Keyes, UK
TEMED	Sigma-Aldrich, Steinheim, Germany
Triton X-100	Techmate Ltd, Milton Keyes, UK
Trizma	Sigma-Aldrich, Steinheim, Germany
Tween 20	Techmate Ltd, Milton Keyes, UK
Yeast extract	Fluka, Gillingham, UK
DNA markers 1kb DNA Ladder	New England Biolabs, Hitchin, UK
Prestained Protein Marker (broad range)	New England Biolabs, Hitchin, UK

2.1.3 Biological Strains	**Source**
Escherichia coli DH5α	Thermo Fisher Scientific, UK
Leishmania tarentolae PARROT TarII	Marc Oullete, McGill, University, Canada
Leishmania mexicana MNYC/BZ/62/M379, clone 2	Wiese group

2.1.4 Plasmids

	Source
pLEXSY-ble2.1	Jena Bioscience
pLPhSAP2SAG1	this work
pLPhSAP2CYC18	c
pBME -Amp-SAG1Lt	Biomatik, Canada
pBME -Amp-Cyc18Lt	Biomatik, Canada
pUCCYC18SAG1SAP	Biomatik, Canada
pSSUSAP2mod2C	M. Wiese group
pSSUHygSAP1	M. Wiese group
pVAX-SAG1	this work
pVAX-Cyc18	Craig W. Roberts
pVAX-SAG1	Craig W. Roberts

2.1.5 Media and Buffers

Ammonium Persulphate (10%)	10% (w/v) Ammonium Persulphate dissolved in ddH_2O
Agarose gel loading buffer (10×)	0.1 M EDTA pH 8.0 0.1% (w/v) bromophenol blue 0.1% (w/v) xylenecyanol 0.5 × TBE 50% (v/v) glycerol
Carbenicillin stock (100 mg/mL)	100 mg/mL dissolved in ddH_2O and filter sterilised
Coomassie Staining Solution	0.3% (w/v) Coomassie Brilliant Blue R250 45% (v/v) methanol 10% (v/v) acetic acid 45% (v/v) ddH_2O
Coomassie Destaining Solution	30% (v/v) methanol 60% (v/v) ddH_2O 10% (v/v) acetic acid
iFCS	FCS heated to 56°C for 45 min then filter sterilised

Immunoblot Blocking Solution	5% (w/v) Milk Powder in PBST 20 mM Tris-HCl pH 7.5
Immunoblot transfer buffer	25 mM Trizma 150 mM glycine 10% (v/v) methanol
LB Agar	1.5% (w/v) agar-agar in LB Medium. Autoclaved, allowed to cool to <50°C, sterile antibiotics added if required, and poured into plates.
LB Medium	1% (w/v) bacto tryptone 0.5% (w/v) yeast extract 1% (w/v) NaCl Autoclaved, allowed to cool to <50°C, sterile antibiotics added if required
PBS (10×)	137 mM NaCl 10.1 mM Na_2HPO4 2.7 mM KCl 1.8 mM KH_2PO4
PBST	0.2% (v/v) Tween-20 in 1 × PBS
Resolving Gel Buffer for SDS-PAGE (4×)	1.5 M Tris-HCl pH 8.8 0.4% (w/v) SDS
SDM79 Medium for Promastigote Culture	10% (v/v) iFCS 7.5 µg/ml hemin in SDM Medium Filter-sterilised
SDS-PAGE Running Buffer (10×)	0.25 M Tris-HCl pH 8.3 1.92 M Glycine 1% (w/v) SDS
SDS-PAGE Sample Buffer (SSB) (5×)	250 mM Tris-HCl pH6.8 50% (v/v) glycerol 10% (w/v) SDS 0.5% (w/v) bromophenol blue 200 mM DTT
Stabilate Freezing Solution for *Leishmania*	10% (v/v) sterile DMSO 90% (v/v) iFCS

Stacking Gel Buffer for SDS-PAGE (4×)	0.5 M Tris-HCl pH 6.8 0.4% (w/v) SDS
TBE (5×)	0.45 M Tris-HCl 0.45 M boric acid 10 mM EDTA pH 8.0
Tris-Buffered Saline (TBS) (10×)	200 mM Tris-HCl pH 7.5 1.37 M NaCl
TBST	0.2% (v/v) Tween-20 in 1 × TBS
TENS solution	10 mM Tris-HCl pH 8.0 1 mM EDTA 100 mM NaOH 0.5% (w/v) SDS

2.1.6 Enzymes

Restriction endonucleases	New England Biolabs, UK Promega, Southampton, UK
RNase A (bovine pancreas)	Roche Diagnostics, Mannheim, Germany
Taq DNA polymerase	Roche, Sigma-Aldrich, Gillingham, UK
T4 DNA ligase	Roche Diagnostics, Mannheim, Germany

2.1.7 Kits

NucleoSpin Extract II	Bioline, UK
Isolate II genomic DNA	Bioline, UK
NucleoBond Xtra Midi Kit	Macherey & Nagel, Germany
DNA Gigaprep	Macherey & Nagel, Germany
The Mouse Th Cytokine panel	Biolegend, London, UK
Mouse inflammation panel	Biolegend, London, UK

2.2. Methods

2.2.1 Molecular Biology Methods

2.2.1.1 Restriction and Endonuclease Analyses

All restriction enzymes, buffers and temperature conditions were used as recommended by the manufacturer's instructions. Reactions were prepared using 1 µl of DNA (1-2 µg/µl) and 5-10 U of the appropriate restriction enzyme for midiprep DNA digestion. 4 µl of DNA (~0.4 µg/µl) with 10-15 U of appropriate enzyme in a total volume of 15 µl for miniprep DNA digestion. 2 µg of RNase A was added to miniprep digests. The samples were incubated for 3 h at 37°C. DNA fragments were separated by gel electrophoresis using 0.8-1% (w/v) agarose at 120 V in 0.5 × TBE running buffer for 60 min. Nucleic acids were visualised by UV illumination under low intensity UV light (λ =365 nm) as ethidium bromide (0.3 µg/ml) was used in the gel for staining. Preparative digests were set in total 100 µl using 10 µl of midi-prep DNA (1-2 µg / µl) and 10-15 U enzyme. The DNA bands of interest were extracted and isolated using a NucleoSpin Extract II kit, and eluted in 30 µl ddH_2O.

2.2.1.2 Dephosphorylation of 5'-ends of DNA Fragments

In cases where endonuclease digestion had resulted in blunt ends or complementary overhangs of the linearised vector molecule it was necessary to dephosphorylate the 5'-ends to reduce the likelihood of spontaneous self-ligation. The purified DNA from the restriction digest mixture was treated with shrimp alkaline phosphatase (SAP) (Roche Diagnostics, Mannheim, Germany). The eluted DNA was dissolved in 25.5 µl ddH_2O, 3 µl of the provided 10 × SAP buffer and 1.5 U SAP were added, and incubated at 37°C for 2 hours. The enzyme was inactivated by heating the mixture to 65°C for 20 minutes.

2.2.1.3 Plasmid Concentration Using Nanodrop

To determine the concentration and purity of a plasmid, 1 µl of the plasmid solution was analysed using the Nanodrop 2000 (Thermo Fisher Scientific, USA). Nucleic acids monitoring option was selected from the Nanodrop software, 1 µl of double distilled water was used to blank the instrument. 1 µl of plasmid DNA was used to measure the DNA concentration in ng/µl and to determine sample purity (Absorbance ration 260 nm / 280 nm).

2.2.1.4 Ligation of DNA Fragments

Fragments from cleaved plasmid constructs were ligated using 0.8 µl T4 ligase and 1.5 µl 10× T4 ligase buffer (Roche Diagnostics, Mannheim, Germany) in a ratio of 3:1 insert DNA: vector. The ligation reaction was prepared in pre-cooled PCR tubes, after addition of all reaction components the PCR tube was vortexed and centrifuged at 1,000 × g, 4°C for 60 s. The ligation reaction volume of 15 µl was incubated at 13°C overnight.

2.2.1.5 Transformation of *E. coli* DH5α cells

5 µl of a ligation was mixed with 200 µl *E. coli* DH5α competent cells and incubated on ice for 1 h. Then the mixture of plasmid and *E. coli* DH5α was heat shocked at 42°C for 90 s and returned to ice for 5 min. *E. coli* DH5α cells were revived by adding 800 µl of LB broth and shaking for 1 h at 37°C. 200 µl volume of *E. coli* DH5α transformed cells were aseptically plated on LB agar containing the selective antibiotic carbenicillin (100 µg/ml).

2.2.1.6 DNA Miniprep (TENS method, Zhou *et al*., 1990)

Plasmids were purified from *E. coli* DH5α cell cultures using the TENS method. 6 – 12 single colonies of transformed cells were used to inoculate 6-12 tubes with 3ml of LB broth each containing the selective antibiotic Carbenicillin (100 µg/ml) and incubated for a period between 16 –18 h at 37°C, shaking at 220 rpm. Next day, the cultures for which growth was observed were streaked onto LB agar plates containing Carbenicillin (100 µg/ml) and incubated overnight at 37°C. 1.5 mL of culture were harvested in Eppendorf tube at 15,800 × g for 30 s, supernatant was poured and the sediment cells were resuspended in the remaining supernatant about 100 µl. Plasmids were purified from cultures by addition of 300 µl of TENS and the cell suspension was vortexed at medium speed for 4 s then immediately placed on ice. Subsequently, 150 µl of 3M Sodium Acetate buffer (pH5.2) was added and the mixture was vortexed at medium speed for 3 s and placed on ice again. Cells were sedimented at 15,800 ×g at 4°C for 15 min, and clear supernatant was transferred to a new 1.5 mL tube, the spin step was repeated twice to collect clear supernatant. 900 µl of 100% ice-cold ethanol were added to precipitate the DNA, then the mixture was centrifuged at 15,800 ×g at 4°C for 15 min. DNA pellet was washed with 70% ice cold ethanol solution and centrifuged for 15 min at 11,000 × g, 4°C. The supernatant was removed in sterile conditions under laminar airflow hood. DNA pellet was left to dry for 15-20 min, then DNA pellet was re-suspended in 30 µl of ddH_2O.

2.2.1.7 Preparation of Glycerol Stocks

500 µl of an overnight bacterial culture was mixed with 500 µl of sterile glycerol in a sterile cryo-tube (Nunc).The tube was vortexed to ensure adequate mixing, incubated for 30 min on ice and then stored at -80°C.

2.2.1.8 DNA Midiprep Culture Inoculation and Plasmid Purification Using Macherey and Nagel Kits

A single colony of a positive miniprep clone was used to inoculate 100 ml of LB broth with Carbenicillin (100 µg/ml) and incubated 16–18h at 37°C, 220 rpm. The overnight 100 ml culture was split between two 50 ml sterile tubes and spun for 15 min at 4,000 × g, 4°C. Subsequently the plasmid DNA was purified from the culture by using the Nucleobond® Xtra Midi High copy plasmid protocol (Macherey-Nagel, Germany) until the elution step of the plasmid DNA. The eluate was distributed between six 1.5 ml micro centrifuge tubes; 833 µl of eluate was mixed with 583 µl of isopropanol at room temperature, before being centrifuged for 30 minutes at 15,800 × g and 4°C. The supernatant was decanted and the DNA pellets were washed with 1 ml of 70% ethanol then centrifuged for 10 min at 15,800 × g, 4°C. The pellets were air-dried and resuspended in a total volume of 120 µl ddH_2O. The DNA was stored at -20°C.

2.2.1.9 DNA Gigaprep Culture Inoculation and Plasmid Purification using Macherey and Nagel Kits

A endotoxin–free plasmid DNA purification kit was used from Macherey & Nagel, Germany. The plasmid DNA was purified from bacterial culture (2 liter of LB medium with kanamycin (50 µg/ml) incubated for 16-18h at 37°C, 220 rpm. The overnight culture was split between two sterile tubes and centrifuged for 15 min at 6,000 × g, 4°C using a high speed centrifuge (Beckman Coulter, US). Pellets of the bacterial cells were resuspended in 120 ml of S1 buffer, then a 120 ml of S2 buffer was added and the suspension was incubated for 5 min at room temperature. 120 ml of pre-cooled S3 buffer was added and immediately mixed for 6-8 times until the off-white homogenous suspension was formed. The lysate then was poured into the NucleoBondR Bottle Top Filter (A ×10,000) using

the supplied vacuum. The clear bacterial lysate from later step was loaded onto the giga column after it was equilibrated with 100 ml of N2 buffer. Two washing steps with N3 (4 × 150 ml) and N4 (3 × 130 ml) was applied with discarding the flow-through each time. DNA was eluted with 100 ml of N5 buffer and 70 ml of room-temperature isopropanol was added and centrifuged at 15,000 × g for 30 min at 12°C. 10 ml of 70% ethanol was added to the pellet, vortexed briefly and centrifuged at 15,000 × g for 10 min at room temperature. Ethanol was carefully removed and the pellet was allowed to dry, then the DNA pellets were resuspended in TE-EF buffer. DNA concentration and purity was measured by nanodrop then it was stored at -20°C.

2.2.1.10 DNA Sequencing

DNA sequencing was performed by (Source Bioscience, Motherwell). Samples for sequencing were prepared according to the company's instructions. The NCBI BLAST server was used for sequence similarity analyses.

2.2.2 Cell Culture

2.2.2.1 Culture of *Leishmania*

L. mexicana and *L. tarentolae* wild type promastigotes were grown at 27°C without CO_2 in complete SDM79 media and re-inoculated every 3-4 days.

2.2.2.2 *Leishmania* Cell Counting

Leishmania promastigotes were counted after fixing using a haemocytometer and a 1:100 dilution (5 µl from the culture in the late logarithmic phase was added to 495 µl fixing solution). The haemocytometer cover slide was moistened and applied to the

haemocytometer chamber until Newton rings could be observed. 1: 100 dilution of fixed cell culture was pipetted under the cover slide of the haemocytometer. Fixed cells were observed under a light microscope with a 40× objective lens focused on the haemocytometer grid lines. *Leishmania* cells were counted on both side of the haemocytometer slide, and the averaged cell counts used to calculate cell density per milliliter. Total cell density calculated using: number of cells/ml = number of counted cells × dilution factor (100) × 10^4.

2.2.2.3 *Leishmania* Stabilates for Long-term Storage

10 ml of late-log phase *Leishmania* promastigote culture (density range of 2×10^7 cells/ml to 1×10^8 cells/ml) was centrifuged at 1,800 × g 4°C for 30 minutes. The supernatant was discarded and the pellet resuspended in 1.5 ml stabilate freezing solution for *Leishmania*, distributed in 500 µl aliquots in cryo tubes and frozen in the gaseous phase of liquid nitrogen. Tubes were submerged in liquid nitrogen 24 hrs later.

2.2.2.4 Electroporation of *Leishmania tarentolae*

Transfection of linearised DNA into *L. tarentolae* cells was performed using a Nucleofector II (Amaxa Biosystems, Lonza, Germany). 100 µl of cell electroporation buffer was prepared by combining 82 µl human T cell solution and 18 µl supplement solution provided in the Nucleofector II kit. A maximum volume of 10 µl of purified linearised plasmid DNA was mixed with the electroporation buffer before the cells were resuspended in it. *L. tarentolae* promastigotes were grown until a density of approximately 3×10^7 cells/ml. The cells were harvested by centrifugation for 2 min, at 5,600 × g and 4°C. The supernatant was gently removed from sedimented *L. tarentolae* cells and immediately replaced with 100 µl electroporation buffer by gentle pipetting. The

re-suspended cells were transferred to a pre cooled electroporation cuvette with no air bubbles present. Cells were electroporated using Nucleofector II program V-033 or X-001 and left on ice for 10 minutes. Electroporated cells were transferred from the cuvette to 10 ml SDM79 medium supplemented with 8% v/v heat-inactivated fetal calf serum, 0.3% v/v hemin and incubated at 27°C for 24 hours. On the second day, the electroporated cells were diluted 1:2 and 1:40 with supplemented SDM79 medium and the appropriate selective antibiotic (40 µM Puromycin) was added to the diluted cell cultures. 1:2 and 1:40 dilutions were pipetted into 96 well plates using a multi-channel pipette (200 µl per well) and incubated at 27°C for 10-14 days until turbid wells indicated growth of recombinant parasites.

2.2.2.5 Scale up of Cell Lines

96 well plates were visually inspected for signs of turbidity after incubation for 10-14 days. Turbid wells were checked using an inverted microscope to confirm the presence of *Leishmania* cells in the well. 200 µl of positive clones identified on the 96 well plate were transferred to 12 well plates containing 2 ml supplemented SDM79 medium with the appropriate selective antibiotic and incubated at 27°C for 72 h until turbidity was visibly observed in the well. Subsequently 40 µl from positive clones identified in 12 well plates were used to inoculate 10 ml of supplemented SDM79 medium with the appropriate selective antibiotic and the culture incubated at 27°C for approximately 96 h until the culture reached the late logarithmic growth phase.

2.2.2.6 Isolation of Genomic DNA from *L. tarentolae* Cells

The “isolate II genomic DNA kit from Bioline, UK” was used to isolate genomic DNA from *L. tarentolae* cells following the manufacturer’s instructions. Briefly, 3 ml of a

stationary phase culture of *Leishmania* promastigotes (approximately 10^7 parasites) was centrifuged at 15,800 × g for 30 seconds and sedimented cells were resuspend in 200 µl lysis buffer GL. 25 µl proteinase K and 200 µl lysis buffer G3 were added and the sample was incubated at 70°C for 10-15 min. 210 µl of 100% ethanol was added. For each sample a spin column was placed into collection tube and centrifuged at 11,000 × g for 1 min. After two wash steps the genomic DNA pellets were resuspended in 100 µl TE buffer and stored at 4°C until required. The concentration of genomic DNA was determined using a NanoDrop2000® (Thermo Scientific, Wilmington USA).

2.2.2.7 Polymerase Chain Reaction (PCR)

PCRs were performed using the Expand High Fidelity PCR System from Roche. Reactions were carried out in a 25 µl PCR tube using a *PE* thermocycler. Sequences of forward and reverse primers are showed in the table below:

Primers	Sequence
LeishSSU. for	5'-GATCTGGTTGATTCTGCCAGTAG-3'
pLexyup1.rev	5'-CCTACGTCAATCGCAGACCT-3'
SAP2mod2C.for	5'- AGCGACGTCCCTTCCTTCAA-3'
SAG1-2 rev	5'-CCACTACTGCAGCGGCACGA-3'
Cyc18.rev	5'- CTGGTGGTTCTCGAAGTCGC-3'

The reaction mixture was composed of: template 10-200 ng, forward primer (10 µM) 1 µl, reverse primer (10 µM) 1 µl, 10 × PCR buffer (15 mM $MgCl_2$) 5 µl, dNTPs (10 mM) 1 µl, HiFidelity DNA Polymerase 0.8 µl, ddH_2O was used to attain a final volume to 25 µl. PCR

temperature Sequence: initial denaturation step 94°C 3 min, then 30 cycles consisting of 94°C for 45 sec, 52°C for 30 sec, 72°C for 60 sec and a final extension step at 72°C for 7 min and then 4°C.

2.2.2 Culture of *T. gondii*

2.2.2.1 Maintenance and Passaging of Transfected *T. gondii* Prugniaud Strain

Tachyzoites were grown *in vitro* by infecting human foreskin fibroblast cells (HFF) in 10 ml of DMEM supplemented with L-glutamine (Invitrogen, UK), 125 µg penicillin/streptomycin (Sigma, USA), 125 µg Amphotericin B and 10% foetal calf serum (PAA,UK) in 75 cm^3 flasks (Corning, UK) at 37°C in 5% CO_2. When tachyzoites had multiplied and invaded 95% of the HFFs the flask was scraped using a cell scarper (TPP, Trasadingen, Switzerland) and poured into 50 ml tube. 1 ml of parasites were added to a confluent flask of HFF containing 9 ml of complete DMEM medium. This was incubated at 37°C in 5% CO_2.

2.2.2.2 Maintenance of *T. gondii* Beverley (RRA) Strain

Beverley cysts were maintained *in vivo* at the Strathclyde Institute of Pharmacy and Biomedical Sciences, by passage of infective brain tissue homogenates through outbred CD1 albino mice. Briefly, infected mice were sacrificed by CO_2 inhalation and the brain removed prior to homogenisation by repeated passing through a 21 gauge needle, containing 2 ml of sterile PBS (Lonza, UK), to obtain an even suspension. 15 µl of brain suspension was placed on a microscope slide and the cysts enumerated by light microscopy.

2.2.2.3 Maintenance of *T. gondii* RH Strain *in vivo*

In vivo stocks of RH *T. gondii* were maintained in BALB/c mice. Mice were infected intraperitoneally with RH tachyzoites. Approximately, four days later mice were sacrificed by CO_2 inhalation and tachyzoites harvested by intraperitoneal washout with 1 ml ice-cold PBS. Tachyzoites were counted in a haemocytometer, mice injected with 2,000 tachyzoites intraperitoneally.

2.2.2.4 *Toxoplasma* Lysate Antigen Preparation (TLA)

T. gondii tachyzoites (RH strain) were grown HFFs cells. The flasks content were scraped and poured in 50 ml falcon tube and then centrifuged at 216 × g for 10 minutes at 4°C. The pellet was resuspended in 500 µl of distilled water. The preparation was freeze-thawed, sonicated before passing through 25-gauge needle. The last step repeated 8-10 times (freeze/thaw/syringing process). Following centrifugation at 2,000 × g for 5 min, the supernatant was filtered through 0.22 nm filter, collected and diluted 1:10 with PBS. The protein concentration was measured using a Bradford's assay (Bradford, 1976).

2.2.3 Protein Expression and Purification Methods

2.2.3.1 Secreted Acid Phosphatase Assay

An acid phosphatase assay was used for protein expression detection in *Leishmania* cell-free culture supernatants, which were prepared from 25 µl culture at late logarithmic stage by sedimentation at 5600 × g, 4°C, 2 min. The total volume per well of a 96-well plate was 100 µl composed of 20 µl supernatant/media sample, 70 µl (50 mM *p*-nitrophenylphosphate in 100 mM sodium acetate pH 5.0). The assay was incubated at 37°C for 30 min. The reaction was stopped by addition 10 µl of 2M sodium hydroxide

solution leading to a visible colour change (yellow, positive reaction; pink, no activity). Absorbance readings for the assay were measured at 405 nm using a spectrophotometer (Spectramax M5, Molecular Devices, USA).

2.2.3.2 Production of Recombinant Protein from *L. tarentolae*

Production of recombinant protein was carried out using modifications of the method described by (Fritsche, 2007). 1-liter culture was used to scale up the amount of protein. The culture was incubated at 27ºC with agitation at 100 rpm using a shaking incubator until the parasites reached log phase 1×10^7 cells/ml. The parasite density and the effect of cell density on recombinant protein expression was monitored daily. Cell culture supernatants were harvested by centrifugation at 2,500 × g, 4ºC, 15 min.

2.2.3.3 Ammonium Sulfate (AS) Precipitation

Proteins were purified by adding the ammonium sulfate to obtain the desired salt concentration followed by centrifugation. Culture supernatants were taken from late log phase parasite cultures. *Leishmania* cultures were harvested by centrifugation at 2,500 × g for 20 min at 4ºC. Subsequently, solid AS was added to the culture supernatant with mixing to give 20%, 30%, 40%, 50%, and 60% saturation as shown in figure 2.1. 60 min was allowed for precipitation to happen. The pelleted protein could be readily solubilised using standard buffers.

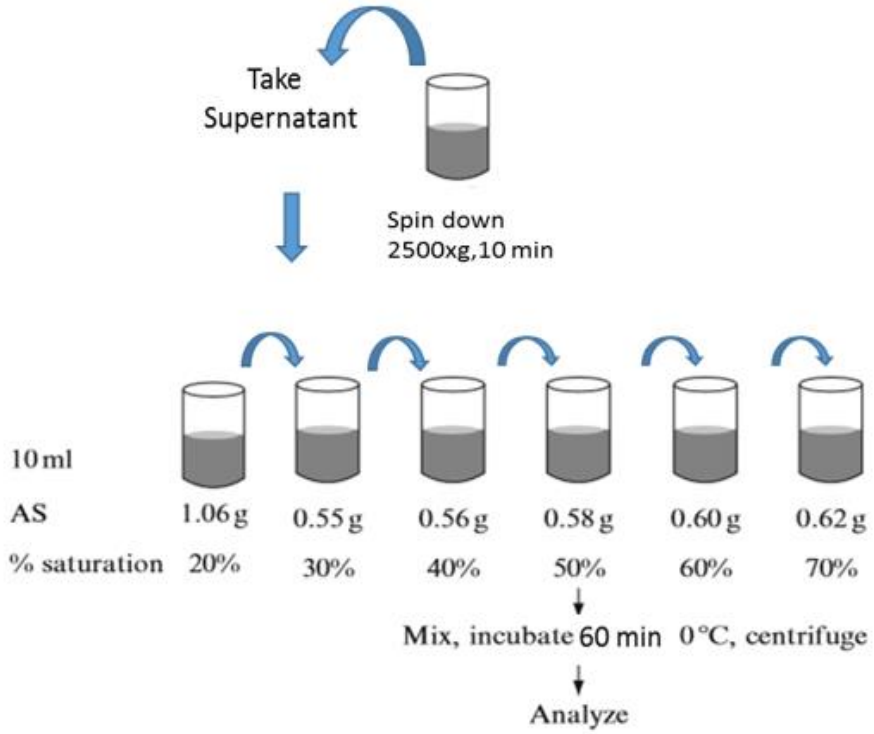


Figure 2. 1 Ammonium Sulfate Precipitation test.

Modified from (Burgess, 2009)

2.2.3.4 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Proteins were separated by SDS-PAGE consisting of a 10% (v/v) resolving gel and 4% (v/v) stacking gel. Protein samples were diluted in sample buffer with DDT (40 mM), boiled at 95°C for 10 min. Samples were electrophoresed at 30 mA/gel until the front dye had left the gel (~1hr). A protein marker was also loaded into one of the lanes of the gel. Gel running buffer was used in electrophoresis. Gels were placed in Coomassie stain for

20 min. Then destaining solution was applied on the gel 4-5 times for 20 minutes until the protein bands were clearly visible.

2.2.3.5 Immunoblot Analysis

The production of SAG1 and Cyc18 proteins by transfected *L. tarentolae* promastigotes was determined using immunoblot analysis. Proteins were transferred from a 10% SDS-gel to an Immobilon-P Polyvinylidene Difluoride (PVDF) membrane by electroblotting using a Fastblot B33/B34 (Biometra) system at a current of 4 mA/cm^2 of gel for 90 min. Prior to setting up the electro blot six Whatman papers, cut to the size of the resolving gel, were soaked in transfer buffer. The PVDF membrane was activated in 100% methanol for 1 min then soaked in transfer buffer for 5 min. After transfer the membrane was incubated for one hour at 37°C in blocking solution (5% (w/v) milk powder in PBST, 20 mM Tris-HCl pH 7.5). This was then replaced with blocking solution containing the primary monoclonal antibody (mAb) LT8.2 (1:2,000) or anti-His antibody (1:5,000) in blocking solution and incubated one hour with gentle agitation at 37°C. The membrane was then washed four times for 5 min with 1 × PBST at room temperature before goat anti-mouse IgG HRP- linked1:2,000-1:5,000 from DAKO, Hamburg, Germany) was added in blocking solution and incubated for one hour at 37°C. Three times washes for 5 min were carried out with 1 × PBST and two with 1 × PBS. The blot was incubated and exposed to X-ray films with solutions from the SuperSignal West Pico Chemiluminescent Substrate Kit (Fischer Scientific, Loughborough, UK) or 5 min-1hr.

2.2.3.6 Immunofluorescence Microscopy

Leishmania cell culture samples were prepared and fixed on a 10 well-masked slide. Each sample well on the slide was prepared by washing with 50 µl of 70% ethanol

followed by the application of 20 µl of 0.1 mg/ml polylysine in 1× PBS and incubation at room temperature for 15 min. After the incubation was completed each individual well on the slide was washed with 50 µl per well 1× PBS solution twice. 20 µl of total cell culture or cell culture supernatant in a cell density range of 1-2 × 10^7 cells/ml was fixed to each well with 20 µl of 4% *p*-formaldehyde solution by incubation for 15 min at room temperature. After the incubation was completed each well was washed once for 15 min at room temperature with 50 µl per well 1× PBS solution, 50 mM NH_4Cl, 0.1% Saponin. 50 µl per well of blocking buffer (1× PBS solution, 2% Bovine Serum Albumin (BSA), 1× PBS, 0.1% Saponin) was applied to the wells and incubated for further 15 min at room temperature. 20 µl of the primary antibody mAb LT8.2 in a 1:40 dilution in blocking buffer was applied for 1h at room temperature followed by two wash steps with 50 µl per well 1× PBS solution. Then the wells were incubated with 20 µl per well of a 1:500 dilution of the secondary antibody (goat anti mouse IgG -conjugated fluorescent tag) in blocking buffer at room temperature for 1 h. The slide was washed three times with 50 µl 1× PBS solution. 10 µl Mowiol/DABCO solution was applied to each well before the slide was sealed with a cover slip and left to dry for 15 min. Fluorescence was detected using an Epifluorescence microscope at 60× magnification.

2.2.3.7 Determination of Protein Levels in Samples

The protein concentration was determined using the Bio-Rad protein assay reagent. Briefly, 10 µl of protein standards (BSA 0.1-1 mg/ml) or 10 µl of recombinant protein sample were added to appropriate wells of a 96 well ELISA plate. 200 µl of Bio-Rad protein assay reagent, pre-diluted 1:5 with distilled water, were added to each sample. The absorbance of the samples was measured at O.D. 595 nm using a spectramax spectrophotometer (Bio-Tek, E-800, USA). The concentration of the unknown sample

was determined from the standard curve plotted using the protein standards by linear regression. In all cases, a correlation coefficient of > 0.97 was obtained.

2.2.4 Immunological Studies

2.2.4.1 Mice

BALB/c (20-25 g) in-house inbred mice supplied from the Strathclyde University were used for vaccine studies. Experimental groups consisted of five female mice (age 6-8 weeks old) per group. The experiments were conducted with sufficient sample sizes to have at least an 80% power to detect the differences at 5% level of significance. For DNA vaccination studies, mice were injected intramuscularly with 100 µl of 2 mg/ml plasmid solution (50 µl in each anterior tibialis). Mice received three immunisation doses at two week intervals for challenge experiments.

2.2.4.2 Caging

Each cage contained 5 mice with bedding, nesting material, food and water ad libitum. The room was maintained at a constant temperature and it was held on a 12 hours light and dark cycle.

2.2.4.3 Infection

The Beverley strain (type II) of *T. gondii* was used for oral infection. Mice were infected with10 cysts of Beverley strain in 200 µl of PBS delivered by gavage.

2.2.4.4 Ethical Approval

All the animals studies were carried out in accordance to guidelines of United Kingdom Home Office approval at the University of Strathclyde. All works were covered by local ethical requirements under license PPL60/4568, “Treatment & prevention of Toxoplasmosis”.

2.2.4.5 Tail Bleeding and Sera Preparation

Blood was collected into a heparinised capillary tube from the tail vein of mice at designated time points. Plasma was obtained following centrifugation at 2,100 × g for 10 minutes and stored at -20°C until further use.

2.2.4.6 ELISA (Enzyme- Linked Immunosorbent Assay)

The humoral response was assessed by measuring IgG1 and IgG2a antibody titers by ELISA. Briefly, ELISA plates were coated with TLA protein at 5 µg/ml in 50 mM sodium carbonate buffer, pH 9 overnight at 4°C with 100 µl in each well. The plates were washed 3 times with PBS containing 0.05% Tween 20. After the plates were blocked with 5% BSA in PBS at 200 µl/well and incubated for 1 h at 37 °C. Serum was serially diluted in blocking buffer and 100 µl added to each well. The plates were incubated at 37 °C for 1 h followed by 3 washes with 0.05% Tween-20 in PBS 0.01 M. Goat anti-mouse IgG1 and IgG2a (polyclonal) conjugated with horse radish peroxidase (HRP) (Invitrogen, USA) was added at 100 µl to each well after dilution 1:5,000 in blocking buffer to detect the bound antibodies after incubation at 37°C for 1 h. 100 µl of substrate composed of 10 ml of sodium acetate pH 5.5, 100 µl of Trimethyl benzidine (TMB), 5 µl H_2O_2 was used followed by incubation at 37°C for 30 min. To stop the reaction 50 µl of 10% (v/v) H_2SO_4 was added

to each well and the plate was read at 450 nm on a Spectramax spectrophotometer (Bio-Tek, E-800, USA).

2.2.4.7 Generation of Bone Marrow Derived Macrophages

Bone marrow was harvested from the femur and tibia of individual mice by flushing the bones with 5 ml of DMEM medium using a 25G needle. The cell suspension was passed through a cell strainer with an additional 5 ml of DMEM per bone. The suspension was centrifuged at 200 × g for 5 min. Precipitated cells were resuspended with complete DMEM (supplemented with 20% iFCS, 30% L-cell conditioned medium, 2% penicillin/streptomycin (100 µg/ml) and 2% L-glutamine). 10 ml of cell suspension from one bone was plated and incubated at 37°C in 5% CO_2. On day 3 another 10 ml of fresh, pre-warmed macrophage medium was added to each dish. On day 7, the medium was removed, discarded and fresh L-cell supplemented medium added. On day, 10 the macrophages were harvest from the plates using a cell scrapper and transferred to a 50 ml centrifuge tube. Cells were pelleted by centrifugation at 200 × g for 5 min and the pellets resuspended in 2 ml of complete RPMI medium (10% FCS, 2 mM L-Glutamine, P/S 100 µg/ml). Cells were counted by Trypan Blue exclusion using a haemocytometer. Following the appropriate dilution, the cells plated out and incubated overnight at 37°C in 5% CO_2 to allow the macrophages to adhere to the new plates. Cells were used for DNA transfection the following day.

2.2.3.3 DNA Transfection Assay

X-tremeGENE 9 DNA Transfection Reagent (Roche, Sigma-Aldrich, Gillingham, UK) address was used to transfect plasmid DNA with concentration 0.5-0.0625 µg/ml into macrophages cell lines. The DNA to transfection reagent ratio was (ratio 6:1 µl/µg DNA) as recommended in manufacturer's instructions (*http://www.powerful-transfection.com.)*

2.2.3.4 Cytokines Determination

Cytokine levels in the cell supernatants were determined by ELISA assay using anti mouse cytokine antibodies and cytokine standards. Briefly, a 96 well ELISA plate was coated with 50 µl/well of the appropriate rat anti-mouse anti-cytokine antibody (IL-12; clone:15.6 and IFN-ɣ; clone: DB-12), 2 µg/ml, in coating buffer (PBS pH 9). Plates were incubated overnight at 4ºC and then washed three times in wash buffer (PBS pH 7.4 containing 0.05% v/v tween-20). Plates were then blocked by adding 150 µl PBS pH 7.4 containing 10% v/v FCS to the appropriate wells of the plate and plates were incubated for 1 hour at 37ºC. Plates were washed as before and then 50 µl of cell supernatant or cytokine standard (serially diluted from 20 ng/ml with 10% v/v FCS in PBS pH 7.4) were added to the appropriate wells of the plate before incubating as before for 2 hours. Plates were washed as before and then 100 µl of the appropriate rat anti-mouse biotin anti-cytokine antibody (1 µg/ml, 10% v/v FCS in PBS pH 7.4) were added to the appropriate wells of the plate. Plates were incubated for 1 hour as before and then washed three times. Streptavidin alkaline phosphate conjugate (100 µl, 1:4,000 in 10% v/v FCS in PBS pH 7.4) was added to the appropriate wells of the plate before incubation for 1h at 37ºC. Plates were washed as above and then 100 µl of p-nitrophenyl phosphate disodium salt hexahydrate substrate (1 mg/ml in glycine buffer [0.1 M glycerin, 2 mM magnesium dichloride, 1 mM Zinc, pH 10.4]) were added to the appropriate wells of the plate.

Then the plate was incubated at room temperature in the dark for 20-60 minutes. The absorbance of the samples at 405nm was measured by Spectramax (Bio-Tek, E-800, USA) and the amount of cytokine (ng/ml) in the cell supernatants was determined from the standard curve plotted from standards run on the same plate. In each case, linear regression analysis of the standards gave a correlation coefficient of > 0.97. The mean cytokine production (ng/ml ± SE) for each treatment was determined.

2.2.3.5 Splenocytes Stimulation Assay

Mice were sacrificed by cervical dislocation and the spleens removed aseptically with forceps and placed into ice-cold 1×PBS in 1.5 ml centrifuge tubes. Single cell suspensions were created by pushing the spleens though a 70 µm cell strainer (BD Bioscience, UK), with ice-cold sterilised PBS wash buffer (10% FCS in sterile PBS). The cell suspension was centrifuged at 300 × g for 5 minutes and the supernatant discarded. The resulting pellets were resuspended in 3 ml of pre-warmed (37°C) RBS lysis buffer (Sigma, UK) which was used to lyse red blood cells. Then 5 ml of complete RPMI was added to neutralise the lysis buffer and re-centrifuged at 300 × g for 5 minutes and this was repeated twice to ensure the entire lysis buffer had been removed. The supernatant was discarded and the cells suspended with 3 ml complete RPMI. Cell concentrations were determined by mixing cell suspension 1:10 with Trypan blue solution and cells counted by using a haemocytometer and were viewed at 400× magnification on a microscope (Nikon Eclipse E400 Microscope, Nikon UK limited, London, UK). Medium containing 100 µl (0.5×10^7 cells/ml) were added to the appropriate wells of a 96 well tissue plate. Cells in triplicate wells stimulated with 100 µl cell of TLA (10 µg/ml) or left unstimulated as controls. The plates were incubated at 37°C with 5% CO_2 for 72h then stored at -20°C for cytokine determination as required.

2.2.3.7 Brain Homogenisation for Bead Array

Brain tissue was collected aseptically and placed on dry ice. 500 µl of fresh Halt protease and phosphatase inhibitor cocktail (Thermo Scientific™, UK) was diluted 1:1,000 in PBS and added to half brain samples. Brain tissue was minced into small sections, homogenised with T25 basic homogeniser and placed again on dry ice. Homogenates were centrifuged at 4,000× g at 4 °C for 15 min, and the resulting supernatants were collected in fresh tubes and stored at -20 °C for cytokine determination.

2.2.3.8 Bead Array

The Mouse Th cytokine and inflammation bead arrays (Biolegend, London, UK) were used to determine the cytokine levels in cell culture supernatants and brain homogenates. Cell culture supernatants were used without dilution. The Th cytokine panel allows simultaneous quantification of 13 mouse cytokines, including IL-2, 4, 5, 6, 9, 10, 13, 17A, 17F, 21, 22, IFN-ɣ and TNF-α, which are collectively secreted by Th1, Th2, Th9, Th17, Th22 and T follicular cells. The inflammation panel used for determining cytokine levels in brain homogenates after 2-fold dilution with assay buffer. 13 mouse cytokines, including IL-1α, IL-1β, IL-6, IL-10, IL-12p70, IL-17A, IL-23, IL-27, MCP-1, IFN-β, IFN-ɣ, TNF-α, and GM-CSF were measured corresponded to cytokines that are produced by innate immune cells. The standards and samples were assayed in duplicate according to the manufacturer's instructions (www.biolegend.com/legendplex). Data were analysed by using BioLegend's LEGENDplex software to determine the concentration of cytokines.

2.2.3.8 *In Vivo* Toxoplasma Luciferase Imaging

BALB/c mice were injected intraperitoneally with 20,000 luciferase expressing tachyzoites (Prugniaud) in 400 µl volumes. For imaging, a fresh stock of Luciferin at 15

mg/ml in PBS was prepared and filter sterilised using a 0.2 µm filter. Each mouse was injected with 150 mg luciferin/kg body weight (IP injection volume was 200 µl luciferin per a mouse). Mice were anesthetised with 3% isoflurane and imaged on an open filter with medium binning for 1 minute, 20 minutes after administration of luciferin. The total flux (photons/second) for each mouse was determined by using Living Image® Version 4.5 Software.

2.2.3.10 Statistical Analysis

The differences among all the groups were determined with a one-way ANOVA. The level of significance was set as less than 5% ($P < 0.05$). Non-parametric data from the *in vitro* and *in vivo* studies were analysed using the Mann Whitney U test and Kruskal Wallis, to assess differences in means between vaccinated and control mice. The statistical package GraphPadPrism 5.0® (Graph Pad Software, Inc., San Diego, CA, USA) for Windows was used for statistical analysis (www.graphpad.com).

Part I: Protein Expression

Chapter 3: Detection of Recombinant Filamentous *L. mexicana* SAP2 Fused to *T. gondii* Proteins (SAG1 and Cyc18) in *L. tarentolae* Culture Supernatants

3.1 Introduction

3.1.1 The secreted Acid Phosphatase of *Leishmania mexicana*

Leishmania mexicana is a protozoan parasite of the genus *Leishmania*, which are the causative agents of a range of diseases called leishmaniasis mostly found in tropical and subtropical regions (Dey *et al.*, 2014). *L. mexicana* insect stage promastigotes secrete a phosphomonoesterase in the form of a filamentous complex. Two single copy genes *lmsap1* and *lmsap2*, encode for two proteins of acid phosphatases with the distinctive features of a different length of the serine/threonine-rich regions. In the SAP1 protein this region is 32 amino acids long, whereas the serine/threonine repeats are composed of 383 amino acids in SAP2 (Wiese *et al.*, 1995). Both SAP1 and SAP2 are enzymatically active and assemble to form the secreted acid phosphatase filaments (Stierhof *et al.*, 1998; Wiese *et al.*, 1995). The monoclonal antibody (mAb) LT8.2 binds to an eleven amino acid long linear epitope in the COOH-terminal tail of SAP1 and SAP2 (Wiese *et al.*, 1999). The filaments are most likely assembled in the flagellar pocket (Figure 3.1).

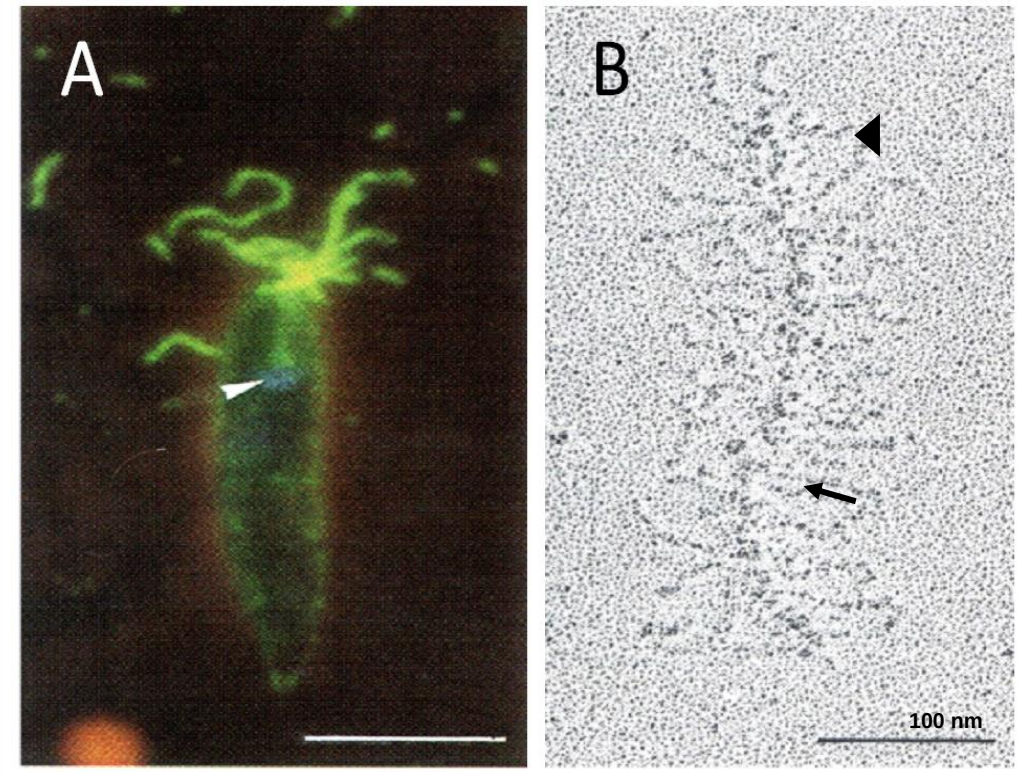


Figure 3. 1 Filamentous acid phosphatase.

(A) *Leishmania* promastigote with secreted acid phosphatase filaments emerging from the flagellar pocket shown in green, the white arrowhead refers to the kinetoplast of the promastigote. **(B)** Electron micrograph of acid phosphatase filament showing the central chain and side projections, short filaments of SAP1 (arrowhead); long filaments of SAP2 (black arrow). Size bar, 5 µm in A and 100 nm in B (adapted from Stierhof *et al*., 1998).

3.1.2 *Leishmania tarentolae*

Leishmania tarentolae, is a trypanosomatid protozoan parasite living as promastigotes mainly in the lumen of the intestine of lizards such as the gecko (*Tarentola annularis*) (Wilson & and Southern, 1979). In contrast to pathogenic *Leishmania*, *L. tarentolae* is distinguished by lacking 250 genes that are expressed in the intracellular amastigote stage. Hence, *L. tarentolae* has been used for heterologous gene expression and recombinant protein production (Raymond *et al.*, 2012). *L. tarentolae* has been used as protein expression system for both cytoplasmic and secreted proteins (see chapter 1).

3.2 Aims and Objectives

The aim of this chapter is to determine the effectiveness of a novel eukaryotic filamentous protein expression system based on the *L. mexicana* SAP2 in *L. tarentolae* for vaccine development.

1- Generation of plasmid constructs for expression of filamentous secreted acid phosphatase 2 fused with SAG1 (SAP2SAG1) or Cyc18 (SAP2Cyc18) using two different expression plasmids, pSSUSAP2MOD2C and pLEXSY-ble2.1.

2- Expression of the recombinant proteins using the plasmids mentioned above in *L. tarentolae*. Immunofluorescence microscopy and immunoblot analysis using the specific mAb LT8.2 and anti-hexahistidine tag antibodies.

3.3 Results

3.3.1 Plasmid Constructs

Two plasmid vectors pSSUSAP2MOD2C and pLEXSY-ble2.1 were used to generate the protein expression constructs. These plasmids contain two sequences derived from the 18S rRNA gene locus, a sequence of a modified secreted acid phosphatase 2 from *L. mexicana* (SAP2), the sequences of genes of interest (SAG1 and Cyc18 from *T. gondii)*, the sequences of antibiotic resistance genes for selection in prokaryotes and eukaryotes, plasmid propagation sequences in prokaryotes, and the sequences required for gene expression in *Leishmania* (Figure 3.2).

