Novel Approaches to Whole Parasite Blood-Stage Malaria Vaccine Development

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Submitted in fulfilment of the requirements of the degree of
Doctor of Philosophy

February 2018
Statement of originality

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

Signature redacted

(Signed).

Aloysious Ssemaganda
Dedication

To my parents Mr. and Mrs. Muwonge who toiled to ensure that I got a good education and encouraged me to aim for greater horizons

and

To my lovely wife Julia and awesome children Hazel, Liam and Cristian

Thank you for all the love and patience
Acknowledgements

Firstly, I would like to express sincerest gratitude to Prof. Michael Good for the opportunity to work in his lab and for the continuous support, motivation as well as immense knowledge accorded to me throughout this PhD journey. I could not have asked for a better supervisor and mentor. To my associate supervisors Prof. Sue Berners-Price and Dr. Danielle Stanisic, your guidance and support especially in the development of this thesis was highly appreciated.

Special thanks go to members of the Good group especially Leanne Low for the assistance in the lab and constructive discussions that have been critical to the successful completion of my PhD. My appreciation also goes to Ashwini Giddam who was very instrumental in the initiation of the liposome vaccine studies that form the bulk of this thesis and to Emma Langshaw, thank you very much for reading my first drafts, you got me into the writing mode.

My appreciation to Griffith University for the tuition and living allowance scholarships and to National Health and Medical Research Council, Australia for generously funding the research, without which this work would not have happened.

Deep thanks to the Ugandans in Queensland community particularly, Arnold Bainomugisa, John Nsamba, Ismail Sebina and Ronald Musenze, you guys gave me a home away from home. The sports arguments, soccer outings and cooking always made me look forward to the weekend hook-ups.

To my awesome family Julia, Hazel, Liam and Cristian, being away from you for nearly four years has been the most challenging part of this journey and my life. Your love, patience, continuous encouragement and moral support were immeasurable and for that, I dedicate this work to you.
To all who contributed in one way or another, I am greatly indebted, and may God reward you abundantly.
Acknowledgement of published paper included in this thesis

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Included in this thesis is a published paper in Chapter 4 which is co-authored with other researchers. My contribution to each co-authored paper is outlined at the front of the relevant chapter. The bibliographic details published for this paper including all authors, are:

ALL PAPERS INCLUDED ARE CO-AUTHORED

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Chapter 4: Gold (I) phosphine compounds as parasite attenuating agents for malaria vaccine and drug development.

Appropriate acknowledgements of those who contributed to the research but did not qualify as authors are included in the paper.

Signed

(Signed).

Aloysious Ssemaganda

Principal Supervisor: Prof. Michael F. Good
Other publications during candidature not included in this thesis


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Prof. Michael F. Good
Conceived the studies, advised with data interpretation, reviewed all thesis chapters and overall PhD supervision.
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<th>Full Form</th>
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<tbody>
<tr>
<td>AF</td>
<td>Auranofin</td>
</tr>
<tr>
<td>AMA</td>
<td>Apical membrane antigen</td>
</tr>
<tr>
<td>APCs</td>
<td>Antigen Presenting Cells</td>
</tr>
<tr>
<td>AS01</td>
<td>Adjuvant System 01</td>
</tr>
<tr>
<td>AS02</td>
<td>Adjuvant System 02</td>
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<tr>
<td>ARC</td>
<td>Animal Resource Centre</td>
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<tr>
<td>AUC</td>
<td>Area Under the Curve</td>
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<tr>
<td>BAFF</td>
<td>B cell activating factor</td>
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<tr>
<td>BCA</td>
<td>Bicinchoninic Acid</td>
</tr>
<tr>
<td>CHMI</td>
<td>Controlled Human Malaria Infection</td>
</tr>
<tr>
<td>CPM</td>
<td>Counts Per Minute</td>
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<td>CR1</td>
<td>Complement Receptor 1</td>
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<td>DSPC</td>
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<td>ECM</td>
<td>Experimental Cerebral Malaria</td>
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<td>HTLV</td>
<td>Human T-cell Lymphotropic Virus</td>
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<td>Abbreviation</td>
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<td>-----------</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrogen Chloride</td>
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<tr>
<td>hRBCs</td>
<td>Human Red Blood Cells</td>
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<td>ICAM</td>
<td>Intercellular Adhesion Molecule</td>
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<td>LLPCs</td>
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<td>LOD</td>
<td>Limit of Detection</td>
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<tr>
<td>MBCs</td>
<td>Memory B Cells</td>
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<td>MFI</td>
<td>Median Fluorescent Intensity</td>
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<tr>
<td>MPLA</td>
<td>Monophosphoryl Lipid A</td>
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<tr>
<td>MSP</td>
<td>Merozoite Surface Protein</td>
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<tr>
<td>NADPH</td>
<td>Nicotinamide Adenine Dinucleotide Phosphate</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer</td>
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<td>NKT</td>
<td>Natural Killer T cells</td>
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<tr>
<td>NL</td>
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<td>NLR</td>
<td>NOD-like receptor</td>
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<td>PfEMP</td>
<td>\textit{Plasmodium falciparum} Erythrocyte Membrane Protein</td>
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<td>\textit{Plasmodium falciparum} Reticulocyte-binding protein Homologue 5</td>
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<td>PfTrxR</td>
<td>\textit{Plasmodium falciparum} thioredoxin reductase</td>
</tr>
<tr>
<td>pRBCs</td>
<td>Parasitized Red Blood Cells</td>
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<td>pRBC/ml</td>
<td>Parasitized Red Blood Cells per millilitre</td>
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<td>PS</td>
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<tr>
<td>QGPGAP</td>
<td>Glutamine-glycine-proline-glycine-alanine-proline</td>
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<tr>
<td>QS-21</td>
<td><em>Quillaja saponaria</em> Molina, fraction 21</td>
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<tr>
<td>RBCs</td>
<td>Red Blood Cells</td>
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<td>RESA</td>
<td>Ring-infected erythrocyte surface antigen</td>
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<tr>
<td>Rifins</td>
<td>Repetitive interspersed family</td>
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<td>RON2</td>
<td>Rhopty Neck Protein 2</td>
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<td>ROS</td>
<td>Reactive Oxygen Species</td>
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<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
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<tr>
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<td>Real-time quantitative PCR</td>
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<td>SAP1</td>
<td>Sporozoite Asparagine-rich Protein 1</td>
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<td>Severe Combined Immunodeficient</td>
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<td>SEM</td>
<td>Standard Error of the Mean</td>
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<td>Standard Operating Procedure</td>
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<td>Stevor</td>
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<td>Transmission Blocking Vaccine</td>
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<td>Thrombospondin-Related Adhesion Protein</td>
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<td>UISs</td>
<td>Upregulated Infectious Sporozoites</td>
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<td>Uganda Virus Research Institute-International AIDS Vaccine Initiative</td>
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<td>VCAM-1</td>
<td>Vascular adhesion molecule-1</td>
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Synopsis

The development of blood-stage vaccines capable of reducing severe disease remains a critical component of malaria eradication efforts. Such a vaccine will hypothetically reduce gametocyte densities which will in turn reduce transmission. An effective blood-stage vaccine will also bridge the gap between partially effective pre-erythrocytic vaccines such as RTS, S and control measures such as anti-malarial drugs and insecticide-treated bed nets that are threatened by resistance as well as inappropriate use.