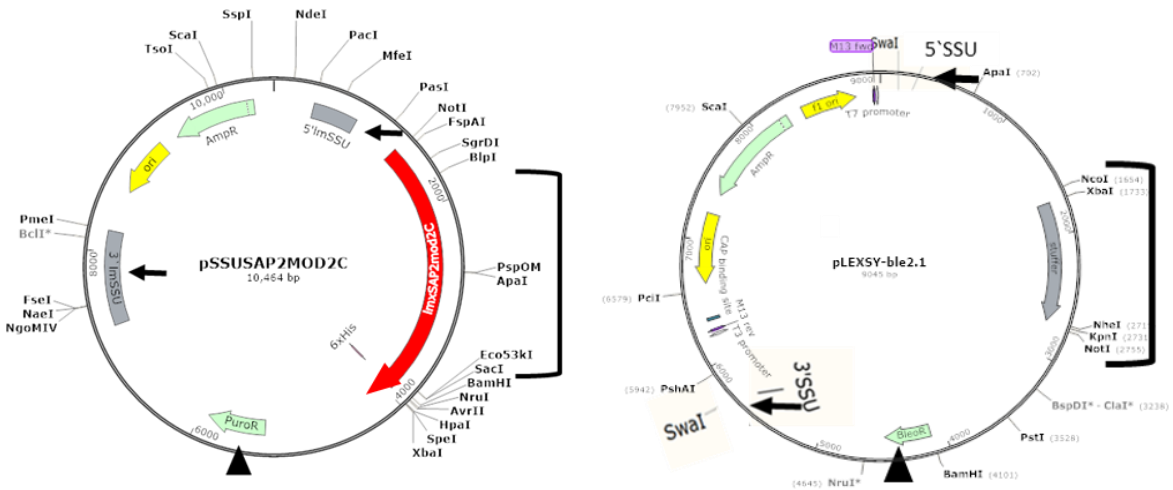


Figure 3. 2 pSSUSAP2MOD2C and pLEXSY-ble2.1 for gene expression in *L. tarentolae.*

5' and 3' small subunit of *L. tarentolae* rRNA genes (SSU) (black arrows); SAP2 (brackets); antibiotic resistance gene for selection in eukaryotes (arrowhead).

3.3.2 Generation of pSSUPacSAP2SAG1 and pSSUPacSAP2Cyc18

pUCCYCSAGSAP has the sequences that makes the structure of SAG1 same made by (He *et al.*, 2002). The expression constructs pSSUPacSAP2SAG1 and pSSUPacSAP2Cyc18 were generated by cleaving pSSUSAP2MOD2C and pUCCYCSAGSAP with AvrII + NruI to isolate the 10451 bp, 763 bp, and 541 bp fragments (Figure 3.3). The 763 bp and 541 bp fragments were ligated with the 10451 bp to generate pSSUPacSAP2SAG1 and pSSUPacSAP2Cyc18, respectively (Figure 3.4).

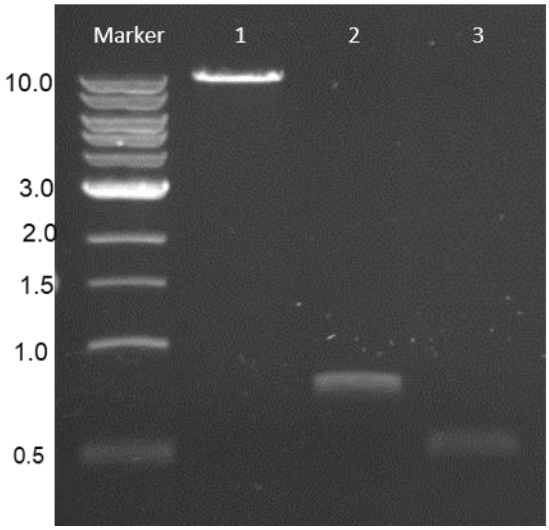


Figure 3. 3 Confirmation of purity and size of isolated AvrII/NruI fragments.

Lane 1, 10451 bp fragment from pSSUSAP2MOD2C; lane 2, 763 bp fragment from pUCCYCSAGSAP; lane 3, 541 bp fragment from pUCCYCSAGSAP. DNA size marker in kb.

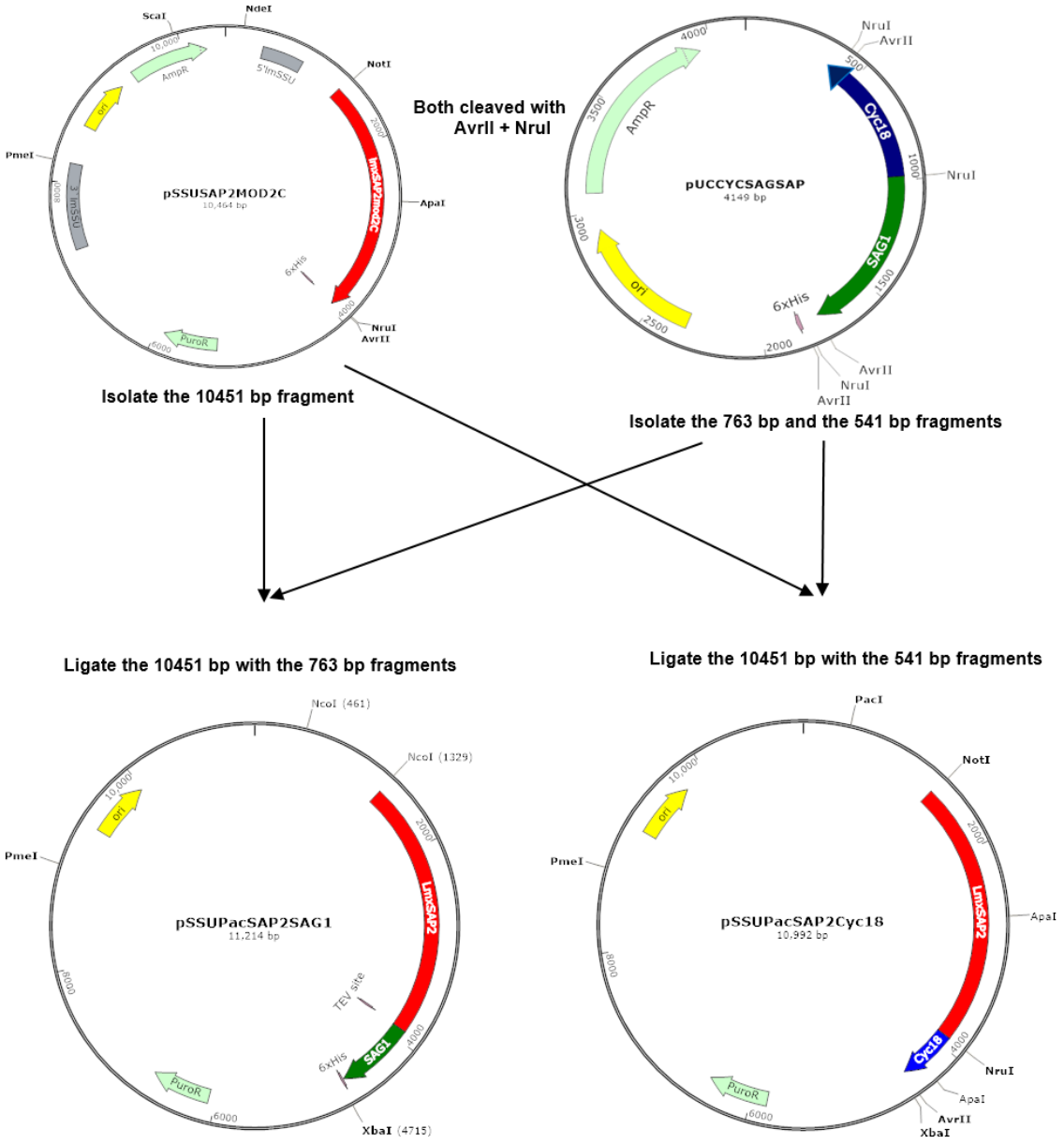


Figure 3. 4 Generation of pSSUPacSAP2SAG1 and pSSUPacSAP2Cyc18.

pSSUPacSAP2SAG1 and pSSUPacSAP2Cyc18 were cleaved with XbaI + MfeI, ApaI and NruI + NotI to confirm their correct identities. A detailed summary of expected fragment sizes is shown in table 3.1 and figure 3.5.

Table 3. 1 Restriction analysis of pSSUPacSAP2SAG1 and pSSUPacSAP2Cyc18.

Enzymes	pSSUPacSAP2SAG1 (lanes 1-3)	pSSUPacSAP2Cyc18 (lanes 4-6)
ApaI	9214 bp, 1688 bp, 288 bp and 24 bp	9400 bp and 1592 bp
NruI + NotI	8669 bp and 2545 bp	8447 bp and 2545 bp
XbaI + MfeI	7166 bp and 4048 bp	7166 bp and 3826 bp

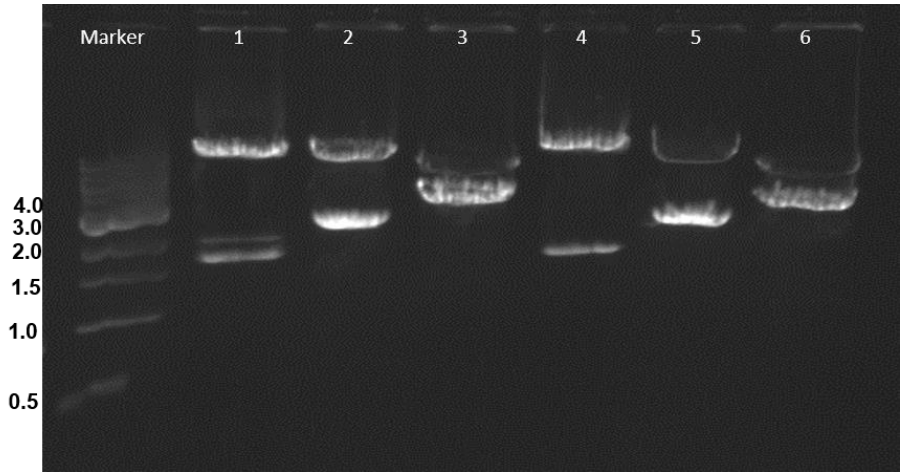


Figure 3. 5 Restriction analysis of pSSUPacSAP2SAG1 (lanes 1-3) and pSSUPacSAP2Cyc18 (lanes 4-6).

Lanes 1 and 4, ApaI; lanes 2 and 5, NruI+NotI; lanes 3 and 6, XbaI+MfeI. DNA size marker in kb.

3.3.3 Generation of pLPhSAP2SAG1 and pLPhSAP2Cyc18

A 7984 bp fragment was isolated from pLEXSY-ble2.1 cleaved with NcoI + NheI. pSSUPacSAP2SAG1 and pSSUPacSAP2Cyc18 were cleaved by NcoI + XbaI and the 3386 bp and 3164 bp fragments isolated, respectively (Figure 3.6). pLPhSAP2SAG1 was produced from ligation of 3386 bp fragment with the 7984 bp, while pLPhSAP2Cyc18 was generated from ligation of the 3164 bp fragment with the 7984 bp fragment (Figure 3.7).

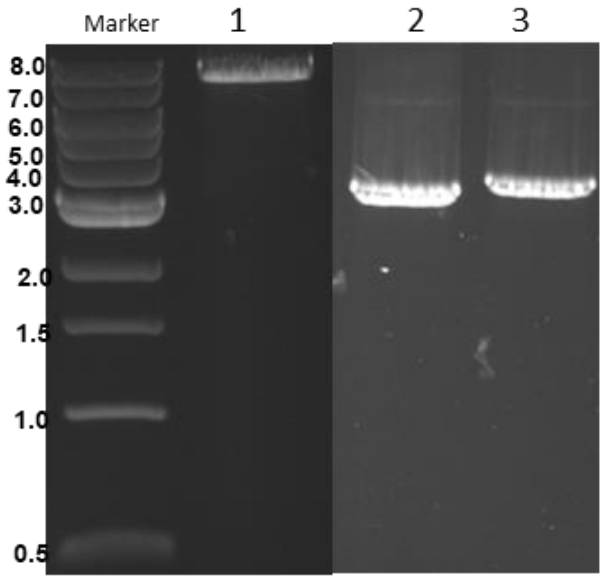


Figure 3. 6 Confirmation of purity and size of isolated fragments from pLEXSY-ble2.1, pSSUPacSAP2SAG1 and pSSUPacSAP2Cyc18.

Lane 1, 7984 bp fragment from pLEXSY-ble2.1; lane 2, 3164 bp fragment from pSSUPacSAP2Cyc18; lane 3, 3386 bp fragment from pSSUPacSAP2SAG1. DNA size marker in kb.

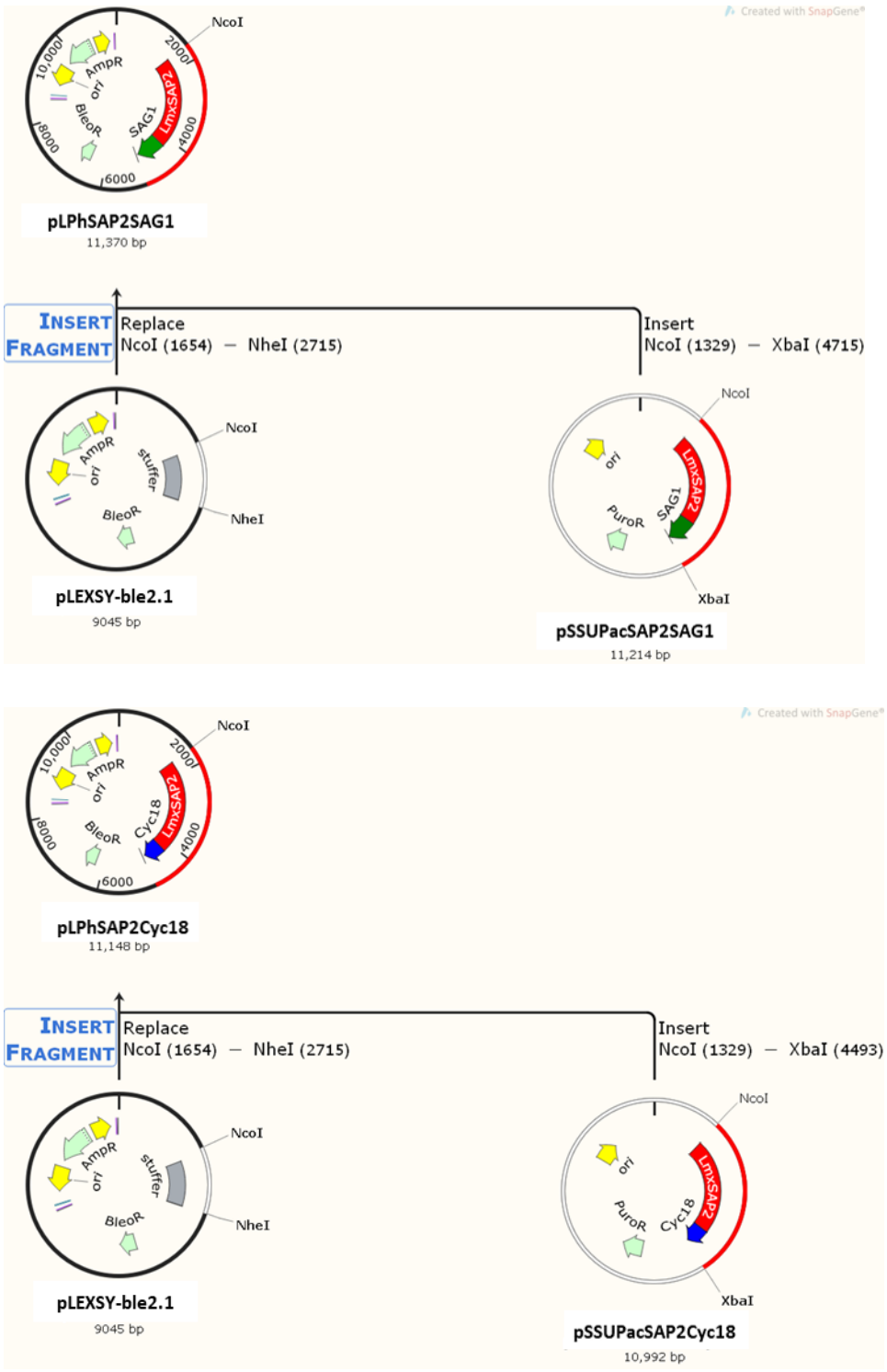


Figure 3. 7 Generation of pLPhSAP2SAG1 and pLPhSAP2Cyc18 from pLEXSY-ble2.1.

pLPhSAP2SAG1 and pLPhSAP2Cyc18 were transformed into *E. coli* DH5α, amplified, isolated and cleaved with BamHI, NotI and HindIII to confirm the identities of the plasmids (Table 3.2 and figure 3.8).

Table 3. 2 Restriction analysis of pLPhSAP2Cyc18 and pLPhSAP2SAG1.

Enzymes	pLPhSAP2Cyc18 (lane 1-3)	pLPhSAP2SAG1 (lane 4-6)
BamHI	2001 bp and 9147 bp	2223 bp and 9147 bp
NotI	3174 bp and 7974 bp	3396 bp and 7974 bp
HindIII	5094 bp and 6054 bp	5094 bp and 6276 bp

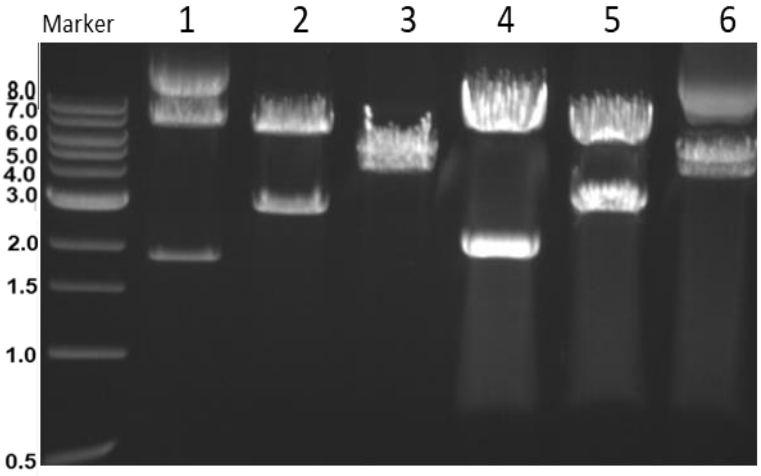


Figure 3. 8 Restriction analysis of pLPhSAP2Cyc18 (lanes 1-3) and pLPhSAP2SAG1 (lanes 4-6).

Lanes 1 and 4, BamHI; lanes 2 and 5, NotI; lanes 3 and 6, HindIII. DNA size marker in kb.

3.3.4 Generation of Linear DNA Fragments for Electroporation

Four linear fragments were isolated under sterile condition for electroporation. pSSUPacSAP2SAG1 and pSSUPacSAP2Cyc18 were cleaved with PmeI/PacI allowing the isolation of 8556 bp and 8334 bp DNA fragments, respectively. Whereas, pLPhSAP2SAG1 and pLPhSAP2Cyc18 were cleaved with SwaI to isolate the 8506 bp and 7207 bp DNA fragments, respectively. Figure 3.9 A and B show the confirmation of the fragment sizes.

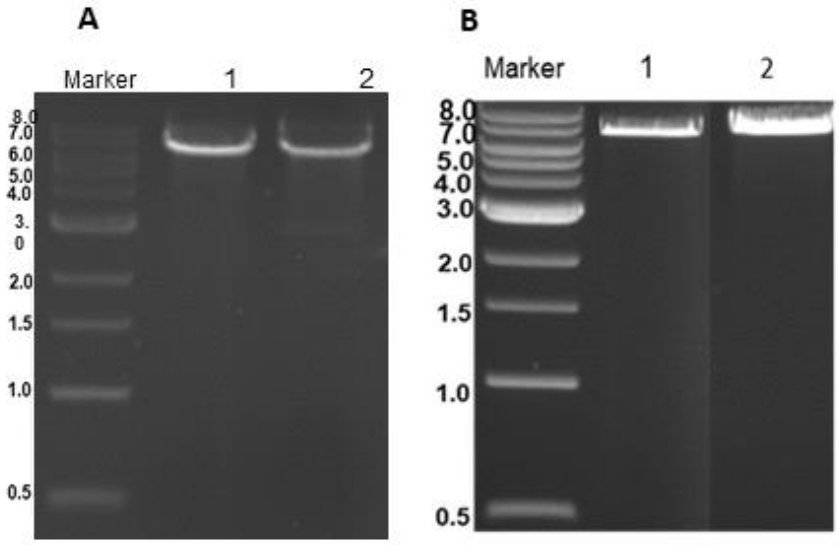


Figure 3. 9 Generation of linear DNA for electroporation.

(A) pSSUPacSAP2SAG1 and pSSUPacSAP2Cyc18 cleaved with PmeI + PacI resulted in 8556 bp and 8334 bp DNA fragments. **(B)** pLPhSAP2SAG1 and pLPhSAP2Cyc18 cleaved with SwaI resulted in 7207 bp and 8506 bp DNA fragments, respectively. DNA size marker in kb.

3.3.5 Transfection of *L. tarentolae* Promastigotes

Electroporation was performed to generate cell lines of *L. tarentolae* for expression of SAP2SAG1 and SAP2Cyc18 fusion proteins. Isolated linear fragments were transfected into *L. tarentolae* promastigotes. Turbid wells were obtained from 1:40 dilution of the electroporated cells on 96 well plates; numbers are 12, 9, 5 and 4 for PacSAP2SAG1, PacSAP2Cyc18, PhleoSAP2SAG1 and PhleoSAP2Cyc18, respectively. Four positive cell lines were chosen for each construct to scale up in 10 ml cultures for further analysis.

Table 3. 3 Number of turbid wells in 96 well plate after 10-14 days of incubation of electroporated cells at 27°C under selective pressure.

Transfected constructs	**No. of turbid wells in 1:40 dilution**
PacSAP2SAG1	12:96
PacSAP2Cyc18	9:96
PhleoSAP2SAG1	5:96
PhleoSAP2Cyc18	4:96

3.3.6 Confirmation of Integration of Constructs into the 18S rRNA Gene Locus by Polymerase Chain Reaction (PCR)

To test the correct integration of the construct into the rRNA gene locus specific oligonucleotide primers were chosen for PCR in order to amplify fragments of distinctive size (Table 3.4 and figure 3.10). Amplification of an 862 bp DNA fragment proved the correct integration of SAP2SAG1 and SAP2Cyc18 constructs into the rRNA gene locus (Figure 3.11 A). In a second PCR, a 696 bp amplicon confirmed the fusion of the secreted acid phosphatase gene (SAP2) to SAG1 coding region (Figure 3.11 B). The fusion of secreted acid phosphatase to Cyc18 was shown by a 333 bp amplicon (Figure 3.11 C). Oligos SAP2MOD2C.Fw2 and Cyc18.Rv3 by chance amplified a band in *L. tarentolae* wild type, which also appeared in parasites carrying PacSAP2Cyc18 and PhleoSAP2Cyc18, which served as an excellent control in the PCR (Figure 3.11 C).

Table 3. 4 Oligonucleotide sequences used in PCR and expected fragment sizes for each PCR reaction.

Primers	Sequence	Size
LeishSSU.Fw1	5'-GATCTGGTTGATTCTGCCAGTAG-3'	862 bp
pLexyup1.Rv1	5'-CCTACGTCAATCGCAGACCT-3'	
SAP2MOD2C.Fw2	5'-AGCGACGTCCCTTCCTTCAA-3'	696 bp
SAG1.Rv2	5'-GTGAAGTGGTTCTCCGTCGG-3'	
SAP2MOD2C.Fw2	5'-AGCGACGTCCCTTCCTTCAA-3'	333 bp
Cyc18.Rv3	5'-CTGGTGGTTCTCGAAGTCGC-3'	

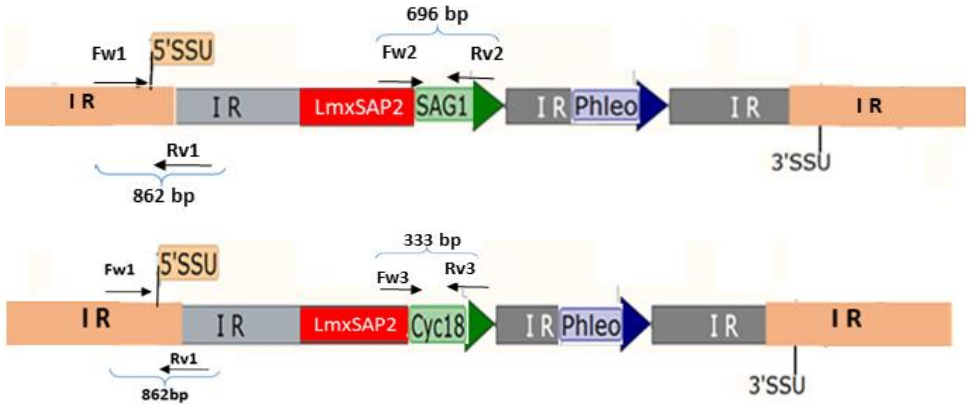


Figure 3. 10 Schematic representation of the different PCR reactions; oligonucleotide sequences used and expected fragments for each reaction.

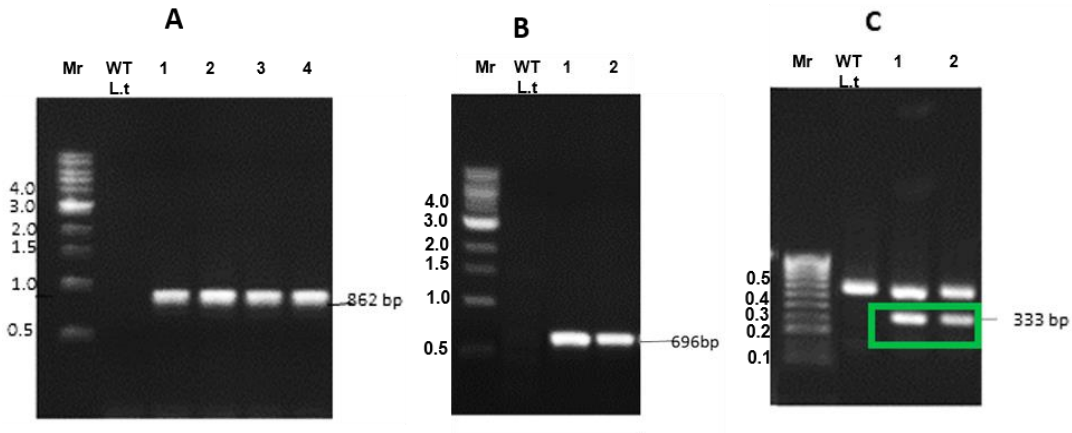


Figure 3. 11 PCR verification for correct integration of PacSAP2SAG1, PacSAP2Cyc18, PhleoSAP2SAG1 and PhleoSAP2Cyc18 into the rRNA gene locus of *L. tarentolae.*

(A) Integration of SAP2/SAG1 and SAP2/Cyc18 constructs into the *L. tarentolae* rRNA gene locus. Lanes 1-4, 862 bp amplicon reflects the correct integration. **(B)** Fusion of SAG1 to SAP2 for both PacSAP2SAG1 and PhleoSAP2SAG1. Lanes 1 and 2, the 696 bp fragments, confirmed the fusion of SAG1 to SAP2. **(C)** Fusion of Cyc18 to SAP2 for both PacSAP2Cyc18 and PhleoSAP2Cyc18. Lanes 1 and 2, the 333 bp amplicons validate the fusion of Cyc18 to SAP2 (green box). *L. t* WT is *L. tarentolae* wild type (negative control). DNA size marker in kb in A and B and 100 bp in C.

3.3.7 Secreted Acid Phosphatase Activity Detection in the Culture Supernatants of Recombinant *L. tarentolae* Promastigotes

Secreted acid phosphatase (SAP2) activity in culture supernatants was used to determine the presence of the recombinant fusion proteins. A quantitative spectrophotometric assay was performed to measure the SAP2 activity from different culture supernatants of recombinant *L. tarentolae* promastigotes grown in SDM79 medium supplemented with 10% iFCS and 7.5 µg/ml hemin. For comparison purposes, the supernatants of two controls, *L. mexicana* wild type (positive control) and *L. tarentolae* wild type (negative control), were also measured. Four cell lines of *L. tarentolae* promastigotes transfected with PacSAP2SAG1 and one cell line of *L. tarentolae* promastigotes transfected with PacSAP2Cyc18 showed enzymatic activity (Figure 3.12 A). *L. tarentolae* promastigotes transfected with PhleoSAP2SAG1 presented four clones with enzymatic activity, *L. tarentolae* transfected with PhleoSAP2Cyc18 did not result in any clones showing acid phosphatase activity (Figure 3.12 B).

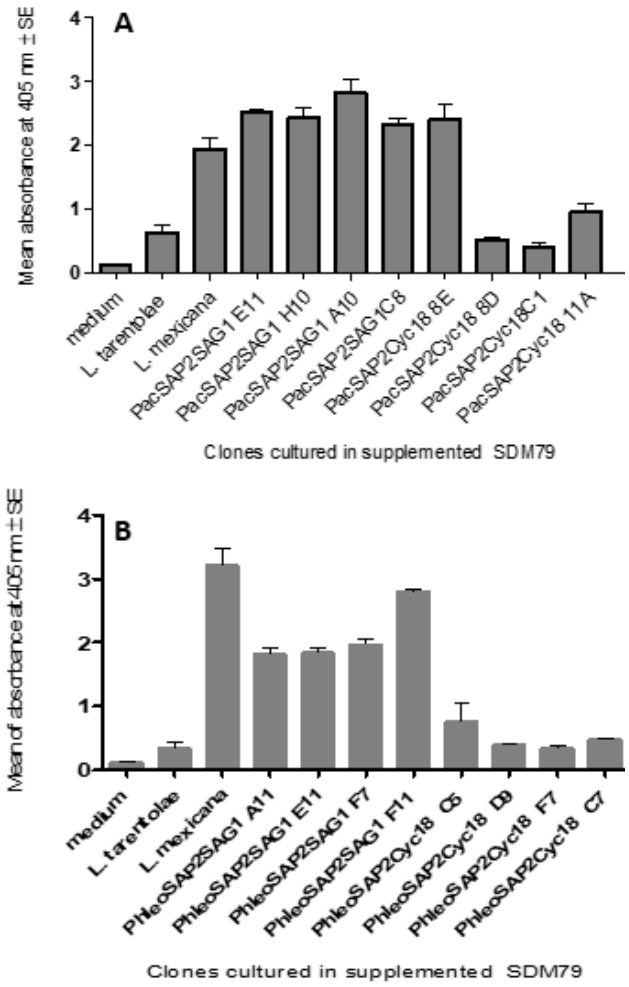


Figure 3. 12 Secreted acid phosphatase activity in supplemented culture supernatants of *Leishmania* promastigotes.

(A) *L. tarentolae* promastigotes transfected with PacSAP2SAG1 and PacSAP2Cyc18. (B) *L. tarentolae* promastigotes transfected with PhleoSAP2SAG1 and PhleoSAP2Cyc18. *L. mexicana* and *L. tarentolae* were used as positive and negative controls, respectively. Measurements were done in triplicate.

To determine the levels of recombinant protein in supplemented culture supernatants, the cells were grown over three days and the cell density and enzymatic activity were measured. At day one a low cell density and enzymatic activity was found. The second and third day presented similar enzymatic activity, but the cell density on day three suggested that the cells were already in stationary growth phase (Figure 3.13). Therefore, for the following experiments the second day of culture was selected to enrich the fusion protein from the culture supernatants of recombinant cells.

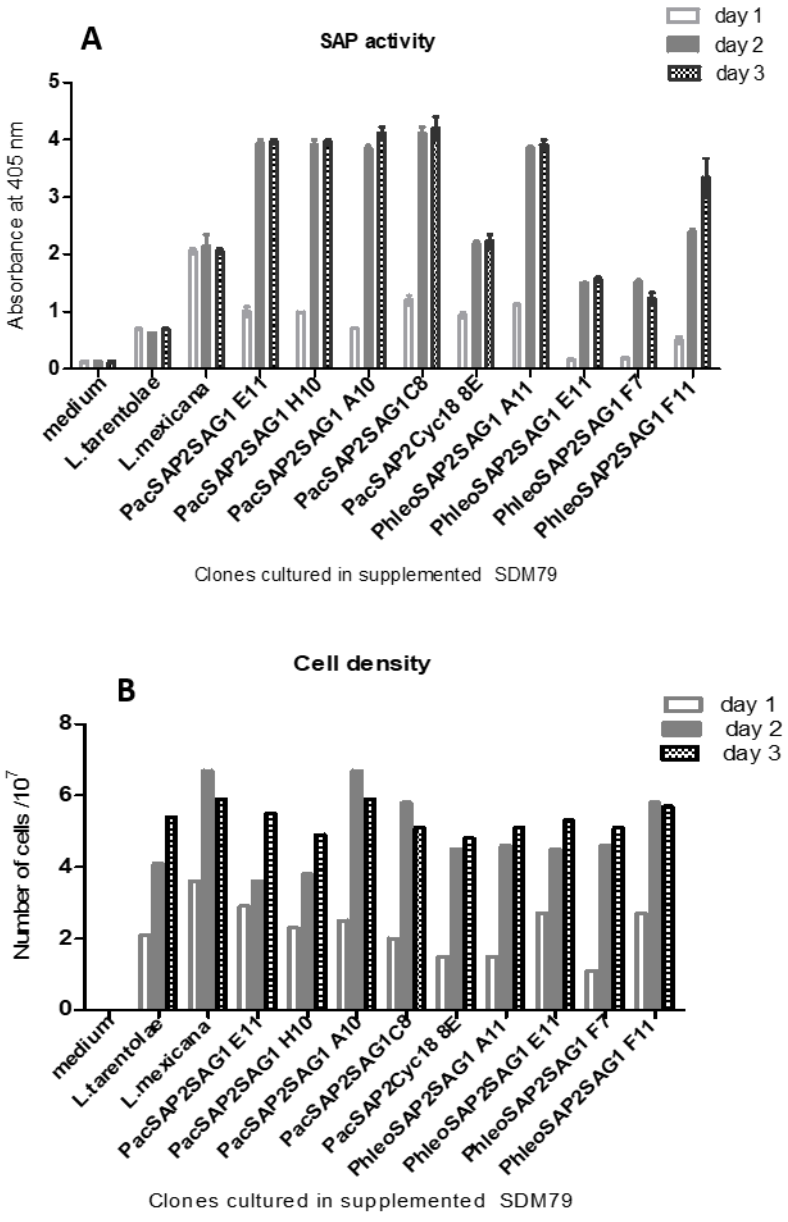


Figure 3. 13 Time course of secreted acid phosphatase activity in culture supernatants.

(A) Enzyme activity of recombinant *L. tarentolae* promastigotes from supplemented culture supernatants followed over three days. Measurements were done in triplicate. **(B)** Cell density over three days of incubation.

In order to make purification of the fusion protein from culture supernatants as simple as possible, it was attempted to grow the promastigotes in non-supplemented SDM79 medium. Four *L. tarentolae* cell lines transfected with the PacSAP2SAG1 fragment (E11, H10) or the PhleoSAP2SAG1 fragment (A11, F11), one *L. tarentolae* cell line transfected with PacAP2Cyc18 (E8), *L. tarentolae* and *L. mexicana* wild type were selected to grow in supplemented and non-supplemented SDM79 medium to determine the enzyme activity in the media. When compared, no activity was observed from promastigotes cells growing in non-supplemented media for two days; in contrast, growing cells in supplemented media showed high enzymatic activity (Figure 3.14).

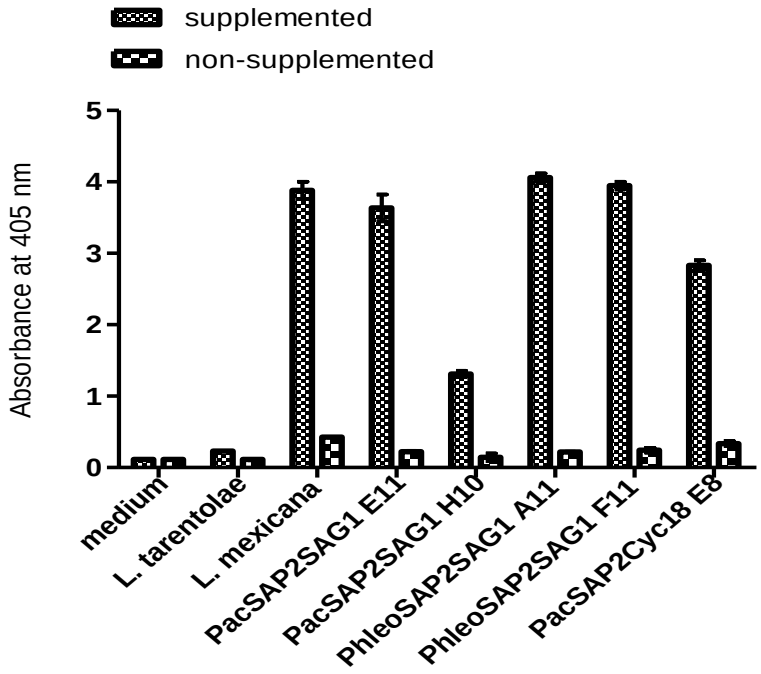


Figure 3. 14 Comparison of secreted acid phosphatase levels from culture supernatants of *Leishmania* promastigotes grown in supplemented and non-supplemented cultures.

WT *L. tarentolae* and *L. mexicana* were used as negative and positive controls, respectively. Measurements were done in triplicate.

3.3.8 Detection of Recombinant Protein in Culture Supernatants by Immunofluorescence Microscopy

Immunofluorescence analysis of cells and cell culture supernatants was used to detect the SAP2SAG1 and SAP2Cyc18 fusion protein secreted by *L. tarentolae* promastigotes into their culture supernatants. *L. mexicana* wild type promastigotes secrete large amounts of secreted acid phosphatase, which can be detected using the mAb LT8.2 and hence served as positive control. *L. tarentolae* promastigotes were not found to show any acid phosphatase activity in their culture supernatants and therefore showed no material detectable with mAb LT8.2 (negative control). Supernatants from *L. tarentolae* promastigotes transfected with PacSAP2SAG1 and PacSAP2Cyc18 showed less fluorescence than those from *L. mexicana* (Figure 3.15). However, *L. tarentolae* PhleoSAP2SAG1 promastigotes showed more fluorescent filaments in their culture supernatant.

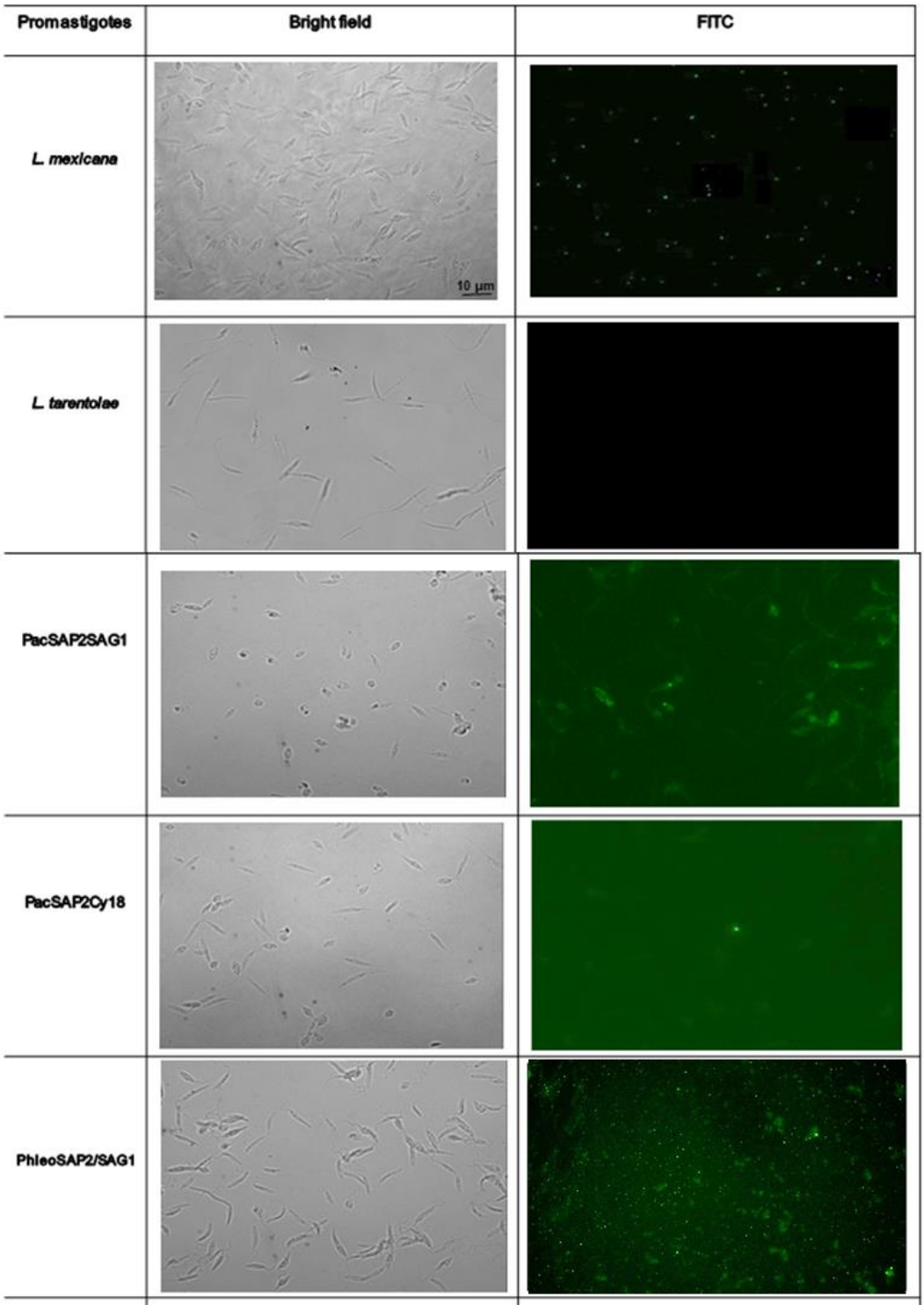


Figure 3. 15 Immunofluorescence analysis of *Leishmania* promastigotes in medium using mAb LT8.2 to detect secreted acid phosphatase.

Bright field microscopy using 40× magnification on the left and fluorescence microscopy using 488 nm excitation with FITC filter at the same magnification on the right. Size bar, 10 µm.

3.3.9 Detection of Recombinant Fusion Proteins in Culture Supernatants from Recombinant *L. tarentolae* Promastigotes by Immunoblot Analysis

Immunoblot analysis was used to detect the recombinant SAP2SAG1 and SAP2Cyc18 fusion proteins in the sediment of culture supernatants of recombinant *L. tarentolae* promastigotes after enrichment by ultracentrifugation using an anti-hexahistidine tag antibody and mAb LT8.2. A GSTLT8.2 epitope fusion protein of around 28 kDa served as a positive control for the mAb LT8.2 (Figure 3.16 C). mAb LT8.2 detected strong bands around 175 kDa in culture supernatants and promastigote cells for PacSAP2SAG1 and PacSAP2Cyc18 (Figure 3.16 B and C). The anti-hexahistidine tag antibody detected two bands in the same samples from culture supernatants (Figure 3.16 D). The 175 kDa band indicated the expression of full length SAP2SAG1 and SAP2Cyc18 fusion proteins, whereas the second, smaller bands are most likely SAG1 (30 kDa) and Cyc18 (18 kDa). *L. mexicana* samples showed a band of 89 kDa corresponding to SAP1 (Wiese *et al.*, 1999), while no band was detectable when using *L. tarentolae* (negative control).

L. tarentolae transfected with the PhleoSAP2SAG1 construct showed a strong band around 175 kDa from cell pellets using mAb LT8.2 (Figure 3.17 B). The anti-hexahistidine tag antibody detected a 175 kDa full length protein and a 30 kDa truncated protein in cells and supernatants (Figure 3.17 C and D).

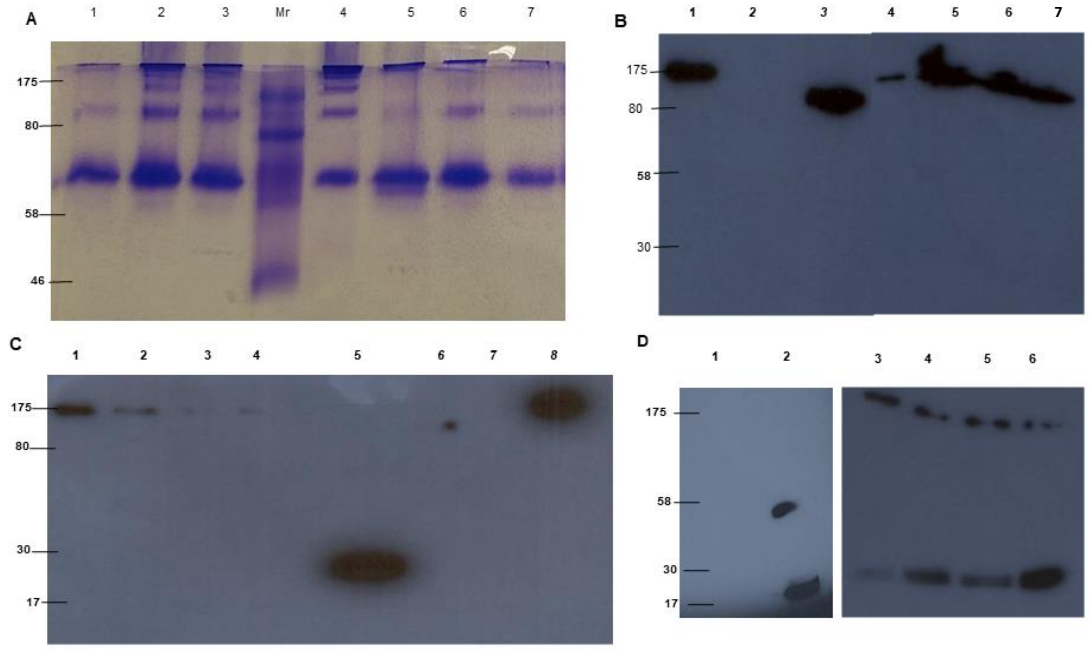


Figure 3. 16 Immunoblot analysis of culture supernatants and cells from *L. tarentolae* promastigotes expressing SAP2SAG1 and SAP2Cyc18.