Herein, novel approaches to the development of whole parasite blood-stage malaria vaccines were examined. Firstly, clinical isolates of *P. falciparum* were successfully collected from volunteers with malaria living in Uganda. These clinical isolates are valuable reagents for preliminary evaluation of malaria vaccine candidate prior to deployment for further testing in large-scale clinical trials. The cryopreserved isolates were successfully revived *in vitro*, and culture-adapted. Preliminary anti-malarial drug susceptibility characterisation revealed that these parasites were sensitive to chloroquine and Riamet as well as gold(I) phosphine compounds auranofin and [Au(d2pype)₂] Cl. However, further characterisation on a larger panel of established anti-malarial drugs will be required especially if these isolates are to be released for use in CHMI studies.

Secondly, the utility of gold(I) phosphines as potential parasite attenuating agents for blood-stage malaria vaccine and drug development was examined. It was shown that gold(I) phosphines inhibited parasite growth following short-term *in vitro* culture. Furthermore, when mice were inoculated with completely attenuated parasites, no protection was observed following challenge with a wild-type malaria infection. However, protective immunity was attained in some experiments when
mice were inoculated with partially attenuated parasites. Future studies will 
therefore be required to optimise the reproducibility of achieving partial 
attenuation in order to further develop this approach for an effective blood-stage 
malaria vaccine. *In vivo,* gold(I) phosphines were unable to inhibit parasite growth 
indicating that pharmacokinetic studies will be required to examine bioavailability 
following administration. Furthermore, these compounds need to be better 
designed to more efficiently target and selectively inhibit the parasite *in vivo.*
The ultimate test of an effective malaria vaccine is the ability to protect against 
infection from multiple parasite species/strains. Therefore, a heterologous model 
was developed to assess the immunogenicity and protective efficacy of liposomes 
formulated with a mannose lipid core peptide, F3, (also referred to as mannosylated 
hereonin) containing human parasite, *P. falciparum,* antigens in a mouse model. To 
address one of the challenges to the development of whole parasite blood-stage 
vaccines, the induction of anti-red blood cell antibodies, it was shown that using a 
novel immunomagnetic method, red cell membranes were efficiently depleted from 
parasite antigen. When mice were immunised with liposomes containing red cell 
membrane-depleted parasite antigen, induction of antibodies against the surface of 
the red blood cell was significantly reduced compared with control mice that 
received injections of intact normal red blood cells. Furthermore, it was shown that 
depletion of red cell membranes from parasite antigen did not affect the 
immunogenicity of liposome formulations. These data strongly support the use of 
this method to minimize the induction of anti-red blood cell antibodies in vaccinees. 
To address storage and cold-chain maintenance limitations of vaccine products 
especially in malaria endemic regions, liposomes were lyophilized. Immunological 
assessments showed that lyophilization did not affect the induction of humoral immune responses but altered the cytokine response to a predominantly Th1
response compared to a balanced Th1/Th2 response observed following immunisation with freshly prepared liposomes. Therefore, optimisation of freeze-drying protocols will be required in order to achieve optimal cytokine responses consistently following immunisation with lyophilized liposomes.

Vaccine efficacy studies revealed that freshly prepared mannosylated liposomes containing *P. falciparum* antigens, in some experiments, induced strong species-transcending protective immunity following challenge, with no direct correlate of immune protection. Future studies will thus be required to optimise formulations to attain reproducible protective efficacy.

A whole parasite multi-stage liposome-based vaccine was then formulated with different combinations of F3, PHAD (a synthetic analogue of monophosphoryl Lipid A (MPLA), the circumsporozoite repeat peptide derived from the rodent parasite, *P. yoelii* (QGPGAP)$_4$ and blood-stage Py17x antigens. Immunisation of mice resulted in low (QGPGAP)$_4$-specific antibody titres and potent parasite-specific antibody responses which were associated with liposomes containing PHAD. Additionally, the parasite–specific antibody responses strongly correlated with protection against a homologous wild-type challenge infection.

Collectively, the data presented in this thesis lays the foundation for future studies on the use of gold(I) phosphines as parasite attenuating agents as well as anti-malarial drugs. Additionally, these studies advance our understanding of the immune responses elicited following immunisation with liposomes containing inactivated whole parasite antigens and inform the design of future vaccine formulations suitable for human use. Importantly, the data strongly support transition of whole parasite liposome-based vaccines to human trials.
Chapter 1: Introduction and literature review
1.0 Introduction

Malaria, a ruinous infectious disease caused by *Apicomplexan* parasites of the genus *Plasmodium*, remains a global health problem with transmission still being reported in over 100 countries (World Health Organisation, 2017). The populations most at risk are children under the age of five in Sub-Saharan Africa with the highest mortality and morbidity being reported in this age group and region (World Health Organisation, 2017). Malaria is generally a curable and preventable disease with currently available anti-malarial drugs as well as insecticide-treated mosquito nets (ITNs) and indoor residual spraying (IRS). These control measures are, however, faced with drug and insecticide-resistance as well as inappropriate use indicating an urgent need for the improvement and development of new control measures (Alonso & Tanner, 2013).

The best hope for ending malaria globally, particularly in endemic areas, is through the development and administration of a safe, affordable and effective vaccine. Over the years, several approaches to malaria vaccine design have been considered, with limited success (Hoffman et al., 2002; Roestenberg et al., 2009; J. Cohen, Nussenzweig, Nussenzweig, Vekemans, & Leach, 2010). The most advanced malaria vaccine to date is the sub-unit vaccine, RTS, S, which has received a positive scientific opinion from European Medicines Agency (EMA) and World Health Organisation (WHO) and has been licenced for use in a WHO pilot implementation program in 3 African countries (World Health Organization, 2017). However, this vaccine has shown partial efficacy in phase III trials and the immune response elicited is short-lived (Olotu et al., 2013; RTS S Clinical Trials Partnership, 2015). Sub-unit vaccines in general, have demonstrated poor efficacy mainly due to antigenic polymorphism and failure to maintain long-lived immune responses. In addition, antigens used in
sub-unit vaccines may not be conserved across species or strains and therefore may not provide cross-strain/species immunity.