(A) Coomassie-stained 10% SDS-PAGE. Lane 1, *L. tarentolae*; lane 2, *L. mexicana*; lane 3, PacSAP2Cyc18; lanes 4-7, PacSAP2CSAG1 (A10, H11, E11, C8). **(B)** mAb LT8.2 immunoblot from total cell lysate. Lane 1, PacSAP2Cyc18; lane 2, *L. tarentolae*; lane 3, *L. mexicana*; lanes 4-7, PacSAP2CSAG1 (A10, H11, E11, C8). **(C)** mAb LT8.2 immunoblot from culture supernatants. Lanes 1-4, PacSAP2CSAG1 (E11, A10, C8, H10); lane 5, GSTLT8.2; lane 6, *L. mexicana*; lane 7, *L. tarentolae*; lane 8, PacSAP2Cyc18. **(D)** Anti-hexahistidine tag immunoblot from culture supernatants. Lane 1, *L. tarentolae*; lane 2, PacSAP2Cyc18; lanes 3-6, PacSAP2CSAG1 (E11, A10, H10, C8). Protein size marker in kDa.

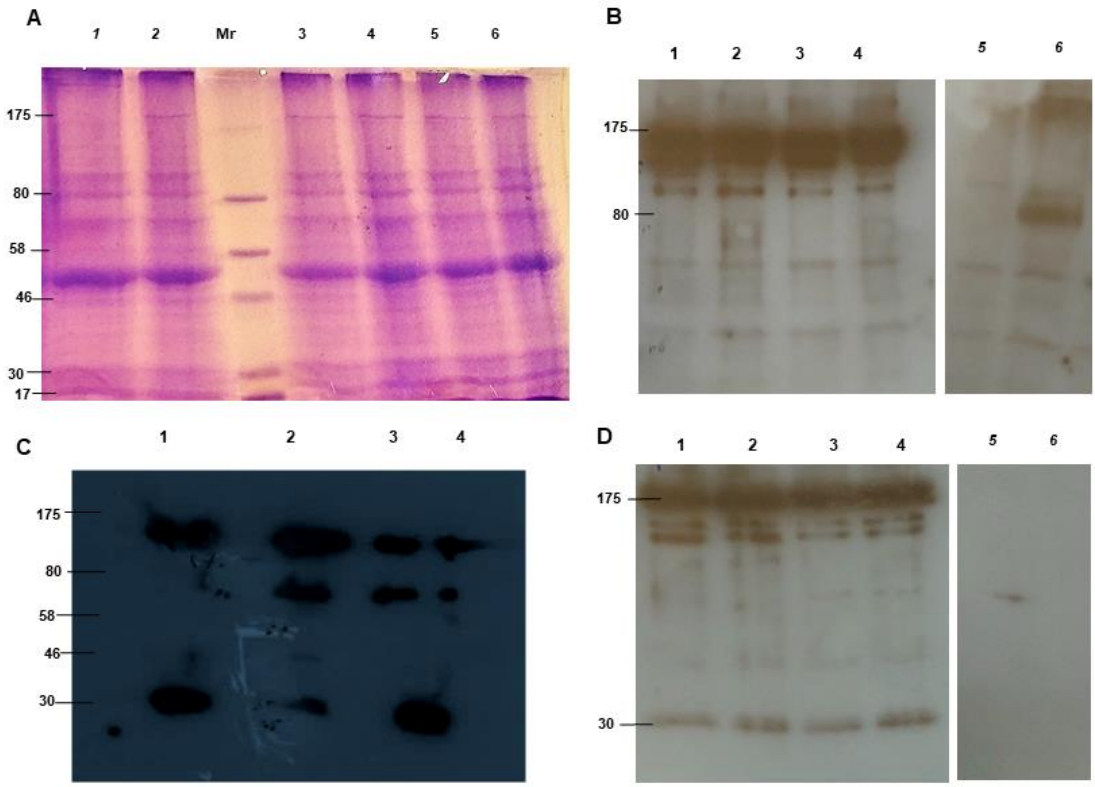


Figure 3. 17 Immunoblot analysis of *L. tarentolae* carrying PhleoSAP2SAG1.

(A) Coomassie-stained 10% SDS-PAGE. Lane 1, *L. tarentolae*; lane 2, *L. mexicana*; lane 3-6, PhleoSAP2SAG1 (A11, E11, F7, F11). **(B)** mAb LT8.2 immunoblot of promastigote cells. Lane 1-4, PhleoSAP2SAG1 (A11, E11, F7, F11); lane 5, *L. tarentolae*; lane 6, *L. mexicana*. **(C)** Anti-hexahistidine tag immunoblot of supernatants. Lane 1-4, PhleoSAP2SAG1 (A11, E11, F7, F11). **(D)** Anti-hexahistidine tag immunoblot of promastigote cells. Lane 1-4, PhleoSAP2SAG1 (A11, E11, F7, F11); lane 5, *L. tarentolae*; lane 6, *L. mexicana*. Protein size marker in kDa.

3.4 Discussion

3.4.1 DNA Constructs Generation

Because of its unique features, *L. tarentolae* has previously been used to express secreted proteins. These features entail, easy growth of parasites, protein purification from culture supernatants, and the presence of eukaryotic post-translational modifications (Kovtun *et al.*, 2011; Basile & Peticca, 2009). We have constructed a novel filamentous protein expression system using a DNA cassette for integration into the 18S ribosomal RNA gene locus using two different expression vectors (pSSU and pLEXSY-ble2.1). Different proteins have been successfully expressed in high levels using pSSU constructs in *Leishmania*, e.g. β–galactosidase and green fluorescent protein (Mißlitz *et al.*, 2000). pLEXSY has been used in *L. tarentolae* to produce and secrete small proteins, like human laminin-332 and c-reactive protein into the culture supernatant (Dortay *et al.*, 2011; Phan *et al.*, 2009). SAP2 from *L. mexicana* including its N-terminal phosphatase domain, the serine/threonine-rich repeats and the mAb LT8.2 epitope was used as a carrier for *T. gondii* proteins, SAG1 and Cyc18, which were genetically fused to its C-terminus. The identities of SAG1 and Cyc18 in the generated constructs were confirmed by restriction enzyme analyses as shown in figures 3.6 and 3.9 as well as by Sanger sequencing.

3.4.2 Efficiency of Electroporation

Trypanosomatids can be efficiently transfected by electroporation of *in vitro* cultivated promastigotes (Beverley & Clayton, 1993). Moreover, selection of recombinant parasites can easily be performed using antibiotic resistance genes, such as the genes for puromycin acetyl transferase and phleomycin binding protein, which were also used in

this investigation. To generate permanent and stable transfectants, linear DNA has to be used for chromosomal integration (Beverley & Clayton, 1993). However, when using higher DNA amounts homologous integration of multiple copies of the transfected DNA could be observed (Kianmehr *et al.*, 2016). The recombinant DNA cassette is integrated into the *L. tarentolae* genome through homologous recombination (Kianmehr *et al.*, 2016). To confirm integration into the small subunit rRNA gene locus of the *L. tarentola*e genome by homologous recombination diagnostic PCRs were performed. The PCR results proved both the fusion of SAG1 and Cyc18 genes to the SAP2 gene and the integration into the proposed site (Figure 3.11). It is important to note that transcription in this chromosomal location is under the control of RNA polymerase I, which allows high level transcription (Clayton, 1999; Teixeira, 1998).

3.4.3 Gene Expression in *L. tarentolae*

SAP of *L. mexicana* promastigotes was previously expressed in *L. donovani* (Ilg *et al.*, 1991) and *L. major* (Wiese *et al.*, 1999). It is easier to isolate and purify recombinant proteins if they are exported outside the cell. In this study, filaments composed of SAP2SAG1 and SAP2Cyc18 fusion proteins were generated and expressed in *L. tarentolae*. To improve protein yield transfection of *L. tarentolae* with multiple copies of the gene of interest integration into different gene loci could have been attempted (Breitling *et al.*, 2002). The recombinant constructs for gene expression were integrated into the 18S small subunit ribosomal RNA gene locus to achieve strong transcription. Interestingly gene regulation, in trypanosomatids, occurs on a post-transcriptional level through intergenic untranslated regions (UTRs) (Clayton, 1999). Therefore, the choice of suitable UTRs is crucial for the construction of an efficient expression cassette suitable for large-scale recombinant protein production (Breitling *et al.*, 2002). The expression

plasmid, pSSU, has the cysteine proteinase B (CPB2.8) intergenic regions, which were shown to result in high expression levels in *L. mexicana* (Mißlitz *et al.*, 2000). However, the SAP activity data in this study indicate a moderate expression level in *L. tarentolae.* The commercial plasmid, pLEXSY-ble2.1, contains three different untranslated regions (UTR1, UTR2 and UTR3), which were supposed to increase the expression level of recombinant proteins in *L. tarentolae*. UTR1 belongs to the adenine phosphoribosyl transferase gene of *L. tarentolae*; UTR2 is derived from the intergenic region of the calmodulin cluster and the UTR3 intergenic region is from the dihydrofolate reductase-thymidylate synthase gene locus of *L. major*. The LEXSY system has been successfully used to express small secreted proteins, like IFN-ɣ (Davoudi *et al*. 2011) and IL-29 (Taromchi *et al*. 2013).

Previous study purified the SAP protein from culture supernatant and concentrated by ultracentrifugation at 100,000 × g for 2h (Ilg *et al.*, 1991). In order to purify the SAP from *L. tarentolae* promastigote*,* ultracentrifugation was used at 200,000 × g for 3h to precipitate the filamentous proteins from culture supernatant then the recombinant proteins were detected by measuring SAP activity, immunofluorescence and immunoblot analysis.

3.4.4. Secreted Acid Phosphatase Activity

An advantage of the SAP1 and SAP2 fusion protein system is the ease of detecting protein expression by a phosphatase reporter assay directly from cell culture supernatants. The expression level of the SAP2 fusion proteins in *L. tarentolae* was tested by measuring the secreted acid phosphatase activity in the culture supernatant. Reasonable enzymatic level of the SAP2 fusion proteins from culture supernatant of *L. tarentolae* growing in 10% iFCS demonstrated the successful expression of SAP2SAG1

and SAP2Cyc18 in *L. tarentolae*. The secretion signal sequence (ER import signal) of *L. mexicana* secreted acid phosphatase was used to produce different proteins (reviewed, Basile & Peticca, 2009). To avoid interference of proteins, present in iFCS (mainly bovine serum albumin) with the purification of the recombinant protein, recombinant parasites were grown in non-supplemented media. No phosphatase activity was detectable in culture supernatants from these cells confirming the importance of iFCS in the culture medium. In fact, serum is required for *L. tarentolae* growth and for their use as expression system. An alternative medium, which does not require the addition of iFCS, would be more useful for protein purification purposes. Recently, a new serum- and protein-free medium for promastigote growth over multiple passages was established in which, essential nutrients were added individually for sustaining *L. mexicana* promastigote growth (Nayak *et al.*, 2018). This medium might prove useful for production and purification of SAP2 fusion proteins from *L. tarentolae* culture supernatants.

3.4.5 Immunofluorescence Analyses

The detection of the recombinant proteins by immunofluorescence confirmed successful secretion of the recombinant proteins into culture supernatants. When comparing the amount of green fluorescence obtained from *L. mexicana* wild type, which served as a positive control, SAP2SAG1 and SAP2Cyc18 expressed from pSSU showed less fluorescence. On the other hand, SAP2SAG1 expressed using pLEXSY-ble2.1 revealed more filamentous structures along with stronger green fluorescence signals. There was also a slight difference in expression levels between SAG1 and Cyc18 from cell lines transfected with pSSU. The SAP2SAG1 cell lines consistently showed higher levels of expression than the SAP2Cyc18 cells. SAG1 is a prototypic member of a superfamily of surface glycoproteins, which is mostly located at the surface of *T. gondii* (He *et al.*, 2002).

This makes it more likely to be secreted normally through the secretory pathway of other protozoan parasites like *L. tarentolae*.

A possible explanation for the low enzymatic activity of SAP2Cyc18 in the culture supernatant is that Cyc18 is a cytosolic protein and normally found in cells of all tissues in mammals (Wang & Heitman, 2005). Therefore, it might not be stable when moved along the secretory pathway.

3.4.6 Immunoblot Analyses

Immunoblot analyses results of SAP2SAG1 and SAP2Cyc18 using the mAb LT8.2 showed strong bands around175 kDa from supernatants and cell pellets. The signal in the cell pellets is most likely derived from fusion protein retained in the flagellar pocket or from protein in transit in the secretory pathway. Two bands were detected in culture supernatants when using the anti-hexahistidine tag antibody. The 175 kDa bands correspond to the full length recombinant fusion protein, the bands at 30 kDa most likely indicate SAG1 and the 18 kDa band corresponds to Cyc18. Hence, the *T. gondii* proteins SAG1 and Cyc18 have been cleaved off from the fusion partner SAP2. Most secretory proteins undergo post-Golgi proteolytic cleavages that yield the mature, active proteins. Proteolytic processing is catalysed by various proteases, some of which are unique to the regulated secretory pathway. Generally, proteolytic maturation occurs in secretory or transport vesicles carrying proteins from the trans-Golgi network to the cell surface (Lodish *et al.*, 2000). SAP2 forms a filamentous complex with long serine/threonine-rich regions, which are highly glycosylated (Wiese *et al.*, 1995). These modifications will protect the protein against proteolytic degradation. Likewise, N-linked glycans are present on the N-terminal phosphatase domain of SAP2 protecting this part of the protein against degradation. Therefore, proteolytic cleavage most likely occurs following on from

the serine/threonine-rich domain leading to the generation of SAG1 and Cyc18 proteins (either 30 kDa or 18 kDa). Because of the presence of the hexahistidine tag at the very C-terminus of the construct these small proteins are detectable using the anti-hexahistidine tag antibody. It is interesting to note that the cleaved off proteins remain associated with the filaments even through the process of ultracentrifugation. SAG1 is known to form a dimer (He *et al.*, 2002), this could explain the presence of cleavage off SAG1 associated SAG1 still covalently bound to SAP2. Cyc18 has the ability to form a ternary complex, which could explain an extra band with a size of 54 kDa detectable using the anti- hexahistidine tag antibody on the blot in figure 3.16 D (Wang & Heitman, 2005). Similar to SAG1, the property of forming a trimer allows purification of free Cyc18 along with Cyc18 fused to the SAP2 filaments. However, the culture supernatants more likely contained both a truncated and full fusion proteins.

The data gathered by the use of enzyme activity measurement, immunoblot and immunofluorescence analyses in this study provide convincing evidence that the proteins of interest have been expressed successfully. Jena Bioscience stated that their pLEXSY-ble2.1 system might be able to deliver protein yields of up to 500 mg/L of culture. Protein amounts obtained for SAP2SAG1 and SAP2Cyc18 using pSSU or pLEXSY-ble2.1 were not determined because of too high serum contaminations in the samples. However, it can be said from the results from the Coomassie-stained gels that the amount of protein is more in the μg than in the mg range. To improve the protein expression levels of the filamentous fusion proteins, SAP2 protein was replaced with SAP1, which has a shorter serine/threonine-rich region, and expected to be expressed more efficiently was used to carry *T. gondii* proteins SAG1 and Cyc18.

3.5 Conclusions

- *Leishmania* codon-optimised genes were successfully generated for SAG1 and Cyc18, cloned as fusion constructs into pSSU and pLEXSY-ble2.1 and introduced into *L. tarentolae* to generate recombinant cell lines expressing filamentous fusion proteins SAP2SAG1 and SAP2Cyc18.
- PCR confirmed that the expression cassettes were successfully integrated into the small ribosomal subunit RNA gene locus.
- Immunoblot and immunofluorescence analyses demonstrated expression and secretion of recombinant filamentous fusion proteins into the culture supernatants of *L. tarentolae* promastigotes.
- Expression levels for the fusion proteins were deemed not to be sufficiently high. Therefore, a new construct using pLEXSY-ble2.1 and the gene for *L. mexicana* SAP1 will be generated as described in next chapter.

Chapter 4: Expression and Purification of *Toxoplasma gondii* Proteins (SAG1 and Cyc18) fused to *Leishmania mexicana* secreted acid phosphatase 1 in *Leishmania tarentolae*

4.1 Introduction

The goal of this chapter is to generate the constructs for expression of SAP1SAG1 and SAP1Cyc18. LT8.2 is a monoclonal antibody that recognises a linear epitope of SAP1, which is derived from *L. mexicana* (Wiese *et al*., 1995; Ilg *et al*., 1991). SAP1 is genetically fused to the N-terminus of SAG1 and Cyc18 allowing expression of a fusion construct. The presence of SAP1 will result in a multimeric, filamentous protein. SAG1 and Cyc18 originate from *T. gondii*. A hexahistidine tag is located at the C-terminus of each fusion protein. The whole DNA construct is designed for integration into the ribosomal small subunit (SSU) RNA gene locus of *L. tarentolae*. The amino acid sequences of the recombinant proteins are presented in detail in figure 4.1.

SAP1SAG1

MASRLVRVLAAAMLVAAAVSVDARFVVRMVQVVHRHGARSALIDDNTTEICGTLYPCGEL
TGEGVEMVRAIGEFARSRYNNLSLVESPLFPSTRYNSSVVHTRSTHTQRTIQSATAFLRG
LFQDDYFYPVVYSTNRTTETLLSTDAVPSVVGRSWLDNPALHAALNPVIDEHLSWDAIQS
AAKDAWVEGLCADYNARTNCVLDMYDVAAAFEAAGRLDNATNLKAVYPGLQEVNAAWFKY
VFSWNHTSKLDLTQGSASQNLAQTVLANINAHRLSPSYNMFQYSAHDTTVTPLAVTFGDQ
GETTMRPPFAVTIFVELLQDTADASGWYVRLIRGNPVKAADGTYVFQESGIKAYCIDEAG
NKYLAHTGICPLNSFRRMVDYSRPAVADGHCAMTQTQYSNMDCPRTIADNKPVPSRCWLY
RHVCPSKACPDSYILSAVDHQCYPGPDVTNPTSSSSSEGTTTSSSSSSSKSTSSSDVPSF
KKPANWSPRVGSENLYFQSRPLVANQVVTCPDKKSTAAVILTPTENHFTLKCPKTALTEP
PTLAYSPNRHICPAGTTSSCTSKAVTLSSLIPEAEDSWWTGDSASLDTAGIKLTVPIEKF
PVTTQTFVVGCIKGDDAQSCMVTVTVQARASSVVNNVARCSYGANSTLGPVKLSAEGPTT
MTLVCGKDGVKVPQDNNHYCSGTTLTGCNEKSFKDILPKLSENPWQGNASSDNGATLTIN
KEAFPAESKSVIIGCTGGSPEKHHCTVQLEFAGPRGGHHHHHH

SAP1Cyc18

MASRLVRVLAAAMLVAAAVSVDARFVVRMVQVVHRHGARSALIDDNTTEICGTLYPCGEL
TGEGVEMVRAIGEFARSRYNNLSLVESPLFPSTRYNSSVVHTRSTHTQRTIQSATAFLRG
LFQDDYFYPVVYSTNRTTETLLSTDAVPSVVGRSWLDNPALHAALNPVIDEHLSWDAIQS
AAKDAWVEGLCADYNARTNCVLDMYDVAAAFEAAGRLDNATNLKAVYPGLQEVNAAWFKY
VFSWNHTSKLDLTQGSASQNLAQTVLANINAHRLSPSYNMFQYSAHDTTVTPLAVTFGDQ
GETTMRPPFAVTIFVELLQDTADASGWYVRLIRGNPVKAADGTYVFQESGIKAYCIDEAG
NKYLAHTGICPLNSFRRMVDYSRPAVADGHCAMTQTQYSNMDCPRTIADNKPVPSRCWLY
RHVCPSKACPDSYILSAVDHQCYPGPDVTNPTSSSSSEGTTTSSSSSSSSKSTSSSDVPSF
KKPANWSPRVGSENLYFQSRMKLVLLFLALAVSGAVRKAYMDIDIDGEHAGRIILELRED
IAPKTVKNFIGLFDKYKGSVFHRIIPDFMIQGGDFENHQGTGGHSIYGRRFDDENFDLKH
ERGVISMANAGPNTQGSQFFITTVKTEWLDGRHVVFGKITTESWPTVQAIEALGGSGGRP
SKVAKITDIGLLEHHHHHH

Figure 4. 1 Sequences of the recombinant protein monomers SAP1SAG1 and SAP1Cyc18.

SAP1 (pink) with the mAb LT8.2 epitope located at its C-terminal end (yellow) followed by the TEV protease cleavage site (grey); SAG1 (green); Cyc18 (blue) (*T. gondii* proteins). Hexahistidine tag (red) at the C-terminal end of the fusion construct.

4.2 Results

4.2.1 pLPhSAP1sSAG1 and pLPhSAP1sCyc18 Plasmid Constructs

The eukaryotic protein expression system pLEXSY-ble2.1 from Jena Bioscience (https://www.jenabioscience.com/) was used in this study. It has three different untranslated (intergenic) regions; UTR1, derived from a transferase gene from *L. tarentolae;* UTR2, derived from the calmodulin cluster (camCB); UTR3, the intergenic region of dihydrofolate reductase-thymidylate synthase gene of *L. major*. These three UTR regions have been found to result in an optimised protein expression in the *L. tarentolae* host LEXSY P10. Multiple cloning sites allow alternative fusion of the target genes to a region coding for the signal peptide of secreted acid phosphatase of *L. mexicana* for secretion of proteins, or production of cytosolic proteins. Secreted acid phosphatase 1 (SAP1) was used in this work fused to the proteins of interest, which are *T. gondii* surface antigen protein 1 (SAG1) and Cyclophilin 18 (Cyc18). pLPhSAP1sSAG1 and pLPhSAP1sCyc18 plasmids were generated in two cloning steps using the plasmids pLPhSAP2SAG1, pLPhSAP2Cyc18 and pSSUHygSAP1 (Fig. 4.2 and 4.3).

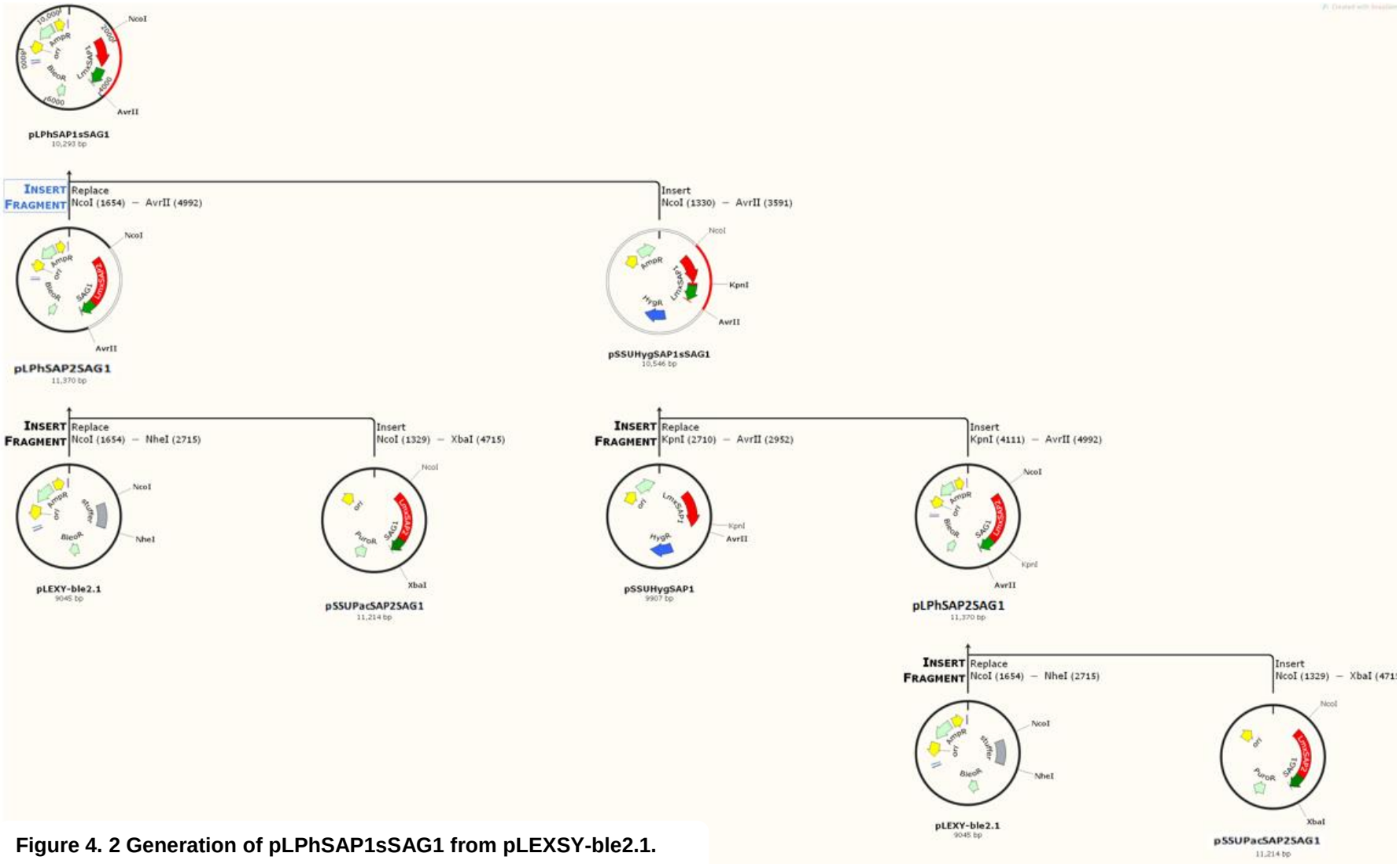


Figure 4. 2 Generation of pLPhSAP1sSAG1 from pLEXSY-ble2.1.

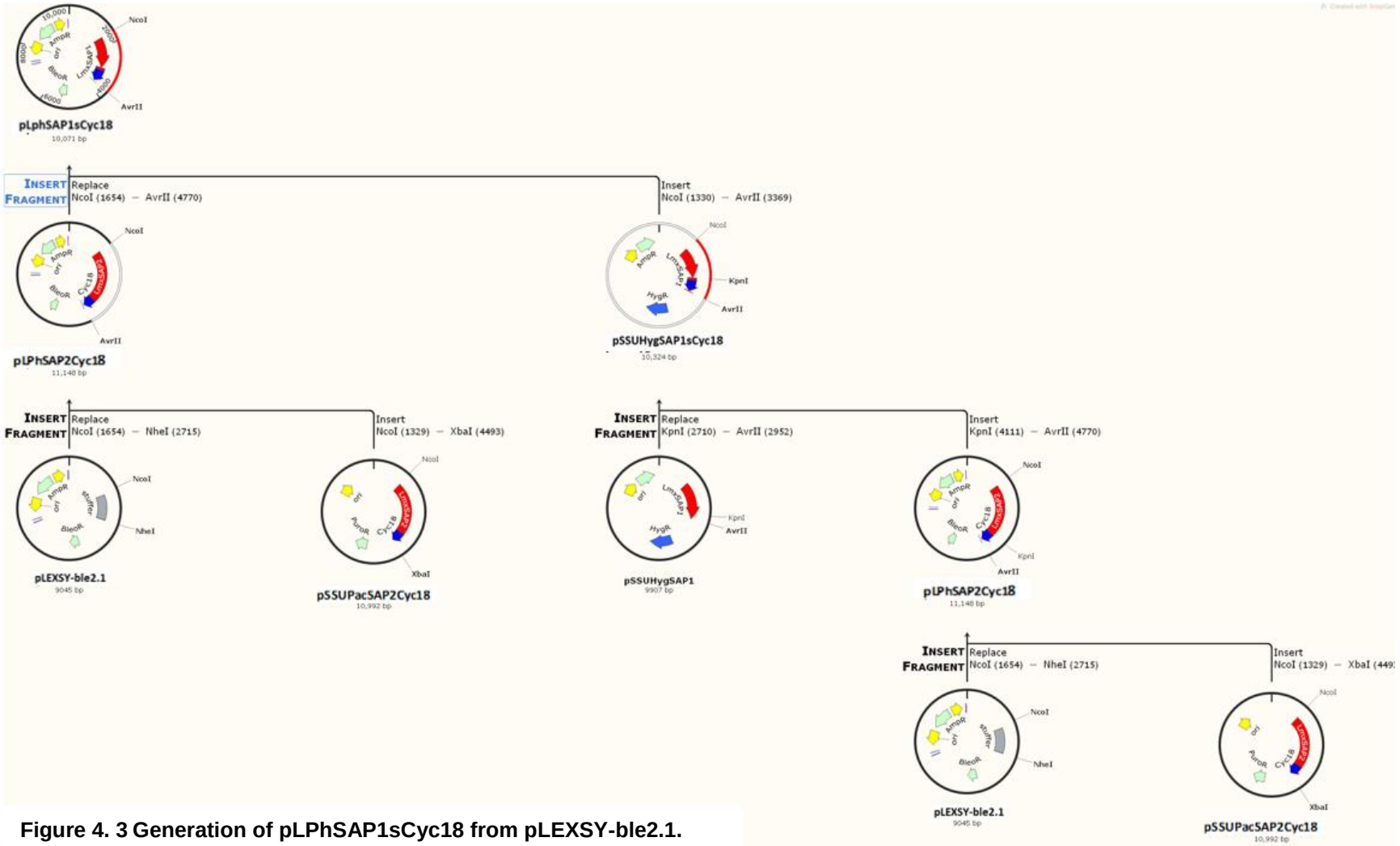


Figure 4. 3 Generation of pLPhSAP1sCyc18 from pLEXSY-ble2.1.

The first cloning step involved cleaving pSSUHygSAP1, pLPhSAP2SAG1 and pLPhSAP2Cyc18 (parent plasmids) with KpnI and AvrII restriction enzymes allowing the isolation of 9665 bp, 881 bp and 659 bp fragments, respectively.

The 881 bp and 659 bp fragments were ligated with the 9665 bp fragment to generate pSSUHygSAP1sSAG1 and pSSUHygSAP1sCyc18, respectively. After transformation into *E. coli* DH5α the plasmid DNA was isolated and a sample cleaved with the restriction enzymes BamHI, NcoI, and XhoI in separate reactions. Table 4.1and the figure 4.4 show the expected band sizes for pSSUHygSAP1sSAG1 and pSSUHygSAP1sCyc18.

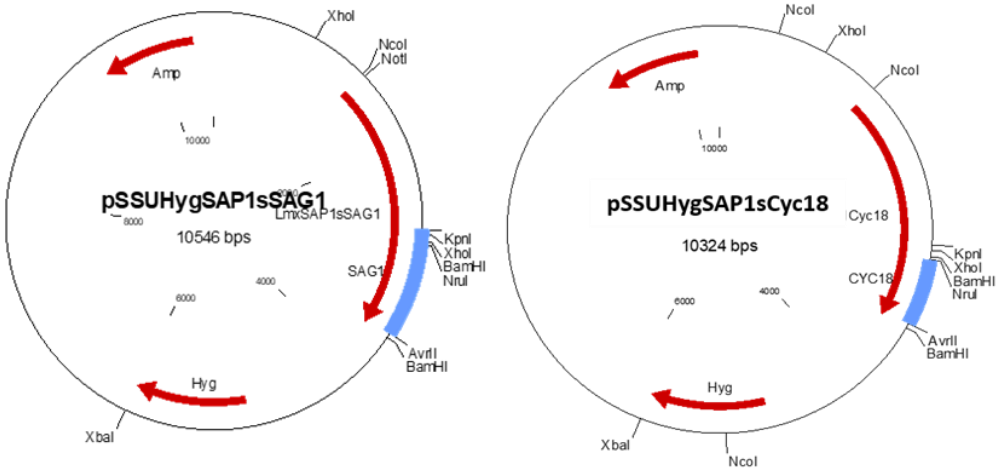


Table 4. 1 Expected fragmentation of pSSUHygSAP1sSAG1 and pSSUHygSAP1sCyc18.

Enzymes	pSSUHygSAP1sSAG1 (lanes 1-3)	pSSUHygSAP1sCyc18 (lanes 4-6)
BamHI	828 bp and 9718 bp	606 bp and 9718 bp
NcoI	869 bp, 4005 bp and 5672 bp	869 bp, 3738 bp and 5672 bp
XhoI	1894 bp and 8652 bp	1894 bp and 8430 bp

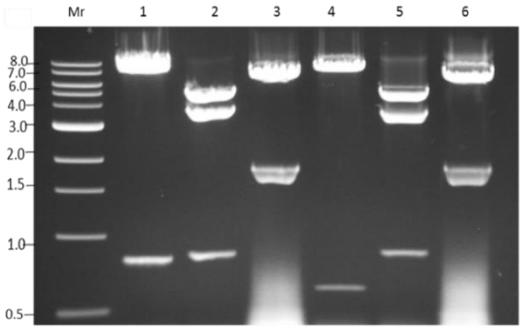


Figure 4.4 Restriction analysis of pSSUHygSAP1sSAG1 and pSSUHygSAP1sCyc18.

Lanes 1-3, pSSUHygSAP1sSAG1; lanes 4-6, pSSUHygSAP1sCyc18. Lanes 1 and 4, BamHI; lanes 2 and 5, NcoI; lanes 3 and 6, XhoI. Mr, DNA size marker in

The second cloning step was conducted by cleaving pLPhSAP2SAG1, pLPhSAP2Cyc18, pSSUHygSAP1sSAG1 and pSSUHygSAP1sCyc18 with NcoI and AvrII. The resulting 8032 bp, 2261 bp and 2039 bp DNA fragments were isolated. Each of the 2261 bp and 2039 bp fragments were ligated with the 8032 bp fragment to generate pLPhSAP1sSAG1 and pLPhSAP1sCyc18 and transformed into *E. coli* DH5α. The two plasmids were isolated and a sample cleaved with HindIII + BamHI, SwaI and NcoI, respectively. Table 4.2 and figure 4.5 illustrate a detailed summary of the expected fragment sizes and the actually observed fragments on an agarose gel.

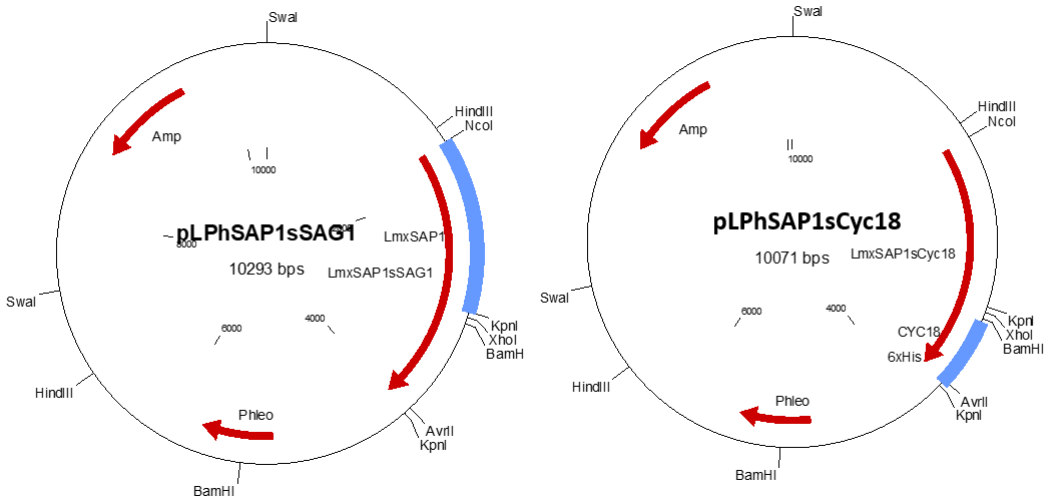


Table 4. 2 Expected fragmentations of pLPhSAP1sSAG1 and pLPhSAP1sCyc18.

Enzymes	pLPhSAP1sSAG1 (lanes 1-3)	pLPhSAP1sCyc18 (lanes 4-6)
HindIII + BamHI	1378 bp,1598 bp, 2223 bp and 5094 bp	1378 bp, 1598 bp, 2001 bp and 5094 bp
SwaI	2864 bp and 7429 bp	2864 bp and 727 bp
NcoI	10293 bp	10071 bp

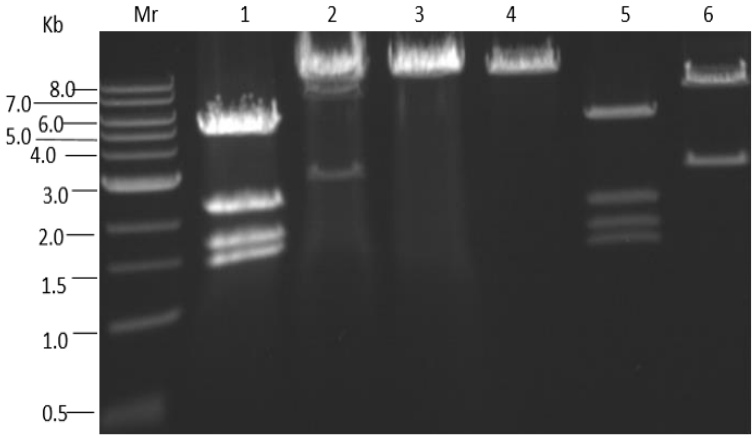


Figure 4.5 Restriction analysis of pLPhSAP1sSAG1 and pLPhSAP1sCyc18.

Lanes 1-3, pLPhSAP1sSAG1; lanes 4-6, pLPhSAP1sCyc18. Lanes 1 and 5, HindIII & BamHI; lanes 2 and 6, SwaI; lanes 3 and 4, NcoI. Mr, DNA size marker in kb.

4.2.2 Expression of Recombinant Protein

Expression of the recombinant protein in *L. tarentolae* was expected after transfection of the linear constructs into *L. tarentolae*. Positive clones were selected by adding the appropriate antibiotic (phleomycin 40 µg/ml) to the culture.

4.2.2.1 Preparation of Linear DNA Fragments for Electroporation into *L. tarentolae*

pLPhSAP1sSAG1 was cleaved with SwaI resulting in two fragments of 7429 bp and 2864 bp. pLPhSAP1sCyc18 was cleaved with the same enzyme resulting in two fragments of 7207 bp and 2864 bp. The 7429 bp and 7207 bp fragments were isolated under sterile conditions for electroporation into *L. tarentolae* (Figure 4.6 A and B).

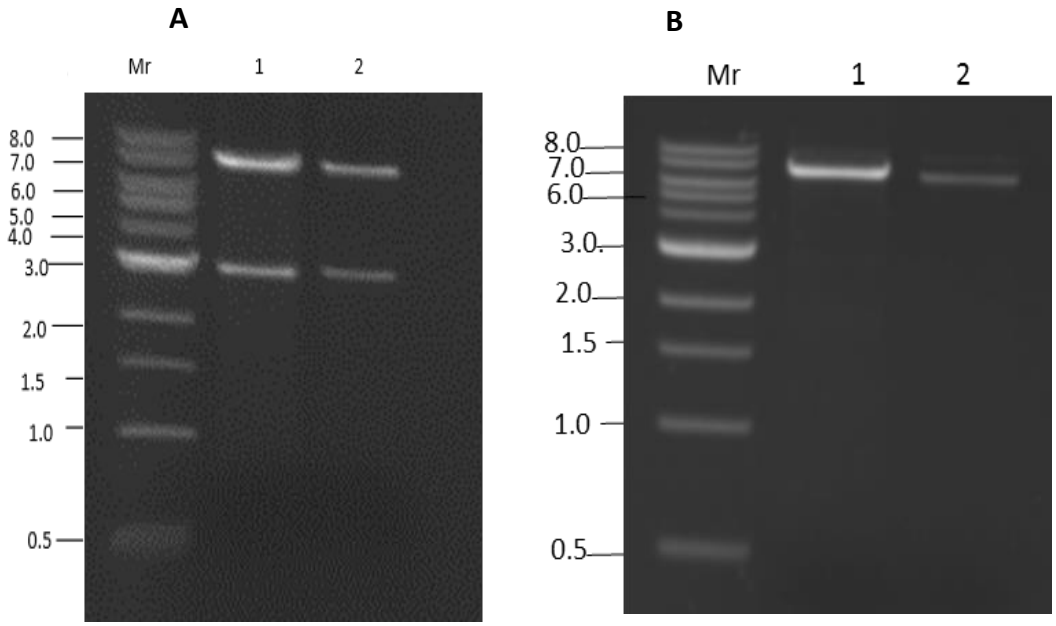


Figure 4. 6 Generation of linear DNA fragments for integration into the rRNA gene locus of *L. tarentolae*.

(A), SwaI digest of pLPhSAP1sSAG1 and pLPhSAP1sCyc18. Expected fragmentations are 7429 bp + 2864 bp and 7207 bp + 2864 bp (lane 1 and 2). **(B)**, isolated linear fragments. Lane 1, 7429 bp; lane 2, 7207 bp. Mr, DNA size marker in kb.

4.2.2.2 Transfection of *L. tarentolae* Promastigotes

Electroporation was used to generate cell lines of *L. tarentolae* for protein expression of SAP1SAG1 and SAP1Cyc18. Linear sterile DNA fragments were transfected into *L. tarentolae* promastigotes. The result of the electroporations is shown in table 4.3. 86 and 67 turbid wells were obtained from the 1:2 dilution of pLPhSAP1sSAG1 and pLPhSAP1sCyc18, respectively. For the 1:40 dilution, numbers are 12 and 8 for pLPhSAP1sSAG1 and pLPhSAP1sCyc18, respectively. The cell lines grown in the 1:40 dilution are most likely originated from one single cell each and therefore represent clones. Four cell lines from 1:40 dilution were chosen to scale up in 10 ml culture with phleomycin for further analysis.

Table 4. 3 Results of transfection of each cell line and the number of positive wells with visible turbidity in 96-well plate after 10-14 incubation days at 27°C.

transfected construct	No. of turbid wells in 1:2 dilution	No. of turbid wells in 1:40 dilution
SAP1sSAG1	86:96	12:96
SAP1sCyc18	67:96	8:96

4.2.2.3 Confirmation of Integration into SSU Gene Locus Using PCR

Genomic DNA was isolated from *L. tarentolae* WT and from one positive clone to confirm the integration. PCR was performed to analyse the correct integration of the respective constructs into the rRNA gene locus. The oligonucleotides and the size of the expected amplicons are shown in the table 4.4 and figure 4.7. The PCR products are shown on an agarose gel; no visible band was detectable for *L. tarentolae* WT, which served as a negative control. Amplification of an 862 bp fragment confirmed the integration of SAG1 and Cyc18 constructs into rRNA gene locus of *L. tarentolae*. The 628 bp and 333 bp fragments in the PCR reaction proved the correct fusion of the secreted acid phosphatase (SAP1) to SAG1 and Cyc18, respectively (Fig. 4.8). As expected, *L. tarentolae* WT did also not show any amplified fragment in the second PCR.

Table 4.4 Oligonucleotide sequences used in PCR and expected fragment sizes for each PCR reaction.

LeishSSU (Fw1)	5'-GATCTGGTTGATTCTGCCAGTAG-3'	862 bp
pLexyup1 (Rv1)	5'-CCTACGTCAATCGCAGACCT-3'	
SAP2mod2C (Fw2)	5'-AGCGACGTCCCTTCCTTCAA-3'	628 bp
SAG1-2 (Rv2)	5'-CCACTACTGCAGCGGCACGA-3'	
SAP2mod2C (Fw3)	5'-AGCGACGTCCCTTCCTTCAA-3'	333 bp
Cyc18 (Rv3)	5'-CTGGTGGTTCTCGAAGTCGC-3'	

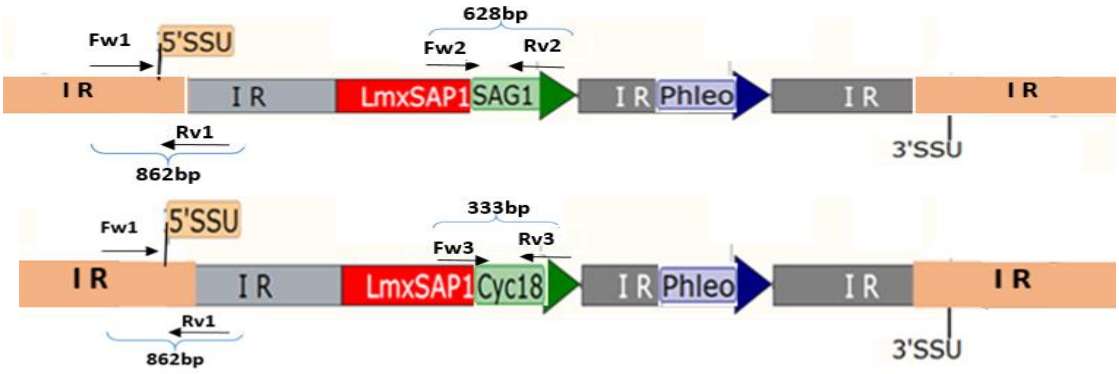


Figure 4. 7 Schematic representation of the different PCR reactions; oligonucleotide sequences used and expected fragments for each reaction.

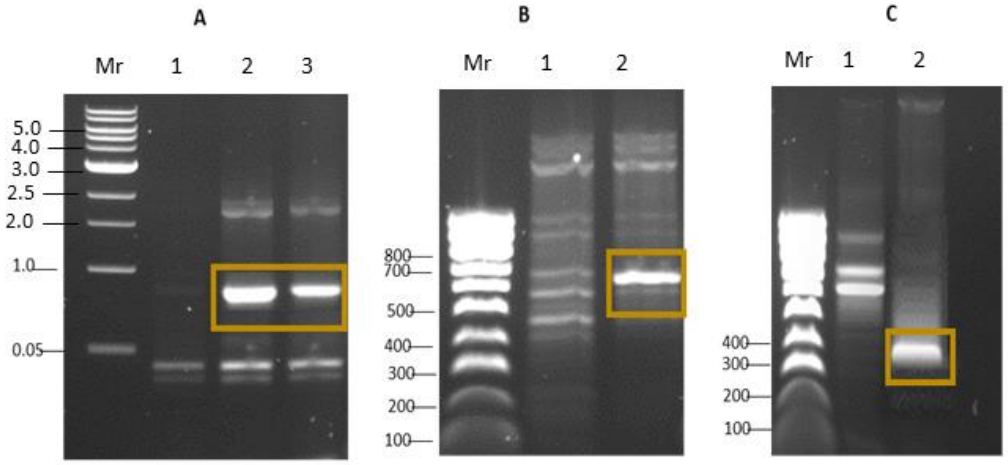


Figure 4.8 Verification of correct integration of fragments derived from pLPhSAP1sSAG1 and pLPhSAP1sCyc18 into the rRNA gene locus by PCR.

(A) Integration of SAG1 and Cyc18 constructs into the *L. tarentolae* rRNA gene locus. Lane 1, *L. tarentolae* WT; lanes 2 and 3, *L. tarentolae* clones with integrated SAG1 and Cyc18 (expected band size 862 bp). **(B)** PCR fragment to prove fusion of SAP1 to SAG1. Lane 1, *L. tarentolae* WT; lane 2, *L. tarentolae* with integrated SAG1 (expected band size 628 bp). **(C)** PCR fragment to prove fusion of SAP1 to Cyc18. Lane 1, *L. tarentolae* WT; lane 2, *L. tarentolae* with integrated Cyc18 (expected band size 333 bp). Mr is the1kb or 100 bp ladder.