The whole organism approach is a fast re-emerging alternative and is believed to address some of the challenges associated with sub-unit vaccines (Good, 2011). Recently, vaccination with irradiated sporozoites (PfSPZ) (Seder et al., 2013; Epstein et al., 2017; Ishizuka et al., 2016; Lyke et al., 2017; Sissoko et al., 2017) and infection with *P. falciparum* sporozoites with chemoprophylaxis (PfSPZ-cVac) (Mordmuller et al., 2017) has demonstrated tremendous promise in controlled human malaria infection studies. In addition, immunisation of mice with chemically attenuated blood-stage parasites was shown to elicit potent cross-species immune responses and protected mice against a wild-type challenge infection (Good et al., 2013; Raja et al., 2016). Such studies demonstrate the feasibility of using the whole organism approach availing a novel avenue for vaccine development to expand the pipeline of vaccine products in the bid to meet the goal of a licensed malaria vaccine by 2030 (Malaria Vaccine Funders Group, 2013).

### 1.1 Malaria epidemiology

Annually, about half of the world’s population is at risk of malaria and transmission continues to occur in over 100 countries (World Health Organisation, 2017). In 2016, approximately 216 million cases were reported compared to 237 million cases in 2010 with the 90% of the cases being reported in Africa (World Health Organisation, 2017).

*Plasmodium falciparum* remains the most prevalent parasite in Africa, accounting for 99% of cases reported while *P. vivax* is predominant in South America, South-East Asia and Eastern Mediterranean regions representing 64%, 30% and 40% of malaria cases reported (World Health Organisation, 2017). Between 2010 and
2016, there was a significant decline in the global incidence rate of malaria from 76 to 63 cases per 1000 population at risk. South-East Asia recorded the highest decline in malaria incidence (48%) followed by South America (22%) and Sub-Saharan Africa (20%) (World Health Organisation, 2017).

An estimated 445,000 deaths occurred in 2016, down from 584,000 deaths reported in 2013, with Africa bearing the biggest brunt - approximately 91% (World Health Organisation, 2014, 2017). The reduction in malaria incidence and deaths over the years has been attributed to improved surveillance systems with more countries reporting less than 10,000 cases per year and steadily progressing towards complete elimination (World Health Organisation, 2017). Additionally, there has been an increased uptake of prevention strategies such as IRS and ITNs which increased to 80% in 2016 from 50% in 2010 in Sub-Saharan Africa (World Health Organisation, 2017).

However, there are some challenges to the complete eradication of malaria which include multi-drug resistance reported in South-East Asia as well as resistance to pyrethroids, insecticides used in ITNs, which increased from 71% in 2010 to 81% in 2016 (World Health Organisation, 2017). The other challenge is the emergence of *P. falciparum* histidine-rich protein 2 gene (PfHRP2) deletions which contributes to false negative test results when HRP2-based rapid diagnostic tests (RDTs) are used for malaria diagnosis. These deletions have been extensively reported in South America (Gamboa et al., 2010; Abdallah et al., 2015; Akinyi Okoth et al., 2015) but are less frequent in high transmission areas of Africa. However, further monitoring is required (Koita et al., 2012; World Health Organisation, 2017). These challenges and alarming statistics underscore the need for new improved treatment and control interventions as well as development of a safe and effective vaccine for complete eradication of this devastating disease.
1.2 Life-cycle of *Plasmodium* species

Anopheline mosquitoes transmit *Plasmodium*, the causative agent of malaria, as they partake a blood meal from a vertebrate host. The malaria parasite is an obligate intracellular organism, meaning it is unable to survive outside its host. Therefore, in order to complete its life-cycle, the parasite needs a definitive host, in this case the *Anopheles* mosquito, and an intermediate vertebrate host for the sexual and asexual reproductive stages, respectively. Over 150 species of *Plasmodium* have been identified in mammals, reptiles and birds (Sinden & Gilles, 2002; Centers for Disease Control and Prevention, 2013). The life-cycle of *Plasmodium* parasites can be broadly divided into 3 stages: the mosquito stage which occurs in the vector; the pre-erythrocytic stage which occurs in the vertebrate host’s liver; and the erythrocytic stage which occurs in the blood of the vertebrate host.

1.2.1 Mosquito stage

The life-cycle begins in the midgut of the Anopheline mosquito, where fertilization of sexual gametes results in the formation of a zygote, which matures within 24 hours into a motile ookinete. The ookinete migrates across the midgut, developing into an oocyst at the basal membrane of the midgut epithelium. The oocyst differentiates into thousands of sporozoites within 7 to 15 days, depending on the species of *Plasmodium*. The sporozoites then migrate to the acinar cells of the mosquitoes’ salivary glands ready for inoculation into a vertebrate host (Fig. 1.1) (Fujioka & Aikawa, 1999; Crompton et al., 2014).

1.2.2 Pre-erythrocytic stage

The pre-erythrocytic stage starts when sporozoites from the mosquitoes’ salivary glands are inoculated into the skin of the host and traverse the skin capillaries before migrating to the liver (Fig. 1.1). In the liver, the sporozoites invade
hepatocytes in which they develop into hepatic schizonts within 5-15 days, depending on the infecting species. The hepatic schizonts subsequently differentiate into merozoites, which are released into the blood circulation following schizont rupture through the budding of merosomes, then invade other red blood cells (RBCs) (Fig. 1.1) (Crompton et al., 2014). However, some *Plasmodium* species such as *P. vivax* and *P. ovale*, may also develop into hypnozoites which cause a latent infection in the liver for weeks or even years prior to relapsing into an active blood-stage infection (Fujioka & Aikawa, 1999).

1.2.3 Erythrocytic stage

The erythrocytic stage is initiated when merozoites, released from the liver, traverse the blood stream and invade erythrocytes (Fig. 1.1). At this stage, the parasite undergoes erythrocytic schizogony, in which the parasite matures from its’ early trophozoite (ring) form through to the late trophozoite stage and eventually into a schizont. The parasite modifies the host erythrocyte for its survival, resulting in the rupture and release of mature merozoites, which can then invade more red blood cells. This process occurs every 48 (for tertian parasites) to 72 (for quartan parasites) hours depending on the *Plasmodium* species (Fujioka & Aikawa, 1999; Crompton et al., 2014). It’s during this stage that the clinical symptoms of malaria manifest. Clinical symptoms of uncomplicated malaria include fevers, chills, sweating, headaches, nausea, body aches and general body weakness. However, if left untreated, uncomplicated malaria can rapidly develop into severe malaria leading to multiple complications such as severe anaemia, acute respiratory distress, seizures, renal failure, coma and eventually death (Anstey & Price, 2007). During the erythrocytic stage, some merozoites undergo gametocytogony, following invasion of the erythrocytes, differentiating into male and female gametocytes; a
process that takes 4 to 10 days. The mosquito ingests these gametocytes during a blood meal and this continues the parasite life-cycle (Fig. 1.1) (Fujioka & Aikawa, 1999).

Figure 1.1. Life-cycle of the malaria parasite *Plasmodium falciparum* (Sauerwein, Roestenberg, & Moorthy, 2011). Reproduced with permission from Springer Nature © 2011.

1.3 Human *Plasmodium* parasites

To date, there are 6 species of *Plasmodium* with the capacity to infect humans: *P. falciparum*, *P. vivax*, *P. ovale curtisi*, *P. ovale wallikeri*, *P. malariae* and *P. knowlesi*.

1.3.1 *Plasmodium falciparum*

*Plasmodium falciparum*, the most pathogenic of all human *Plasmodium* species, is the main cause of morbidity and mortality worldwide (Centers for Disease Control
and Prevention, 2012). It is mainly found in tropical and sub-tropical regions as development in the mosquito host is affected by temperature (Sinden & Gilles, 2002). The parasite has a synchronous 48-hour erythrocytic cycle with the ability to cause chronic infections. *P. falciparum* has the ability to adhere to and sequester in blood vessels causing severe disease such as cerebral and placental malaria, which are often fatal (Sinden & Gilles, 2002; Centers for Disease Control and Prevention, 2012).

### 1.3.2 *Plasmodium vivax*

*Plasmodium vivax* is found mainly in the temperate regions of Asia, Latin America and certain parts of Africa and is the second most pathogenic human malaria parasite (Mueller et al., 2009). Its minimal distribution in Africa was long believed to be due to lack of the duffy antigen receptor on RBCs, an important receptor required for erythrocyte invasion by *P. vivax*. Recent data however, suggests *P. vivax* is also able to infect individuals that do not express the duffy antigen receptor on their RBCs (Mendes et al., 2011). *P. vivax* forms hypnozoites in the liver, which can be reactivated weeks or months after the initial infection, resulting in blood-stage infection. Activation of latent hypnozoites has been associated with concurrent systemic parasitic or bacterial infections (Shanks & White, 2013). *P. vivax* undergoes a 48-hour erythrocytic cycle and its merozoites have been shown to preferentially invade reticulocytes (Kitchen, 1938; Sinden & Gilles, 2002).

### 1.3.3 *Plasmodium ovale*

*Plasmodium ovale* exists as 2 distinct species, *P. ovale curtisi* and *P. ovale wallikeri* (Sutherland et al., 2010). *Plasmodium ovale* is prevalent in tropical Africa, specifically West Africa, and has also been found in the islands of the Western Pacific (Sinden & Gilles, 2002). Similar to *P. vivax*, *P. ovale* manifests a tertian erythrocytic
cycle and has latency periods of up to 4 years (Collins & Jeffery, 2005). *Plasmodium ovale* infections are characterised by low parasitaemia, hence the difficulty with its diagnosis. The parasite is often found in mixed infections with other *Plasmodium* species (Sinden & Gilles, 2002; Centers for Disease Control and Prevention, 2012).

### 1.3.4 *Plasmodium malariae*

*Plasmodium malariae* is a globally distributed parasite found in both tropical and sub-tropical regions of the world, particularly in Africa, Guyana and parts of India (Sinden & Gilles, 2002). This parasite undergoes a 72-hour erythrocytic cycle and exhibits slow growth in both the human and mosquito host. If left untreated, *P. malariae* establishes a chronic infection, which can last for several years in the host and can cause kidney disorders such as proteinuria, hypoalbuminemia and oedema (Sinden & Gilles, 2002; Collins & Jeffery, 2007; Centers for Disease Control and Prevention, 2012).

### 1.3.5 *Plasmodium knowlesi*

*Plasmodium knowlesi*, whose intermediate host is known to be macaque monkeys, has also been reported to cause malaria in humans, especially in South-East Asia (Centers for Disease Control and Prevention, 2012). This parasite causes severe fatal disease and can easily be misdiagnosed as *P. falciparum* or the less virulent *P. malariae* by conventional microscopy, given the similarities of the blood-stage schizonts and early trophozoites, respectively (Kantele & Jokiranta, 2011). It undergoes a 24-hour erythrocytic cycle, hence the rapid progression from uncomplicated to severe disease, which can be fatal if left untreated. The transmission dynamics of *P. knowlesi* are yet to be established (Contacos et al., 1968; Chin et al., 2011; Centers for Disease Control and Prevention, 2012).
1.4 Murine models of malaria

Murine malaria parasites have been widely utilised to study several areas of malariology (Stephens et al., 2012). To date, 4 murine Plasmodium species are available for use in modelling the different aspects of human malaria parasite infection and they are: P. chabaudi, P. berghei, P. yoelii and P. vinckei.

1.4.1 Plasmodium chabaudi

Plasmodium chabaudi is one of the most widely used rodent parasites to model and investigate the pathogenesis and immunological mechanisms of human malaria infection (Stephens et al., 2012). It is composed of well characterised strains, P. chabaudi (clones AS and CB) and P. chabaudi adami, which have a synchronous erythrocytic cycle and exhibit different levels of virulence in the host and varying levels of sequestration and transmissibility to mosquitoes (Gilks et al., 1990; Bell et al., 2006; Gadsby et al., 2009; Stephens et al., 2012). The P. chabaudi AS clone causes a non-lethal infection in BALB/c, C57BL/6, CBA/Ca mice and a lethal infection in A/J and DBA/2J mouse strains while P. chabaudi adami manifests a mild to non-lethal infection in BALB/c and C57BL/6 mice (Sanni et al., 2002). Plasmodium chabaudi displays some marked differences compared to human parasites such as: sequestration occurring in the liver rather than the brain (hence it cannot be used as a model for cerebral malaria); the need for higher levels of parasitaemia for the manifestation of pathogenic symptoms; and the development of hypothermia rather than the fever that is observed in human malaria infections (Stephens et al., 2012). Plasmodium chabaudi adheres to the placenta and hence can be used as a rodent model for placental malaria (Stephens et al., 2012).

1.4.2 Plasmodium berghei

Plasmodium berghei consists of the P. berghei ANKA and P. berghei K173 strains and
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has been used as an experimental model for cerebral malaria in C57BL/6 mice (Mackey et al., 1980; Basir et al., 2012). BALB/c mice have however been reported to be resistant to the development of cerebral malaria and have thus been used as controls in experiments (Dekossodo & Grau, 1993; Sanni et al., 2002). The parasite is asynchronous and infects both reticulocytes and mature RBCs (Sanni et al., 2002).

1.4.3 *Plasmodium yoelii*

*Plasmodium yoelii* consists of 3 clones - *P. yoelii* 17X non-lethal (*Py17XNL*), *P. yoelii* 17X lethal (*Py17X*) and *P. yoelii* YM. *Py17XNL* and *Py17X* have identical antigenic and genetic background but the latter is highly virulent and causes a lethal infection in mice while the former causes a self-limiting, nonlethal infection (Yoeli et al., 1975). *P. yoelii* preferentially infects reticulocytes and mature RBCs and has been widely used to test malaria vaccine candidates (Narum et al., 2000; Kawabata et al., 2002; Wykes & Good, 2009). Infection with the *P. yoelii* 17XNL clone manifests as a low to mild level infection and has been widely used in the study of immune mechanisms and pathogenesis such as experimental cerebral malaria. However, some strains of *P. yoelii* 17XNL have been reported to cause a fatal infection due to cerebral malaria in CF1 and A/J mice within 6 to 7 days post intraperitoneal inoculation (Krishna et al., 1983; Sanni et al., 2002).