4.2.3 Detection of Recombinant Proteins in the Culture Supernatant

The recombinant protein was detected in the culture supernatant of *L. tarentolae* clones transfected with SAP1 fusion constructs by following the different assays as shown in the schematic diagram below:

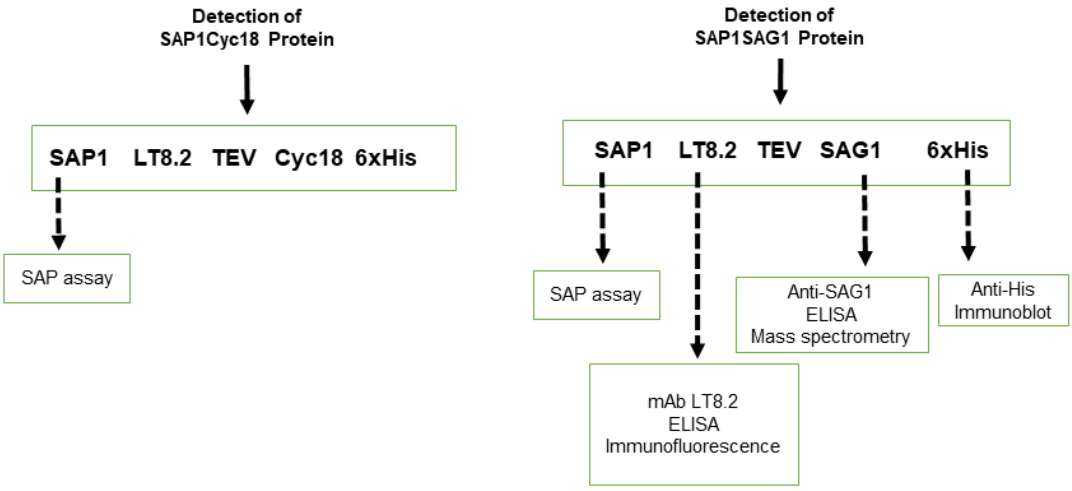


Figure 4.9 Schematic diagram showing experiments conducted to prove protein expression.

Secreted acid phosphatase activity assay, immunofluorescence and immunoblot analysis. SAP1 was detected by measuring secreted acid phosphatase (SAP) levels in culture supernatants. The LT8.2 epitope was detected by immunofluorescence and ELISA analysis. SAP1SAG1 was detected by mass spectrometry analysis. The hexahistidine (6×His) tag was used for protein detection by immunoblot analysis**.**

4.2.3.1 Determination of Secreted Acid Phosphatase Activity in Culture Supernatants of *L. tarentolae*

Secreted acid phosphatase (SAP) activity was determined in dilutions of culture supernatants to confirm the expression of recombinant protein from recombinant *L. tarentolae* promastigotes. *p*-nitrophenylphosphate was used as a substrate to determine the phosphatase activity in different samples by measuring the absorbance at 405 nm. Promastigote cultures at late logarithmic growth phase were used for determination of enzyme activity from the culture supernatant. *L. mexicana* WT culture supernatant served as a positive control for SAP activity. *L. tarentolae* WT culture supernatant was the negative control. Four cell lines of *L. tarentolae* promastigotes transfected with the SAP1SAG1 construct (D9, F7, D11 and D5) and two cell line of *L. tarentolae* promastigotes carrying the SAP1Cyc18 construct (A11, D5) showed a significant enzymatic activity in SDM79 supplemented with 10% iFCS (Figure 4.10 A). Enzymatic activity was determined from SAP1SAG1 (D9) and SAP1Cyc18 (A11) after decreasing the amount of iFCS to 2%. Culture supernatants from SAP1SAG1 and SAP1Cyc18 clones with 2% iFCS showed higher activity than that harvested from *L. mexicana* WT for all cell lines grown in different percentages of iFCS in SDM79 media (Figure 4.10 B).

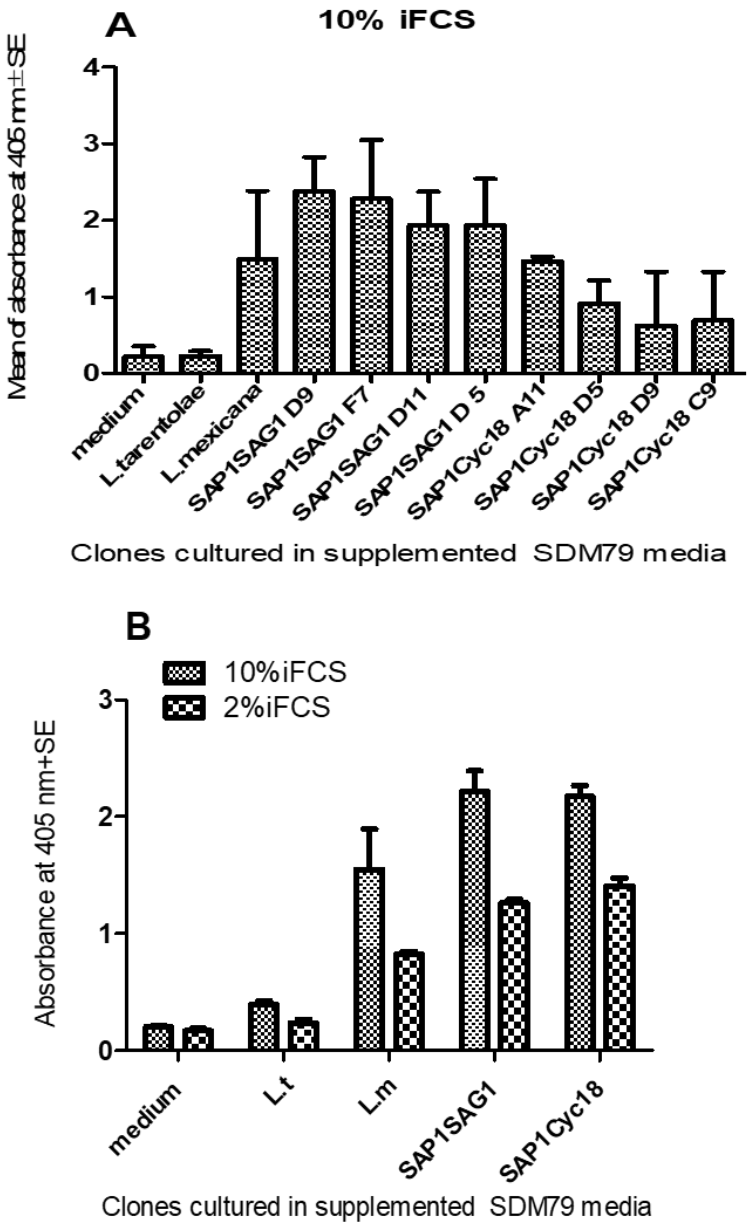


Figure 4.10 Secreted acid phosphatase (SAP) activity in culture supernatants of *L. tarentolae* promastigotes.

(A) SAP activity of SAP1SAG1 and SAP1Cyc18 in 10% iFCS supplemented culture. **(B)** Evaluation of SAP activity of SAP1SAG1 and SAP1Cyc18 in media containing 10% and 2% iFCS.

4.2.3.2 Enrichment of Recombinant SAP1SAG1 Protein by Ammonium Sulfate Precipitation (AS)

In order to enrich the SAP1SAG1 fusion protein AS salt precipitation was attempted. Figure 4.11 shows that a protein of the expected size for recombinant SAP1SAG1 precipitated in the presence of 60% (w/v) AS. Partial purified SAP1SAG1 protein revealed a significant enzymatic activity after ammonium sulfate precipitation using 60% saturation (Figure 4.11 A). Most of the recombinant protein was precipitated from the culture supernatant at 60% AS concentration as displayed in figure 4.11. The protein precipitation experiment was also done with culture supernatants from *L. tarentolae* WT grown in the same conditions as the SAP1SAG1 expressing *L. tarentolae* (2% of iFCS). Figure 4.11 B confirms that the strong band around 64 kDa obtained using 40-60% AS with culture supernatant from *L. tarentolae* WT is BSA. Whereas, the band around 82 kDa from *L. tarentolae* expressing SAP1SAG1, enriched with 60% of AS confirms the previous finding of it being the SAP1SAG1 recombinant protein.

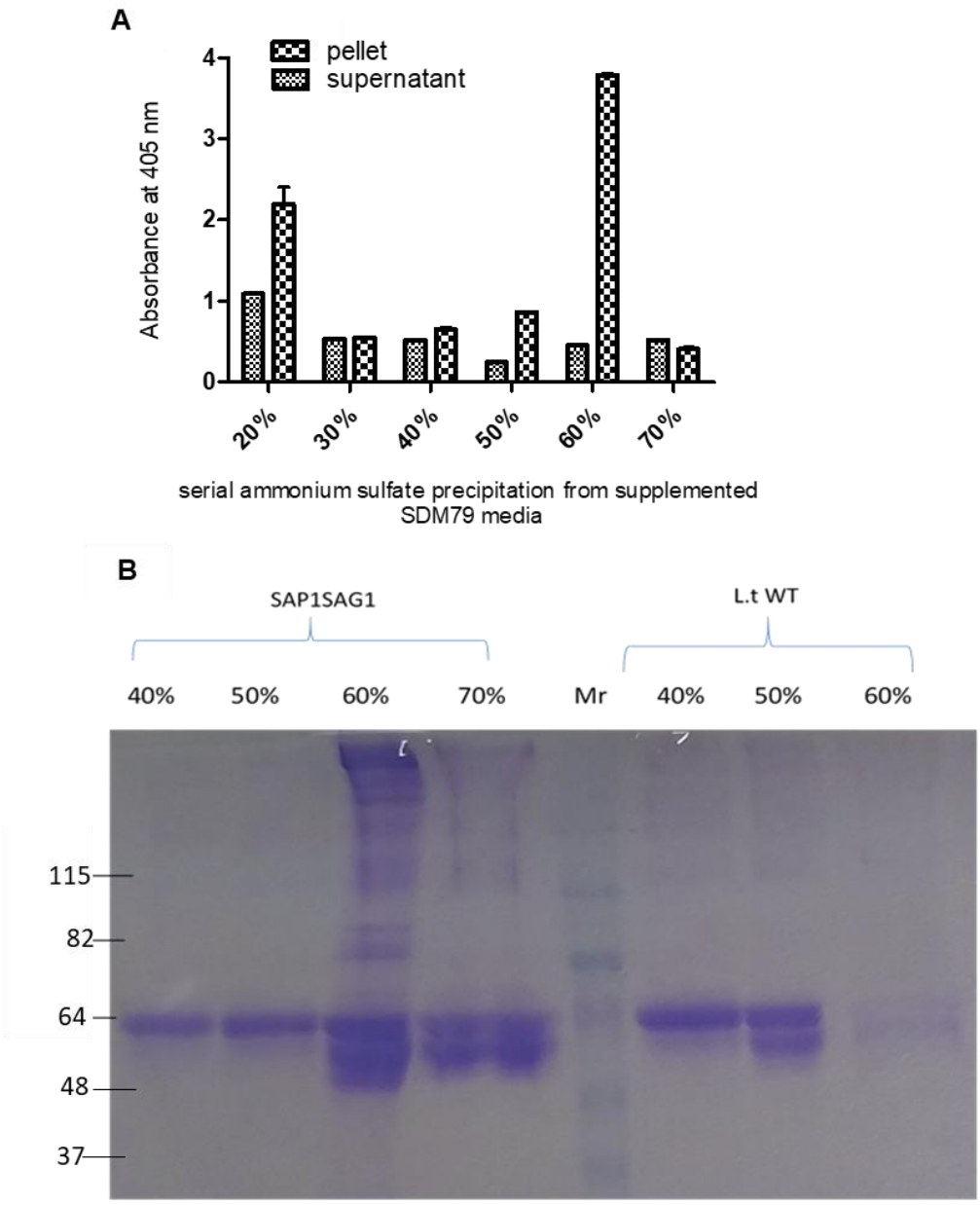


Figure 4.11 Ammonium sulfate enrichment of SAP1SAG1.

(A) SAP activity in resuspended pellet after ammonium sulfate precipitation using the indicated percentages of salt. **(B)** Coomassie-stained 10% SDS-PAGE of *L. tarentolae* cell lines. An 82 kDa band represents the SAP1SAG1 recombinant protein; the 64 kDa band is BSA from the *L. tarentolae* culture media.

4.2.3.3 Immunoblot Analysis to Confirm the Identity of the SAP1SAG1 Fusion Protein

Immunoblot analysis using an anti-hexahistidine-tag antibody for samples, which were obtained from a promastigote culture supernatant after differential precipitation with ammonium sulfate (20-70%), showed strong bands around 82 kDa corresponding in size to the SAP1SAG1 fusion protein (Figure 4.12 B). As the hexahistidine-tag is located at the C-terminal end of the fusion construct the detected protein is the full-length fusion protein. This procedure was repeated with Alexa flour 680 secondary antibody and an infrared immunoblot scanner (LI-COR) detected a fluorescence signal of the same size (Figure 4.12 C). No reaction with an anti-His antibody was obtained for a sample derived from *L. tarentolae* WT indicating the specificity of the antibody (Figure 4.12 C).

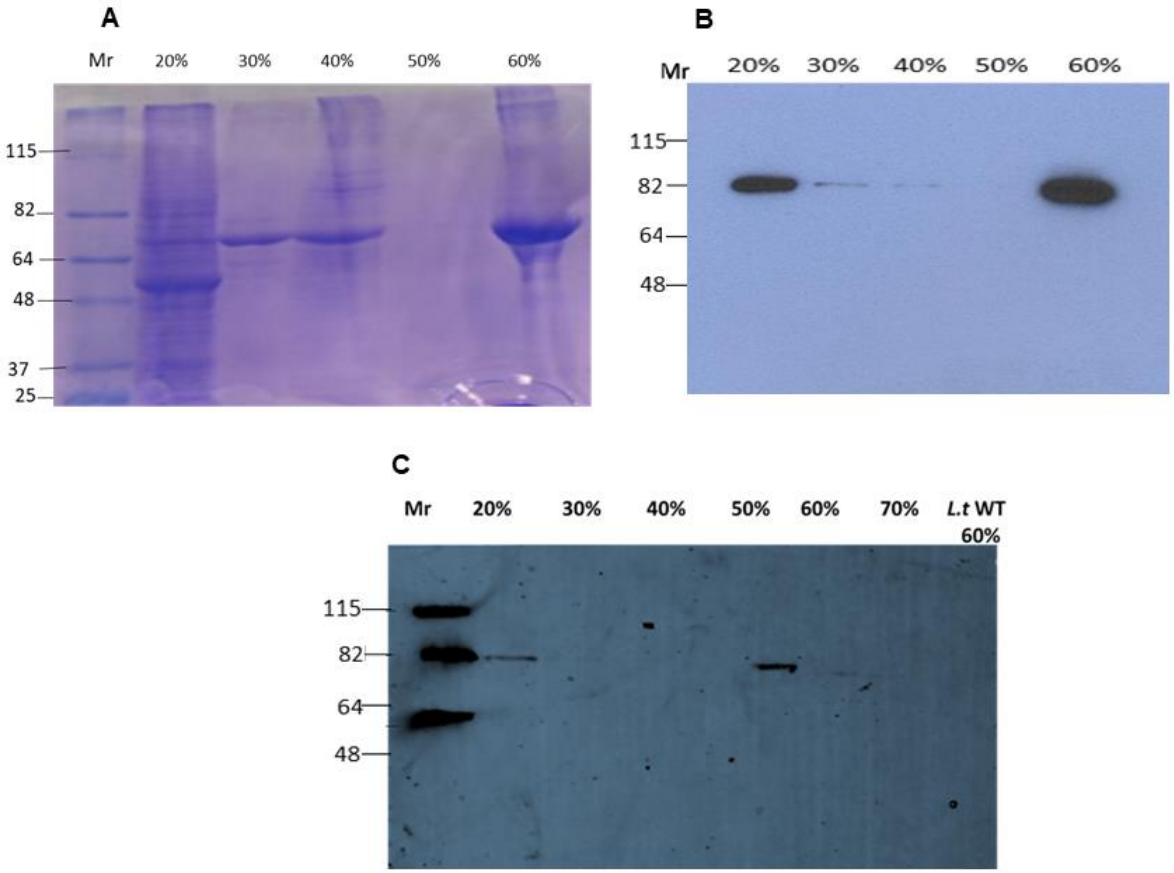


Figure 4.12 Anti-His-tag immunoblot analysis of *L. tarentolae* promastigotes expressing SAP1SAG1 obtained from culture supernatants after differential ammonium sulfate precipitation.

(A) Coomassie-stained 10% SDS-PAGE SAP1SAG1 purified with different AS saturations: Strong band refers to SAP1SAG1 protein after 60% AS precipitation. **(B)** Immunoblot analysis: Strong bands are visible for 20% and 60% AS corresponding to SAP1SAG1. **(C)** Infrared immunoblot: The fluorescence signal confirms the SAP1SAG1 protein from 60% AS saturation; lane 7, *L. tarentolae* WT shows no reaction.

4.2.3.4 ELISA

For further confirmation of expression and purification of the full-length recombinant protein SAP1SAG1 from the 60% AS enrichment an ELISA was carried out. The ELISA plate was coated with SAP1SAG1 protein and then exposed to the LT8.2 and anti-SAG1 antibodies. The measurements for LT.8.2 showed a strong reaction for SAP1SAG1 enriched by 60% AS (Figure 4.13 A). Similarly, the measurements for specific anti-SAG1 antibody detected the presence of SAP1SAG1 fusion protein (Figure 4.13 B).

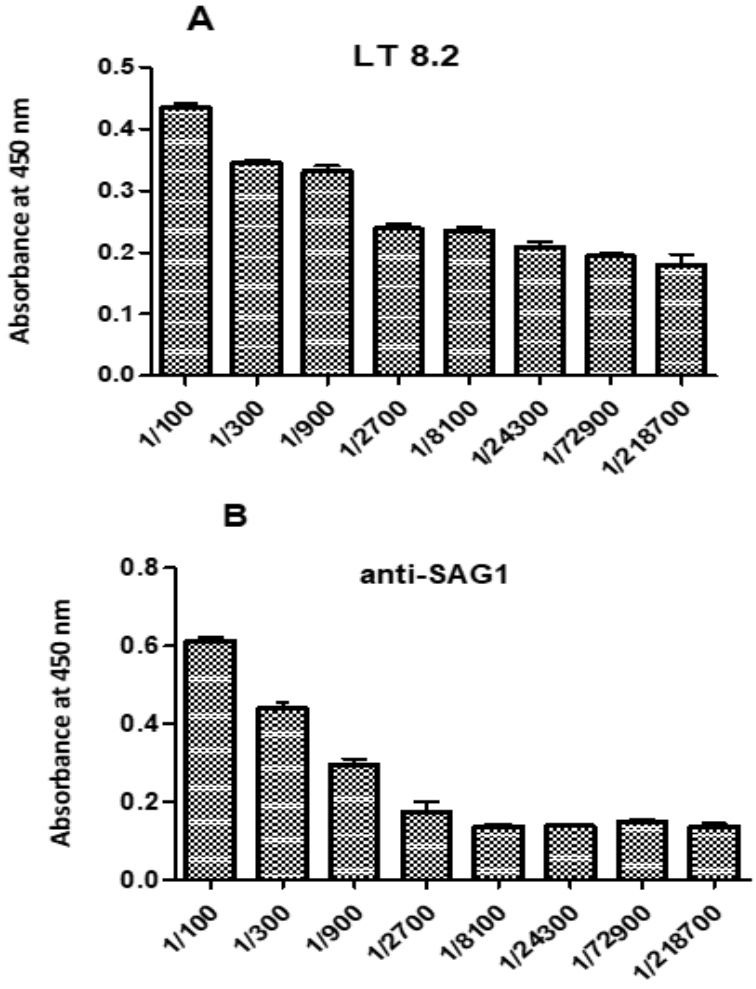


Figure 4.13 ELISA demonstrates LT8.2 antibody and anti-SAG1 antibody recognise recombinant SAP1SAG1protein obtained by 60% ammonium sulphate enrichment.

Plates were coated with SAP1SAG1 protein and the reactivity of LT8.2 and anti-SAG assessed. **(A)** Demonstrates that serial dilutions of mAb LT8.2 recognise SAP1SAG1. **(B)** Demonstrates serial dilutions of anti-SAG1recognises the recomninant SAP1SAG1 protein.

4.2.3.5 Determination of the Concentration of Purified Protein

The culture volume for the *L. tarentolae* SAP1SAG1 expression cell line was scaled up to 1 liter to increase the yield of recombinant protein. SAP1SAG1 protein was enriched using ammonium sulfate precipitation and then the protein concentration was determined. A standard curve using 0 – 1 mg/ml BSA was plotted to determine the concentration of unknown protein samples (Figure 4.14). Enrichment with AS resulted in 3 ml of SAP1SAG1 solution at a concentration of 1.16 mg/ml.

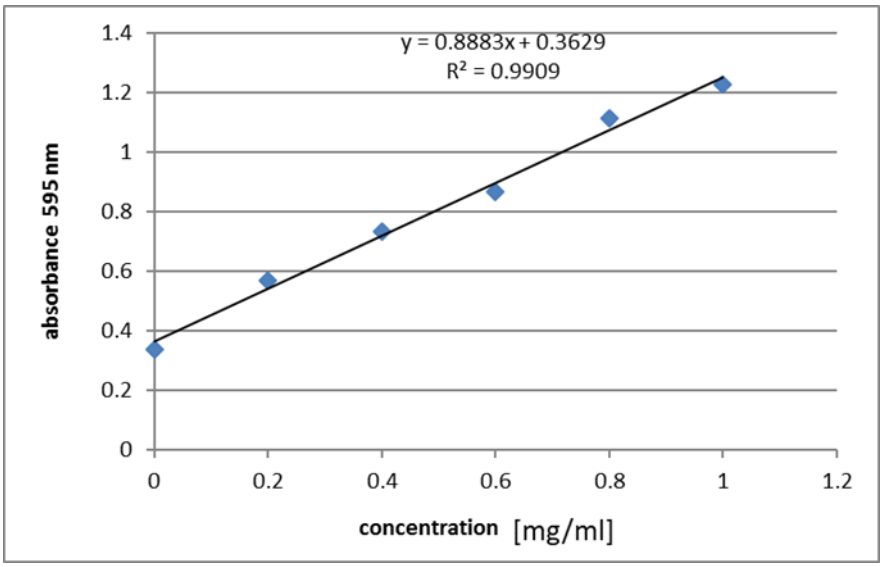


Figure 4.14 Standard curve for BSA in Bradford assay (Bradford, 1976).

4.2.3.6 Immunofluorescence Analysis

To directly visualise the expression and secretion of recombinant SAP1SAG1 fusion protein by *L*. *tarentolae* clones, immunofluorescence analysis from cells and cell culture supernatants (cells at a density of 1-2 × 10^7 cells/ml were fixed with 2% *p*-formaldehyde) was performed. The secreted acid phosphatase (SAP1) of *L. mexicana* naturally carries the mAb LT8.2 epitope, which is also found in the SAP1SAG1 fusion protein and hence could be used for protein detection in the culture supernatant. Bright field images were taken to visualise the *Leishmania* promastigotes. *L. tarentolae* WT does not show any fluorescence staining (negative control). *L. mexicana* WT shows bright green structures in the supernatant supplemented with 2% iFCS indicating the presence of secreted acid phosphatase filaments (positive control). *L. tarentolae* SAP1SAG1 recombinant promastigotes showed strong green structures in the culture supernatant, which were even brighter than those found in *L. mexicana* WT (Figure 4.15 E and F). Figure 4.15 G shows the filamentous protein secreted from the flagellar pocket of a promastigote cell. 60% AS precipitation led to a concentrated solution of SAP1SAG1 molecules as shown by the increase in fluorescent filaments (Figure 4.15 H).

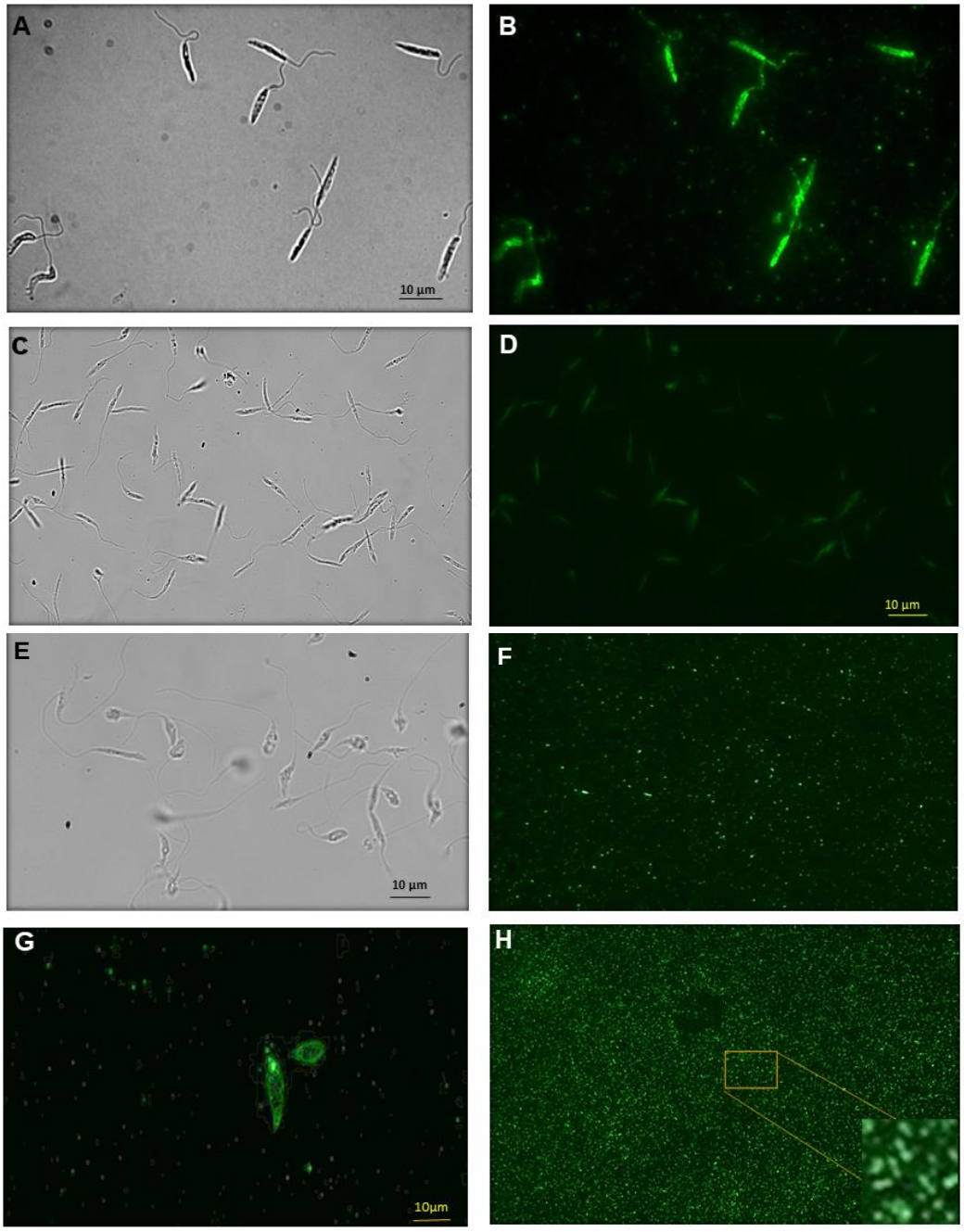

A
10 µm
B
C
D
10 µm
E
10 µm
F
G
10µm
H

Figure 4.15 Immunofluorescence analysis of *Leishmania* promastigotes and culture supernatants with mAb LT8.2.

(**A**) *L. mexicana* WT bright field; (**B**) *L. mexicana* WT FITC (positive control); (**C**) *L. tarentolae* WT bright field; (**D**) *L. tarentolae* WT FITC (negative control); (**E**) *L. tarentolae* expressing SAP1SAG1 bright field; (**F**) *L. tarentolae* expressing SAP1SAG1 FITC; (**G**) *L. tarentolae* expressing SAP1SAG1 showing the filamentous protein secreted from the flagellar pocket; (**H**) SAP1SAG1 protein from 60% AS precipitation. Size bar, 10 μm.

4.2.3.7 Mass Spectrometry (MS) Analysis of the Putative SAP1SAG1 Fusion Protein

A Coomassie blue-stained band was cut from a lane of an SDS-PAGE containing the 60% AS precipitate of a culture supernatant of *L. tarentolae* expressing SAP1SAG1 and was analysed by mass spectrometry to confirm its identity (Glasgow Polyomics Facility, College of Medical, Veterinary & Life Sciences, University of Glasgow). Data was investigated using Batch-Tag Web (http://msviewer.ucsf.edu/prospector/mshome.htm) against user protein sequences. Amino acid sequences detected by MS confirmed that the purified protein is SAP1SAG1 (Figure 4.16 A). The sequences highlighted with red indicate similarity between the sequences in the table with nine peptides matching the sequences of the secreted acid phosphatase part and one peptide matching SAG1 (Figure 4.16 A and B).

Num Unique	% Cov	Best Disc Score	Best Expect Val
10	23.7	3.86	1.2e-7

A

m/z	DB Peptide	Variable Mods	Score	Expect
845.7803	LDLTQGSASQNLAQTVLANINAHR		34.1	1.2e-7
873.4684	TTETLLSTDAVPSVVGR		30.8	2.2e-6
743.3601	AADGTYVFQESGIK		27.9	6.6e-6
1039.1920	TALTEPPTLAYSPNRHICPAGTTSSCTSK		19.4	8.4e-5
503.7821	AIGEFARSR		20.4	7.9e-4
1106.2434	SALIDDNTTEICGTLYPCGELTGEGVEMVR	Oxidation@28	16.1	0.0034
1040.5336	SRYNNLSLVESPLFPSTR		15.8	0.0036
1044.5543	TNCVLDMYDVAAAFEAAGR	Oxidation@7	15.7	0.0071
601.3333	YLAHTGICPLNSFRR		15.9	0.0088
449.2740	LIRGNPVK		15.8	0.032

B

Protein MW: 82439.3 **Protein pI:** 6.5 **Protein Length:** 763 **Index:** 1

1 MASRLVRVLA AAMLVAAAVS VDARFVVRMV QVVHRHGARS **ALIDDNTTEI CGTLYPCGEL TGEGVEMVRA IGEFARSRYN**

81 **NLSLVESPLF PSTR**YNSSVV HTRSTHTQRT IQSATAFLRG LFQDDYFYPV VYSTNR**TTET LLSTDAVPSV VGR**SWLDNPA

161 LHAALNPVID EHLSWDAIQS AAKDAWVEGL CADYNAR**TNC VLDMYDVAAA FEAAGR**LDNA TNLKAVYPGL QEVNAAWFKY

241 VFSWNHTSK**L DLTQGSASQN LAQTVLANIN AHR**LSPSYNM FQYSAHDTTV TPLAVTFGDQ GETTMRPPFA VTIFVELLQD

321 TADASGWYVR **LIRGNPVKAA DGTYVFQESG IK**AYCIDEAG NK**YLAHTGIC PLNSFRR**MVD YSRPAVADGH CAMTQTQYSN

401 MDCPRTIADN KPVPSRCWLY RHVCPSKACP DSYILSAVDH QCYPGPDVTN PTSSSSSEGT TTSSSSSSSK STSSSDVPSF

481 KKPANWSPRV GSENLYFQSR PLVANQVVTC PDKKSTAAVI LTPTENHFTL KCPK**TALTEP PTLAYSPNRH ICPAGTTSSC**

561 **TSK**AVTLSSL IPEAEDSWWT GDSASLDTAG IKLTVPIEKF PVTTQTFVVG CIKGDDAQSC MVTVTVQARA SSVVNNVARC

641 SYGANSTLGP VKLSAEGPTT MTLVCGKDGV KVPQDNNHYC SGTTLTGCNE KSFKDILPKL SENPWQGNAS SDNGATLTIN

721 KEAFPAESKS VIIGCTGGSP EKHHCTVQLE FAGPRGGHHH HHH

Figure 4.16 MS analysis of SAP1SAG1 fusion protein.

(A) m/z, mass to charge ratio; DB peptide, peptide sequence; variable mods; methionine oxidation modification; score, mascot score; expect, expected value.**(B)** MS results against protein of interest. Matching peptides of recombinant SAP1SAG1 are highlighted in bold red.

4.3 Discussion

4.3.1 Generation of DNA Constructs

In the present study two plasmids were successfully constructed, pLPhSAP1sSAG1 and pLPhSAP1sCyc18 (see figures 4.2 and 4.3), containing the open reading frame of *L. mexicana* SAP1 (Secreted Acid Phosphatase 1) fused with genes of interest from *T. gondii* (SAG1 or Cyc18) and the phleomycin resistance antibiotic marker. SAP1 forms a filamentous protein secreted from the flagellar pocket of *L. mexicana* promastigotes (Stierhof *et al.*, 1998; Wiese *et al.*, 1995). SAP1 including its N-terminal phosphatase domain, was used as a carrier for *T. gondii* proteins, SAG1 and Cyc18, which were genetically fused to its C-terminus. Hence, *L. tarentolae* has been used for heterologous gene expression and recombinant protein production (Raymond *et al.*, 2012). In this study *L. tarentolae* was used as protein expression system for secretion of eukaryotic recombinant proteins SAP1SAG1 and SAP1Cyc18 into culture supernatants.

4.3.2 Gene Expression in *L. tarentolae*

L. tarentolae promastigotes were successfully transfected with pLPhSAP1sSAG1 and pLPhSAP1sCyc18 constructs by using electroporation. Selection of recombinant parasites can easily be performed using phleomycin binding protein, which was also used in this investigation to generate permanent and stable transfectants (Beverley & Clayton, 1993). *L. tarentolae* cell lines can be grown at high and stable growth rates. Promastigotes can be cultivated to a maximum cell density of 1×10^8 parasites/ml after 4-5 days of incubation in supplemented cultures (Fritsche *et al*., 2007). Integration of the recombinant DNA cassette into the *L. tarentolae* genome through homologous

recombination (Kianmehr *et al.*, 2016), was confirmed by diagnostic PCRs. PCRs were carried out to detect the correct integration of the expression constructs into the 18S small subunit (SSU) rRNA gene locus of *L. tarentolae*. A second PCR was required to prove the fusion of SAP1 with SAG1 or Cyc18 as explained in figure 4.8. Both PCRs were positive for the clones analysed and confirmed the existence of the intact expression construct in the correct genomic location. It is important to note that transcription is under the control of RNA polymerase I, which allows high level transcription (Clayton, 1999; Teixeira, 1998).

4.3.3 Secreted Acid Phosphatase Activity

The expression level of recombinant proteins SAP1SAG1 and SAP1Cyc18 in *L. tarentolae* was determined using the phosphatase activity in culture supernatants. High enzymatic activity was obtained from recombinant *L. tarentolae* expressing SAP1SAG1 and less from recombinant *L. tarentolae* transfected with SAP1Cyc18 when grown in media supplemented with 10% iFCS and hemin. To minimise the effect of bovine serum albumin on the purification of the recombinant protein, the amount of iFCS in the culture media was reduced to 2% (v/v). One clone each of *L. tarentolae* expressing SAP1SAG1 or SAP1Cyc18 was selected and grown in 2% iFCS and hemin. When comparing phosphatase activity high enzymatic activity was detected in culture supernatants of cell lines expressing SAP1SAG1 and SAP1Cyc18, even higher than what was found for *L. mexicana* wild type. As the activity was highest for SAP1SAG1 expressing cells this cell line was chosen to purify the recombinant protein from culture supernatant and to further analyse protein production using immunofluorescence and immunoblotting.

4.3.4 Enrichment of Recombinant SAP1SAG1 by Ammonium Sulfate

The basic theory of using AS salt is that the solubility of globular proteins increases upon the addition of salt (< 15%), an effect termed salting-in. At higher salt concentrations, protein solubility usually decreases, leading to precipitation; this effect is termed salting-out. SAP1SAG1 is a multisubunit protein complex forming filaments. According to the literature, complex or high molecular weight proteins could be purified from culture media using different percentages of ammonium sulfate salt (AS) and the large multiprotein complexes can often be salted out with < 20% saturation (Wingfield, 2016). For example, Interleukin-1β was purified in higher salt concentration between 50-77% AS saturation for precipitation; IgG has been precipitated from blood with 40-45% of AS (Wingfield, 2016). In this work, we employed a modified method of ammonium sulfate precipitation to purify SAP1SAG1 from culture supernatants by stepwise addition of solid AS salt between 20-70% (v/v). *L. tarentolae* was grown in SDM79 media supplemented with 2% iFCS and hemin, which are essential for their growth. This still allowed a final cell density of 5×10^7 parasites/ml.

Acid phosphatase activity was determined in precipitates and supernatants from SAP1SAG1 culture supernatants after sequential addition of AS (Figure 4.11 A). Phosphatase activity in precipitates using 60% AS saturation from culture supernatants of recombinant parasites grown in 2% iFCS was higher than the activity determined for samples obtained from the *L. mexicana* positive control. Protein precipitation was also done with culture supernatants from *L. tarentolae* wild type grown in the same conditions as the SAP1SAG1 expressing *L. tarentolae* (2% of iFCS). Figure 4.11 B shows that the strong band around 64 kDa obtained using 40-60% AS is present here as well and is most likely BSA. Whereas, the band around 82 kDa from *L. tarentolae* expressing

SAP1SAG1 enriched with 60% AS is the SAP1SAG1 recombinant protein. Time constraints of the project did not permit to purify SAP1Cyc18 and do immunofluorescence microscopy and immunoblot analysis of the cell line expressing SAP1Cyc18 recombinant filaments.

4.3.5 Immunofluorescence and Immunoblot Analysis

Immunofluorescence analysis using mAb LT8.2 showed that the filamentous protein can be detected by epifluorescence microscopy in promastigote cultures and in the 60% AS precipitate, but was absent in *L. tarentolae* wild type. Secretion of SAP1SAG1 filaments from the flagellar pocket of recombinant *L. tarentolae* using mAb LT 8.2 was detected matching what is known about secretion of SAP filaments in *L. mexicana* (Figure 4.15). High acid phosphatase activity, a band of the correct size in immunoblot analysis and visualisation of filaments in immunofluorescence microscopy all match up for the 60% AS sediment. For some extent, some cells showed autofluorescence, which could have been caused by the long exposure time that was used to visualise the filaments around the cells.

To determine the expression of the fusion protein, immunoblot analysis was performed using an anti-hexahistidine antibody recognising the very C-terminal end of the fusion protein. The strong bands around 82 kDa shown on the immunoblot identified SAP1SAG1 in the 60% AS precipitations. From the reaction with the anti-hexahistidine antibody it can be concluded that the full-length SAP1SAG1 was expressed, secreted, and enriched from the *L. tarentolae* cell cultures. If the protein was truncated on transit through the secretion pathway, another smaller band would have shown on the blot, as the hexahistidine tag is located only at the C-terminal end (Figure 4.12). 20% AS precipitated the multisubunit

filamentous form of SAP1SAG1 whereas 60% AS led to the precipitation of monomeric protein.

4.3.6 ELISA

The availability of an anti-SAG1 antibody allowed to carry out an ELISA to confirm the presence of SAG1 as part of the recombinant fusion protein enriched in the 60% AS precipitate. The mAb LT.8.2 was used as a positive control in the ELISA. ELISA is a rapid, sensitive and specific assay that has been used as a diagnostic tool in medicine and biotechnology to detect the presence of a ligand (commonly a protein) in a liquid sample using specific antibodies directed against the protein to be measured. In this study, the immunodominant antigens of the SAP1SAG1 from 60% AS precipitate are binding to the specific mAb LT.8.2 and anti-SAG1 antibodies. The subsequent reaction produced a detectable signal for the fusion protein.

These results confirmed the immunofluorescence microscopy, and immunoblot and mass spectrometry analyses. As an anti-Cyc18 antibody is not available, an ELISA was not conducted for detection of recombinant SAP1Cyc18.

4.3.7 Mass Spectrometry (MS) Analysis

Mass spectrometry analysis was carried out in order to confirm the identity of SAP1SAG1. Mass spectrometry is a powerful technique with different applications in chemistry, biology and clinical medicine. It determines the molecular weight of compounds by separating molecule ions based on their mass and charge. Recently MS has become one of the most important methods for protein studies in terms of identification and sequencing. The sequences of SAP1SAG1 peptides were identified by mass

spectrometry (MS) identifying nine peptides of SAP1 and one peptide that belongs to SAG1 (He *et al.*, 2002) (accession code 1KZQ), which are highlighted with red colour in figure 4.18 A and B confirmed the identity of SAP1SAG1.

4.3.8 Concentration of SAP1SAG1

The concentration of SAP1SAG1 in culture supernatant was 3.48 mg/L. This observation agreed with previous studies, which stated that the level of protein expression in *L. tarentolae* could vary between 0.1 and 30 mg/L of culture. For example, the expression level for green fluorescent protein (GFP) (Basile & Peticca, 2009), interferon-gamma (IFN-ɣ) (Davoudi *et al.*, 2011), and human coagulation factor VII (Mirzaahmadi *et al.*, 2011) has been achieved to be 30, 9.5 and 1 mg/L of culture, respectively.

Probably the lowering of the iFCS to 2% might lower the expression levels of recombinant *L. tarentolae,* which resulted in decreasing the concentration of recombinant protein in the culture supernatants. And to increase the valuable using the ammonium sulfate in protein purification, using the ammonium sulfate first for partial purification of recombinant SAP1SAG1 then using the ion exchange chromatography may be useful to get rid of the iFCS fraction.

Further investigations are required to increase the amount of SAP1SAG1 production. A possibility might be to place the gene of interest under the control of a foreign promoter like the T7 RNA polymerase promoter, which has been shown that the protein yield was higher than a polymerase I promoter in *L. tarentolae* (Fritsche *et al.*, 2007; Kushnir *et al.*, 2005). Alternatively, *L. tarentolae* could be grown in other media that does not require iFCS or hemin as supplementary factors. For example, BHI media was used to produce darbepoetin alfa from *L. tarentolae* T7-TR cells (pLEXSY_Iblecherry3 vector), in which

tetracycline was used as an inducer to increase the expression level to 51.2 mg/ml of culture medium by adding 5 µg/ml (Kianmehr *et al.*, 2016).

The amount of SAP1SAG1 obtained was disappointingly low (approximately 3mg). As a single experiment would require approximately 1mg this would not allow sufficient vaccination studies to be performed and replicated. Moreover, recombinant SAP1Cyc18 was not yet produced and purified. It was therefore decided not to proceed to *in vivo* work with this protein. The second reason for not using the recombinant protein in a vaccination study is that the purified protein might still contain a low amount of protein from iFCS. Moreover, taking into account that the main goal was to be able to use Cyc18 as a natural adjuvant it was decided to generate a DNA vaccine for both SAG1 and Cyc18 to allow continuation to the immunisation part of the project.

4.5 Conclusions

- pLPhSAP1sSAG1 and pLPhSAP1sCyc18 were successfully constructed and linear DNA-fragments derived from them were transfected into *Leishmania* by electroporation.

- PCRs detected the correct integration of the intact expression constructs into the 18S small subunit (SSU) rRNA gene locus of *L. tarentolae*.

- Enzyme activity, immunoblot and immunofluorescence analyses confirmed that *L. tarentolae* promastigotes successfully expressed, secreted SAP1SAG1 and the protein could be enriched with 60% AS from the culture supernatant.

- ELISA confirmed the presence of SAG1 as part of the recombinant fusion protein.

- Mass spectrometry analysis confirmed the identity of SAP1SAG1.

- Low amount of partially purified SAP1SAG1 and time constraints for optimization of SAP1Cyc18 purification prevented production of these proteins for immunisation

Part Ⅱ: Immunological Studies

Chapter 5: *Toxoplasma gondii* Cyclophilin 18 (TgCyc18) as Natural Adjuvant for a Vaccine to Prevent Toxoplasmosis

5.1 Introduction

5.1.1 DNA Vaccine

DNA vaccines can effectively elicit humoral and cellular immunity and have specifically been demonstrated to induce cytotoxic $CD8^+$ T cells (Gülçe Iz *et al.*, 2014). In addition, DNA vaccines offer other advantages including ease of manufacture, their stability, and inexpensive production. However, most importantly they inherently produce proteins in a eukaryotic system and therefore are more likely to undergo similar post translational modification as they would in eukaryotic pathogens (reviewed, Xenopoulos & Pattnaik, 2014). Despite these theoretical advantages of DNA vaccines, their development has not been particularly successful and there is no DNA vaccines currently licensed for use in humans. However, a DNA vaccine has been approved for prevention of West Nile Disease in horses (CDC, 2005). The reasons for the lack of a functional DNA vaccine in humans could include: (i) their poor uptake into host cells and low expression efficiency, (ii) a lack of appropriate adjuvants (Ivory & Chadee, 2004).

SAG1 is a surface antigen of *T. gondii* and is considered a main antigenic protein. It is important for adhesion of tachyzoite to host cells and therefore their invasion of cells (Grimwood & Smith, 1996). Moreover, SAG1 has a number of known $CD4^+$ and $CD8^+$ T cell epitopes that are recognised by different haplotypes of human histocompatibility complex (Mendes *et al.*, 2013). Therefore, a number of studies have examined the ability of SAG1 to afford protection as part of a vaccine with varying degrees of success (see table 1.2).

5.1.2 *T. gondii* Cyclophilin 18 (TgCyc18)

T. gondii Cyclophilin 18 (TgCyc18) is a naturally occurring chemokine mimic that interacts with CCR5 receptor. CCR5 is expressed on macrophages and dendritic cells and normally binds CCL3, CCL4, CCL5, CCL8, CCL11, CCL14 and CCL16 (reviewed, Marques *et al.*, 2013). In addition, upon ligation macrophages and dendritic cells have been shown to produce IL-12 (Aliberti *et al.*, 2003). IL-12 induces NK cells to produce IFN-ɣ and directs the differentiation of Th1 cells and cytotoxic $CD8^+$ T cells. Consequently, IL-12 is important for the control of *T. gondii* infection (Aliberti *et al.*, 2016). Cyc18 has also been shown to induce the production of nitric oxide (NO), IL-6, IL-12 and low levels of TNF-α which have also been demonstrated to mediate protection against *T. gondii* infection (Ibrahim *et al.*, 2009). Moreover, CCR5-deficient mice infected with *T. gondii* have decreased IL-12 production, reduced NK cell migration, higher parasite burdens and increased mortality relative to wild-type mice (Aliberti *et al.*, 2016). It has thus been suggested that TgCyc18 is important in directing a protective immune response to *T. gondii.*

5.2 Aims

The following studies were performed to determine if the addition of TgCyc18 to recombinant SAG1 DNA vaccine could improve its efficacy against *T. gondii* infection. Specifically, to determine:

1. The ability of a vaccine containing SAG1 and Cyc18 genes cloned into pVAX-1 plasmids to protect mice against challenge with *T. gondii.*
2. The ability of plasmid to increase the immune response against a SAG1 DNA vaccine and specifically direct it towards a Th1 response.