1.4.4 *Plasmodium vinckei*

*Plasmodium vinckei* is comprised of *P. vinckei vinckei*, which causes a lethal infection and *P. vinckei petteri*, which causes a non-lethal infection in mice. Both parasites infect mature RBCs and have been mainly used in chemotherapeutic studies and investigations into disease pathogenesis as well as immune mechanisms (Sanni et al., 2002).
Collectively, murine models of malaria are the mainstay to understanding host-parasite genetics, genomics, immunobiology, disease pathogenesis as well as drug and vaccine development.

1.5 Immunity to malaria

1.5.1 Pre-erythrocytic stage immunity to malaria

The pre-erythrocytic stage of malaria infection is clinically quiescent, probably due to the low number of sporozoites inoculated by the mosquito during a blood meal. During this stage, studies in mice have shown that antibodies can control infection through immobilization of sporozoites thereby reducing their interaction with host cells as well as inhibiting sporozoite motility and subsequent invasion of hepatocytes (Chatterjee et al., 1995; Sultan et al., 1997). Field studies in humans have demonstrated the existence of liver-stage specific antibodies to *P. falciparum* in natural infection; however, their role remains unclear (John et al., 2003; John et al., 2005; Offeddu et al., 2012).

To date, the best model that has enabled studying mechanisms of pre-erythrocytic immunity has utilised irradiated sporozoites in both humans and animals. Radiation-attenuated sporozoites, at an appropriate dose, retain the capacity to infect hepatocytes but cannot develop into an erythrocytic infection. Studies in rodent models involving inoculation of radiation-attenuated sporozoites, have demonstrated that antibodies were involved in the enhanced clearance of sporozoites, reduced sporozoite motility and inhibition of hepatocyte invasion (Vanderberg & Frevert, 2004; Sinnis & Coppi, 2007).

Cell-mediated pre-erythrocytic immunity, following inoculation of radiation-attenuated sporozoites, has also been shown to contribute to the induction of sterilizing immunity to *Plasmodium* infection in both mice and humans. In human
volunteers, both CD4+ and CD8+ T cell responses against pre-erythrocytic antigens have been observed (Nussenzweig et al., 1967; Krzych et al., 1995; Hoffman et al., 2002; Seder et al., 2013; Ishizuka et al., 2016; Epstein et al., 2017; Lyke et al., 2017; Sissoko et al., 2017). Studies in rodent models however, indicate that a network of cellular mechanisms mediate immunity to pre-erythrocytic infection (Fig. 1.2).

Initial studies in mice immunised with irradiated Py17XL sporozoites indicated that cytotoxic CD8+ T cell mediated protection against a wild-type challenge of infectious sporozoites (Weiss et al., 1988). Further studies indicated that IL-12 from liver antigen presenting cells (APCs) (such as macrophages, dendritic cells and monocytes) stimulated CD8+ T cells and NK cells to produce interferon (IFN)-γ. IFN-γ in turn induces the infected hepatocytes to produce nitric oxide (NO), which subsequently kills the parasite in the hepatocyte (Seguin et al., 1994; Doolan & Hoffman, 1999; Sinnis & Nardin, 2002). In addition, the balance between IL-10, IL-2, IL-12 and IFN-γ cytokines results in an inflammatory response that contributes to pre-erythrocytic immunity (Hollingdale & Krzych, 2002; Steers et al., 2005).
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 Controlled human malaria infection (CHMI) studies, involving experimental sporozoite inoculation via infectious mosquito bites with chemoprophylaxis provides another model for investigating pre-erythrocytic immunity to malaria. These studies have shown that a protective effector memory T cell response to pre-erythrocytic antigens predominantly characterised by the production of IFN-γ, tumor necrosis factor (TNF), and IL-2 was induced (Roestenberg et al., 2009; Roestenberg et al., 2011; Bijker et al., 2013; Mordmuller et al., 2017). Furthermore, IgG antibodies against *P. falciparum* sporozoites, purified from plasma samples obtained from CHMI trials were shown to inhibit liver stage infection in a humanized

Figure 1.2. Mechanisms of pre-erythrocytic cell-mediated immunity in mice following inoculation of irradiated sporozoites (Doolan & Hoffman, 1999). Reproduced with permission from The American Association of Immunologists, Inc. © 1999.
liver-chimeric mouse model (Roestenberg et al., 2009; Bijker et al., 2013; Behet et al., 2014).

However, there has been no evidence of naturally acquired immunity to pre-erythrocytic malaria. This was demonstrated in earlier epidemiological studies that indicated that naturally acquired antibodies to sporozoites did not induce sterilizing immunity to malaria (Hoffman et al., 1987). Recent evidence in mice suggests that exposure to wild-type blood-stage malaria infection disrupts the development of humoral pre-erythrocytic immunity (Keitany et al., 2016) and this might explain why it is difficult to induce immunity to pre-erythrocytic antigens following vaccination in malaria endemic areas. In these studies, it was found that infection with a genetically attenuated parasite, *P. yoelii* fabb/f− (*Pyfabb/f−*), which arrests late in liver stage development and does not cause blood-stage infection, but not wild-type *P. yoelii* infection, stimulated robust Circumsporozoite Protein (CSP)-specific antibody responses (Vaughan et al., 2009; Keitany et al., 2016). Additionally, wild-type *P. yoelii* infection averted the development of germinal centre B cells which in turn altered the quality of the CSP-specific memory B cells, resulting in a weakened secondary response to a sporozoite challenge (Keitany et al., 2016). These data hence raise concerns about the efficacy of pre-erythrocytic vaccines in endemic areas where individuals are constantly exposed to blood stage infections and might explain the lack of naturally acquired pre-erythrocytic immunity (Keitany et al., 2016).