5.3 Experimental Design

5.3.1 Vaccination

Female BALB/c mice 6-8 weeks old were divided randomly into five groups of 5 mice each. Groups were given: pVAX-1 (empty plasmid), pVAX-SAG1, pVAX-Cyc18, pVAX-SAG1 and pVAX-Cyc18 (pVAX-SAG1/Cyc18) or left unvaccinated as a negative control. For vaccination, mice were injected intramuscularly with 100 µl of 2 mg/ml of plasmid (50 µl in each anterior tibialis). Mice received three immunisation doses at two week intervals for challenge experiments. Mice received two immunisation doses at two week intervals for splenocyte re-stimulation assays.

5.3.2 Challenge experiment

T. gondii tachyzoites (Luciferase-expressing Prugniaud strain) type II were used for challenge. Three weeks after the last vaccination, all immunised groups and non-vaccinated control group were challenged intraperitoneally with 200 µl (2×10^4 tachyzoites/mouse). The experiment was repeated twice.

5.3.3 Evaluation the immune response

IgG subsets were measured after 21 days of last vaccination by ELISA to evaluate the humoral immune response. Parasite abundance was monitored by bioluminescence at 4, 6 and 8 days post infection. Mouse body weights and survival rates were measured until the conclusion of the experiment. Splenocytes were obtained from vaccinated and negative control mice two weeks following vaccination, to determine their cytokine productions (as described in 2.2.3.8).

5.4 Results

5.4.1 Construction and Preparation of pVAX-SAG1 and pVAX-Cyc18

To generate pVAX-SAG1, pBSAG1 and pVAX-SAG4.2 were cleaved with EcoRI and NotI which resulted in 1098 bp, 2924 bp, 630 bp and 2972 bp fragments. The 1098 bp and 2972 bp fragments were isolated and ligated together (Figure 5.1). While, pVAX-Cyc18 was kindly provided by Prof. Craig W. Roberts. Both plasmids were verified with appropriate restriction analyses. Figure 5.2 illustrates the final plasmid maps, expected fragment sizes of restriction analyses and the actually detected fragments on an agarose gel.

For vaccination, preparative plasmid preparations of pVAX-SAG1 and pVAX-Cyc18 were obtained by using Macherey and Nagel endotoxin free Giga kit (as described in 2.2.1.10).

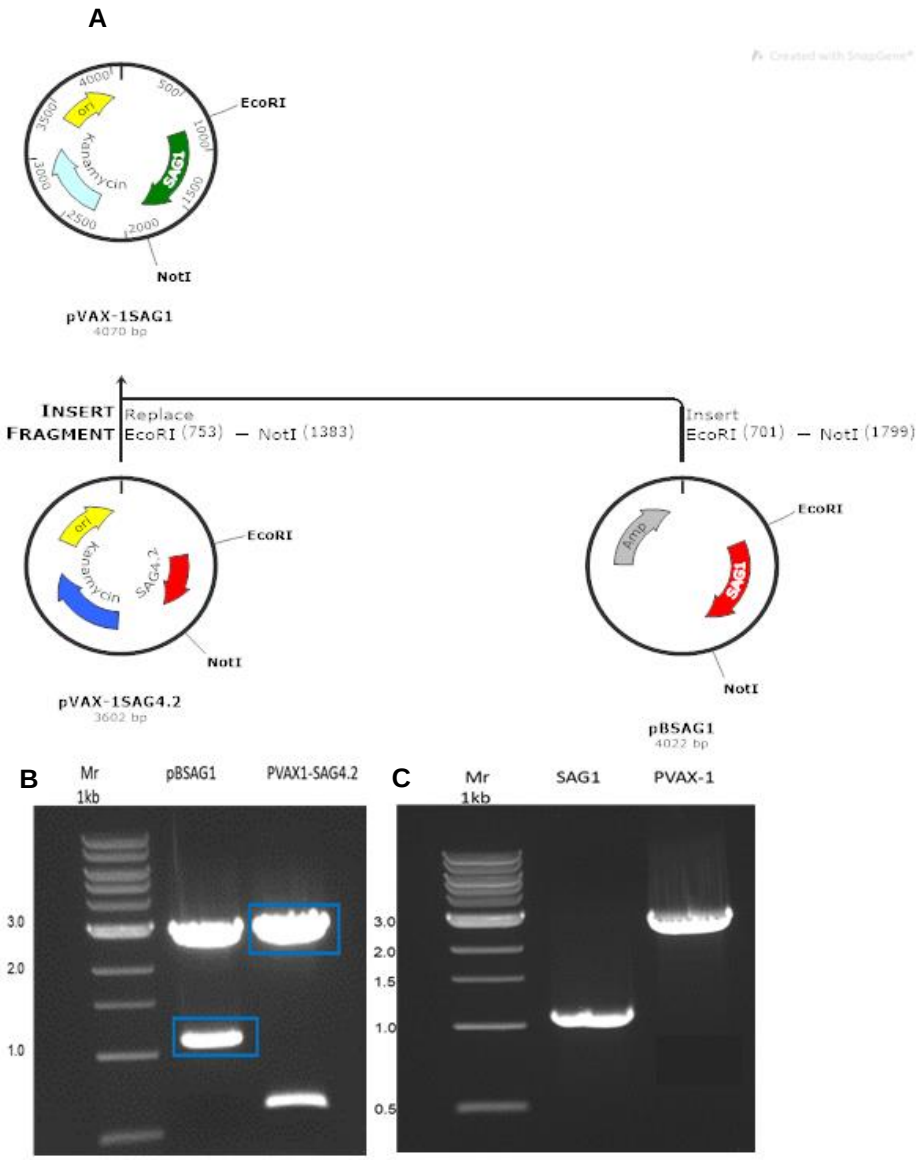
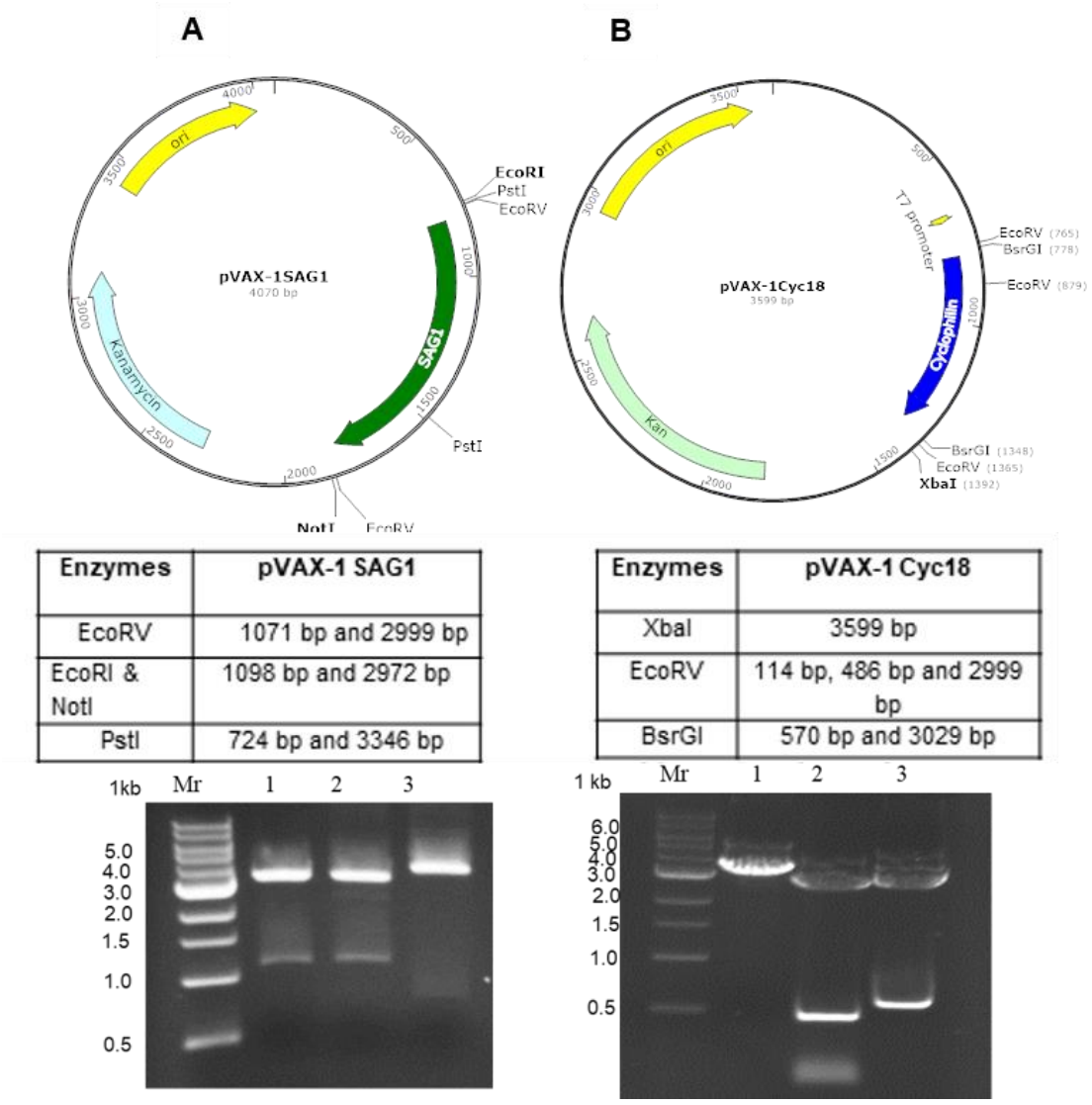


Figure 5. 1 Generation of pVAX1-SAG1.

(A) Strategy for generation of pVAX-SAG1. **(B)** The SAG1 coding region (1098bp) was obtained from pBSAG1 by digesting with EcoRI and NotI. The pVAX1 plasmid backbone (2972bp) was obtained from pVAX1-SAG4.2 digesting with EcoRI and NotI. **(C)** Restriction analysis was used to confirm successful ligation of the SAG1 coding region into pVAX1. Mr, DNA size marker in kb.



Enzymes	pVAX-1 SAG1
EcoRV	1071 bp and 2999 bp
EcoRI & NotI	1098 bp and 2972 bp
PstI	724 bp and 3346 bp

Enzymes	pVAX-1 Cyc18
XbaI	3599 bp
EcoRV	114 bp, 486 bp and 2999 bp
BsrGI	570 bp and 3029 bp

Figure 5. 2 Characterisation of recombinant plasmids pVAX-SAG1 and pVAX-Cyc18 by restriction analysis.

(A) Top, pVAX-SAG1 map; middle, table of expected of fragments sizes using different restriction enzymes; bottom, agarose gel of pVAX-SAG1 restriction analysis. Lane 1, EcoRV; lane 2, EcoRV & NotI; lane 3, PstI . **(B)** Top, pVAX-Cyc18 map, middle, table of fragments sizes using different restriction enzymes; bottom, agarose gel of pVAX-Cyc18 restriction analysis. Lane 1, XbaI; lane 2, EcoRV; lane 3, BsrGI . Mr, DNA size marker in kb.

5.4.2 Transfection of Macrophages with Recombinant DNA and IL-12 Production

Recombinant DNA of pVAX-SAG1, pVAX-Cyc18 and pVAX-1 were transfected into macrophage cultures and incubated for 24h and 48h. ELISA was carried out to measure IL-12 from culture supernatants. Macrophages transfected with pVAX-1 expressed low IL-12 levels after 24h incubation period. Macrophages transfected with 0.25 µg/ml or higher of either pVAX-SAG1 or pVAX-Cyc18 stimulated IL-12 production after 24h. Whereas at low levels of plasmid (< 0.125 µg/ml) only the pVAX-Cyc18 and not the pVAX-SAG1 induce significant levels of IL-12 (Figure 5.3 A). At 48h incubation, a similar pattern was observed with both pVAX-SAG1 and pVAX-Cyc18 being able to induce IL-12 when given at high concentrations, but only pVAX-Cyc18 being able to induce IL-12 at low concentrations (Figure 5.3 B).

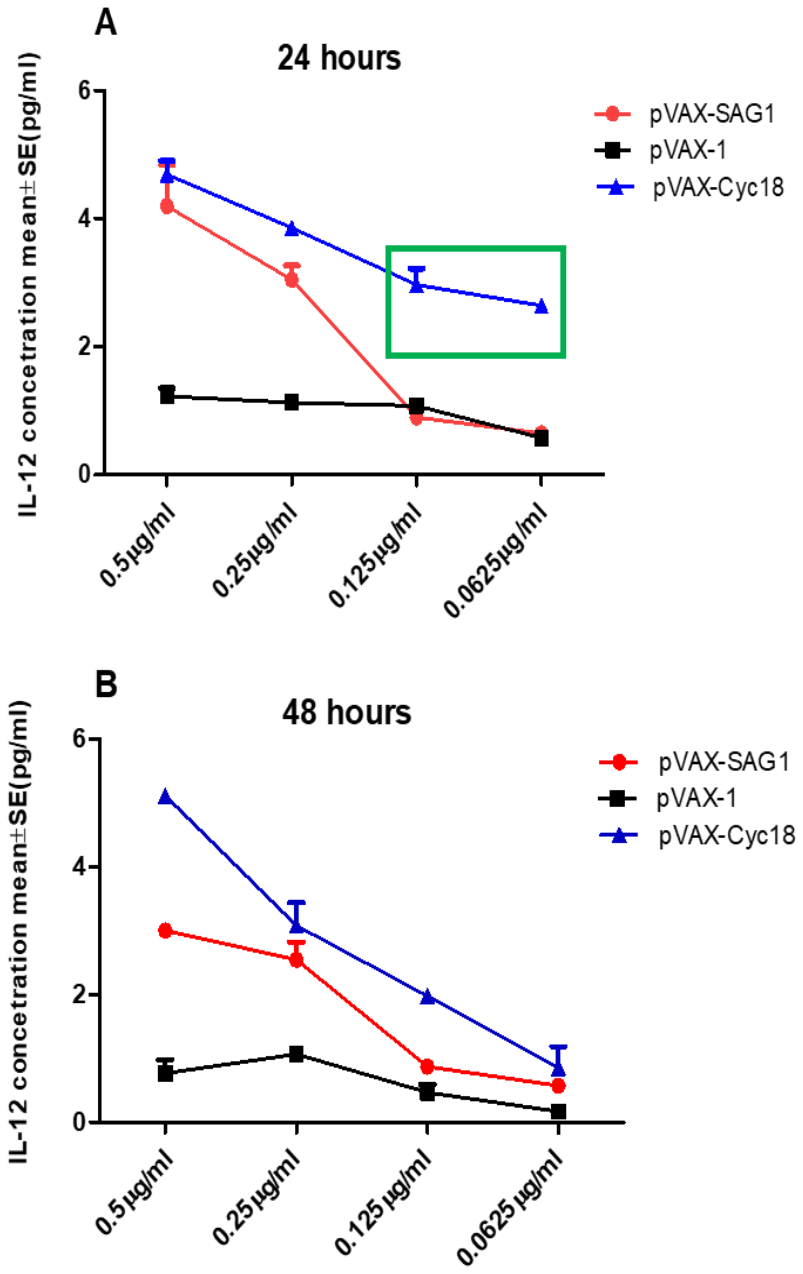


Figure 5. 3 IL-12 production from culture supernatants induced by transfected recombinant DNA into macrophages with different concentrations.

(A) Incubated for 24 h **(B)** Incubated for 48 h**.** Each value represents the mean ± the standard error of data from triplicate samples. The results are representative of three repeated experiments with similar results. .Macrophages transfected with 0.125ug/ml of pVAX-Cyc18 produced high levels of IL-12, relative to those transfected with pVAX-SAG1 or pVAX-1 at 24 hours post incubation (Green box)

5.4.3 Assessment of Parasite Number in Mice by Bioluminescence

Bioluminescence indicative of live parasites was evident in the abdomen of mice from day 4 post infection. Vaccination of mice with empty pVAX-1 did not confer any protection relative to control non-vaccinated animals with mice in each group showing similar distribution of parasites and kinetics of parasite multiplication. Mice vaccinated with pVAX-SAG1, pVAX-Cyc18 or pVAX-SAG1/Cyc18 had similar parasite distributions, but markedly reduced parasite numbers, as measured by bioluminescence, relative to control non-vaccinated mice or empty pVAX-1 vaccinated mice (Figure 5. 4).

At day 4 and 6 post infection had no significant difference in total flux was observed between control and vaccinated mice with the exception of mice vaccinated with pVAX-SAG1/Cyc18, which had significantly lower total flux, measured than control unvaccinated mice (Figures 5.5 A and B). Mice vaccinated with SAG1/Cyc18 had significantly reduced total flux than control mice vaccinated with pVAX-1 alone at day 8 post infection (Figures 5.5 C). The area under the curve was calculated for as an indicator of relative parasite numbers in each individual mouse over the course of the 8 day infection (Figure 5.5 D). Mice vaccinated with pVAX-1 had no significant difference in parasite number compared with non-vaccinated control mice. pVAX-SAG1 or pVAX-SAG1/Cyc18 vaccinated mice showed significantly lower numbers of parasites empty plasmid pVAX-1 vaccinated mice (Figure 5.5).

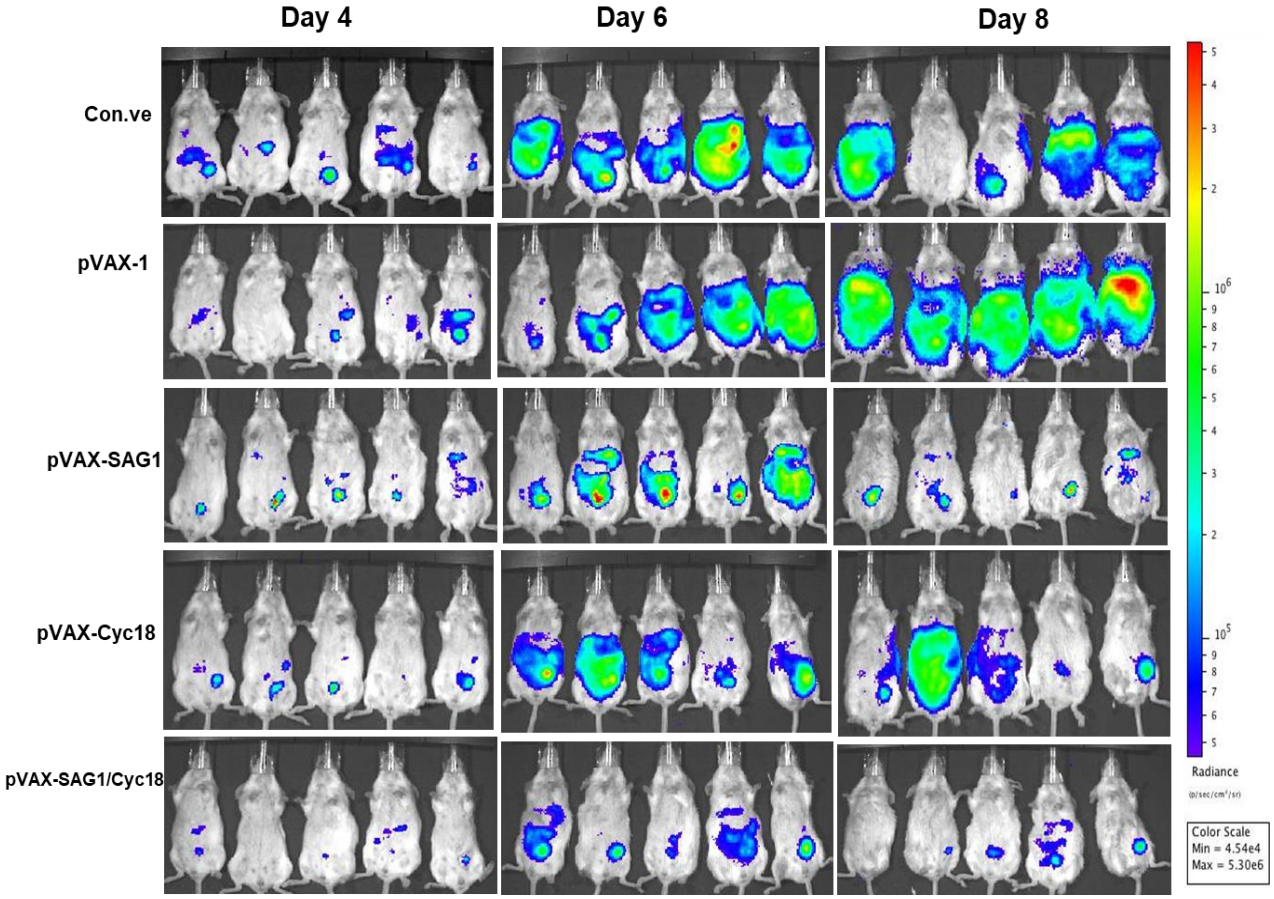


Figure 5. 4 Parasite distribution and relative abundance as determine by bioluminescence in control and vaccinated mice infected with luciferase-expressing *T. gondii* on day 4, 6 and 8 post infection.

Mice vaccinated with pVAX-SAG1/Cyc18 or pVAX-SAG1 had less bioluminescent signal than control non-vaccinated mice and mice vaccinated with empty plasmid.

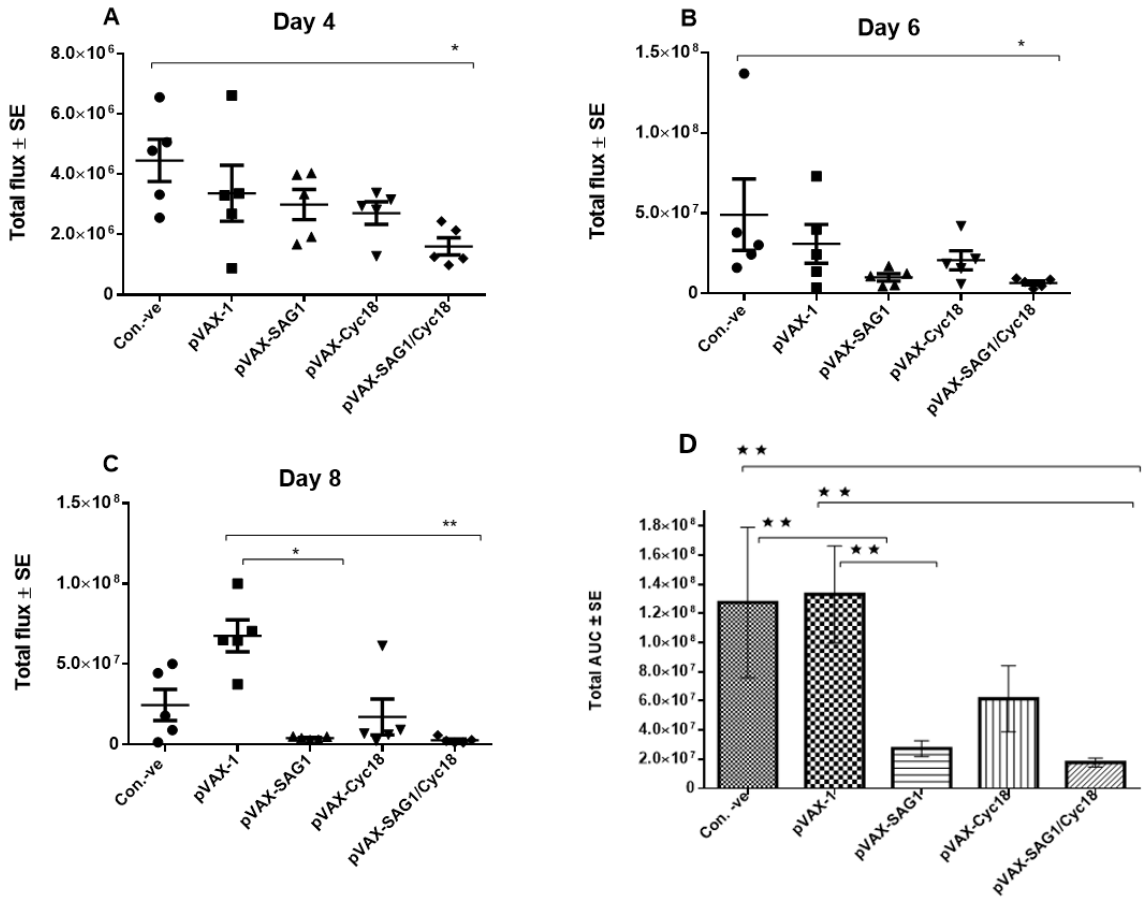


Figure 5. 5 Relative parasite numbers in control and vaccinated mice as determine by IVIS and computed as area under the curve (AUC) over 4-8 days post infection from the data presented in figure 5.4.

pVAX-SAG1/Cyc18 and pVAX-SAG1 vaccinated mice had significantly reduced number of parasites compared with control non-vaccinated and empty pVAX-1 mice. **<p 0.01.

5.4.4 Survival Rate

Vaccination with pVAX-SAG1/Cyc18 and pVAX-SAG1 provided significant protection ($p<0.0001$ for each comparison) with 100% of mice surviving infection against *T. gondii* tachyzoites infection. Mice vaccinated with pVAX-Cyc18 showed 80% protection ($p<0.01$). Mice vaccinated with empty plasmid or negative control mice (left unvaccinated) had 40% mortality (Figure 5.6).

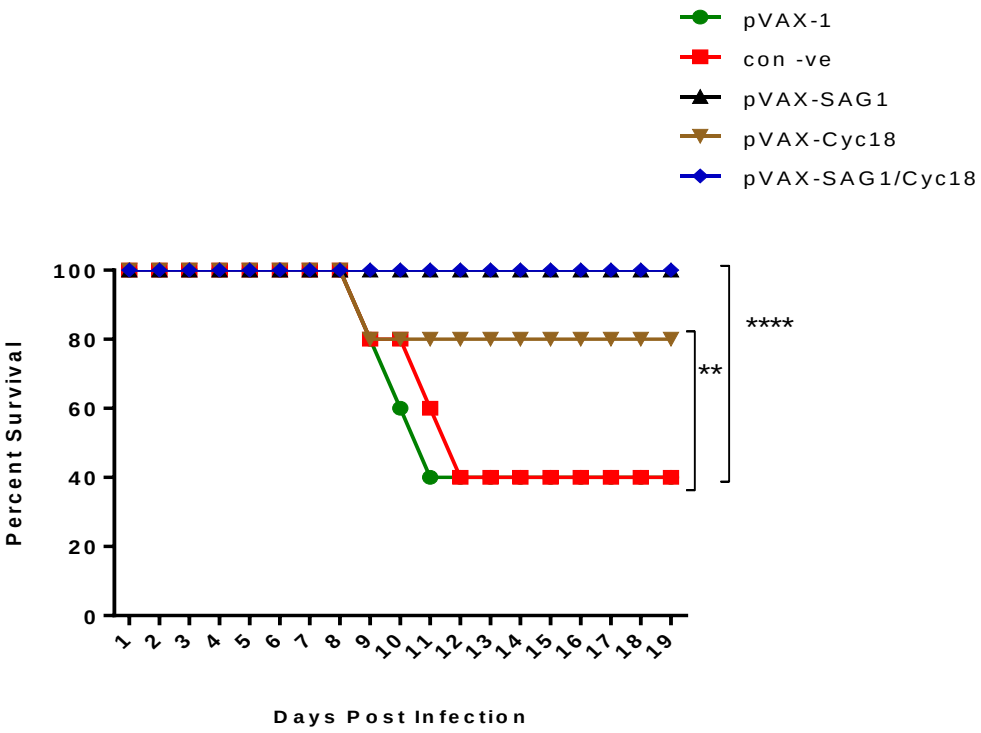


Figure 5.6 Survival curves of BALB/c mice groups following challenge infection. Mice vaccinated with pVAX-SAG1/Cyc18 and pVAX-SAG1 showed significant protection against challenge compared with control non-vaccinated and empty plasmid pVAX-1 groups. The experiment was performed twice. Each group contains five mice. Survival rates were monitored until 20 days post infection. **<p 0.01, ***<p 0.001.

5.4.5 Body Weight Differences

The 2 mice out of the 5 control non-vaccinated group of mice that survived to the end of the study lost over 5% of their body weight and never recovered to pre-infection weights (Figure 5.7 A). Similarly, the pVAX-1 vaccinated mice had the similar levels of mortality and again surviving mice failed to recover weight (Figure 5.7 B). The pVAX-SAG1 vaccinated mice which had zero mortality and although all mice exhibited weight loss, 2 mice recovered to within 5% of their starting weight by the end of the study (Figure 5.7 C). Four out of the 5 pVAX-Cyc18 vaccinated mice survived and 1 mouse recovered to within 5% of starting weight (Figure 5.7 D). pVAX-SAG1/Cyc18 vaccinated mice had zero mortality and mice recovered significantly to within 5% of the starting weight than mice vaccinated with pVAX-SAG1 alone (Figure 5.7 E and F). As a proportion of mice died in many groups comparison of mean weight loss between groups is not always meaningful. However, mice vaccinated with pVAX-SAG1/Cyc18 or pVAX-SAG1 each had 100% survival allowing meaningful comparison. Mice vaccinated with pVAX-SAG1/Cyc18 had significantly less weight loss than those vaccinated with pVAX-SAG1.

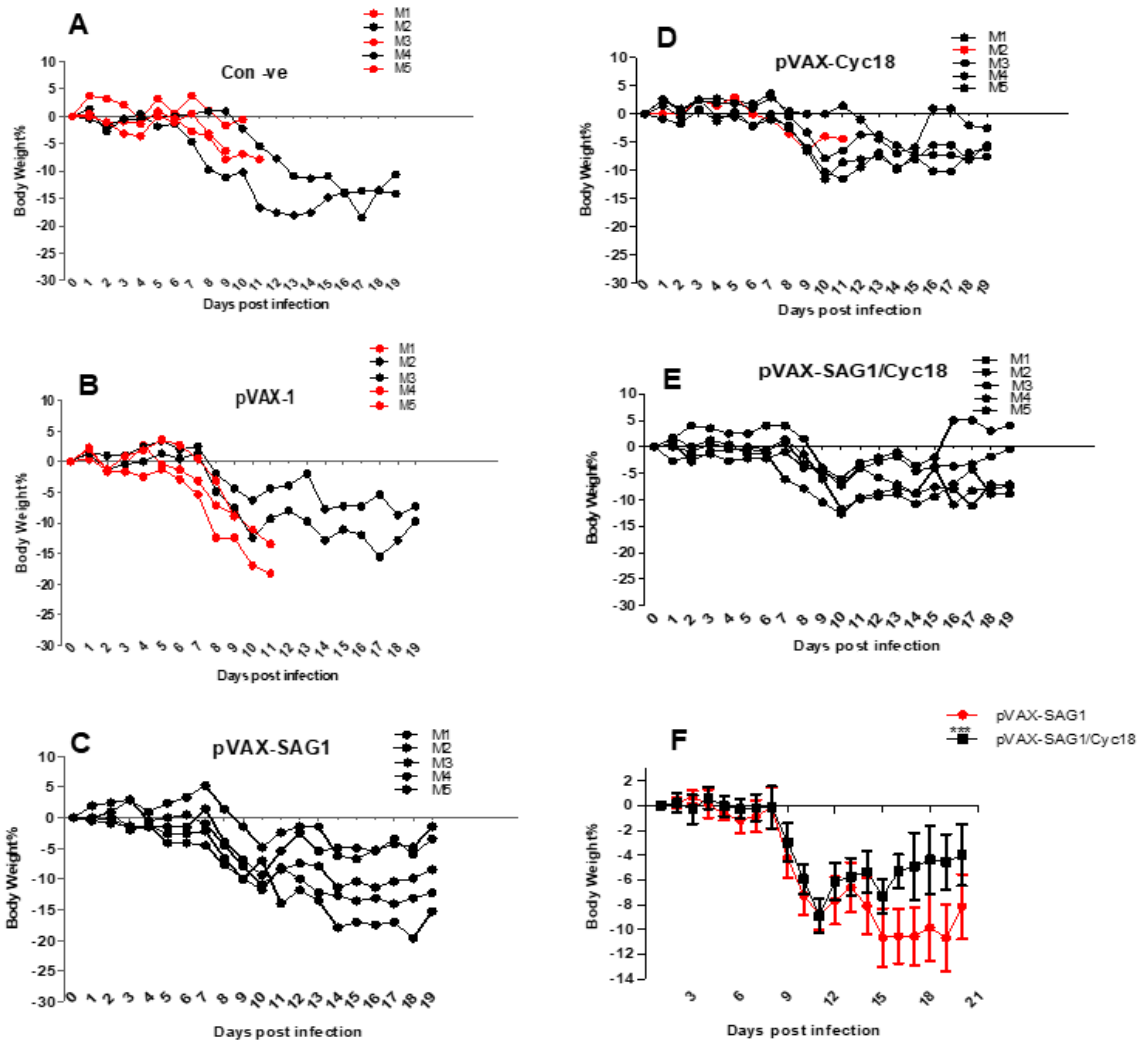


Figure 5.7 The effect of vaccination on weight of BALB/c mice following challenge infection. Mice were left unvaccinated as controls (A) or vaccinated with pVAX-1 (B), pVAX-SAG1 (C), pVAX-Cyc18 (D), pVAX-SAG1/Cyc18 (E) before challenge with *T. gondii.* Mice that died or had to be culled due to reaching agreed end-points are in red while mice surviving to the end of the study (day 20) are in black. Mice vaccinated with pVAX-SAG1/Cyc18 had significantly (***<p 0.001) less weight loss than those vaccinated with pVAX-SAG1 (F). The experiment was performed twice. Each group contains five mice.

5.4.6 Measurements of IgG Subclass Levels from Serum of BLAB/c Mice

End- point titer for *T. gondii* specific IgG1 was low (endpoint < 1/100) in control non-vaccinated mice and empty pVAX-1 vaccinated. pVAX-SAG1 vaccinated mice had a mean end point titre of 4500 ± 1505.9. pVAX-Cyc18 and pVAX-SAG1/Cyc18 had significantly lower endpoint titres (2780 ± 1443 and 3060 ± 1322.7, respectively) than pVAX-SAG1vaccinated animals (Figure 5.8 A).

T. gondii specific IgG2a was not observed in serum of control non-vaccinated and empty pVAX-1 vaccinated mice (endpoint < 1/100). Mice vaccinated with pVAX-SAG1 but not pVAX-Cyc18 exhibited significantly ($p< 0.0049$) increased endpoint titres (8820 ± 4128 and 1122.497 ± 501.996, respectively), compared pVAX-1 and non-vaccinated mice. Mice vaccinated with pVAX-SAG1/Cyc18 had a significantly ($p<0.0055$) higher endpoint titre (64800 ± 46577.93) than pVAX-1 vaccinated and control non-vaccinated mice (Figure 5.8 B).

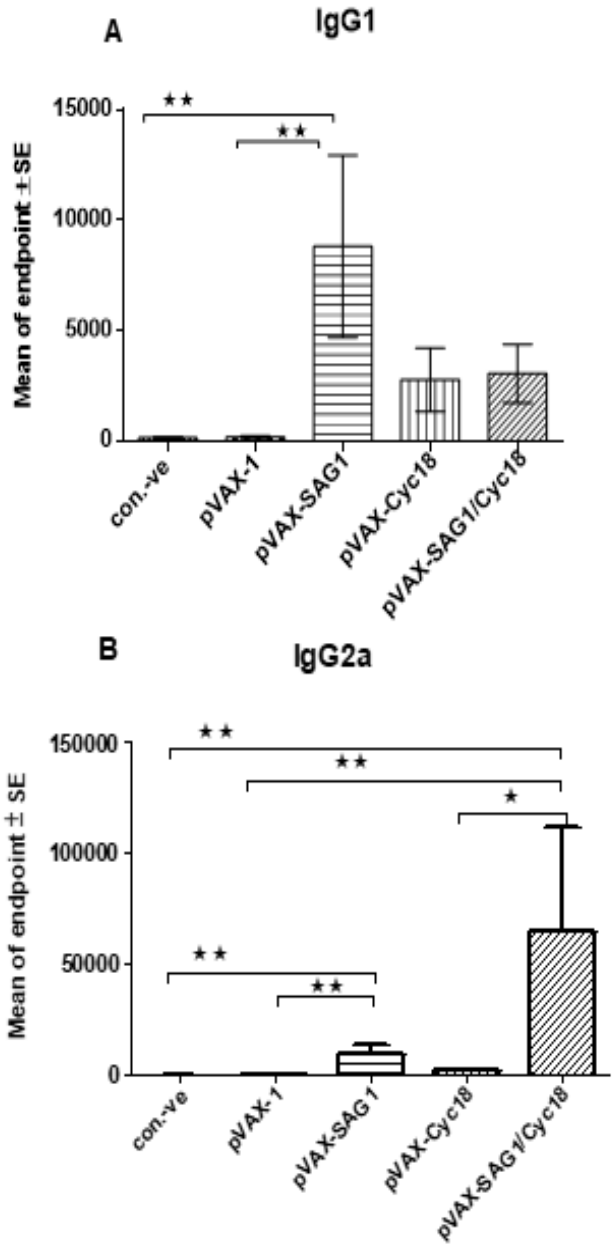
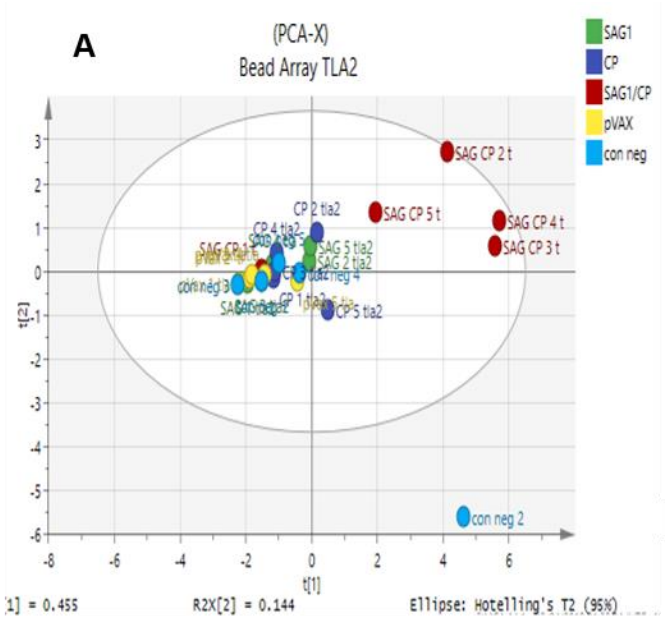


Figure 5. 8 Determination of the specific anti *T. gondii* IgG subclass from serum samples of vaccinated and control mice by ELISA.

(A) IgG1 isotype, **(B)** IgG2a isotype. This experiment was repeated twice. Asterisk shows level of significance (*<p 0.05, **<p 0.01) between the groups. Immunised mice, n=5

5.4.7 Splenocytes Stimulation Assay

To determine the quality of the immune response induced by vaccine preparations, splenocytes were isolated (2 weeks after the final vaccination) and stimulated with 10 µg/ml of TLA. Cytokine production was measured by multiplex cytokine bead array and flow cytometry. A PCA demonstrated no clear separation between non-stimulated cells, but there was a clear separation of stimulated cells in pVAX-SAG1/Cyc18 group from control non-vaccinated, pVAX-1, pVAX-SAG1, and pVAX-Cyc18 groups (Figure 5.9 A). Consequently, OPLAS-DA scores was employed to determine the relative contributions of the individual cytokine components and visualised in the form of a VIP table (Figure 5.9 B). IL-6, IFN-ɣ, IL-22, TNF-α, IL-9 and IL-17a had the highest scoring VIP values suggesting potential differences in Th17, Th1 and TFH cell expansion.



B

Var ID (Primary)	M1.VIP[1+1+0]
IL-6	1.27563
IFN-γ	1.25621
IL-22	1.22103
TNF-α	1.20881
IL-9	1.16429
IL-17a	1.07523
IL-5	1.05176
IL-10	1.04566
IL-2	0.955187
IL-4	0.798018
IL-21	0.748586
IL-13	0.124809
IL-17f	0.0778729

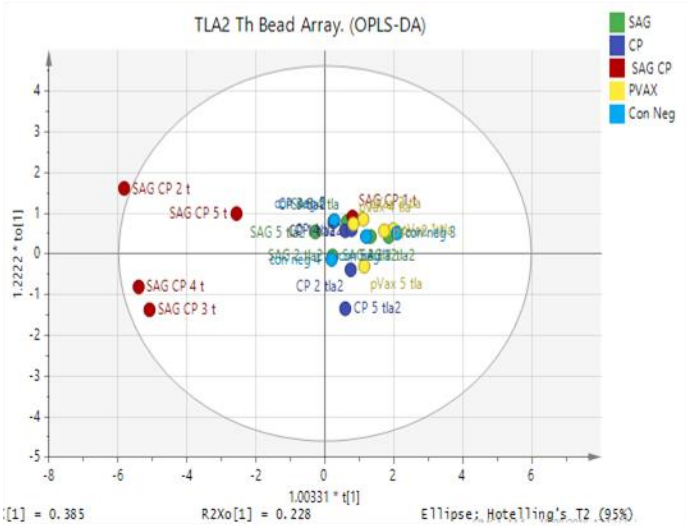


Figure 5. 9 Multivariate analysis of cytokine production in splenocytes isolated from control and vaccinated mice.

(A) PCA plot demonstrates separation of mice vaccinated with pVAX-SAG1/Cyc18 from other groups **(B)** OPLAS-DA plot and VIP table demonstrate the relative importance of cytokines to this separation. Groups: pVAX-SAG1 (Green); pVAX-Cyc18 (purple); pVAX-SAG1/Cyc18 (red); pVAX-1(yellow); control negative (blue).

Splenocytes from control non-vaccinated mice and mice vaccinated with empty plasmid pVAX-1 produced low levels of IFN-ɣ and TNF-α in response to TLA stimulation. Splenocytes from mice vaccinated with pVAX-SAG1/Cyc18 produced significantly higher levels of IFN-ɣ and TNF-α, than negative control and empty plasmid groups when stimulated with TLA (Figure 5.10 A and B). Splenocytes production of IL-2 was similar in all groups with the exception of mice receiving empty pVAX-1 where IL-2 production was low (Figure 5.10 C). These results indicate induction of Th1 cells in mice vaccinated with pVAX-SAG1/Cyc18.

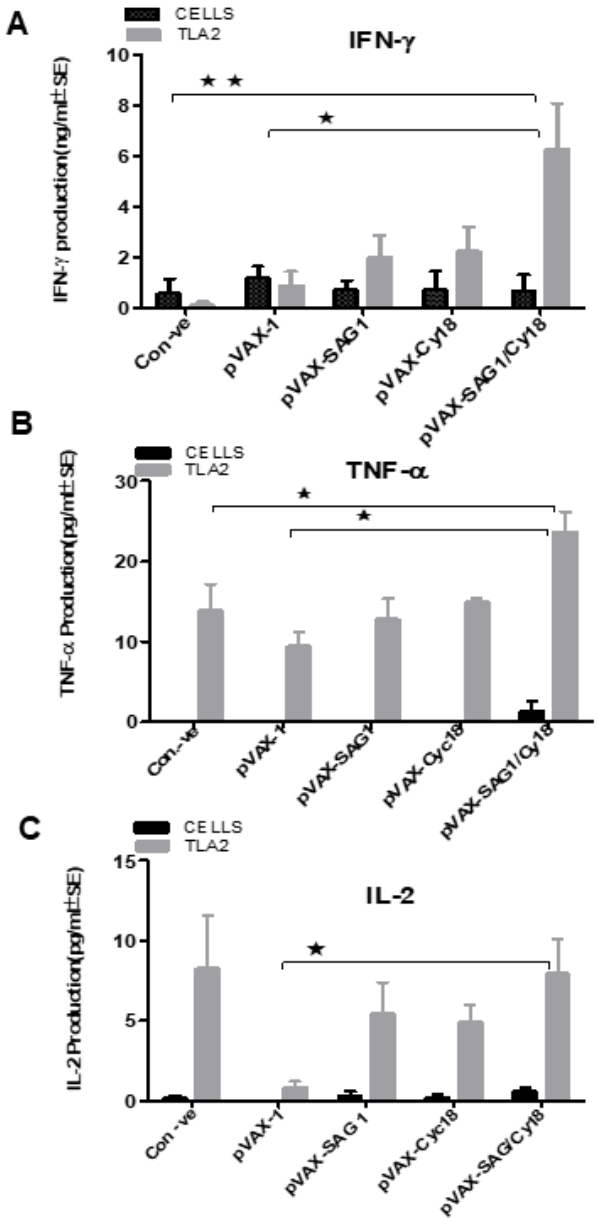


Figure 5.10 Relative Th1 cell cytokine production in splenocytes isolated from control and vaccinated mice two weeks after the final immunisation. Splenocytes were left unstimulated (black) or were stimulated with 10 µg/ml of TLA (grey) and supernatants collected at 72 hours post stimulation.

(A and B) Mice vaccinated with pVAX-SAG1/Cyc18 showed a significant production of IFN-ɣ and TNF-α than control non-vaccinated and pVAX-1 vaccinated mice. **(C)** IL-2 exhibited a significant production in mice vaccinated with pVAX-SAG1/Cyc18 than mice vaccinated with pVAX-1. *<p 0.05, **<p 0.01 significance between the groups. Immunised mice, n=5.

Splenocytes obtained from control non-vaccinated and mice vaccinated with VAX-1 showed very low productions of IL-17a, IL-17f and IL-22 after stimulated with TLA (Figure 5.11 A, B and C). In contrast, splenocytes from mice vaccinated with pVAX-SAG1/Cyc18 showed a significant production of IL-17a, IL-17f and IL-22 cytokines versus negative control and empty plasmid groups. Furthermore, splenocytes from mice vaccinated with pVAX-SAG1/Cyc18 had significantly increased levels of IL-22 production compared with splenocytes from non-vaccinated control mice, pVAX-1 vaccinated mice, mice vaccinated with pVAX-SAG1 or pVAX-Cyc18. These results induction of Th17 cells in mice vaccinated with pVAX-SAG1/Cyc18. These results:

Splenocytes collected from negative non-vaccinated mice, and mice vaccinated with pVAX-1, pVAX-SAG1 and pVAX-Cyc18 groups produced low levels of IL-9, IL-6 and IL-21. Mice vaccinated with pVAX-SAG1/Cyc18 showed a significant high level of IL-9 than mice vaccinated with pVAX-1 and pVAX-SAG1. Similarly, mice vaccinated with pVAX-SAG1/Cyc18 showed a significant level of IL-6 production than control non-vaccinated, pVAX-1, pVAX-SAG1 and pVAX-SAG1/Cyc18 (Figure 5.12 A and B). These results also offer an indicator of TFH cells stimulation.

No significant differences in IL-5, IL-4 and IL-10 production from splenocytes of vaccinated mice and non-vaccinated mice was observed following TLA stimulation. (Figure 5.13).

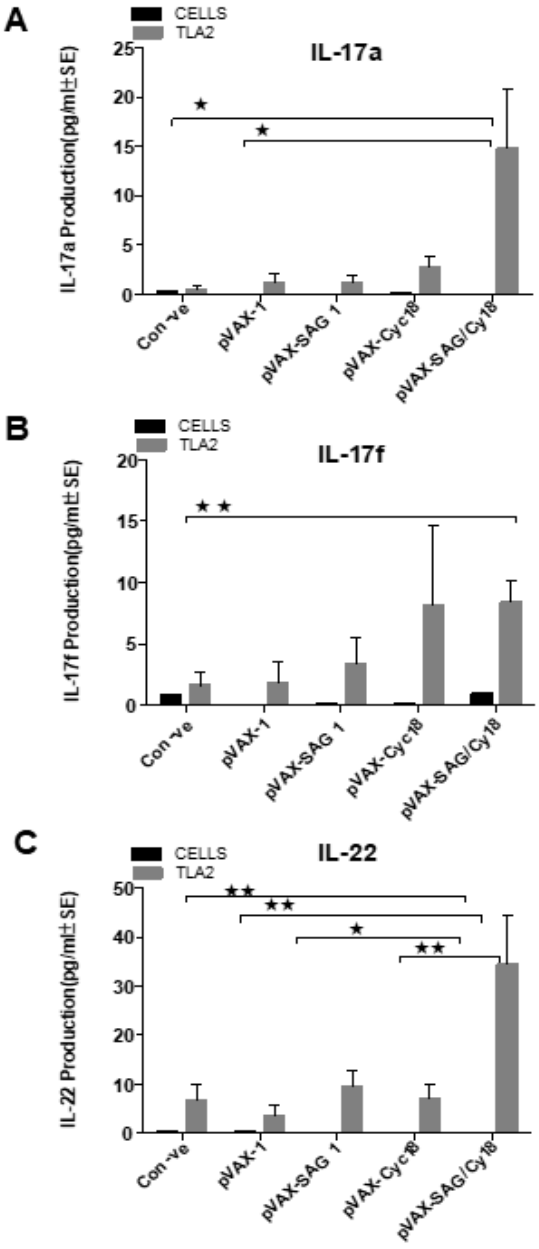


Figure 5. 11 Relative Th17 cell cytokine production in splenocytes isolated from control and vaccinated mice two weeks after the final immunisation. Splenocytes were left unstimulated (black) or were stimulated with 10 µg/ml of TLA (grey) and supernatants collected at 72 hours post stimulation.

(A) Mice vaccinated with pVAX-SAG1/Cyc18 showed a significant production of IL-17a than control non-vaccinated and pVAX-1 vaccinated mice. **(B)** Mice vaccinated with pVAX-SAG1/Cyc18 showed a significant production of IL-17f than control negative. **(C)** Mice vaccinated with pVAX-SAG1/Cyc18 showed a significant production of IL-22 than control non-vaccinated negative, mice vaccinated with pVAX-1, pVAX-SAG1 and pVAX- Cyc18, respectively. *<p 0.05 and **<p 0.01 significance between the groups. Immunised mice, n=5.

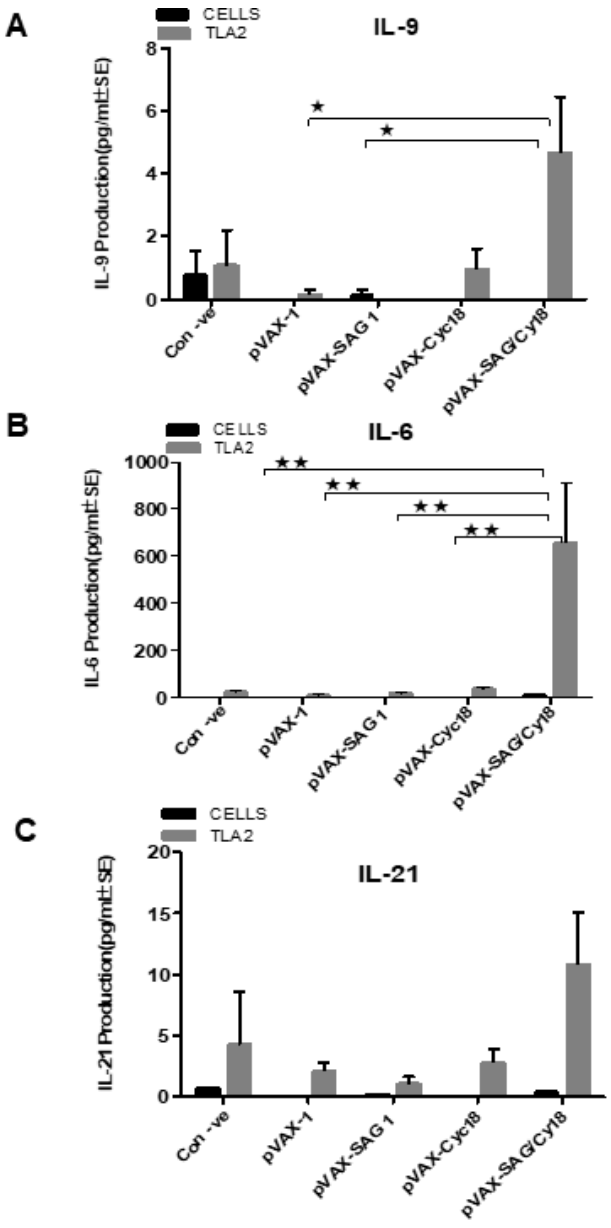


Figure 5.12 Relative TFH cell cytokine production in splenocytes isolated from control and vaccinated mice two weeks after the final immunisation. Splenocytes were left unstimulated (black) or were stimulated with 10 µg/ml of TLA (grey) and supernatants collected at 72 hours post stimulation.

(A) Mice vaccinated with pVAX-SAG1/Cyc18 showed a significant production of IL-9 than mice vaccinated with pVAX-1 and pVAX-SAG1. **(B)** Mice vaccinated with pVAX-SAG1/Cyc18 showed a significant production of IL-6 than control negative, pVAX-1, pVAX-SAG1 and pVAX-Cyc18. **(C)** Mice vaccinated with pVAX-SAG1/Cyc18 showed high but not significant of IL-21 production. *<p 0.05 and **<p 0.01 significance between the groups. Immunised mice, n=5.

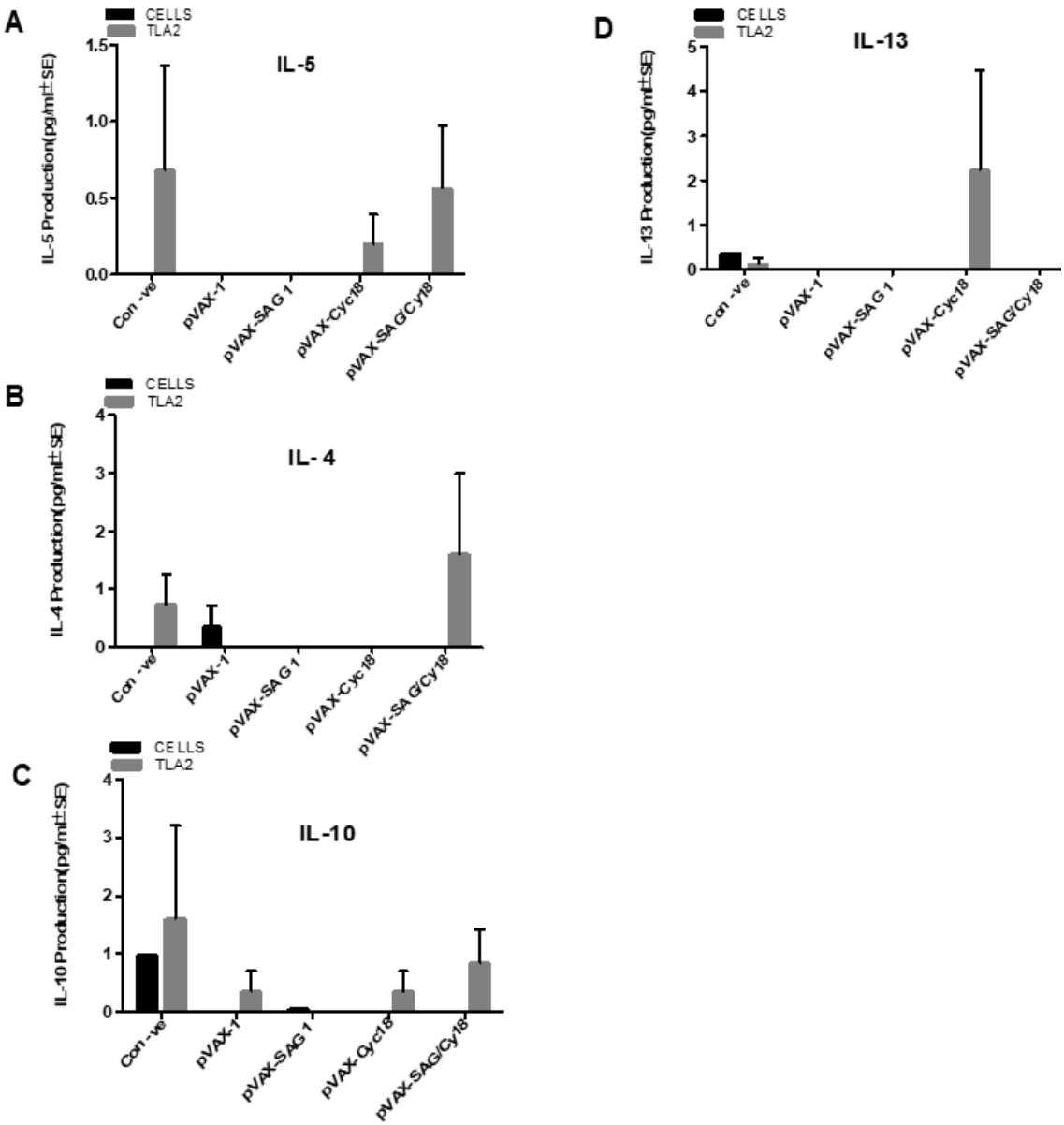


Figure 5.13 Relative Th2 cell cytokine production in splenocytes isolated from control and vaccinated mice two weeks after the final immunisation. Splenocytes were left unstimulated (black) or were stimulated with 10 µg/ml of TLA (grey) and supernatants collected at 72 hours post stimulation.

(A) Mice in control non-vaccinated group showed higher production of IL-5 than vaccinated mice. **(B)** Negative non-vaccinated mice showed high IL-4 production as well as mice vaccinated with pVAX-SAG1/Cyc18. **(C)** Mice in control non-vaccinated group showed higher production of IL-10 than vaccinated mice. **(E)** Mice vaccinated with pVAX-Cyc18 showed high production of IL-13.

5.5 Discussion

5.5.1 DNA Vaccine

In spite of a great deal of research testing multiple vaccine candidates as part of a DNA vaccine there is still no vaccine that gives sterile immunity in mice. SAG1 has been used in multiple studies with varying degrees of success in a number of murine models and has epitopes that can be recognised by the host immune cells, makes it a hopeful vaccine candidate (He *et a*l., 2002). More recently, SAG1 or epitopes derived from SAG1 have been used experimentally with other genes or epitopes as part of a multi-component vaccine, sometimes with more success (Cao *et al.*, 2015; Liu *et al.*, 2009). However, vaccines also depend on the choice of adjuvant which play a key factor in dictating the quality and effectiveness of the immune response and development of memory after vaccination (reviewed, Lee and Nguyen, 2015). Of note, there are few experimentally effective adjuvants for use in DNA vaccines although CPG motifs have been used with some success (reviwed, McCluskie, Weeratna & Davis, 2000). CPG is defined as single-stranded DNA molecules that contain a cytosine triphosphate deoxynucleotide and are considered a pathogen-associated molecular pattern (PAMP) and is known to bind Toll-Like Receptor 9 (TLR9), which is constitutively expressed only in B cells and dendritic cells (Bauer & Wagner, 2002). The current study was undertaken to explore the use of TgCyc18, a *T. gondii* derived gene as a natural adjuvant to increase augment and direct the immune response to the tachyzoite expressed surface antigen SAG1. As TgCyc18 is known to be a chemokine mimic that binds CCR5 and induces IL-12 production the hypothesis is that inclusion of this component would favour a Th1 response and the development of $CD8^+$ CTL cells. Notably, CCR5 is important for development of protective immunity during a natural *T. gondii* infection as mice deficient in this receptor

have increased parasite multiplication, reduced survival and reduced NK and $CD8^+$ cell migration (Bonfá *et al.*, 2014; Khan *et al.*, 2006b; Luangsay *et al.*, 2003).

5.5.2 TgCyc18 Potential as an Adjuvant in a DNA Vaccine

The initial *in vitro* results in this current study demonstrated that transfection of macrophages derived from mouse bone marrow with pVAX-Cyc18 successfully induced IL-12 production following DNA transfection with pVAX-Cyc18. In contrast, pVAX-1 and pVAX-SAG1 were only able to induce IL-12 at relatively high concentrations as a likely consequence of endogenous CPG motifs and consequent TLR signaling. This is consistent with the original observation that TgCyc18 derived from tachyzoite extract can induce production of IL-12 from mouse dendritic cells through CCR5 in *in vitro* (Aliberti *et al.*, 2003). The ability of pVAX-Cyc18 to induce IL-12 *in vitro* provided the confidence to test this plasmid as an adjuvant *in vivo*.

5.5.3 IgG1 and IgG2a Results as Indicator of Stimulation of Specific Humoral Immune Response

Production of IgG subsets is an indicator of the quality of T-helper immune responses. IgG2a production is dependent on IFN- ɣ and thus and an indicator of Th1 activation while IgG1 is generally associated with IL-4 production and Th2 activation (Snapper and Paul., 1987). Mice vaccinated with pVAX-SAG1/Cyc18 had increased levels of IgG2a compared with mice vaccinated with pVAX-SAG1 indicating an increased Th1 response as a likely consequence of *T. gondii* Cyclophilin 18 induced IL-12 production from dendritic cells or macrophages resulting in IFN- ɣ production from NK cells, which acts in concert with IL-12 to induce Th1 cells. These results were encouraging as previous DNA vaccine trials that resulted in augmentation of the IgG2a subclass in the sera of immunised mice were

found to induce protection (Hassan *et al.*, 2014; Mévélec *et al.*, 2005; Couper *et al.*, 2003; Fachado *et al.*, 2003). Examination of IgG sub types is generally undertaken to better understand the cytokine environment and T cell activation. However, the humoral response has also been demonstrated to promote phagocytosis and activate the classical complement pathway to kill intracellular parasites (Croix *et al.*, 1996). IgG2a is generally perceived to more efficiently induce opsonisation and complement fixation than IgG1 (Ey & Jenkin, 1980). It has been found that production of IgG in late stages of *T. gondii* infection is related with prolonged survival and reduced brain cyst numbers (Hester *et al*., 2012). Furthermore, specific IgG can cross the placental barrier within 3-4 weeks after infection to decrease the congenital toxoplasmosis prevalence (Nielsen *et al.*, 2005).

5.5.4 Cellular Mediated Immunity Response

To better understand the ability of inclusion of a plasmid encoding *T. gondii* cyclophilin18 in a vaccine to influence T cell differentiation the cytokine profiles of T-lymphocytes derived from the splenocytes of vaccinated mice were examined using a 13-plex cytometric bead array. PCA analysis demonstrated robust separation between mice vaccinated with pVAX-SAG1/Cyc18 and all other groups of mice. Orthogonal projection to latent structure for discriminant analyses (OPLS-DA) was used to determine the cytokine levels that most contribute to these differences and these included IL-6, IFN- ɣ, TNF-a, IL-22 and IL-9. Mice immunised with pVAX-SAG1/Cyc18 had significantly increased IFN-ɣ and TNF-α levels compared with control non-vaccinated and pVAX-1 vaccinated mice indicating the preferential differentiation of Th1 cells. This would be predicted to be beneficial to protection as previous studies demonstrate that IFN-ɣ and TNF-α activate macrophage functions and control tachyzoite replication during both acute and chronic phases of infection (Jongert *et al.*, 2010; Alexander *et al.*, 1997).

Splenocytes from mice vaccinated with pVAX-SAG1/Cyc18 also had increased levels of IL-6, IL-9, IL-17a, IL-17f and IL-22 compared with control unvaccinated or pVAX-1 vaccinated mice indicating induction of Th17 and TFH. Th17 and Th22 are likely to be important in protection against *T. gondii* infection as IL-17R-/- and IL-22-/- mice are more susceptible to infection as they develop higher parasite burdens than wild-type control mice (Fonseca *et al.*, 2012). Moreover, IL-17A-deficient C57BL/6 mice exhibited higher rates of mortality than wild-type mice during the acute phase of *T. gondii* infection (Moroda *et al.*, 2017).

Mice vaccinated with pVAX-SAG1/Cyc18 also had high levels of IL-21 suggesting induction of TFH cells. T follicular helper cells are known to be essential for B cell maturation and are one of the main sources of IL-21. IL-21R deficient mice infected with *T. gondii* have been demonstrated to have more active neurological disease than wild type mice (increased tachyzoite numbers and reduced cyst numbers) and a reduction of poly functional $CD8^+$ T cell activation (Moretto *et al.*, 2017).

Taken together, these results that demonstrate preferentially induction of Th1, Th17, Th22 and TFH cells rather than Th2 and Treg cells imply the vaccination protocol is likely to induce an immune response that has been associated with protection against *T. gondii* infection.

5.5.5 Bioluminescent Results

To assess protection against infection vaccinated mice were infected with *T. gondii* tachyzoites expressing the luciferase bioluminescent reported and imaged using an IVIS. Mice vaccinated with pVAX-SAG1/Cyc18 or pVAX-SAG1 had significantly reduced parasite numbers during 4, 6 and 8 days post infection. Similarly, mice vaccinated with

pVAX-SAG1/Cyc18 or pVAX-SAG1 had increased survival compared with other groups of mice. Inclusion of the TgCyc18 gene in the vaccine did not provide significantly enhanced protection relative to pVAX-SAG1 vaccinated mice in terms of parasite multiplication or survival. However, inclusion of TgCyc18 provided significant protection against *T. gondii* infection-induced weight loss, which has been shown to be an indicator of severity of infection.

5.6. Conclusion

The cellular immune response plays an important role in the control of multiplication of a *T. gondii* infection. The current study demonstrates that the cellular and humoral immune response to vaccination with SAG1 DNA can be altered by inclusion TgCyc18. Thus, inclusion of TgCyc18 induced significant *T. gondii*-specific differentiation of Th1, Th17, Th9 and Th22 cells as determined by cytokine profile and preferential production of IgG2a. TgCyc18 inclusion in the SAG1 vaccine, despite clearly altering the immune response generated only had a modest effect on protection against infection over that seen in SAG1 only vaccinated animals. This might be due to the infection model being used in this study that looks at acute infection where the innate immune response plays a significant role and thus differences in the adaptive immune response could be less obvious or important. Therefore, studies described in the next chapter were undertaken to ascertain if there is increased benefit of including TgCyc18 in a model that allowed infection to progress to a chronic infection. Simultaneously, the ability of Cyc18 to adjuvant a cocktail of DNA vaccines including surface antigens from bradyzoite namely, SAG4 and SAG4.2 against cysts challenge for chronic infection is assessed.

Chapter 6: The ability of Cyclophilin 18 (TgCyc18) to adjuvant Bradyzoite Surface Antigens (SAG4 or SAG4.2) and to protect mice from a cyst forming strain of *T. gondii*

6.1 Introduction

6.1.1 Chronic Toxoplasmosis

In the previous chapter, the inclusion of a plasmid containing the TgCyc18 gene as part of a DNA vaccine containing SAG1 was found to augment immunogenicity and protection of mice against challenge with *T. gondii* tachyzoites. However, although vaccination resulted in 100% protection in terms of survival, parasite multiplication could still be observed and this was accompanied by weight loss in all mice. Furthermore, infection in humans or animals is normally through ingestion of tissue cysts or oocysts rather than injection of tachyzoites. Therefore, it is important to examine the efficacy of vaccines following a more clinically relevant challenge such as *T. gondii* cysts administered orally. Furthermore, published studies have demonstrated that the inclusion of bradyzoite expressed antigens can enhance protection of vaccines (Nielsen *et al.*, 2006). Inclusion of such antigens could have 2 possible benefits. Firstly, immune responses generated to bradyzoite antigens could target the bradyzoites as they emerge from the cyst immediately following infection. Secondly, the generated immune response could target parasites as they make the transition from tachyzoite to bradyzoite. Previous work has demonstrated that certain bradyzoite-expressed antigens can provide a degree of protection in various murine models of disease (Table 6.1). For example, vaccination with a combination of a DNA vaccine encoding SAG4 (a *T. gondii* bradyzoite expressed protein) in conjunction with a peptide containing a linear B-cell epitope from SAG4 was found to provide a degree of protection. Vaccinated mice had increased survival time when challenged with RH tachyzoites and 31% less brain cysts when challenged with the Pru strain of *T. gondii* (Zhou & Wang, 2017). In another study, immunisation with a multicomponent DNA vaccine composed of plasmids encoding SAG1 and GRA4 with a

plasmid encoding GM-CSF as an adjuvant was able to provide significant protection against acute mortality and reduce brain cyst burden in chronic infection following challenge with the 76K strain of *T. gondii* (Mévélec *et al.*, 2005).

6.1.2 *T. gondii* SAG4 and SAG4.2

SAG4 and SAG4.2 are part of an antigenically family of SAG-related sequences (SRS). The structure of SAG1 has been solved and it reveals homodimeric protein complex with a deep grove between that has been postulated to bind sulfated proteoglycans (He *et al.*, 2002). Consistent with this hypothesis, *T. gondii* was previously demonstrated to use sulfated proteoglycans to attach and invade host cells (Carruthers *et al.*, 2000). More recently the structure determination of BSR4 revealed a similar homodimeric arrangement, but with a distinct binding site suggested that it binds a distinct ligand (Crawford *et al.*, 2009). It is likely that other family members including SAG3 and SAG5 have a similar structure and play similar roles in host cell attachment (reviewd, Rezaei *et al.*, 2019). Some members of the SRS family are known to be differentially expressed by tachyzoites and bradyzoites. SAG4 and SAG4.2 are known to be expressed in the bradyzoite stage, but not the tachyzoite stage of *T. gondii*. Tissue cysts containing bradyzoites can be detected within six to seven days following infection with tissue cysts and are supposed to persist throughout the life of the host. However, cysts can also be detected in visceral organs including the lungs, brain, liver and kidneys as well as the bone marrow (Dubey, *et a*l 1998). SAG4 and SAG4.2 antigens would therefore be among the first bradyzoite specific SRS antigens to be seen by the immune system following ingestion of tissue cysts.

Table 6.1 Examples of vaccine studies that included bradyzoite expressed antigens

Vaccine Type	Tachyzoite component	Bradyzoite component	Animal model	Challenge strain of *T .gondii*	Results	Reference
T.g.HSP30/bagl	Not found	T.g.HSP30	C57BL/6 BALB/c mice	Cyst of Fukaya strain (orally)	Both C57BL/6 and BALB/c mice produce IgG antibodies, reduced brain cysts and significant survival rate than non-vaccinated mice	(Mun *et al.*, 1999)
pGRA4 and pSAG1mut GM-CSF DNA vaccine	SAG1 GRA4	GRA4	C57BL/6 Swiss OF1 mice	Cyst of 76K type II strain (orally)	87% survival in vaccinated mice versus 0% in control non-vaccinated Lower number of cerebral cysts	(Mévélec *et al.*, 2005)
pGEX-MAG1 and pGEX-BAG1 DNA vaccine	MAG1	MAG1 BAG1	C3H/HeN mice	30 cysts of avirulent *T. gondii* SSI 119 strain (orally)	Reduce brain cyst number to 62% and induce production of specific IgG2a antibodies	(Nielsen *et al.*, 2006)
rROP2, rGRA5 and rGRA7 recombinant proteins (I/N)	rROP2, rGRA5 and rGRA7	rROP2, rGRA5 and rGRA7	BALB/c mice	50 cysts from the VEG strain (orally)	partial protection against brain cyst formation	(Igarashi *et al.*, 2008)

liposome-encapsulated GRA4 DNA vaccine	GRA4	GRA4	C57BL/6 BALB/c mice	ME 49 cysts (orally) RH tachyzoite (I/P)	High levels of IgG, IFN-ɣ and IL-2 production, and prolong survival rate Longer survival time	(Chen *et al.*, 2009)
GRA1 and SAG1 DNA vaccine	GRA1 and SAG1	GRA1	BALB/c mice	tachyzoites of RH strain (I/p)	High levels of IgG, IFN-ɣ and IL-2 production	(Wu *et al.*, 2012)
Multi-epitopes DNA vaccine of SAG1, GRA2, GRA7 and ROP16 with pRANTES adjuvant	SAG1, GRA2, GRA7 and ROP16	GRA2, GRA7 and ROP16	BALB/c mice	RH strain tachyzoites (I/P)	high levels of IFN-ɣ and IgG2a, and low levels of IL-4 or IL-10	(Cao *et al.*, 2015)
TgMIC1, TgMIC4 and TgMIC6 proteins	TgMIC1, TgMIC4 TgMIC6	TgMIC1, TgMIC4	C57BL/6 mice	80 cysts of the ME49 (orally)	reduced brain cyst numbers and increased of survival time after challenge	(Pinzan *et al.*, 2015)
pSAG4 DNA vaccine and SAG4 peptide	Not found	SAG4	BALB/c mice	RH strain tachyzoites (I/P) 20 cysts of PRU strain (orally)	Increased IgG and IFN-ɣ Brain cyst number is 31% of the number in PBS injected mice.	(Zhou & Wang, 2017)

6.2 Aims

The studies in the previous chapter suggested that the inclusion of TgCyc18 to a recombinant DNA vaccine including the SAG1 gene could induce protection against *T. gondii* tachyzoites. Therefore, in this chapter the aim is to investigate if the inclusion of bradyzoite expressed genes into this multicomponent DNA vaccine provides protection against *T. gondii* cyst challenge.

Specifically, by:

1. Determine if inclusion of SAG4 or SAG4.2 to the DNA vaccine comprising SAG1 and TgCyc18 provides better protection against weight loss, mortality and cyst formation.
2. Compare the immune response of control and vaccinated mice following oral challenge with *T. gondii* cysts.

6.3 Experimental Design

6.3.1 Vaccination

Female BALB/c mice 6-8 weeks old were divided randomly into five groups of 5 mice each. Mice were vaccinated with single plasmids, or combinations of 2 or 3 mixed plasmids. Specifically, groups of mice given left non-vaccinated as controls or given pVAX-1(empty plasmid), pVAX-SAG1/Cyc18, pVAX-SAG1/Cyc18/SAG4, or pVAX-SAG1/Cyc18/SAG4.2. For vaccination, mice were injected intramuscularly with plasmid DNA (50 µl in each anterior tibialis at a concentration of 2 mg/ml). Mice received three immunisation doses at two week intervals before challenge with *T. gondii.*

6.3.2 Challenge experiment:

Three weeks after the last vaccination, vaccinated and non-vaccinated mice were challenged orally with 20 cysts/mouse of *T. gondii* (Beverley strain).

6.3.3 Evolution the immune response

Protective immune responses were evaluated by determination of cytokine levels in brain after the sacrifice day by using multiplex cytokine bead array and flow cytometry. Mouse body weights and survival rates were measured until the end of the experiment.

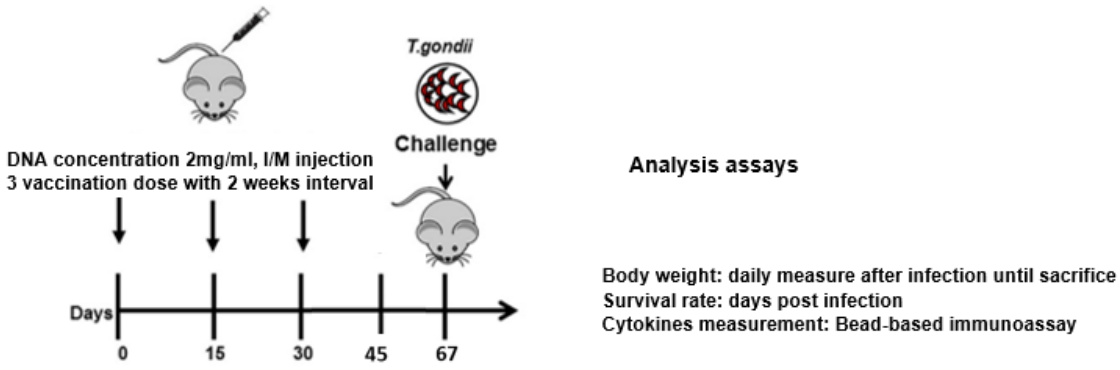


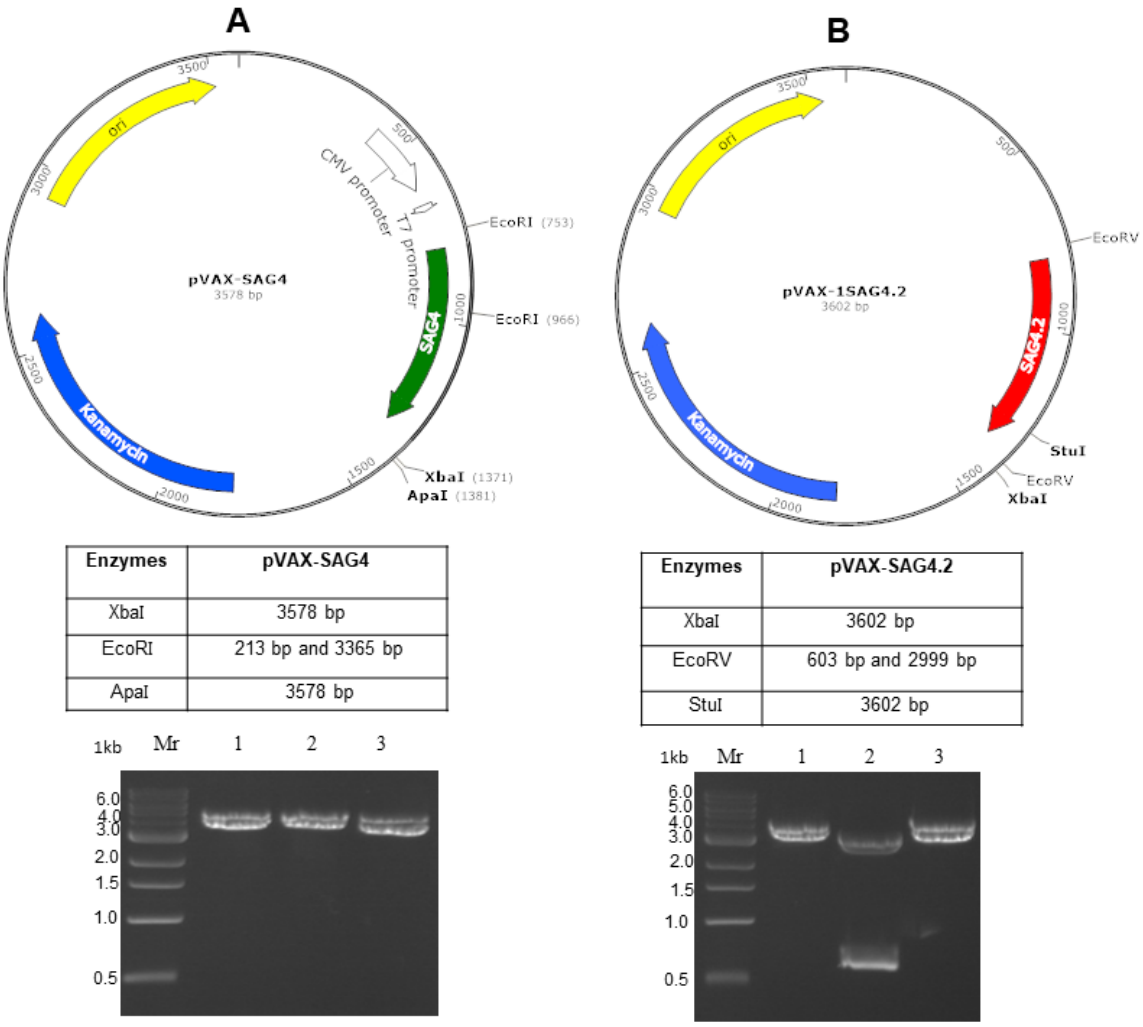
Figure 6. 1 Experimental design for vaccination and challenge infection with cysts of *T. gondii*.

6.4 Results

6.4.1 Preparation of pVAX-SAG4 and pVAX-SAG4.2 Constructs

pVAX-SAG4 and pVAX-SAG4.2 were kindly provided by prof. Craig W Roberts. The two plasmids were transformed into *E. coli* DH5α, amplified, isolated and cleaved to confirm their identities as shown in figure 6.2.

pVAX-SAG4 was cleaved with XbaI, EcoRI and ApaI resulted in 3578 bp, 213 bp,3365 bp and 3578 bp fragments (Figure 6.2 A). While pVAX-SAG4.2 was cleaved with XbaI, EcoRV and StuI resulted in 3602 bp, 603 bp, 2999 bp and 3602 bp fragments (Figure 6.2 B). For vaccination, both plasmids were purified and obtained by using Macherey and Nagel endotoxin free Giga kit.



Enzymes	pVAX-SAG4
XbaI	3578 bp
EcoRI	213 bp and 3365 bp
ApaI	3578 bp

Enzymes	pVAX-SAG4.2
XbaI	3602 bp
EcoRV	603 bp and 2999 bp
StuI	3602 bp

Figure 6. 2 Characterisation of recombinant plasmids pVAX-SAG4 and pVAX-SAG4.2 by restriction analysis.

(A) Top, pVAX-SAG4 map; middle, table of expected of fragments sizes using different restriction enzymes; bottom, agarose gel of pVAX-SAG4 restriction analysis. Lane 1, XbaI; lane 2, EcoRI; lane 3, ApaI . **(B)** Top, pVAX- SAG4.2 map, middle, table of fragments sizes using different restriction enzymes; bottom, agarose gel of pVAX- SAG4.2 restriction analysis. Lane 1, XbaI; lane 2, EcoRV; lane 3, StuI . Mr, DNA size marker in kb.

6.4.2 Survival Rate

From day 15 post infection, onwards the death of mice was observed. Mice vaccinated with pVAX-1 and non-vaccinated groups had 40% and 60% of survival rate, respectively. Mice vaccinated with SAG1/Cyc18/SAG4 and pVAX-SAG1/Cyc18 had 60% survival rate.

In contrast, significant protection was seen in mice vaccinated with SAG1/Cyc18/SAG4.2 relative to control non-vaccinated or pVAX-1 vaccinate mice, with 80% of mice surviving infection with *T. gondii* bradyzoites ($p<0.001$ and 0.01, respectively) (Figure 6.3).

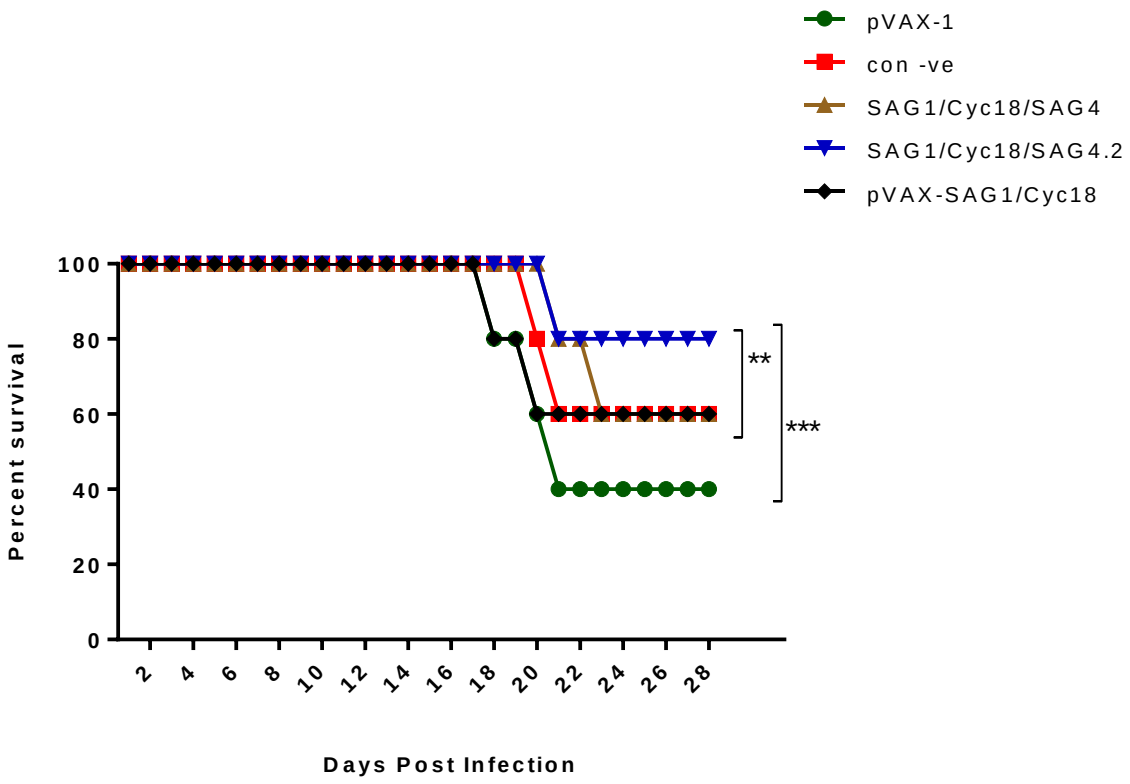


Figure 6. 3 Survival curves of BALB/c mice groups following orally challenged with 20 cysts/mouse.

Mice vaccinated with SAG1/Cyc18/SAG4.2 showed 80% protection rate against challenge infection. Each group contains five mice. **<p 0.01, ***<p 0.001.

6.4.3 Body Weight

The body weights of all groups of mice after challenge infection were measured daily for the duration of the experiment. 2 mice out of the 5 control non-vaccinated group of mice lost over 5% of their body weight and never recovered to pre-infection weights (Figure 6.4 A). The pVAX-1 vaccinated mice had high levels of mortality with the one surviving mouse recovering weight (Figure 6.4 B). 2 out of the 5 mice vaccinated with SAG1/Cyc18 or SAG1/Cyc18/SAG4 mice recovered to within 5% of their starting weight by the end of the study. In contrast, 4 out of the 5 pVAX-SAG1/Cyc18/SAG4.2 vaccinated mice survived and all recovered to within 5% of starting weight (Figure 6.4 D). The mean weights of surviving mice over the course of the experiment is shown in Figure 6.4 F although this has to be interpreted with caution as it excludes mice that died or had to be euthanized.

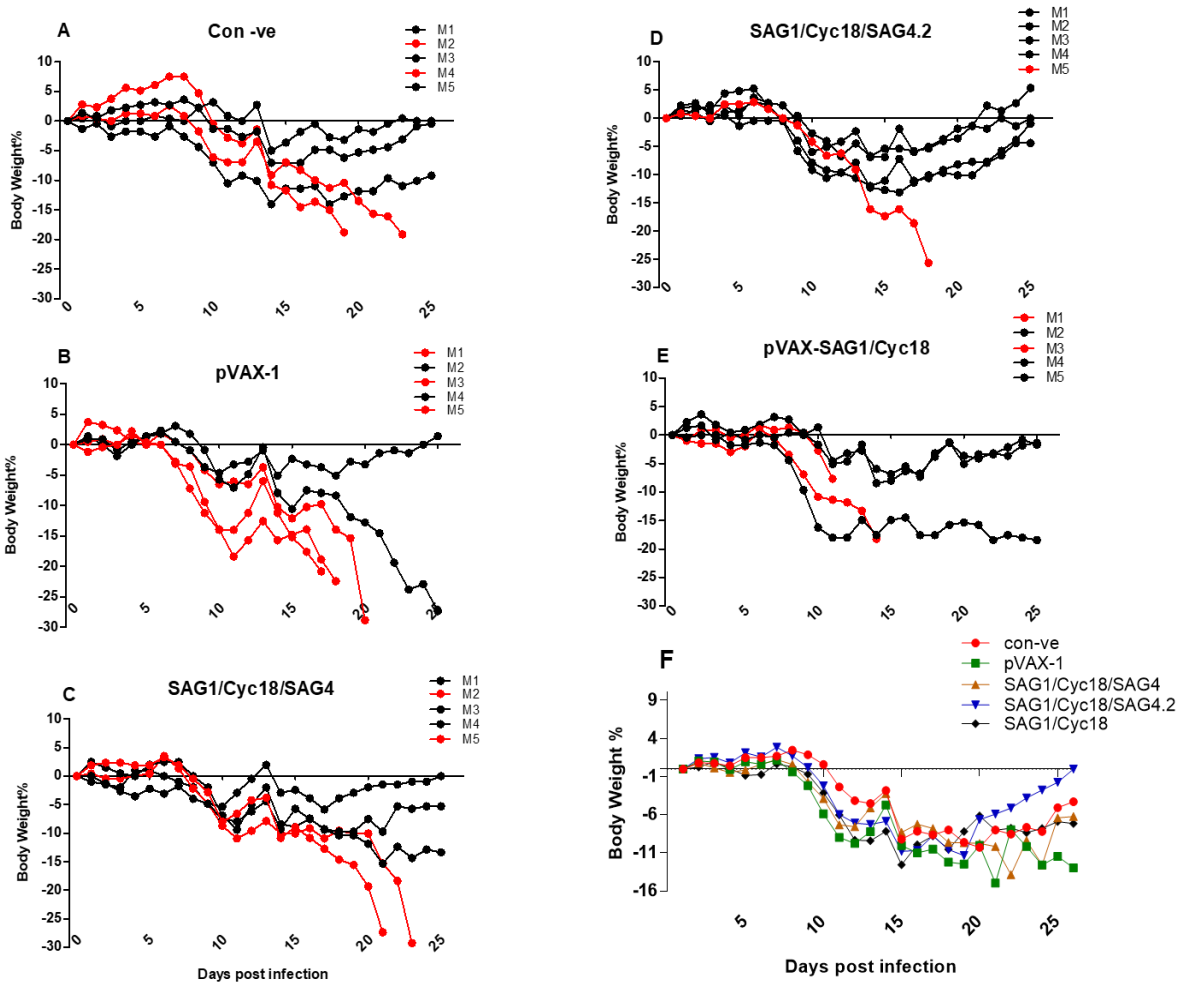


Figure 6. 4 Survival curves and body weight differences of BALB/c mice groups following challenge infection with *T. gondii* cysts orally.

Mice vaccinated with pVAX-SAG1/Cyc18/SAG4.2 showed a significant protection against body weight loss.

6.4.4 Detection of Cytokine Levels from Brain Tissue

To determine the inflammation response in brains of vaccinated and non-vaccinated mice, a multiplex cytokine bead array was used. Levels of cytokines were generally low in all groups of mice. However, mice vaccinated pVAX-1 without a gene insert generally had lower levels of cytokine compared with control non-vaccinated mice (this was significant for GM-CSF and IL-17a). In contrast, mice vaccinated with SAG1/Cyc18/SAG4 generally had increased levels of cytokines relative to control non-vaccinated mice (this was significant for: GM-CSF, IFN-ɣ, IFNβ, MCP-1, TNF-α, IL-1α, IL-1β, IL-6, IL-12p70, IL-17a and IL-23) (Figures 6. 5 and 6.6).

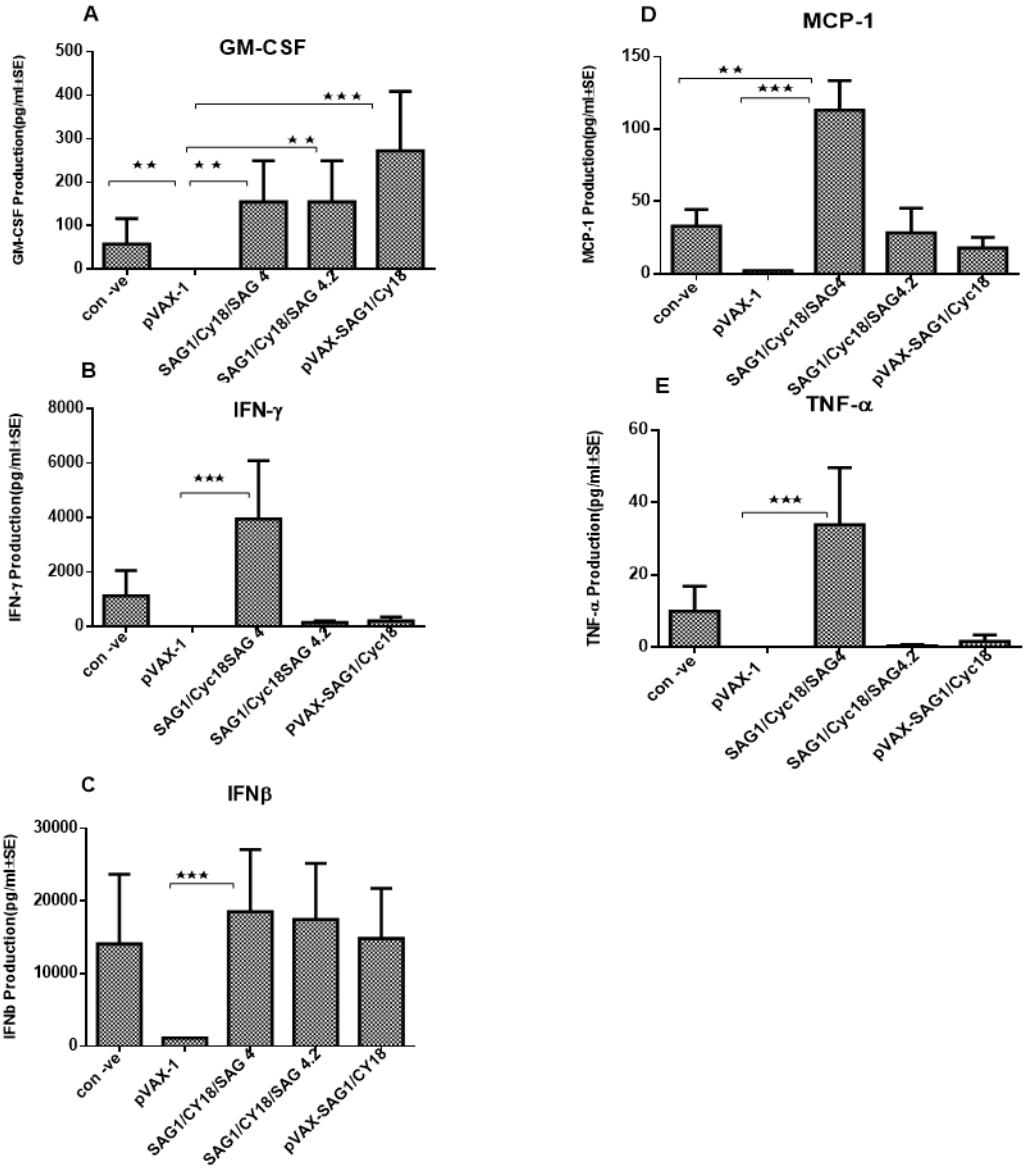


Figure 6. 5 Levels of GM-CSF, IFN-ɣ, IFNβ, MCP-1 and TNF-α from brain tissue of vaccinated and control negative groups following challenge infection.