### 1.5.2 Erythrocytic stage immunity to malaria

During this stage, clinical signs and symptoms of malaria manifest as the parasites invade and replicate in RBCs (Fig. 1.1). Studies in animal models have demonstrated that the innate immune system is involved in the initial recognition
of blood-stage parasites, promotion of inflammation, inhibition of parasite growth and potentiation of the adaptive immune response (Stevenson & Riley, 2004; Crompton et al., 2014). Several innate immune cells such as dendritic cells (DCs), macrophages, mast cells, neutrophils, NK cells, natural killer T (NKT) cells, and γδ T cells have been implicated in this initial immune response (Fig. 1.3) (Urban et al., 2005; Hansen et al., 2007; Porcherie et al., 2011; Stevenson et al., 2011). During this phase of the immune response, innate immune cells initiate the inflammatory cascade through the binding of pattern recognition receptors (PRRs) on their surface to pathogen-associated molecular patterns (PAMPs) on parasitized red blood cells (pRBCs) or to parasite products in the blood stream, such as hemozoin, following RBC rupture (Fig. 1.3). This interaction of PAMPs and PRRs results in the production of pro-inflammatory cytokines by the APCs such as, IL-12 (p70), IFN-γ and TNF as well as regulatory cytokines IL-10 and TGF-β that have been implicated in immunity and pathogenesis to blood-stage malaria infection (Stevenson et al., 2011; Crompton et al., 2014).
Antibodies play a role in immunity against the erythrocytic stage of malaria in humans, as it has been shown that passive transfer of immunoglobulin from immune donors resulted in reduction of parasitaemia and clinical disease among semi-immune patients from East Africa as well as non-immune Thai patients (Cohen et al., 1961; McGregor et al., 1963; Sabchareon et al., 1991). Antibodies may function by inhibiting merozoite invasion of RBCs (Wahlin et al., 1984), binding to pRBCs and enhancing clearance by the spleen (Udeinya et al., 1981; Treutiger et al., 1992), as well as opsonizing pRBCs, resulting in phagocytosis by macrophages (Bouharoun-Tayoun et al., 1995; Perlmann & Troye-Blomberg, 2002).
Cell-mediated immunity against the erythrocytic stage is primarily mediated by CD4+ T cells, as demonstrated in both murine and human models. Studies showed that mice depleted of CD4+ T cells developed very high parasitaemia and were unable to control the infection compared to mice depleted of CD8+ T cells, which developed mild parasitaemia that subsequently resolved, indicating a clear role of CD4+ T cells (Suss et al., 1988; Podoba & Stevenson, 1991). Additionally, adoptive transfer of CD4+ T cells was shown to confer protection and control parasitaemia in immunodeficient mice (Amante & Good, 1997). Further investigation of the role of CD4+ T cells indicated that during the acute phase of infection, there was a significant upregulation of an IFN-γ-specific CD4+ T cell (Th1) response followed by an antibody mediated IL-4-specific CD4+ T cell (Th2) response during the chronic phase (Langhorne et al., 1989). These data indicate that early activation of Th1 cells is able to control the infection via effector mechanisms such as macrophages, followed by a Th2 response which activates B cells to clear the parasite in the later stages of the infection in mice (Langhorne et al., 1989; Perez-Mazliah & Langhorne, 2014).

In human studies, the role of CD4+ T cells was demonstrated when volunteers were infected with low doses of blood-stage P. falciparum followed by drug cure (Pombo et al., 2002). In this study, volunteers were strongly protected against a homologous challenge infection and immunity was mediated by an IFN-γ-specific CD4+ T cell response and nitric oxide synthase (NOS) in the absence of detectable antibodies (Pombo et al., 2002). Another study showed that stimulation of T cells obtained from children living Papua New Guinea resulted in parasite specific- IFN-γ and -TNF responses which were associated with protection against clinical episodes malaria (Robinson et al., 2009). More recently, studies in African children demonstrated that CD4+ T cells may play an important modulatory role in the development of
blood-stage immunity (Jagannathan et al., 2014; Boyle et al., 2015). In these studies, IFN-γ/IL-10+ specific-CD4+ T cell responses were observed among children heavily exposed to malaria although this response did not correlate with protection from subsequent clinical episodes indicating that CD4+ T cells may play an important immunomodulatory role in the pathogenesis childhood malaria (Jagannathan et al., 2014). Additionally, induction of IL-10-producing CD4+ T cells amongst highly exposed children may interfere with the development of immunity which may have implications for vaccine development (Boyle et al., 2015).

T follicular helper cells (Tfh) are a subset of CD4+ T cells, capable of providing B cell help as well as activating follicular B-cell responses (Breitfeld et al., 2000; Scherli et al., 2000; Kim et al., 2001). Recent studies in mice have shown that Tfh cells play a critical role in controlling P. chabaudi blood-stage infection via activation of IL-21 mediated responses (Perez-Mazliah et al., 2017). Therefore, since humoral responses are critical to the erythrocytic stages of Plasmodium, an in-depth understanding of the activation and maintenance of Tfh cells during malaria will be critical in designing blood-stage vaccines (Perez-Mazliah et al., 2017).

Regulatory T cells (Tregs) are another CD4+ T cell subset implicated in the maintenance of immune homeostasis and control of excessive pathogen-driven inflammatory responses (Stockinger et al., 2004; Tang & Bluestone, 2008). In Plasmodium infection, uncontrolled production of pro-inflammatory cytokines is associated with pathology in both mice and humans (Dodoo et al., 2002; Omer & Riley, 1998; Day et al., 1999; Li et al., 1999) and anti-inflammatory cytokines (TGF-β and IL-10) are known to be critical in the modulation of this inflammatory response (Fig. 1.3) (Omer & Riley, 1998; Li et al., 1999; Couper et al., 2008; Scholzen et al., 2009). The immunomodulatory function of IL-10 and TGF-β has been associated with Tregs whose role in rodent malaria remains unclear. Some studies
have shown that Tregs are critical in the control of pro-inflammatory responses associated with pathology (Nie et al., 2007; Cambos et al., 2008) while other studies have associated upregulation of Tregs with detrimental outcomes (Hisaeda et al., 2004; Amante et al., 2007; Nie et al., 2007). These discrepancies were mainly dependent on the rodent parasite strains utilised in the study (Finney et al., 2010). However, clinical studies have shown that infection with malaria resulted in upregulation of Tregs which in turn was associated with parasite load and subsequent disease severity amongst these individuals (Walther et al., 2005; Jangpatarapongsa et al., 2008; Minigo et al., 2009; Bueno et al., 2010).

The role of CD8+ T cells in the defence against blood-stage parasites remains unclear due to the fact that mature pRBCs do not express MHC class I molecules. However, in vitro studies have shown that both P. falciparum and P. vivax are able to invade erythroblasts, immature erythrocytes that possess a nucleus and express MHC class I molecules (Panichakul et al., 2007; Tamez et al., 2009). Additionally, studies in mice demonstrated that blood-stage parasite antigens were cross-presented by the CD8-α DCs, inducing parasite-specific CD8+ T cell responses capable of lysing APCs (Lundie et al., 2008). These findings indicated the possible relevance of CD8+ T cells in erythrocytic immunity to Plasmodium. Indeed, additional studies in mice have demonstrated that parasitized erythroblasts activated CD8+ T cells, in an antigen-specific manner (Imai et al., 2013). This contact dependent Fas – Fas Ligand (FasL) interaction of CD8+ T cells with the parasitized erythroblasts results in the exposure of phosphatidylserine (PS) on erythroblast surface (Imai et al., 2015). Cells displaying PS on their surface are rapidly phagocytosed by macrophages. Thus, CD8+ T cells in conjunction with macrophages are able to mediate immunity to a blood-stage malaria infection in mice (Imai et al., 2015). Earlier studies in a P. yoelii infection model demonstrated that professional APCs might cross-present parasite-
derived peptides on MHC class I to CD8+ T leading to cytotoxicity through the production of IFN-γ, perforin and granzyme B (Fig. 1.3) (Imai et al., 2010). Furthermore, parasite-specific CD8+ T cells have also been shown to clear infected reticulocytes, which express MHC class I molecules via the secretion of IFN-γ and expression of granzyme B (Horne-Debets et al., 2013).