*<p 0.05, **<p 0.01 and *** <p 0.001 significance between the groups. Immunised mice, n=5.

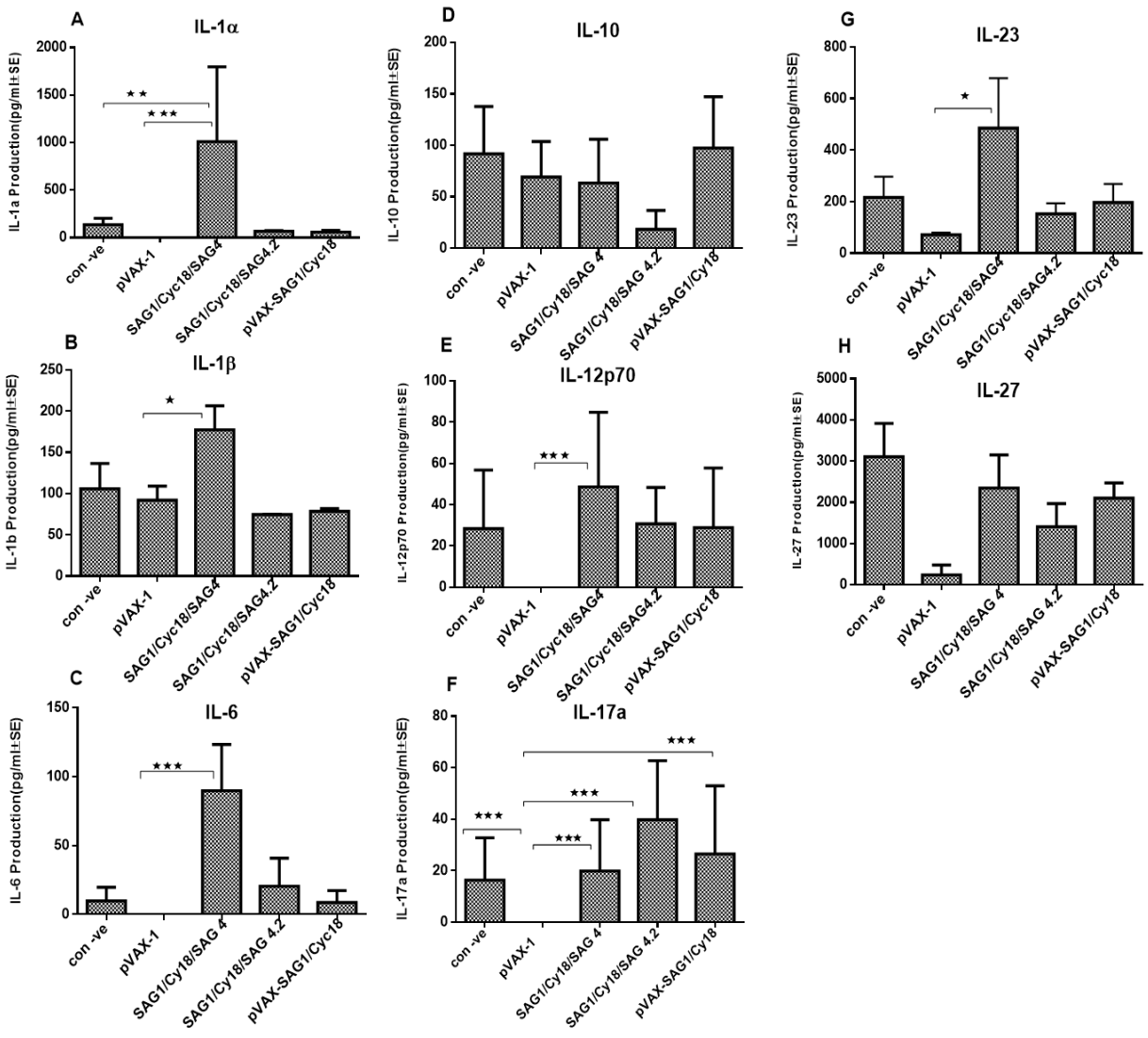


Figure 6. 6 Levels of IL-1α, IL-1β, IL-6, IL-10, IL-12p70, IL-17a, IL-23 and IL-27 from brain tissue of vaccinated and non-vaccinated groups following challenge infection.

*<p 0.05, **<p 0.01 and *** <p 0.01 significance between the groups. Immunised mice, n=5.

6.5 Discussion

Chapter 5 demonstrated that the ability of a plasmid containing TgCyc18 gene to act as an adjuvant and augment the immune response to DAN vaccination with SAG1. This resulted in an augmented Th1 response including increased IFN-ɣ production and higher IgG2a levels than in mice vaccinated with pVAX-SAG1 alone. There was no significant difference in the parasite numbers in mice vaccinated with TgCyc18 and SAG1 in combination and mice vaccinated with SAG1 alone. However, mice receiving pVAX-Cyc18 and pVAX-SAG1 in combination had reduced weight loss compared with mice receiving the pVAX-SAG1 vaccination alone. This suggested that the TgCyc18 induced alteration to the immune response following vaccination was functionally protective. Therefore, the studies described in this chapter were to determine if this vaccination procedure would provide protection in against a cyst forming strain of *T. gondii* and whether the addition of bradyzoite expressed genes into this multicomponent DNA vaccine provided further protection. SAG4 and SAG4.2 were chosen as bradyzoite expressed antigens as they are members of the SRS family similar to SAG1 which has already been shown to be promising vaccine candidate.

Previous studies showed that including bradyzoite antigens in a DNA vaccination against *T. gondii* can be effective and reduce brain cyst formation and elicit a Th1 type immune response as determined by IgG2a production (Nielsen *et al.*, 2006). Other studies have been demonstrated that multi-gene vaccines that included tachyzoite and bradyzoite components could increase survival time and reduce the number of brain cysts in immunised mice (Zhou & Wang, 2017; Mévélec *et al.*, 2005).

Mice vaccinated with SAG1/Cyc18/SAG4.2 had significant protection with 80% of surviving time against *T. gondii* bradyzoites infection compared with mice vaccinated with pVAX-1 and non-vaccinated mice, which had 40% and 60% of survival rate, respectively.

The four mice vaccinated with SAG1/Cyc18/SAG4.2 recovered to within 5% of starting weight whereas recovery was less obvious or only seen in some non-vaccinated mice or mice vaccinated with empty plasmid. SAG4 has been demonstrated to contain immune protective epitopes in a previous study. A vaccine comprising DNA and a SAG4 peptide containing predicted B cell and MHC II epitopes increased the survival time of mice compared with control mice challenged with tachyzoites or bradyzoites of *T. gondii* (Zhou & Wang, 2017).

The cytokine levels from brain tissue after challenge infection were examined using a 13-plex cytometric bead array designed to measure inflammation. The intracerebral immune reactions and the *in vivo* regulation of microglial activity are still poorly understood and the CNS infections, most reported information concerning the immune response in *T. gondii* CNS infection originates from murine models. Several studies demonstrated the effects of *T. gondii* infection to cause toxoplasmic encephalitis for better understanding the immune response in CNS. Tachyzoite parasites appear to infect astrocytes, neurons, and microglial cells, possibly with different affinities followed by $CD4^+$ and $CD8^+$ T cell influx in a process, which is critical for control of *T. gondii* CNS infection. It has been demonstrated that toxoplasmic encephalitis induces IL-12p40, iNOS, IL-1β, TNF-α largely due to $CD8^+$ T cell interaction (Schlüter *et al.*, 2006). However, it has been shown that neurons containing parasite cysts avoid scrutiny by $CD8^+$ T cells and that observed by using two-photon microscopy of living brain tissue and confocal microscopy of fixed brain sections (Schaeffer

et al., 2009). Interferon-gamma (IFN-ɣ)-dependent cell mediated immune response eventually kills off the majority of the tachyzoites but in some sites including the CNS the tachyzoites convert to bradyzoites. It is difficult to draw firm conclusions from the results due to the small numbers of surviving mice in some groups. However, cytokine levels were generally raised in mice vaccinated with SAG1/Cyc18/SAG4 suggesting that vaccination had increased the inflammatory response and potentially protective immunity to *T. gondii* infection. In contrast, vaccination with pVAX-1 without an insert had a deleterious effect with respect to *T gondii* mortality of infected mice and cytokine levels in the brain were generally low. This may be due to immunosuppression, or the toxic effect of pVAX-1 itself. Alternatively, vaccination of mice with empty pVAX-1 plasmid may induce an inappropriate non-specific stimulation of the immune system as previously described (Wu *et al.*, 2012).

6.6 Conclusions

T. gondii Cyc18 is a promising adjuvant candidate that warrants further investigation and potential development to be included with a DNA vaccine or a conventional. The ability of TgCyc18 to direct the cellular immune response towards a Th1 phenotype could be useful in vaccine for other diseases where this is desirable. As DNA vaccination does not require expression and purification of vaccine candidates to test, it provides a useful tool to find protective components that can be used as part of a multicomponent vaccine.

Chapter 6: General Discussion

The development of a safe and active vaccine against *T. gondii* infection has the potential to alleviate human and animal disease, to provide improved animal welfare and impact public health in a positive manner. Modern vaccines currently in use against other pathogens are typically proteins combined with an appropriate adjuvant to induce and augment their immunogenicity (Döşkaya *et al.*, 2007). As many potential vaccine candidates are glycoproteins they are best expressed in a eukaryotic system to facilitate post-translational modification. Eukaryotic systems are costly and generally provide poor yields. Therefore, in the first part of this work the expression of TgCyc18 and TgSAG1 proteins in *L. tarentolae* as filamentous recombinant proteins was attempted as proof of principle and to generate material for experimental immunisation purposes.

Recombinant proteins have been expressed in a number of different systems for a wide variety of purposes. However, each system has a number of advantages and limitations. Expression in prokaryotic systems, such as *E. coli* tends to be used as a first option as it is inexpensive and capable of high yields. In addition, *E. coli* itself can be relatively easily modified to help with expression, through addition of helper plasmids carrying heterologous tRNAs. However, *E. coli* is not suitable for all applications as it does not cope well with the expression of large proteins, does not perform post-translational modifications such as glycosylation and does not always result in authentically folded proteins. In addition as *E. coli* contains endotoxin, proteins have to be purified to remove this potentially harmful contaminant (Rosano & Ceccarelli, 2014).

Consequently, a number of other systems have been developed that make use of eukaryotic organisms. These systems have been especially important in production of therapeutic proteins, which might need to be glycosylated. Eukaryotic systems include mammalian cell-

culture systems, such as Chinese hamster ovary (CHO), yeast systems such as *Pichia pastoris* and even protozoan systems such as *L. tarentolae* (Niimi, 2012; Demain & Vaishnav, 2009b; Chu & Robinson, 2001). CHO and yeast expression systems are currently used to produce some therapeutic proteins but are expensive (Mattanovich *et al.*, 2012). Mammalian expression systems have for example been used to produce antibodies, complex proteins and proteins for use in functional cell-based assays with post-translational processing and functional activity. Such proteins are produced transiently or through stable cell lines, where the expression construct is integrated into the host genome. As well as the extra cost, mammalian systems have demanding culture conditions (reviewed in Jenkins *et al.*, 2009).

L. tarentolae has been used as a eukaryotic expression systems as it can grow in suspension cultures and can produce grams-per-liter yields with providing more native folding and post-translational modifications, such as glycosylation, as compared to other expression systems. Previous studies have used *L. tarentolae* for recombinant protein production (Niculae *et al.*, 2006; Dortay & Mueller-Roeber, 2010; Kianmehr *et al.*, 2016) and the biological application of these proteins is shown in table 1.4. The studies undertaken in this thesis aimed to improve the *L. tarentolae* system, through fusion of proteins to filamentous proteins that would be secreted into the culture supernatant for easy purification. This study found that the SAG1 fusion protein was secreted into the culture medium and could be enriched by ammonium sulfate precipitation. However, the quantities obtained were insufficient for vaccination studies but similar to those obtained in the commercial *L. tarentolae* system. Consequently, the improvement of this eukaryotic protein expression system still remains a challenge.

The second goal of the work in this thesis was to investigate the use of TgCyc18, a chemokine mimic produced by *T. gondii* that binds CCR5 and induces IL-12 as an adjuvant for vaccination. The need for vaccine adjuvants has become greater due to the development of recombinant sub-unit vaccines. This is because synthetic antigens used in vaccines are generally far less immunogenic than live or killed whole organism vaccines. The first widely used adjuvant for humans was ALUM (Glenny *et al.*, 1926). This aluminum salt is able to improve humoral immunity via Th2 type immune responses but not Th1 type (Comoy *et al.*, 1997). ALUM has previously been used with recombinant *T. gondii* SAG1 antigen expressed in *E. coli* to immunise mice, and induced partial protection against challenge with tachyzoites (Petersen, 1998). To overcome this limitation a practical approach to designing new vaccines is to add additional components to the existing adjuvants. So for example the adjuvant ASO4 (HPV vaccines manufactured by GSK), comprises alum and the TLR4 agonist, monophoshoryl lipid A (MPL) (Garçon *et al.*, 2011). No studies to date have reported that AS04 provides any benefits over ALUM in a *T. gondii* model.

One adjuvant that has recently shown promising results for a *T. gondii* vaccine is Glucopyranosyl lipid adjuvant-stable emulsion (GLA-SE), a synthetic Toll-like receptor (TLR) 4. It is currently being explored as a vaccine adjuvant to enhance immunogenicity and can provide protection against seasonal influenza (Coler *et al.*, 2010). This adjuvant has been found to trigger the innate immune response by activation of dendritic cells to induce production of T-helper 1 (Th1) cell–promoting cytokines, TNF-α, lL-1b, IL-6, and IL-12 (Behzad *et al.*, 2011).

Multi-epitope DNA, protein nanoparticles or peptide vaccines have been successfully used with GLA-SE adjuvant to protect human HLA transgenic mice against *T. gondii* resulting in

increases of cytotoxic $CD8^+$ T cells, IFN-ɣ production and efficient presentation by the major histocompatibility complex in immunised mice. Importantly, vaccinated mice when challenged with *T. gondii* developed fewer cysts than control mice (Cong *et al.*, 2012; El Bissati *et al.*, 2016, 2017).

Other TLR agonists such as CPG oligodeoxynucleotides (CPG ODN) have been used with some success as a vaccine as it is known to bind Toll-Like Receptor 9 (TLR9), which is constitutively expressed only in B cells and dendritic cells (Bauer & Wagner, 2002). The use of CPG ODN as adjuvant for vaccines designed to prevent infectious disease was reviewed in (Shirota & Klinman, 2017). CPG vaccines are appealing for inclusion in DNA vaccines as they can be easily incorporated. Cytokine adjuvants also have potential in DNA vaccination as they can be given with a gene and are able to enhance cellular immune responses (Xiang & Ertl, 1995). Granulocyte-macrophage colony stimulating factor (GM-CSF) was used with a multiantigenic DNA vaccine and was found to enhance the survival time of mice against *T. gondii* infection (Mévélec *et al.*, 2005).

The current study demonstrates that TgCyc18 given as part of a multi-component DNA vaccine has adjuvant potential as it can increase the relative intensity of Th1 induction as measured by IFN-ɣ and IgG2a production and provide some benefit to vaccinated mice in terms of reduced weight loss. The inclusion of further *T. gondii* genes in the vaccine did not offer a dramatic improvement of protection in a cyst forming model of chronic toxoplasmosis. Future studies could investigate the use of further synthetic genes encoding defined epitopes. Alternative approaches might also include changing the route of immunisation. The antigen delivery pathway is a key parameter for the induction of protective immune responses by vaccines against intracellular pathogens. Most candidate vaccines have been

given parenterally to induce systemic immunity by using intramuscular immunisation, which can control toxoplasmosis efficiently (Lunden *et al.*, 1993). Several studies have shown that intranasal immunisation with *T. gondii* protein can induce a systemic immune response and partial protection against chronic lethal *T. gondii* infections and brain cyst formation by stimulation of mucosal immune responses in effective sites, including the gut, genital tract, and nose cavity of the experimental mice (Wang *et al.*, 2014; Igarashi *et al.*, 2008).

This work could be extended in a number of ways to understand the mechanisms of action for the experimental vaccine. For example, host gene expression during the immune responses to *T. gondii* in the brains of mice could be evaluated using RNAseq to determine expression levels. Brain cyst burdens could be assessed in further experiments by immunohistochemistry or through using qPCR.

The knowledge acquired from this study has proven that the inclusion of TgCyc18 as a natural adjuvant in a DNA vaccine formulation can better induce a Th1 response for co-administered genes and offers a new strategy for other vaccines where the induction of cell-mediated immunity is required.

Chapter 7: References

Abdallah, D.S.A., Lin, C., Ball, C.J., King, M.R., Duhamel, G.E. & Denkers, E.Y. (2012) *Toxoplasma gondii* triggers release of human and mouse neutrophil extracellular traps. *Infection and Immunity*. 768–777.

Abdizadeh, R., Maraghi, S., Ghadiri, A.A., Tavalla, M. & Shojaee, S. (2015) Cloning and expression of major surface antigen 1 gene of *Toxoplasma gondii* RH strain using the expression vector pVAX1 in Chinese Hamster Ovary cells. *Jundishapur Journal of Microbiology*. 1–10.

Adams Waldorf, K.M. & McAdams, R.M. (2013) Influence of infection during pregnancy on fetal development. *Reproduction (Cambridge, England)*. R151-62.

Ajioka, J.W., Fitzpatrick, J.M. & and Reitter, C.P. (2001) Ultrastructure of a *Toxoplasma gondi*i tachyzoite. Expert Review in Molecular Medicine. *Expert Review in Molecular Medicine.*

Ajzenberg, D., Collinet, F., Aubert, D., et al. (2015) The rural-urban effect on spatial genetic structure of type II *Toxoplasma gondii* strains involved in human congenital toxoplasmosis, France, 2002-2009. *Infection, Genetics and Evolution*. 511–516.

Ajzenberg, D., Lamaury, I., Demar, M., Vautrin, C., Cabié, A., Simon, S., Nicolas, M., Desbois-Nogard, N., Boukhari, R., Riahi, H., Dardé, M.L., Massip, P., Dupon, M., Preux, P.M., Labrunie, A. & Boncoeur, M.P. (2016) Performance Testing of PCR Assay in Blood Samples for the Diagnosis of Toxoplasmic Encephalitis in AIDS Patients from the French Departments of America and Genetic Diversity of *Toxoplasma gondii*: A Prospective and Multicentric Study. *PLoS Neglected Tropical Diseases*. 1–17.

Alexander, J., Scharton-Kersten, T.M., Yap, G., Roberts, C.W., Liew, F.Y. & Sher, A. (1997) Mechanisms of innate resistance to *Toxoplasma gondii* infection. *Philosophical Transactions of the Royal Society B: Biological Sciences*. 1355–1359.

Aliberti, J., Reis, C., Schito, M., Hieny, S., Wells, T., Huffnagle, G.B. & Sher, A. (2016) CCR5 provides a signal for microbial induced CCR5 provides a signal for microbial induced production of IL-12 by CD8 α + dendritic cells. *Nature immunology*. 83.

Aliberti, J., Reis e Sousa, C., Schito, M., Hieny, S., Wells, T., Huffnagle, G.B. & Sher, a (2000) CCR5 provides a signal for microbial induced production of IL-12 by CD8 alpha+ dendritic cells. *Nature immunology*. 83–87.

Aliberti, J., Valenzuela, J.G., Carruthers, V.B., Hieny, S., Andersen, J., Charest, H., e Sousa, C.R., Fairlamb, A., Ribeiro, J.M. & Sher, A. (2003) Molecular mimicry of a CCR5 binding-domain in the microbial activation of dendritic cells. *Nat Immunol*. 485–490.

Allain, J.P., Palmer, C.R. and Pearson, G. (1998) Epidemiological study of latent and recent infection by *Toxoplasma gondii* in pregnant women from a regional population in the UK. *Journal of infection*. 189–196.

Andrade, G.M., Vasconcelos-Santos, D.V., Carellos, E.V., Romanelli, R., Vitor, R.W., Carneiro, A.C. & and Januario, J.N. (2010) Congenital toxoplasmosis from a chronically infected woman with reactivation of retinochoroiditis during pregnancy an underestimated event? *Jornal de pediatria*. 85–88.

Angus, C.W., Klivington-Evans, D., Dubey, J.P. & Kovacs, J. a (2000) Immunization with a DNA plasmid encoding the SAG1 (P30) protein of *Toxoplasma gondii* is immunogenic and protective in rodents. *The Journal of infectious diseases*. 317–324.

Araujo, F.G., Guptill, D.R. and Remington, J.S. (1988) Azithromycin, a macrolide antibiotic with potent activity against *Toxoplasma gondii*. *Antimicrobial agents and chemotherapy*. 755–757.

Backström, E., Kristensson, K. & Ljunggren, H.G. (2004) Activation of natural killer cells: underlying molecular mechanisms revealed. *Scandinavian journal of immunology*. 14–22.

Badr, M.S., Attia, S.S., El-Sherbiny, W.S., Abd-Allah, M.A., Hefny, H.M. & Salem., and A.N. (2016) Genetic Polymorphism of *Toxoplasma gondii* from Recently Infected Aborted Egyptian Women. *J Egypt.Soc.Parasitol*. 49–55.

Barbosa, I.R., Holanda, de C.X.C.M. & de Andrade-Neto, V.F. (2009) Toxoplasmosis screening and risk factors amongst pregnant females in Natal, northeastern Brazil. *Transactions of the Royal Society of Tropical Medicine and Hygiene*. 377–382.

Barragan, A. & Sibley, L.D. (2002) Transepithelial migration of *Toxoplasma gondii* is linked to parasite motility and virulence. *The Journal of experimental medicine*. 1625–1633.

Basak, A., Shervani, N.J., Mbikay, M. & Kolajova, M. (2008) Recombinant proprotein convertase 4 (PC4) from *Leishmania tarentolae* expression system: Purification, biochemical study and inhibitor design. *Protein Expression and Purification*. 117–126.

Basile, G. & Peticca, M. (2009) Recombinant Protein Expression in *Leishmania tarentolae*. *Molecular Biotechnology*. 273–278.

Bauer, S. & Wagner, H. (2002) *Bacterial CpG-DNA Licenses TLR9 BT - Toll-Like Receptor Family Members and Their Ligands*. In: Bruce Beutler & Hermann Wagner (eds.). Berlin, Heidelberg, Springer Berlin Heidelberg. pp. 145–154.

Baz, M., Samant, M., Zekki, H., Tribout-Jover, P., Plante, M., Lanteigne, A.M., Hamelin, M.E., Mallett, C., Papadopoulou, B. & Boivin, G. (2012) Effects of different adjuvants in the context of intramuscular and intranasal routes on humoral and cellular immune responses induced by detergent-split A/H3N2 influenza vaccines in mice. *Clinical and Vaccine Immunology*. 209–218.

Behnke, M.S., Khan, A., Lauron, E.J., Jimah, J.R., Wang, Q., Tolia, N.H. & Sibley, L.D. (2015) Rhoptry Proteins ROP5 and ROP18 Are Major Murine Virulence Factors in Genetically Divergent South American Strains of *Toxoplasma gondii*. *PLoS Genetics*. 1–22.

Behzad, H., Huckriede, A.L.W., Haynes, L., Gentleman, B., Coyle, K. & Wilschut, J.C. (2011) GLA-SE , a Synthetic Toll-like Receptor 4 Agonist , Enhances T-Cell Responses to Influenza Vaccine in Older Adults. *The Journal of Infectious Diseases*. 466–473.

Ben-Abdallah, M., Bondet, V., Fauchereau, F., Béguin, P., Goubran-Botros, H., Pagan, C., Bourgeron, T. & Bellalou, J. (2011) Production of soluble, active acetyl serotonin methyl transferase in *Leishmania tarentolae*. *Protein Expression and Purification*. 114–118.

Beverley, J.K.A., 1959. (1959) Congenital transmission of toxoplasmosis through successive generations of mice. *Nature*. 1348–1349.

Beverley, S.M. & Clayton, C.E. (1993) Transfection ofLeishmania and *Trypanosoma brucei* by Electroporation. In: John E Hyde (ed.). *Protocols in Molecular Parasitology*. Totowa, NJ, Humana Press. pp. 333–348.

El Bissati, K., Chentoufi, A.A., Krishack, P.A., et al. (2016) Adjuvanted multi-epitope vaccines protect HLA-A*11:01 transgenic mice against *Toxoplasma gondii. JCI Insight*. 1–18.

El Bissati, K., Zhou, Y., Paulillo, S.M., Raman, S.K., Karch, C.P., Roberts, C.W., Lanar, D.E., Reed, S., Fox, C., Carter, D., Alexander, J., Sette, A., Sidney, J., Lorenzi, H., Begeman, I.J., Burkhard, P. & McLeod, R. (2017) Protein nanovaccine confers robust immunity against Toxoplasma. *npj Vaccines*. 1–11.

Bliss, S.K., Butcher, B.A. & and Denkers, E.Y. (2000) Rapid recruitment of neutrophils containing prestored IL-12 during microbial infection. *The Journal of Immunology*. 4515–4521.

Bliss, S.K., Gavrilescu, L.C., Alcaraz, A. and & Denkers, E.Y. (2001) Neutrophil depletion during *Toxoplasma gondii* infection leads to impaired immunity and lethal systemic pathology. *Infection and immunity*. 4898–4905.

Bonfá, G., Benevides, L., Souza, M. do C., Fonseca, D.M., Mineo, T.W.P., Rossi, M.A., Silva, N.M., Silva, J.S. & de Barros Cardoso, C.R. (2014) CCR5 Controls Immune and Metabolic Functions during *Toxoplasma gondii* Infection Markus M. Heimesaat (ed.). *PLoS ONE*. e104736.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*. 248–254.

Breitling, R., Klingner, S., Callewaert, N., Pietrucha, R., Geyer, A., Ehrlich, G., Hartung, R., Müller, A., Contreras, R., Beverley, S.M. & Alexandrov, K. (2002) Non-pathogenic trypanosomatid protozoa as a platform for protein research and production. *Protein Expression and Purification*. 209–218.

Burnett, A.J., Shortt, S.G., Isaac-Renton, J., King, A., Werker, D. and Bowie, W.R. (1998) Multiple cases of acquired toxoplasmosis retinitis presenting in an outbreak. ,. *Ophthalmology*. 1032–1037.

Burrells, A., Bartley, P.M., Zimmer, I.A., Roy, S., Kitchener, A.C., Meredith, A., Wright, S.E., Innes, E.A. & Katzer, F. (2013) Evidence of the three main clonal *Toxoplasma gondii* lineages from wild mammalian carnivores in the UK. *Parasitology*. 1768–1776.

Burrells, A., Benavides, J., Cantón, G., Garcia, J.L., Bartley, P.M., Nath, M., Thomson, J., Chianini, F., Innes, E.A. & Katzer, F. (2015) Vaccination of pigs with the S48 strain of *Toxoplasma gondii* - Safer meat for human consumption. *Veterinary Research*. 1–12.

Burrells, A., Opsteegh, M., Pollock, K.G., Alexander, C.L., Chatterton, J., Evans, R., Walker, R., McKenzie, C.A., Hill, D., Innes, E.A. & Katzer, F. (2016) The prevalence and

genotypic analysis of *Toxoplasma gondii* from individuals in Scotland, 2006-2012. *Parasites and Vectors*. 1–12.

Buyukbaba, B.O., Sonmez, T.G., Keceli, O.S., Sonmez, N., Issever, H. & Tekeli., and F. (2012) [Investigation of combined effectiveness of spiramycin and beta-glucan in mice models of acute toxoplasmosis and determination of IL-10, IL-12 and TNF-alpha levels]. *Mikrobiyol.Bul*. 446-455.

Cañón-Franco, W.A., Araújo, F.A.P., López-Orozco, N., Jardim, M.M.A., Keid, L.B., Dalla-Rosa, C., Cabral, A.D., Pena, H.F.J. & Gennari, S.M. (2013) *Toxoplasma gondii* in free-ranging wild small felids from Brazil: Molecular detection and genotypic characterization. *Veterinary Parasitology*. 462–469.

Cao, A., Liu, Y., Wang, J., Li, X., Wang, S., Zhao, Q., Cong, H., He, S. & Zhou, H. (2015) *Toxoplasma gondii*: Vaccination with a DNA vaccine encoding T- and B-cell epitopes of SAG1, GRA2, GRA7 and ROP16 elicits protection against acute toxoplasmosis in mice. *Vaccine*. 6757–6762.

Carruthers, V.B., Håkansson, S., Giddings, O.K. & David Sibley, L. (2000) *Toxoplasma gondii* uses sulfated proteoglycans for substrate and host cell attachment. *Infection and Immunity*. 4005–4011.

Carson, W.E., Ross, M.E., Baiocchi, R.A., Marien, M.J., Boiani, N., Grabstein, K. & and Caligiuri, M.A. (1995) Endogenous production of interleukin 15 by activated human monocytes is critical for optimal production of interferon-gamma by natural killer cells in vitro. *Journal of Clinical Investigation*. 2578.

CDC (2005) CDC and Fort Dodge Animal Health Achieve First Licensed DNA Vaccine. *[Press Release].Avilable at: https://www.cdc.gov.* Accessed: 30 Aprile 2019.

Çelik, E. & Çalık, P. (2012) Production of recombinant proteins by yeast cells. *Biotechnology Advances*. 1108–1118.

Cha, D.Y., Song, I.K., Lee, G.S., Hwang, O.S., Noh, H.J., Yeo, S.D., Shin, D.W. & Lee, Y.H. (2001) Effects of specific monoclonal antibodies to dense granular proteins on the invasion of *Toxoplasma gondii* in vitro and in vivo. *The Korean journal of parasitology*. 233–240.

Chen, R., Lu, S., Tong, Q., Lou, D., Shi, D., Jia, B., Huang, G. & Wang, J. (2009) Protective effect of DNA-mediated immunization with liposome-encapsulated GRA4 against infection of *Toxoplasma gondii*. *Journal of Zhejiang University SCIENCE B*. 512–521.

Chen, W. (2011) Tregs in immunotherapy: opportunities and challenges. *Immunotherapy*. 911–914.

Chessa, G., Chisu, V., Porcu, R. & Masala, G. (2014) Molecular characterization of *Toxoplasma gondii* Type II in sheep abortion in Sardinia, Italy. *Parasite (Paris, France)*. 6.

Chu, L. & Robinson, D.K. (2001) Industrial choices for protein production by large-scale cell culture. *Current Opinion in Biotechnology*. 180–187.

Clayton, C.E. (1999) Genetic Manipulation of Kinetoplastida. *Parasitology Today*. 372–378.

Coler, R.N., Baldwin, S.L., Shaverdian, N., Bertholet, S., Reed, S.J., Raman, V.S., Lu, X., DeVos, J., Hancock, K., Katz, J.M., Vedvick, T.S., Duthie, M.S., Clegg, C.H., van Hoeven, N. & Reed, S.G. (2010) A synthetic adjuvant to enhance and expand immune responses to influenza vaccines. *PLoS ONE*. 13677.

Comoy, E.E., Capron, A. & Thyphronitis, G. (1997) In vivo induction of type 1 and 2 immune responses against protein antigens. *International Immunology*. 523–531.

Cong, H., Mui, E.J., Witola, W.H., Sidney, J., Alexander, J., Sette, A., Maewal, A., El Bissati, K., Zhou, Y., Suzuki, Y., Lee, D., Woods, S., Sommerville, C., Henriquez, F.L., Roberts, C.W. & McLeod, R. (2012) *Toxoplasma gondii* HLA-B*0702-restricted GRA720-28 peptide with adjuvants and a universal helper T cell epitope elicits CD8+ T cells producing interferon-γ and reduces parasite burden in HLA-B*0702 mice. *Human Immunology*. 1–10.

Couper, K.N., Nielsen, H. V., Petersen, E., Roberts, F., Roberts, C.W. & Alexander, J. (2003) DNA vaccination with the immunodominant tachyzoite surface antigen (SAG-1) protects against adult acquired *Toxoplasma gondii* infection but does not prevent maternofoetal transmission. *Vaccine*. 2813–2820.

Couper, K.N., Roberts, C.W., Brombacher, F., Alexander, J. & Johnson, L.L. (2005) *Toxoplasma gondii*-specific immunoglobulin M limits parasite dissemination by preventing host cell invasion. *Infection and immunity*. 8060–8068.

Couvreur, J., Desmonts, G. and Thulliez, P. (1988) Prophylaxis of congenital toxoplasmosis. Effects of spiramycin on placental infection. *Journal of Antimicrobial Chemotherapy*. 193–200.

Crawford, J., Grujic, O., Bruic, E., Czjzek, M., Grigg, M.E. & Boulanger, M.J. (2009) *Structural Characterization of the Bradyzoite Surface Antigen (BSR4) from Toxoplasma gondii , a Unique Addition to the Surface Antigen Glycoprotein 1-related Superfamily **. 9192–9198.

Croix, D.A., Ahearn, J.M., Ariella, M., Hanfl, S., Kelsoefl, G., Ma, M. & Carroll, M.C. (1996) *Antibody Response to a T-dependent Antigen Requires A ~*.

Cua, D.J., Sherlock, J., Chen, Y., Murphy, C.A., Joyce, B., Seymour, B., Lucian, L., To, W., Kwan, S., Churakova, T., Zurawski, S., Wiekowski, M., Lira, S.A., Gorman, D., Kastelein, R.A. & Sedgwick, J.D. (2003) Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature*. 744–748.

Cutchins, E. & Warren, J. (1956) *Toxoplasma* Infection and Vaccination with Killed *Toxoplasma*. *The American journal of tropical medicine and hygiene.* 197–209.

Daffos, F., Forestier, F., Capella-Pavlovsky, M., Thulliez, P., Aufrant, C., Valenti, D. and Cox, W.L. (1988) Prenatal management of 746 pregnancies at risk for congenital toxoplasmosis. *New England Journal of Medicine*. 271–275.

Dardé, M.L., Bouteille, B. & Pestre-Alexandre, M. (1992) Isoenzyme analysis of 35 *Toxoplasma gondii* isolates and the biological and epidemiological implications. *The*

journal of parasitology. 786–794.

Davoudi, N., Hemmati, A., Khodayari, Z., Adeli, A. & Hemayatkar, M. (2011) Cloning and expression of human IFN-γ in *Leishmania tarentolae*. *World Journal of Microbiology and Biotechnology*.27 pp.1893–1899.

Debierre-Grockiego, F., Campos, M.A., Azzouz, N., Schmidt, J., Bieker, U., Resende, M.G., Mansur, D.S., Weingart, R., Schmidt, R.R., Golenbock, D.T., Gazzinelli, R.T. & Schwarz, R.T. (2007) Activation of TLR2 and TLR4 by glycosylphosphatidylinositols derived from *Toxoplasma gondii*. *J. Immunol*. 1129–1137.

Demain, A.L. & Vaishnav, P. (2009a) Production of recombinant proteins by microbes and higher organisms. *Biotechnology Advances*.27 (3) pp.297–306.

Demain, A.L. & Vaishnav, P. (2009b) Production of recombinant proteins by microbes and higher organisms. *Biotechnology Advances*. 297–306.

Denkers, E.Y., Scharton-Kersten, T., Barbieri, S., Caspar, P. & Sher, A. (1996) A role for CD4+ NK1.1+ T lymphocytes as major histocompatibility complex class II independent helper cells in the generation of CD8+ effector function against intracellular infection. *Journal of Experimental Medicine*. 131–139.

Denkers, E.Y., Yap, G., Scharton-Kersten, T., Charest, H., Butcher, B.A., Caspar, P., Heiny, S. & Sher, A. (1997) Perforin-mediated cytolysis plays a limited role in host resistance to *Toxoplasma gondii*. *The Journal of Immunology*. 1903–1908.

Derouin, F., Jacqz-Aigrain, E., Thulliez, P., Couvreur, J. and Leport, C. (2000) Cotrimoxazole for prenatal treatment of congenital toxoplasmosis? *Parasitology Today*. 254–256.

Dey, R., Natarajan, G., Bhattacharya, P., Cummings, H., Dagur, P.K., Terrazas, C., Selvapandiyan, A., McCoy, J.P., Duncan, R., Satoskar, A.R. & Nakhasi, H.L. (2014) Characterization of Cross-Protection by Genetically Modified Live-Attenuated *Leishmania donovani* Parasites against *Leishmania mexicana*. *The Journal of Immunology*. 3513–3527.

Dimier, I.H. & Bout, D.T. (1998) Interferon-γ-activated primary enterocytes inhibit *Toxoplasma gondii* replication: A role for intracellular iron. *Immunology*. 488–495.

DiPaolo, R.J., Brinster, C., Davidson, T.S., Andersson, J., Glass, D. & Shevach, E.M. (2007) Autoantigen-Specific TGFβ-Induced Foxp3 Regulatory T Cells Prevent Autoimmunity by Inhibiting Dendritic Cells from Activating Autoreactive T Cells. *The Journal of Immunology*. 4685 LP – 4693.

Dortay, H. & Mueller-Roeber, B. (2010) A highly efficient pipeline for protein expression in *Leishmania tarentolae* using infrared fluorescence protein as marker. *Microbial cell factories*. 29.

Dortay, H., Schmöckel, S.M., Fettke, J. & Mueller-Roeber, B. (2011) Expression of human c-reactive protein in different systems and its purification from *Leishmania tarentolae*. *Protein expression and purification*. 55–60.

Döşkaya, M., Kalantari-Dehaghi, M., Walsh, C.M., Hiszczyńska-Sawicka, E., Davies, D.H., Felgner, P.L., Larsen, L.S.Z., Lathrop, R.H., Hatfield, G.W., Schulz, J.R., Gürüz, Y. & Jurnak, F. (2007) GRA1 protein vaccine confers better immune response compared to codon-optimized GRA1 DNA vaccine. *Vaccine*. 1824–1837.

Dubey, J.P. (2009) History of the discovery of the life cycle of *Toxoplasma gondii*. *International Journal for Parasitology*. 877–882.

Dubey, J.P. (2010) 'Toxoplasmosis of Animals and Humans (Second Edition)'. *book*. 112.

Dubey, J.P., Lago, E.G., Gennari, S.M., Su, C. & Jones, J.L. (2012) Toxoplasmosis in humans and animals in Brazil: high prevalence, high burden of disease, and epidemiology. *Parasitology*. 1375–1424.

Dubey, J.P., Lindsay, D.S. & and Speer, C.A. (1998) Structures of *Toxoplasma gondii* tachyzoites, bradyzoites, and sporozoites and biology and development of tissue cysts. ,. *Clinical microbiology reviews*. 267–299.

Dubois, D.J. & Soldati-Favre, D. (2019) Biogenesis and secretion of micronemes in *Toxoplasma gondii*. *Cellular Microbiology*. 1–14.

Dubremetz, J.F. & Ferguson, D.J.P. (2009) The role played by electron microscopy in advancing our understanding of *Toxoplasma gondii* and other apicomplexans. *International Journal for Parasitology*. 883–893.

Dutra, M.S., Bela, S.R., Peixoto-RangelA.L., Fakiola, M., Cruz, A.G., Gazzinelli, A., Quites, H.F., Bahia-Oliveira, L.M., Peixe, R.G., Campos, W.R., Higino-Rocha, A.C., Miller, N.E., Blackwell, J.M., Antonelli, L.R. & Gazzinelli, R.T. (2013) Association of a NOD2 gene polymorphism and T-helper 17 cells withpresumed ocular toxoplasmosis. *J. Infect. Dis.* 152–163.

e Sousa, C.R. e, Hieny, S., Scharton-Kersten, T., Jankovic, D., Charest, H., Germain, R.N. & Sher, A. (1997) In Vivo Microbial Stimulation Induces Rapid CD40 Ligand–independent Production of Interleukin 12 by Dendritic Cells and their Redistribution to T Cell Areas. *The Journal of Experimental Medicine*. 1819–1829.

Ehmen, H.G. and Lüder, C.G., 2019. Long-Term Impact of *Toxoplasma gondii* Infection on Human Monocytes. Frontiers in cellular and infection microbiology, 9, p.235.

Ey, P.L., G.J., R.-J. & Jenkin, C.R. (1980) Isotypes of mouse IgG—I. Evidence for 'non-complement-fixing' IgG1 antibodies and characterization of their capacity to interfere with IgG2, sensitization of target red blood cells for lysis by complement. *Molecular Immunology*. 699–710.

Fachado, A., Rodriguez, A., Angel, S.O., Pinto, D.C., Vila, I., Acosta, A. & Amendoeira, R.R. and Lannes-Vieira, J. (2003) Protective effect of a naked DNA vaccine cocktail against lethal toxoplasmosis in mice. *Vaccine*. 1327–1335.

Feldman, H.A. (1953) Congenital toxoplasmosis e a study of 103 cases. *m. J. Dis.CHILD*. 487.

Ferguson, D.J.P. (2009) *Toxoplasma gondii*: 1908-2008, homage to Nicolle, Manceaux and Splendore. *Memorias do Instituto Oswaldo Cruz*. 133–148.

Ferreira, I.M.R., Vidal, J.E., Costa-Silva, T.A., Meira, C.S., Hiramoto, R.M., Penalva de Oliveira, A.C. & Pereira-Chioccola, V.L. (2008) *Toxoplasma gondii*: Genotyping of strains from Brazilian AIDS patients with cerebral toxoplasmosis by multilocus PCR-RFLP markers. *Experimental Parasitology*. 221–227.

Fonseca, D., Benevides, L., Souza, M. do C., Bonfá, G., Solano-Dias, M., Basso, A., Silva, N., Mineo, T., Ryffel, B. & Silva, J. (2012) Th17, Th22 and regulatory T cells are essential for controlling parasite burden and mucosal inflammation during *Toxoplasma gondii* infection (43.11). *The Journal of Immunology*. 43.11-43.11.

Fox, B.A. & Bzik, D.J. (2015) Nonreplicating, cyst-defective type II *Toxoplasma gondii* vaccine strains stimulate protective immunity against acute and chronic infection. *Infection and Immunity*. 2148–2155.

Frenkel, J.K., Pfefferkorn, E.R., Smith, D.D. & and Fishback, J.L. (1991) Prospective vaccine prepared from a new mutant of *Toxoplasma gondii* for use in cats. *American journal of veterinary research*. 759–763.

Fritz, H.M., Bowyer, P.W., Bogyo, M., Conrad, P.A. & Boothroyd, J.C. (2012) Proteomic analysis of fractionated Toxoplasma oocysts reveals clues to their environmental resistance. *PloS one*. e29955.

Fuhrman, S.A. & Joiner, K.A. (1989) *Toxoplasma gondii*: mechanism of resistance to complement-mediated killing. *The Journal of Immunology*. 940–947.

Garçon, N., Wettendorff, M. & Van Mechelen, M. (2011) Role of AS04 in human papillomavirus vaccine: mode of action and clinical profile. *Expert Opinion on Biological Therapy*. 667–677.

Gavrilescu, L.C., Butcher, B.A., Del Rio, L., T., And, G.A. & Denkers, E.Y. (2004) STAT1 is essential for antimicrobial effector function but dispensable for gamma interferon production during *Toxoplasma gondii* infection. *Infection and immunity*. 1257–1264.

Gazzinelli, R., Xu, Y., Hieny, S., Cheever, A. & Sher, A. (1992) Simultaneous depletion of CD4+ and CD8+ T lymphocytes is required to reactivate chronic infection with *Toxoplasma gondii*. *Journal of immunology*. 175–180.

Gazzinelli, R.T., Wysocka, M., Hayashi, S., Denkers, E.Y., Hieny, S., Caspar, P., Trinchieri, G. & Sher, A. (1994) Parasite-induced IL-12 stimulates early IFN-gamma synthesis and resistance during acute infection with *Toxoplasma gondii*. *Journal of immunology (Baltimore, Md. : 1950)*. 2533–2543.

Gazzinelli, R.T., Wysocka, M., Hieny, S., Scharton-kersten, T., Cheever, A., Kuhn, R., Muller, W., Trinchieri, G. & Sher, A. (1996) In the absence of endogenous IL-10, mice acutely infected with *Toxoplasma gondii* succumb to a lethal immune response dependent on CD4+ T cells and accompanied by overproduction of IL-12, IFN-gamma and TNF-alpha. *The Journal of Immunology*. pp.798-805.

Gigley, J.P., Fox, B.A. & Bzik, D.J. (2009) Cell-Mediated Immunity to *Toxoplasma gondii* Develops Primarily by Local Th1 Host Immune Responses in the Absence of Parasite Replication. *The Journal of Immunology*. 1069–1078.

Van Gisbergen, K.P.J.M., Geijtenbeek, T.B.H. & Van Kooyk, Y. (2005) Close encounters of neutrophils and DCs. *Trends in Immunology*. 626–631.

Glenny, A.T., Pope, C.G., Waddington, H. & Wallace, U. (1926) The antigenic value of toxoid precipitated by potassium alum. *J Pathol Bacteriol*. 38–39.

Godofsky, E.W. (1994) Treatment of presumed cerebral toxoplasmosis with azithromycin. *New England Journal of Medicine*. 575–576.

Godwin, R. (2012) *Toxoplasma gondii* and elevated suicide risk. *Veterinary Record*. 225.

Goldman, M., Carver, R.K. and Sulzer, A.. (1958) Reproduction of Toxoplasma gondii by internal budding. *The Journal of parasitology.* 161–171.

Goldszmid, R.S., Bafica, A., Jankovic, D., Feng, C.G., Caspar, P., Winkler-Pickett, R., Trinchieri, G. & Sher, A. (2007) TAP-1 indirectly regulates CD4+ T cell priming in *Toxoplasma gondii* infection by controlling NK cell IFN-γ production. *The Journal of experimental medicine.* 2591–2602.

Grimwood, J. & Smith, J.E. (1996) *Toxoplasma gondii*: the role of parasite surface and secreted proteins in host cell invasion. *International Journal for Parasitology*. 169–173.

Le Gros, G., Ben-Sasson, S.Z., Seder, R., Finkelman, F.D. & Paul, W.E. (1990) Generation of interleukin 4 (IL-4)-producing cells in vivo and in vitro: IL-2 and IL-4 are required for in vitro generation of IL-4-producing cells. *The Journal of experimental medicine*. 921–929.

Guenter, W., Bielinski, M., Deptula, a, Zalas-Wiecek, P., Piskunowicz, M., Szwed, K., Bucinski, a, Gospodarek, E. & Borkowska, a (2012) Does *Toxoplasma gondii* infection affect cognitive function? A case control study. *Folia Parasitol (Praha)*. 93–98. http://www.ncbi.nlm.nih.gov/pubmed/22779109.