1.6 Immune evasion and modulation by *plasmodium* parasites

1.6.1 Immune evasion

*Plasmodium falciparum* expresses variant antigens on the surface of pRBCs and the switching of these antigens facilitates immune evasion (Scherf et al., 2008). The genes that encode these highly variant multi-gene families include: *var*, *rif*, *stevor*, and *Pfmc-2TM*. These encode for, PfEMP-1, Rifin, Stevor (subtelomeric variable open reading frame), and *Pfmc-2TM* (Maurer's cleft two transmembrane) proteins respectively (Casares & Richie, 2009). PfEMP-1 has been comprehensively studied with respect to immune evasion and is encoded by approximately 60 *var* genes (Su et al., 1995). PfEMP-1 mediates the adhesion of pRBCs to the vascular endothelium of capillaries by binding to host receptors such as CD36, intercellular adhesion molecule (ICAM)-1, thrombospondin (TSP), complement receptor 1 (CR1), vascular adhesion molecule-1 (VCAM-1), and chondroitin sulphate A (CSA). The adherence of the parasite in host capillaries protects the parasites from destruction in the spleen and has been implicated in sequestration and hence severe disease (Pasternak & Dzikowski, 2009). The parasite can evade the host immune system by switching to express a different variant gene; each time antibodies are raised against the existing variant (Jensen et al., 2004; Scherf et al., 2008). Recent studies have demonstrated that RIFINs which are encoded by approximately 200 *rif* genes evade the host immune system by targeting immune inhibitory receptors such as leucocyte
immunoglobulin-like receptor B1 (LILRB1) inhibiting the activation of LILRB1-expressing B cells and NK cells (Saito et al., 2017).

Most of the malaria surface antigens possess tandem repetitive arrays of amino acid motifs in their sequences and polymorphism in these repeats leads to formation of multiple allelic forms of the antigens (Berzins & Anders, 1999). Indeed, several studies in malaria endemic regions have reported multiple allelic forms among blood-stage antigens such as Merozoite Surface Protein (MSP)1, MSP2, glutamate-rich protein (GLURP) and Apical Membrane Antigen (AMA)1 (Escalante et al., 2001; Mwingira et al., 2011; Kiwuwa et al., 2013). This allelic polymorphism enables parasites to evade host recall immune responses elicited by a previous exposure of the same antigen.

### 1.6.2 Immune modulation

*Plasmodium* can also evade the host immune system by modulating the function of CD4+ T and B cells. Indeed, the slow and inefficient acquisition of *P. falciparum*-specific LLPCs and MBCs could be related to parasite driven modulation of B cell differentiation (Dorfman et al., 2005; Asito et al., 2008; Crompton et al., 2014). Immune modulation of B cell function is directed by the interaction of systemic cytokines such as B cell activating factor (BAFF) (a cytokine critical for B cell survival) with B cells, to significantly affect the initiation and maintenance of antibody responses (Nduati et al., 2011; Crompton et al., 2014). Several studies have also shown that *Plasmodium* is capable of modulating the host immune defences by directly altering the function of dendritic cells. *In vitro, P. falciparum*-infected red blood cells were shown to modulate DC function by directly binding to CD36 and thrombospondin (TSP) on the surface of the DCs inhibiting their maturation and subsequently reducing their ability to activate a robust cell-
mediated immune response (Urban et al., 1999). Studies in mice confirmed this finding and showed that indeed blood-stage infection altered DC functions, inhibiting their maturation, inverting the secretion pattern of IL-12/IL-10 and suppressing the ability of DCs to initiate immune responses (Ocana-Morgner et al., 2003). Furthermore, these studies demonstrated that interaction of blood-stage parasites with DCs in vitro, results in the secretion of soluble factors that inhibit CD8^+ T cell activation and the suppression of protective CD8^+ T cell responses against the liver stage in vivo (Ocana-Morgner et al., 2003). It was thus suggested that this modulation of DC function by blood-stage malaria infection suppresses CD8^+ T cell responses and might also explain the lack of naturally acquired pre-erythrocytic immunity to malaria (Ocana-Morgner et al., 2003). Further studies in rodent models indicated that infection with virulent parasite strains resulted in inhibition of DC function via the downregulation of IL-12 secretion and inefficient priming of effector T cell function while non-lethal infections resulted in activation of DCs and subsequent priming of protective immune responses (Wykes et al., 2007; Wykes et al., 2007). These data suggest that careful optimisation needs to be considered for whole blood-stage parasite vaccines for the induction an effective immune response (Stanisic & Good, 2016).

Studies in humans have also shown that malaria infection results in dysfunctional peripheral DCs (Goncalves et al., 2010; Arama et al., 2011; Woodberry et al., 2012; Pinzon-Charry et al., 2013) and reduction in the number of circulating DCs, which was associated with increasing parasite load (Woodberry et al., 2012). The reduction in absolute numbers of DCs could be due to apoptosis as observed in both in vitro (Elliott et al., 2007) and in vivo studies (Woodberry et al., 2012; Pinzon-Charry et al., 2013). DC apoptosis amongst naturally infected individuals was associated with IL-10 production and DC functionality was recouped following anti-
malarial treatment (Pinzon-Charry et al., 2013). These findings indicate that anti-malarial treatment might be necessary at the time of vaccination in endemic areas to enable potentiation of an effective immune response (Stanisic & Good, 2016).

It has also been demonstrated that *P. falciparum* infection results in upregulation of Programmed Cell Death 1 (PD-1), a marker of T cell senescence expressed on the surface of CD4⁺ T cells (Butler et al., 2012; Illingworth et al., 2013) although the relevance of PD-1 in human malaria infection is still not clear. However, in a *P. yoelii* infection, blockade of the PD-1 ligand (PD-L1) and the inhibitory receptor Lymphocyte-Activation Gene 3 (LAG-3) restored CD4⁺ T cell function, expanded Tfh cell numbers and germinal centre B cells, increased antibody levels, and subsequently increased parasite clearance in blood (Butler et al., 2012; Crompton et al., 2014). In addition, PD-1 also mediates exhaustion of parasite-specific CD8⁺ T cells, which in turn affects parasite clearance during acute and chronic blood-stage infection (Horne-Debets et al., 2013).

### 1.7 Malaria vaccines

The development of an effective malaria vaccine has been a focus of research since the 1940s, when inoculation with inactivated sporozoites resulted in protection through induction of cellular and humoral immune responses in domestic fowls (Russell & Mohan, 1942; Hill, 2011). Follow-up studies by Freund and others also showed that monkeys and ducks were protected against *P. knowlesi* and *P. lophurae* following vaccination with killed, adjuvanted parasites (Freund et al., 1945; Freund et al., 1945b). Despite these early promising findings, an effective vaccine for malaria still eludes scientists with only one vaccine, RTS, S, showing promise in large-scale phase III trials but with moderate efficacy (Olotu et al., 2013; RTS S Clinical Trials Partnership, 2015). This calls for continued effort in the search for a
safe and effective vaccine against the malaria parasites, *P. falciparum* and *P. vivax* (Malaria Vaccine Funders Group, 2013). Malaria vaccines are broadly classified as sub-unit and whole parasite vaccines, based on the approach used to develop them.