Gülçe Iz, S., Döşkaya, M., Caner, A., Döşkaya, A.D., Rodriguez, F., Gürüz, Y. & Gürhan, S.I.D. (2014) A novel dual promoter DNA vaccine induces CD8+ response against *Toxoplasma gondii* sporozoite specific surface protein 'sporoSAG' through non-apoptotic cells. *Trials in Vaccinology*. 81–88.

Gupta, S.L., Carlin, J.M., Pyati, P., Dai, W., Pfefferkorn, E.R. & Murphy, M.J. (1994) Antiparasitic and antiproliferative effects of indoleamine 2,3-dioxygenase enzyme expression in human fibroblasts. *Infection and Immunity*. 2277 LP – 2284.

El Hajj, H., Demey, E., Poncet, J., Lebrun, M., Wu, B., Galéotti, N., Fourmaux, M.N., Mercereau-Puijalon, O., Vial, H., Labesse, G. and Dubremetz, J.F. (2006) The ROP2 family of *Toxoplasma gondii* rhoptry proteins: proteomic and genomic characterization and molecular modeling. *Proteomics*. 5773–5784.

Hakimi, M., Olias, P. & David, L. (2017) Crossm Toxoplasma Effectors Targeting Host. *Clinical Microbiology Reviews*. 615–645.

Halonen, S.K., Woods, T., McInnerney, K. & Weiss, L.M. (2006) Microarray analysis of IFN-γ response genes in astrocytes. *Journal of neuroimmunology*. 19–30.

Harrington, L.E., Hatton, R.D., Mangan, P.R., Turner, H., Murphy, T.L., Murphy, K.M. & Weaver, C.T. (2005) Interleukin 17–producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nature Immunology*. 1123.

Hassan, I.A., Wang, S., Xu, L., Yan, R., Song, X. & Li, X. (2014) Immunoglobulin and cytokine changes induced following immunization with a DNA vaccine encoding *Toxoplasma gondii* selenium-dependent glutathione reductase protein. *Experimental Parasitology*. 1–10.

Haumont, M., Delhaye, L., Garcia, L., Mazzu, P., Daminet, V., Bollen, A., Biemans, R., Jacquet, A., Haumont, L.E. & Jurado, M. (2000) *Protective Immunity against Congenital Toxoplasmosis with Recombinant SAG1 Protein in a Guinea Pig Model Protective Immunity against Congenital Toxoplasmosis with Recombinant SAG1 Protein in a Guinea Pig Model*. 4948–4953.

He, X., Grigg, M.E., Boothroyd, J.C. & Garcia, K.C. (2002) Structure of the immunodominant surface antigen from the *Toxoplasma gondii* SRS superfamily. *Nature structural biology*. 606–611.

Hehl, A.B., Basso, W.U., Lippuner, C., Ramakrishnan, C., Okoniewski, M., Walker, R.A., Grigg, M.E., Smith, N.C. & Deplazes, P. (2015) Asexual expansion of *Toxoplasma gondii* merozoites is distinct from tachyzoites and entails expression of non-overlapping gene families to attach, invade, and replicate within feline enterocytes. *BMC Genomics*. 1–16.

Hermann, M., Yéra, H., Villena, I., Cimon, B., Thervet, E. & Benachi, A. (2017) Diagnosis of congenital toxoplasmosis in a renal transplant recipient mother. *Journal of Gynecology Obstetrics and Human Reproduction*. 661–663.

Herrmann, D.C., B??rwald, A., Maksimov, A., Pantchev, N., Vrhovec, M.G., Conraths, F.J. & Schares, G. (2012) *Toxoplasma gondii* sexual cross in a single naturally infected feline host: Generation of highly mouse-virulent and avirulent clones, genotypically different from clonal types I, II and III. *Veterinary Research*. 1–10.

Hester, J., Mullins, J., Sa, Q., Payne, L., Mercier, C., Cesbron-Delauw, M.F. and Suzuki, Y., Hester, J., Mullins, J., Sa, Q., Payne, L., Mercier, C., Cesbron-Delauw, M.-F. & Suzuki, Y. (2012) *Toxoplasma gondii* antigens recognized by IgG antibodies differ between mice with and without active proliferation of tachyzoites in the brain during the chronic stage of infection. *Infection and immunity*. 3611–3620.

High, K.P., Joinera, K. & Robert, E. (1994) Isolation , cDNA Sequences , and Biochemical Characterization of the Major Cyclosporin-binding Proteins of *Toxoplasma gondii*. *The Journal of BIioclinical Chemistry*. 9105–9112.

Hohlfeld, P., Daffos, F., Thulliez, P.A., Aufrant, C., Couvreur, J., MacAleese, J., Descombey, D. and Forestier, F. (1989) Fetal toxoplasmosis: outcome of pregnancy and infant follow-up after in utero treatment. *The Journal of pediatrics*. 765–769.

Hutson, S.L., Mui, E., Kinsley, K., Witola, W.H., Behnke, M.S., El Bissati, K., Muench, S.P.,

Rohrman, B., Liu, S.R., Wollmann, R., Ogata, Y., Sarkeshik, A., Yates, J.R. & McLeod, R. (2010) T. gondii RP promoters & knockdown reveal molecular pathways associated with proliferation and cell-cycle arrest. *PLoS ONE*. e14057.

Hwang, S., Cobb, D.A., Bhadra, R., Youngblood, B. & Khan, I.A. (2016) Blimp-1–mediated CD4 T cell exhaustion causes CD8 T cell dysfunction during chronic toxoplasmosis. *The Journal of Experimental Medicine*. 1799–1818.

Ibrahim, H.M., Bannai, H., Xuan, X. & Nishikawa, Y. (2009) *Toxoplasma gondii* cyclophilin 18-mediated production of nitric oxide induces bradyzoite conversion in a CCR5-dependent manner. *Infection and Immunity*. 3686–3695.

Ibrahim, H.M., Nishimura, M., Tanaka, S., Awadin, W., Furuoka, H., Xuan, X. & Nishikawa, Y. (2014) Overproduction of Toxoplasma gondii cyclophilin-18 regulates host cell migration and enhances parasite dissemination in a CCR5-independent manner. *BMC Microbiology*. 76.

Ibrahim, H.M., Xuan, X. & Nishikawa, Y. (2010) *Toxoplasma gondii* cyclophilin 18 regulates the proliferation and migration of murine macrophages and spleen cells. *Clinical and Vaccine Immunology*. 1322–1329.

Igarashi, M., Kano, F., Tamekuni, K., Machado, R.Z., Navarro, I.T., Vidotto, O., Vidotto, M.C. & Garcia, J.L. (2008) *Toxoplasma gondii*: Evaluation of an intranasal vaccine using recombinant proteins against brain cyst formation in BALB/c mice. *Experimental Parasitology*. 386–392.

Ilg, T., Stierhof, Y.D., Etges, R., Adrian, M., Harbecke, D. & Overath, P. (1991) Secreted acid phosphatase of *Leishmania mexicana*: a filamentous phosphoglycoprotein polymer. *Proceedings of the National Academy of Sciences of the United States of America*. 8774–8778.

Ivory, C. & Chadee, K. (2004) DNA vaccines: Designing strategies against parasitic infections. *Genetic Vaccines and Therapy*. 1–8.

Jacobs, L., Fair, J.R. & and Bickerton, J.. (1954) Adult ocular toxoplasmosis: Report of a parasitologically proved case. *AMA archives of ophthalmology*. 63–71.

Jenkins, N., Meleady, P., Tyther, R. & Murphy, L. (2009a) Strategies for analysing and improving the expression and quality of recombinant proteins made in mammalian cells. *Biotechnology and Applied Biochemistry*. 73.

Jenkins, N., Meleady, P., Tyther, R. & Murphy, L. (2009b) Strategies for analysing and improving the expression and quality of recombinant proteins made in mammalian cells. *Biotechnology and Applied Biochemistry*. 73.

Johnson, L.L. & Sayles, P.C. (2002) Deficient humoral responses underlie susceptibility to *Toxoplasma gondi*i in CD4-deficient mice. *Infection and immunity*. 185–191.

Jones, J.L., Muccioli, C., Belfort, R., Holland, G.N., Roberts, J.M. & Silveira, C. (2006) Recently Acquired *Toxoplasma gondii* Infection, Brazil. *Emerging Infectious Disease journal*. 582.

Jongert, E., Lemiere, A., Van Ginderachter, J., De Craeye, S., Huygen, K. & D'Souza, S. (2010) Functional characterization of in vivo effector CD4+ and CD8+ T cell responses in acute Toxoplasmosis: An interplay of IFN-γ and cytolytic T cells. *Vaccine*. 2556–2564.

Jongert, E., Roberts, C.W., Gargano, N., Förster-Wald, E. & Petersen, E. (2009) Vaccines against *Toxoplasma gondii*: Challenges and opportunities. *Memorias do Instituto Oswaldo Cruz*. 252–266.

Kaneko, Y., Takashima, Y., Xuaun, X., Igarashi, I., Nagasawa, H., Mikami, T. and & Otsuka, H. (2004) Natural IgM antibodies in sera from various animals but not the cat kill *Toxoplasma gondii* by activating the classical complement pathway. *Parasitology*. 123–129.

Kang, H., Remington, J.S. & Suzuki, Y. (2000) Decreased Resistance of B Cell-Deficient Mice to Infection with *Toxoplasma gondii* Despite Unimpaired Expression of IFN- , TNF- , and Inducible Nitric Oxide Synthase. *The Journal of Immunology*. 2629–2634.

Katzer, F., Canton, G., Burrells, A., Palarea-Albaladejo, J., Horton, B., Bartley, P.M., Pang, Y., Chianini, F., Innes, E.A. & Benavides, J. (2014) Immunization of lambs with the S48 strain of *Toxoplasma gondii* reduces tissue cyst burden following oral challenge with a complete strain of the parasite. *Veterinary Parasitology*. 46–56.

Khan, A., C., J., Muccioli, C. & Vallochi, A.L., Rizzo, L.V., Belfort Jr, R., Vitor, R.W., Silveira, C. and Sibley, L.D. (2006a) Genetic divergence of *Toxoplasma gondii* strains associated with ocular toxoplasmosis, Brazil. *Emerg Infect Dis*. 942–949.

Khan, A., Taylor, S., Su, C., Mackey, A.J., Boyle, J., Cole, R., Glover, D., Tang, K., Paulsen, I.T., Berriman, M., Boothroyd, J.C., Pfefferkorn, E.R., Dubey, J.P., Ajioka, J.W., Roos, D.S., Wootton, J.C. & Sibley, L.D. (2005) Composite genome map and recombination parameters derived from three archetypal lineages of *Toxoplasma gondii*. *Nucleic Acids Research*. 2980–2992.

Khan, I.A., Ely, K.H. & Kasper, and L.H. (1991) A purified parasite antigen (p30) mediates CD8 + T cell immunity against fatal *Toxoplasma gondii* infection in mice . *The Journal of Immunology*. 3501–3506.

Khan, I.A., Smith, K.A. & Kasper, L.H. (1988) Induction of antigen-specific parasiticidal cytotoxic T cell splenocytes by a major membrane protein (P30) of Toxoplasma gondii. *The Journal of Immunology*. 3600 LP – 3605.

Khan, I.A., Thomas, S.Y., Moretto, M.M., Lee, F.S., Islam, S.A., Combe, C., Schwartzman, J.D. & Luster, A.D. (2006b) CCR5 is essential for NK cell trafficking and host survival following *Toxoplasma gondii* infection. *PLoS Pathogens*. 0484–0500.

Kianmehr, A., Mahrooz, A., Oladnabi, M., Safdari, Y., Ansari, J., Veisi, K., Evazalipour, M., Shahbazmohammadi, H. & Omidinia, E. (2016) Purification and Characterization of Recombinant Darbepoetin Alfa from *Leishmania tarentolae*. *Molecular Biotechnology*. 566–572.

Kim, K. (2006) Time to screen for congenital toxoplasmosis? *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*. 1395–1397.

Korn, T., Bettelli, E., Gao, W., Awasthi, A., Jäger, A., Strom, T.B., Oukka, M. & Kuchroo, V.K. (2007) IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. *Nature*. 484–487.

Kovtun, O., Mureev, S., Jung, W., Kubala, M.H., Johnston, W. & Alexandrov, K. (2011) Leishmania cell-free protein expression system. *Methods*. 58–64.

Krahenbuhl, J.L., Ruskin, J. & Remington, J.S. (1972) The Use of Killed Vaccines in Immunization Against an Intracellular Parasite: *Toxoplasma gondii*l. *The jounal of Immnology*.

Laboudi, M. & Sadak, A. (2017) Serodiagnosis of Toxoplasmosis: The effect of measurement of IgG avidity in pregnant women in Rabat in Morocco. *Acta Tropica*. 139–142.

Laguia-Becher, M., Martin, V., Kraemer, M., Corigliano, M., Yacono, M.L., Goldman, A. & Clemente, M. (2010) Effect of codon optimization and subcellular targeting on *Toxoplasma gondii* antigen SAG1 expression in tobacco leaves to use in subcutaneous and oral immunization in mice. *BMC Biotechnol*. 52.

Lambert, H., Vutova, P.P., Adams, W.C., Loré, K. and & Barragan, A. (2009) The *Toxoplasma gondii*-shuttling function of dendritic cells is linked to the parasite genotype. *Infection and immunity*. 1679–1688.

Lee, S. & Nguyen, M.T. (2015) Recent Advances of Vaccine Adjuvants for Infectious Diseases. *Immune Network*. 51.

Lekutis, C., Ferguson, D.J.P., Grigg, M.E., Camps, M. & Boothroyd, J.C. (2001) Surface antigens of *Toxoplasma gondi*: variations on a theme. *International Journal for Parasitology*. 1285–1292.

Letscher-bru, V., Pfaff, A.W., Abou-bacar, A., Filisetti, D., Antoni, E., Villard, O., Klein, J. & Candolfi, E. (2003) Vaccination with *Toxoplasma gondii* SAG-1 Protein Is Protective against Congenital Toxoplasmosis in BALB / c Mice but Not in CBA / J Mice. *Society*. 6615–6619.

Lihtech, W.M.. (2005) Toxoplasmosis. *eMedicine.Specialties,Opthalmoloy, Infectious Disease.*

Lindberg, R.E. & Frenkel, J.K. (1977) Toxoplasmosis in nude mice. *The Journal of parasitology*. 219-221.

Liu, M.A. (2011) DNA vaccines: an historical perspective and view to the future. *Immunological Reviews*. 62–84.

Liu, S., Shi, L., Cheng, Y. & Fan, G. (2009) Evaluation of protective effect of multi-epitope DNA vaccine encoding six antigen segments of *Toxoplasma gondii* in mice. *Parasitol Res*. 267–274.

Lodish, H., Berk, A., Zipursky, S.L., Matsudaira, P. & Baltimore, D. (2000) *Molecular Cell Biology*. 4th editio. W. H. Freeman (ed.). New York.

Lorenzi, H., Khan, A., Behnke, M.S., et al. (2016) Local admixture of amplified and diversified

secreted pathogenesis determinants shapes mosaic *Toxoplasma gondii* genomes. *Nature Communications*. p.10147.

Luangsay, S., Kasper, L.H., Rachinel, N., Minns, L.A., Mennechet, F.J.., Vandewalle, A. & Buzoni—Gatel, D. (2003) CCR5 mediates specific migration of *Toxoplasma gondii* primed CD8+ lymphocytes to inflammatory intestinal epithelial cells. *Gastroenterology*. 491–500.

Lunden, A., Lovgren, K., Uggla, A. & Araujo, F.G. (1993) Immune responses and resistance to *Toxoplasma gondii* in mice immunized with antigens of the parasite incorporated into immunostimulating complexes. *Infection and Immunity*. 2639–2643.

Lütjen, S., Soltek, S., Virna, S., Deckert, M. & Schlüter, D. (2006) Organ- and disease-stage-specific regulation of *Toxoplasma gondii*-specific CD8-T-cell responses by CD4 T cells. *Infection and Immunity*. 5790–5801.

Lyons, R.E., McLeod, R. & Roberts, C.W. (2002) *Toxoplasma gondii* tachyzoite-bradyzoite interconversion. *Trends in Parasitology*. 198–201.

MacKenzie, C.R., Spekker, K., Schmidt, S.K., Däubener, W. & Heseler, K. (2008) Antimicrobial and immunoregulatory effects mediated by human lung cells: role of IFN-γ-induced tryptophan degradation. *FEMS Immunology & Medical Microbiology*. 273–281.

Magram, J., Connaughton, S.E., Warrier, R.R., Carvajal, D.M., Wu, C., Ferrante, J., Stewart, C., Sarmiento, U., Faherty, D.A. & Gately, M.K. (1996) IL-12-Deficient Mice Are Defective in IFNγ Production and Type 1 Cytokine Responses. *Immunity*. 471–481.

Marques, R.E., Guabiraba, R., Russo, R.C. & Teixeira, M.M. (2013) Targeting CCL5 in inflammation. *Expert Opinion on Therapeutic Targets*. 1439–1460.

Martins-Duarte, É.S., Carias, M., Vommaro, R., Surolia, N. & de Souza, W. (2016) Apicoplast fatty acid synthesis is essential for pellicle formation at the end of cytokinesis in *Toxoplasma gondii*. *Journal of Cell Science*. 3320–3331.

Masek, K.S. and Hunter, C.. (2013) Pro-Inflammatory Responses in Macrophages during *Toxoplasma gondii* Infection. *In Madame Curie Bioscience Database [Internet]. Landes Bioscience.*

Mattanovich, D., Branduardi, P., Dato, L., Gasser, B., Sauer, M. & and Porro, D. (2012) *Recombinant protein production in yeasts. In Recombinant gene expression*. Humana Press, Totowa, NJ.

McCluskie, M.J., Weeratna, R.D. & Davis, H.L. (2000) The role of CpG in DNA vaccines. *Springer Seminars in Immunopathology*. 125–132.

McFadden, G.I. (2011) The apicoplast. *Protoplasma*. 641–650.

McLeod, R., Frenkel, J.K., Estes, R.G., Mack, D.G., Eisenhauer, P.B. & and Gibori, G. (1988) Subcutaneous and intestinal vaccination with tachyzoites of *Toxoplasma gondii* and acquisition of immunity to peroral and congenital toxoplasma challenge. *The Journal of Immunology*. 1632–1637.

Mendes, É.A., Fonseca, F.G., Casério, B.M., Colina, J.P., Gazzinelli, R.T. & Caetano, B.C. (2013) Recombinant Vaccines against *T. gondi*i: Comparison between Homologous and Heterologous Vaccination Protocols Using Two Viral Vectors Expressing SAG1. *PLoS ONE*. p.e63201.

Mercier, C., Dubremetz, J.F., Rauscher, B., Lecordier, L., Sibley, L.D. & Cesbron-Delauw, M.F. (2002) Biogenesis of Nanotubular Network in Toxoplasma Parasitophorous Vacuole Induced by Parasite Proteins. *Molecular biology of the cell,*. 2397–2409.

Mévélec, M.N., Bout, D., Desolme, B., Marchand, H., Magné, R., Bruneel, O. & Buzoni-Gatel, D. (2005) Evaluation of protective effect of DNA vaccination with genes encoding antigens GRA4 and SAG1 associated with GM-CSF plasmid, against acute, chronical and congenital toxoplasmosis in mice. *Vaccine*. 4489–4499.

Mineo, J.R., McLeod, R., Mack, D., Smith, J., Khan, I.A., Ely, K.H. & Kasper, L.H. (1993) Antibodies to *Toxoplasma gondii* major surface protein (SAG-1, P30) inhibit infection of host cells and are produced in murine intestine after peroral infection. *The Journal of Immunology*. 3951 LP – 3964.

Minot, S., Melo, M.B., Li, F., Lu, D., Niedelman, W., Levine, S.S. & Saeij, J.P.J. (2012) Admixture and recombination among *Toxoplasma gondii* lineages explain global genome diversity. *Proceedings of the National Academy of Sciences*. 13458–13463.

Mirzaahmadi, S., Asaadi-Tehrani, G., Bandehpour, M., Davoudi, N., Tahmasbi, L., Hosseinzadeh, N., Mirzahoseini, H., Parivar, K. & Kazemi, B. (2011) Expression of recombinant human coagulation factor VII by the lizard *Leishmania* expression system. *Journal of Biomedicine and Biotechnology*.

Mißlitz, A., Mottram, J.C., Overath, P. & Aebischer, T. (2000) Targeted integration into a rRNA locus results in uniform and high level expression of transgenes in *Leishmania* amastigotes. *Molecular and Biochemical Parasitology*. 251–261.

Mizbani, A., Taslimi, Y., Zahedifard, F., Taheri, T. & Rafati, S. (2011) Effect of A2 gene on infectivity of the nonpathogenic parasite *Leishmania tarentolae*. *Parasitology Research*. 793–799.

Mondragon, R., Howe, D.K., Dubey, J.P. & Sibley, L.D. (1998) Genotypic Analysis of *Toxoplasma gondii* Isolates from Pigs. *The Journal of Parasitology*. 639–641.

Montoya, J.G. and Liesenfeld, O. (2004) Toxoplasmosis Lancet. *CrossRef*| *PubMed*| *CAS*| *Web of Science® Times Cited, .807*. 1965–1976.

Montoya, J. & Liesenfeld, O. (2004) Toxoplasmosis. *The Lancet*. 1965–1976.

Montoya, J.G. & Remington, J.S. (2008) Clinical Practice: Management of *Toxoplasma gondii* Infection during Pregnancy. *Clinical Infectious Diseases*. 554–566.

Moretto, M.M., Hwang, S.J. & Khan, I.A. (2017) Downregulated IL-21 response and T follicular helper cell exhaustion correlate with compromised CD8 T cell immunity during chronic toxoplasmosis. *Frontiers in Immunology*. 1–12.

Moroda, M., Takamoto, M., Iwakura, Y., Nakayama, J. & Aosaia, F. (2017) Interleukin-17A-

Deficient Mice Are Highly Susceptible to *Toxoplasma gondii* Infection Due to Excessively Induced T. gondii HSP70 and Interferon Gamma Production. *american society for microbiology*. 1–13.

Morrissette, N. (2015) Targeting Toxoplasma tubules: Tubulin, microtubules, and associated proteins in a human pathogen. *Eukaryotic Cell*. 2–12.

Mosmann, T.R., Cherwinski, H., Bond, M.W., Giedlin, M.A. & Coffman, R.L. (1986) Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *The Journal of Immunology*. 2348 LP – 2357.

Mun, H.S., Aosai, F. & Yano, A. (1999) Role of Toxoplasma gondii HSP70 and Toxoplasma gondii HSP30/bag1 in antibody formation and prophylactic immunity in mice experimentally infected with *Toxoplasma gondii*. *Microbiology and Immunology*. 471–479.

Murakami, Y., Hoshi, M., Hara, A., Takemura, M., Arioka, Y., Yamamoto, Y., Matsunami, H., Funato, T., Seishima, M. & Saito, K. (2012) Inhibition of increased indoleamine 2,3-dioxygenase activity attenuates *Toxoplasma gondii* replication in the lung during acute infection. *Cytokine*. 245–251.

Murata, Y., Sugi, T., Weiss, L.M. & Kato, K. (2017) Identification of compounds that suppress *Toxoplasma gondii* tachyzoites & Bradyzoites. *PLoS ONE*. 1–14.

Nayak, A., Akpunarlieva, S., Barrett, M. & Burchmore, R. (2018) A defined medium for Leishmania culture allows definition of essential amino acids. *Experimental Parasitology*. 39–52.

Niculae, A., Bayer, P., Cirstea, I., Bergbrede, T., Pietrucha, R., Gruen, M., Breitling, R. & Alexandrov, K. (2006) Isotopic labeling of recombinant proteins expressed in the protozoan host *Leishmania tarentolae*. *Protein Expression and Purification*. 167–172.

Nie, H., Fang, R., Xiong, B.Q., Wang, L.X., Hu, M., Zhou, Y.Q. & Zhao, J.L. (2011) Immunogenicity and protective efficacy of two recombinant pseudorabies viruses expressing *Toxoplasma gondii* SAG1 and MIC3 proteins. *Veterinary Parasitology*. 215–221.

Niedelman, W., Gold, D.A., Rosowski, E.E., Sprokholt, J.K., Lim, D., Arenas, A.F., Melo, M.B., Spooner, E., Yaffe, M.B. & Saeij, J.P.J. (2012) The rhoptry proteins ROP18 and ROP5 mediate *Toxoplasma gondii* evasion of the murine, but not the human, interferon-gamma response. *PLoS Pathogens*. p.e1002784.

Nielsen, H. V., Di Cristina, M., Beghetto, E., Spadoni, A., Petersen, E. & Gargano, N. (2006) *Toxoplasma gondii*: DNA vaccination with bradyzoite antigens induces protective immunity in mice against oral infection with parasite cysts. *Experimental Parasitology*. 274–279.

Nielsen, H.V., Lauemøller, S.L., Buus, S., Fomsgaard, A. & Christiansen, L. (1999) Complete Protection against Lethal *Toxoplasma gondii* Infection in Mice Immunized with a Plasmid Encoding the Complete Protection against Lethal Toxoplasma gondii Infection in Mice Immunized with a Plasmid Encoding the SAG1 Gene. *Infection and immunity*. 6358–6363.

Nielsen, H.V., Schmidt, D.R. & Petersen, E. (2005) Diagnosis of congenital toxoplasmosis by two-dimensional immunoblot differentiation of mother and child immunoglobulin G profiles. *Journal of Clinical Microbiology*. 711–715.

Niimi, T. (2016) *Leishmania tarentolae for the Production of Multi-subunit Complexes BT - Advanced Technologies for Protein Complex Production and Characterization*. In: M Cristina Vega (ed.). Cham, Springer International Publishing. pp. 155–165.

Niimi, T. (2012) Recombinant Protein Production in the Eukaryotic Protozoan Parasite *Leishmania tarentolae*: A Review Argelia Lorence Arkansas (ed.). *Part of the Methods in Molecular Biology book series (MIMB, volume 824*. 307–315.

Ochiaia, E., Saa, Q., Perkinsa, S., Griggb, M.E. & Suzukia, Y. (2016) CD8+ T cells remove cysts of *Toxoplasma gondii* from the Parasite, mostly by recognizing epitopes commonly expressed by or cross-reactive between type II and type III strains of the parasite. *Microbes Infect.* 517–522.

Olias, P., Etheridge, R.D., Zhang, Y., Holtzman, M.J. & Sibley, L.D. (2016) *Toxoplasma* Effector Recruits the Mi-2/NuRD Complex to Repress STAT1 Transcription and Block IFN-γ-Dependent Gene Expression. *Cell Host & Microbe*. 72–82.

Ong, Y.C., Reese, M.L. & Boothroyd, J.C. (2010) Toxoplasma rhoptry protein 16 (ROP16) subverts host function by direct tyrosine phosphorylation of STAT6. *Journal of Biological Chemistry*. 28731–28740.

Pappas, G., Roussos, N. & Falagas, M.E. (2009) Toxoplasmosis snapshots: Global status of *Toxoplasma gondii* seroprevalence and implications for pregnancy and congenital toxoplasmosis. *International Journal for Parasitology*. 1385–1394.

Parasite, P. (2012) *Recombinant Gene Expression*. 307–315.

Parker, S.J., Roberts, C.W. & Alexander, J. (1991) CD8+ T cells are the major lymphocyte subpopulation involved in the protective immune response to *Toxoplasma gondii* in mice. *Clinical and experimental immunology*. 207–212.

De Paschale, M., Agrappi, C., Clerici, P., Mirri, P., Manco, M.T., Cavallari, S. & Viganò, E.F. (2008) Seroprevalence and incidence of *Toxoplasma gondii* infection in the Legnano area of Italy. *Clinical Microbiology and Infection*. 186–189.

Perkins, E.S. (1961) Uveitis and Toxoplasmosis. *Uveitis and Toxoplasmosis*.

Petersen, E. (1998) Immunization with E. coli produced recombinant T. gondii SAG1 with alum as adjuvant protect mice against lethal infection with *Toxoplasma gondi*i. *Vaccine*. 1283–1289.

Petersen, E. (2007) Toxoplasmosis. *Seminars in Fetal and Neonatal Medicine*. 214–223.

Pfefferkorn, E.R. (1984) Interferon gamma blocks the growth of *Toxoplasma gondii* in human fibroblasts by inducing the host cells to degrade tryptophan. *Proceedings of the National Academy of Sciences*. 908–912.

Phan, H.P., Sugino, M. & Niimi, T. (2009) The production of recombinant human laminin-332 in a *Leishmania tarentolae* expression system. *Protein Expression and Purification*.

79–84.

Pinzan, C.F., Sardinha-Silva, A., Almeida, F., Lai, L., Lopes, C.D., Lourenço, E.V., Panunto-Castelo, A., Matthews, S. & Roque-Barreira, M.C. (2015) Vaccination with recombinant microneme proteins confers protection against experimental toxoplasmosis in mice. *PLoS ONE*. 1–18.

Rabaud, C., May, T., Amiel, C., Katlama, C., Leport, C., Ambroise-Thomas, P. and Canton, P. (1994) Extracerebral Toxoplasmosis in Patients Infected with HIV A French National Survey. *Medicine*. 306–314.

Raymond, F. de´ ric, Boisvert, S. bastien, Roy, G. tan, Ritt, J.-F. ois, Gare´, D.L., Isnard, A., Stanke, M., Olivier, M., Tremblay, M.J., Papadopoulou, B., Ouellette, M. & Corbeil, J. (2012) *Genome sequencing of the lizard parasite Leishmania tarentolae reveals loss of genes associated to the intracellular stage of human pathogenic species*. 1131–1147.

Reese, T.A., Bi, K., Kambal, A., Filali-Mouhim, A., Beura, L.K., Bürger, M.C., Pulendran, B., Sekaly, R.-P., Jameson, S.C., Masopust, D., Haining, W.N. & Virgin, H.W. (2016) Sequential Infection with Common Pathogens Promotes Human-like Immune Gene Expression and Altered Vaccine Response. *Cell Host & Microbe*. 713–719.

Reinhardt, R.L., Liang, H.-E. & Locksley, R.M. (2009) Cytokine-secreting follicular T cells shape the antibody repertoire. *Nature immunology*. 385–393.

Rezaei, F., Sarvi, S., Sharif, M., Hejazi, S.H., Pagheh, A. sattar, Aghayan, S.A. & Daryani, A. (2019) A systematic review of *Toxoplasma gondii* antigens to find the best vaccine candidates for immunization. *Microbial Pathogenesis*. 172–184.

Robben, P.M., Mordue, D.G., Truscott, S.M., Takeda, K., Akira, S. & and Sibley, L.D. (2004) Production of IL-12 by macrophages infected with *Toxoplasma gondii* depends on the parasite genotype. *The Journal of Immunology*. 3686–3694.

Roberts, C.W. & Alexander, J. (1992) Studies on a murine model of congenital toxoplasmosis: Vertical disease transmission only occurs in BALB/c mice infected for the first time during pregnancy. *Parasitology*. 19–23.

Roberts, C.W., Ferguson, D.J.P., Jebbari, H., Satoskar, A., Bluethmann, H. & Alexander, J. (1996) Different roles for interleukin-4 during the course of Toxoplasma gondii infection. *Infection and Immunity*. 897–904.

Roberts, C.W., McLeody, R., Henriquez, F.L. & Alexander, J. (2004) Vaccination against Toxoplasmosis: Current Status and Future Prospects. In: K. Kim & L.M. Weiss (eds.). *Toxoplasma gondii: the model apicomplexan. Perspectives and methods.* 2nd editio. Elsevier. pp. 996–1031.

Rosano, G.L. & Ceccarelli, E.A. (2014) Recombinant protein expression in Escherichia coli: Advances and challenges. *Frontiers in Microbiology*. 1–17.

Sabin, A.B. (1942) Toxoplasmosis. A recently recognized disease of human beings. . *Adv. Pediatr*. 1–53.

Sadak, A., Taghy, Z., Fortier, B. and Dubremetz, J.F. (1988) Characterization of a family of rhoptry proteins of *Toxoplasma gondii*. *Molecular and biochemical parasitology*. 203–211.

Saeij, J.P.J., Arrizabalaga, G. & Boothroyd, J.C. (2005a) Bioluminescence Imaging of *Toxoplasma gondii* Infection in Living Mice Reveals Dramatic Differences between Strains. *Microbiology*. 695–702.

Saeij, J.P.J., Boyle, J.P. & Stanford, and J.C.B. (2005b) Differences among the three major strains of *Toxoplasma gondii* and their specific interactions with the infected host. *Trends in Parasitology*. 476–481.

Saeij, J.P.J., Coller, S., Boyle, J.P., Jerome, M.E., White, M.W. & Boothroyd, J.C. (2007) *Toxoplasma* co-opts host gene expression by injection of a polymorphic kinase homologue. *Nature*. 324–327.

Scanga, C.A., Aliberti, J., Jankovic, D., Tilloy, F., Bennouna, S., Denkers, E.Y., Medzhitov, R. & Sher, A. (2002) Cutting edge: MyD88 is required for resistance to *Toxoplasma gondii* infection and regulates parasite-induced IL-12 production by dendritic cells. *The Journal of Immunology*. 5997–6001.

Schaeffer, M., Han, S.-J., Chtanova, T., van Dooren, G.G., Herzmark, P., Chen, Y., Roysam, B., Striepen, B. & Robey, E.A. (2009) Dynamic Imaging of T Cell-Parasite Interactions in the Brains of Mice Chronically Infected with *Toxoplasma gondii*. *The Journal of Immunology*. 6379–6393.

Schlüter, D., Meyer, T., Strack, A., Reiter, S., Kretschmar, M., Wiestler, O.D., Hof, H. & Deckert, M. (2006) Regulation of Microglia by CD4+ and CD8+ T Cells: Selective Analysis in CD45-congenic Normal and *Toxoplasma gondii*-infected Bone Marrow Chimeras. *Brain Pathology*. 44–55.

Shirota, H. & Klinman, D.M. (2017) CpG Oligodeoxynucleotides as Adjuvants for Clinical Use. *Immunopotentiators in Modern Vaccines*. 163–198.

Shwab, E.K., Jiang, T., Pena, H.F.J., Gennari, S.M., Dubey, J.P. & Su, C. (2016) The ROP18 and ROP5 gene allele types are highly predictive of virulence in mice across globally distributed strains of *Toxoplasma gondii*. *International Journal for Parasitology*. 141–146.

Siachoque, H., Guzman, F., Burgos, J., Patarroyo, M.E. & Gomez Marin, J.E. (2006) *Toxoplasma gondii*: Immunogenicity and protection by P30 peptides in a murine model. *Experimental Parasitology*.114 (1).

Sibley, L.D., Khan, A., Ajioka, J.W. & Rosenthal, B.M. (2009) Genetic diversity of *Toxoplasma gondi*i in animals and humans. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*. 2749–2761.

Sibley, L.D., LeBlanc, A.J., Pfefferkorn, E.R. & Boothroyd, J.C. (1992) Generation of a restriction fragment length polymorphism linkage map for *Toxoplasma gondii*. *Genetics*. 1003–1015.

Snapper, C.M. & Paul, W.E. (1987) Interferong and B cell stimulatory factor-1 reciprocally

regulate immunoglobulin isotype control. *Science*. 944–947.

Sokol, C.L. & Medzhitov, R. (2010) Emerging functions of basophils in protective and allergic immune responses. *Mucosal Immunology*. 129–137.

Solá, R.J. & Griebenow, K. (2011) Glycosylation of Therapeutic Proteins: An Effective Strategy to Optimiza Efficacy. *BioDrugs*. 9–21.

Soldati, D., Dubremetz, J.F. & Lebrun, M. (2001) Microneme proteins: Structural and functional requirements to promote adhesion and invasion by the apicomplexan parasite *Toxoplasma gondii*. *International Journal for Parasitology*. 1293–1302.

Song, P., He, S., Zhou, A., Lv, G., Guo, J., Zhou, J., Han, Y., Zhou, H., Hao, Z. & Cong, H. (2017) Vaccination with toxofilin DNA in combination with an alum-monophosphoryl lipid A mixed adjuvant induces significant protective immunity against *Toxoplasma gondii*. *BMC Infectious Diseases*. 1–11.

Splendore, A. (1908) Un nuovo protozoa parassita deconigli incontrato nelle lesioni anatomiche d'une malattia che ricorda in molti punti il Kala-azar dell'uoma. *Rev Soc Sci Sao*. 109–112.

Stierhof, Y.-D., Wiese, M., Ilg, T., Overath, P., Häner, M. & Aebi, U. (1998) Structure of a filamentous phosphoglycoprotein polymer: the secreted acid phosphatase of *Leishmania mexicana*11Edited by W. Baumeister. *Journal of Molecular Biology*. 137–148.

Su, C., Khan, A., Zhou, P., Majumdar, D., Ajzenberg, D., Darde, M.-L., Zhu, X.-Q., Ajioka, J.W., Rosenthal, B.M., Dubey, J.P. & Sibley, L.D. (2012) Globally diverse *Toxoplasma gondii* isolates comprise six major clades originating from a small number of distinct ancestral lineages. *Proceedings of the National Academy of Sciences*. 5844–5849.

Su, C., SHWAB, E.K., Zhou, P., Zhu, X.-Q. & Dubey, J.P. (2010) Moving towards an integrated approach to molecular detection and identification of *Toxoplasma gondii*. *Parasitology*. 1–11.

Subauste, C.S., Ajzenberg, D. and Kijlstra, A. (2011) *Review of the series "disease of the year 2011: toxoplasmosis" pathophysiology of toxoplasmosis. Ocular immunology and inflammation,*. 297–306.

Suzuki, Y., Claflin, J., Wang, X., Lengi, A. & Kikuchi, T. (2005) Microglia and macrophages as innate producers of interferon-gamma in the brain following infection with *Toxoplasma gondii*. *International journal for parasitology*. 83–90.

Suzuki, Y., Joh, K. & Kobayashi, A. (1987) Macrophage-mediated suppression of immune responses in Toxoplasma-infected mice: III. Suppression of antibody responses to parasite itself. *Cellular Immunology*. 218–225.

Tang, X., Yin, G., Qin, M., Tao, G., Suo, J., Liu, X. & Suo, X. (2016) Transgenic Eimeria tenella as a vaccine vehicle: Expressing TgSAG1 elicits protective immunity against *Toxoplasma gondii* infections in chickens and mice. *Scientific Reports*. 1–9.

Taylor, S., Barragan, A., Su, C., Fux, B., Fentress, S.J., Tang, K., Beatty, W.L., El Hajj, H.,

Jerome, M., Behnke, M.S., White, M., Wootton, J.C. & Sibley, L.D. (2006) A secreted serine-threonine kinase determines virulence in the eukaryotic pathogen *Toxoplasma gondii*. *Science*. 1776–1780.

Teixeira, S.M. (1998) Control of gene expression in Trypanosomatidae. *Brazilian journal of medical and biological research = Revista brasileira de pesquisas médicas e biológicas / Sociedade Brasileira de Biofísica ... [et al.]*. 1503–1516.

Thiébaut, R., Leproust, S., Chêne, G. and Gilbert, R. (2007) Effectiveness of prenatal treatment for congenital toxoplasmosis: a meta-analysis of individual patients' data. *Lancet*. 115–122.

Thomas F. Byrd & Horwitz, M.A. (1989) Interferon gamma-activated human monocytes downregulate transferrin receptors and inhibit the intracellular multiplication of Legionella pneumophila by limiting the availability of iron. *Journal of Clinical Investigation*. 1457–1465.

Vallochi, A.L., Muccioli, C., Martins, M.C., Silveira, C., Belfort, R. & and Rizzo, L.V. (2005) The genotype of *Toxoplasma gondii* strains causing ocular toxoplasmosis in humans in Brazil. *American journal of ophthalmology*. 350–351.

Varella, I.S.., ; M.B.W., Darela;, A.C.., And, L.M.N. & Muller, R.W. (2003) Prevalence de seropositivitypara toxoplasmosis gestantes. *J. Pediatr*. 69–74.

Waller, R.F., Keeling, P.J., Donald, R.G.K., Striepen, B., Handman, E., Lang-Unnasch, N., Cowman, A.F., Besra, G.S., Roos, D.S. & McFadden, G.I. (1998) Nuclear-encoded proteins target to the plastid in *Toxoplasma gondii* and Plasmodium falciparum. *Proceedings of the National Academy of Sciences*. 12352–12357.

Wang, H.-L., Zhang, T.-E., Yin, L.-T., Pang, M., Guan, L., Liu, H.-L., Zhang, J.-H., Meng, X.-L., Bai, J.-Z., Zheng, G.-P. & Yin, G.-R. (2014) Partial Protective Effect of Intranasal Immunization with Recombinant *Toxoplasma gondii* Rhoptry Protein 17 against Toxoplasmosis in Mice Mauricio M. Rodrigues (ed.). *PLoS ONE*. e108377.

Wang, P. & Heitman, J. (2005) The cyclophilins. *Genome biology*. 226.

Wang, Y. & Yin, H. (2014) Research progress on surface antigen 1 (SAG1) of *Toxoplasma gondii*. *Parasites & vectors*. 180.

Ware, C.F., Durham, A., Silver, J.S., Glatman Zaretsky, A., Siwicki, M. & Hunter, C.A. (2012) Infection with *Toxoplasma gondii* Alters Lymphotoxin Expression Associated with Changes in Splenic Architecture. *Infection and Immunity*. 3602–3610.

Weiss, L.M. & and Kim, K. (2014) *Toxoplasma gondii : the model apicomplexan.* second edi. Elsevier.

Weiss, L.M. & Dubey, J.P. (2009) Toxoplasmosis: A history of clinical observations. *International Journal for Parasitology*. 895–901.

Weiss LM, Halonen SK, . (2013) Toxoplasmosis. *Handb Clin Neurol.* 125–45.

Wiese, M., Görcke, I. & Overath, P. (1999) Expression and species-specific glycosylation of *Leishmania mexicana* secreted acid phosphatase in *Leishmania major*. *Molecular and*

Biochemical Parasitology. 325–329.

Wiese, M., Ilg, T., Lottspeich, F. & Overath, P. (1995) Ser/Thr-rich repetitive motifs as targets for phosphoglycan modifications in *Leishmania mexicana* secreted acid phosphatase. *The EMBO journal*. 1067–1074.

Wilkins, M.F., O'Connell, E. & Punga, & W.A. Te (1988) Toxoplasmosis in sheep further evaluation of the ability of a live *Toxoplasma gondii* vaccine to prevent lamb losses and reduce congenital infection following experimental oral challenge. *New Zealand Veterinary Journal*. 86–89.

Wilson, (Iain) R.J.M., Denny, P.W., Preiser, P.R., Rangachari, K., Roberts, K., Roy, A., Whyte, A., Strath, M., Moore, D.J., Moore, P.W. & Williamson, D.H. (1996) Complete Gene Map of the Plastid-like DNA of the Malaria Parasite Plasmodium falciparum. *Journal of Molecular Biology*. 155–172.

Wilson, V. & and Southern, B. (1979) Lizard *Leishmania*. In: W. and Evans D Lumsden (ed.). *Biology of Kinetoplastida*. Academic p. New York,. pp. 242–268.

Wingfield, P.T. (2016) Protein Precipitation Using Ammonium Sulfate. *Curr Protoc Protein Sci.* 1–10.

Wu, X., Lin, J., Lin, X., Chen, J., Chen, Z. & Lin, J. (2012) *Multicomponent DNA vaccine-encoding Toxoplasma gondii GRA1 and SAG1 primes : anti- Toxoplasma immune response in mice*. 2001–2009.

Xenopoulos, A. & Pattnaik, P. (2014) Production and purification of plasmid DNA vaccines: Is there scope for further innovation? *Expert Review of Vaccines*. 1537–1551.

Xiang, Z. & Ertl, H.C.J. (1995) Manipulation of the immune response to a plasmid-encoded viral antigen by coinoculation with plasmids expressing cytokines. *Immunity*. 129–135.

Yap, B.G.S. & Sher, A. (1999) Effector Cells of Both Nonhemopoietic and Hemopoietic Origin Are Required for Interferon (IFN)- /– and Tumor Necrosis Factor (TNF)- α– dependent Host Resistance to the Intracellular Pathogen, *Toxoplasma gondii*. *Journal of Experimental Medicine*. 1083–1091.

Yarovinsky, F., Andersen, J.F., King, L.R., Caspar, P., Aliberti, J., Golding, H. & Sher, A. (2004) Structural Determinants of the Anti-HIV Activity of a CCR5 Antagonist Derived from *Toxoplasma gondii*. *Journal of Biological Chemistry*. 53635–53642.

Yarovinsky, F., Zhang, D., Andersen, J.F., Bannenberg, G.L., Serhan, C.N., Hayden, M.S., Hieny, S., Sutterwala, F.S., Richard A. Flavell, 3, Ghosh, S. & Sher, A. (2005) TLR11 Activation of Dendritic Cells by a Protozoan Profilin-Like Protein. *Science*. 1626–1630.

Yin, L.T., Hao, H.X., Wang, H.L., Zhang, J.H., Meng, X.L. & Yin, G.R. (2013) Intranasal immunisation with recombinant *Toxoplasma gondi*i actin partly protects mice against toxoplasmosis. *PLoS ONE*. 1–8.

York, I.A. & Rock, K.L. (1996) Antigen processing and presentation by the class I major histocompatibility complex. *Annu. Rev. Immunol.* 369–396.

Zhang, N.-Z., Xu, Y., Wang, M. & et al (2016) Vaccination with *Toxoplasma gondii* calcium-

dependent protein kinase 6 and rhoptry protein 18 encapsulated in poly(lactide-co-glycolide) microspheres induces long-term protective immunity in mice. *BMC Infectious Diseases*. 1–11.

Zhang, N.Z., Wang, M., Xu, Y., Petersen, E. & Zhu, X.Q. (2015) Recent advances in developing vaccines against *Toxoplasma gondii*: An update. *Expert Review of Vaccines*. 1609–1621.

Zhao, Y., Huang, B., Huang, S., Zheng, H., Li, Y.Q., Lun, Z.R., Shen, J., Wang, Y., Kasper, L.H. & Lu, F. (2013) Evaluation of the adjuvant effect of pidotimod on the immune protection induced by UV-attenuated *Toxoplasma gondii* in mouse models. *Parasitology Research*. 3151–3160.

Zhou, J. & Wang, L. (2017) SAG4 DNA and peptide vaccination provides partial protection against *T. gondii* infection in BALB/c mice. *Frontiers in Microbiology*. 1–8.

Zhu, J., Yamane, H. & Paul, W.E. (2010) Differentiation of Effector CD4 T Cell Populations. *Annual Review of Immunology*. 445–489.

Zorgi, N.E., Costa, A., Galisteo, A.J., do Nascimento, N. & de Andrade, H.F. (2011) Humoral responses and immune protection in mice immunized with irradiated *T. gondii* tachyzoites and challenged with three genetically distinct strains of *T. gondii*. *Immunology Letters*. 187–196.