**1.7.1 Sub-unit vaccines**

Sub-unit vaccines contain a restricted number of parasite antigens in addition to delivery systems, carrier proteins and adjuvants to enhance immune responses against specific antigen epitopes that are included in the formulations (Rueckert & Guzman, 2012). In the context of malaria, sub-unit vaccines generally target a single life-cycle stage and several candidates have been developed and tested in preclinical and clinical studies.

Transmission-blocking vaccines (TBVs) target the development of the parasites within the definitive host, the mosquito, with the aim of preventing disease transmission within a community. The development of TBVs has primarily focused on 4 *P. falciparum* antigens Pfs230 and Pfs48/45 expressed by gametocytes as well as Pfs25 and Pfs28 expressed on the surface of zygotes (Carter et al., 1984). Clinical evaluation of TBV antigens Pfs25 along with its *P. vivax* orthologue Pvs25 resulted in the induction of transmission blocking antibodies as measured by the standard *in vitro* membrane feeding assay (Wu et al., 2008; Talaat et al., 2016). However, as is the case with most sub-unit antigens, TBV antigens are poorly immunogenic and require potent adjuvants and carrier proteins in order to achieve the high long-lived antibody titres required to effectively block malaria transmission, especially in endemic settings (Hoffman et al., 2015). In line with this, a liposome-based adjuvant formulation containing Pfs25, GLA-LSQ, a TLR-4 agonist, *Quillaja saponaria* Molina, fraction 21 (QS-21) saponin coupled with a carrier protein, tetanus toxoid, effectively induced T follicular helper (Tfh), long-lived plasma cell (LLPC) as well as
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durable antibody responses to Pfs25 in mice providing an attractive avenue for further testing in the clinic (Radtke et al., 2017).

Several erythrocytic stage sub-unit vaccine candidates containing major blood-stage antigens: MSP1, MSP2, MSP3, AMA1, thrombospondin-related adhesion protein (TRAP) and Ring-infected Erythrocyte Surface Antigen (RESA) have been developed and tested either as single antigens or in combination, with limited success in clinical trials (extensively reviewed in (Goodman & Draper, 2010)). However, novel candidate antigens such as *P. falciparum* reticulocyte binding protein homolog 5 (PfRH5) have been identified and are rapidly transitioning to clinical trials (Douglas et al., 2011; Douglas et al., 2015).

One of the most promising blood-stage vaccines, FMP2.1/AS01 or AS02, based on the *P. falciparum* 3D7 AMA1 antigen, demonstrated significant allele-specific efficacy against homologous parasites but no overall efficacy against heterologous clinical malaria (Spring et al., 2009; Thera et al., 2011; Ouattara et al., 2013; Payne et al., 2016). Given the lack of efficacy with this approach, new approaches are currently being explored to improve the quality of AMA1-specific antibodies following vaccination by mimicking the AMA1 structure on the invading merozoite (Srinivasan et al., 2014; Srinivasan et al., 2017). It has been shown that AMA1 interacts with a conserved region of Rhoptry neck protein 2 (RON2) during merozoite invasion (Lamarque et al., 2011; Srinivasan et al., 2011) and peptides that block this interaction inhibit merozoite invasion (Srinivasan et al., 2013). With the assumption that the AMA1–specific antibodies are directed towards the AMA1–RON2 complex to effectively block invasion in vivo, an AMA1–RON2L peptide complex vaccine was developed. Immunisation with this complex induced potent merozoite invasion inhibitory antibodies to homologous and heterologous parasites.
in mice and Aotus monkeys providing the impetus for progression to clinical trials (Srinivasan et al., 2014; Srinivasan et al., 2017).

*Plasmodium falciparum* reticulocyte-binding protein homologue 5 (PfRH5), a blood-stage antigen essential for merozoite invasion of erythrocytes (Baum et al., 2009) is another promising vaccine candidate. Unlike other blood-stage antigens such as AMA1 and MSP1 which are highly polymorphic, PfRH5 is highly conserved with limited diversity amongst clinical *P. falciparum* isolates (Manske et al., 2012). Naturally acquired PfRH5-specific antibodies were found to inhibit parasite growth *in vitro* and the presence of these antibodies was associated with protection from malaria (Tran et al., 2014). Aotus monkeys immunised with a PfRH5-based vaccine were strongly protected against a virulent heterologous *P. falciparum* challenge infection and protection was mediated by anti-PfRH5 antibody responses (Douglas et al., 2015). This vaccine is currently undergoing in phase I clinical trials in the United Kingdom and Tanzania (Coelho et al., 2017).

On the other hand, success has been recorded with pre-erythrocytic stage vaccines with RTS, S, a vaccine based on liver stage antigen Circumsporozoite Protein (CSP), which received a positive scientific opinion from European regulators and WHO for the prevention of malaria in young children in Sub-Saharan Africa (Kaslow & Biernaux, 2015; World Health Organization, 2017). Immunisation with 3 doses of RTS, S reduced clinical malaria episodes by 28% in young children and 18% in infants while administration of a booster dose 18 months later reduced the number of cases of clinical malaria in young children by 36% and in infants by 26% (Olotu et al., 2013; RTS S Clinical Trials Partnership, 2015). The vaccine is currently licenced for use in large-scale, pilot implementation trials in Sub-Saharan Africa (World Health Organization, 2017).
The limited success of sub-unit vaccines has been attributed to allelic and antigenic diversity of the protein sub-units in the vaccine formulations as well as failure to maintain parasite-specific antibody responses (Patarroyo et al., 1988; Genton et al., 2002; Ogutu et al., 2009; Sagara et al., 2009; Sirima, Cousens, & Druihl, 2011; Thera et al., 2011; Ewer et al., 2013). The ideal sub-unit vaccine will therefore have to contain multiple alleles from the same antigen or multiple antigens from the same life-cycle stage or different stages. All these strategies have been tested in animal or human studies albeit with limited success. Studies utilizing a vaccine containing multiple AMA1 alleles demonstrated high anti-AMA1 titres capable of inhibiting the growth of *P. falciparum* strains *in vitro* (Miura et al., 2013). However, this vaccine is yet to be tested in clinical trials. Clinical studies utilizing a vaccine containing multiple blood-stage antigens, MSP1, MSP2 and RESA (‘Combination B’), demonstrated an overall reduction in parasitaemia amongst vaccinated volunteer although the efficacy observed was allele-specific (Genton et al., 2002). A vaccine containing pre-erythrocytic stage antigens (CSP and TRAP) and erythrocytic stage antigens (AMA1 and MSP1) in a prime boost strategy protected rhesus monkeys against *P. knowlesi* challenge infection, although this was short-lived (Jiang et al., 2009). Therefore, new vaccine approaches have been proposed to address diversity of malaria vaccine antigens (*extensively reviewed in* (Ouattara et al., 2015)) and one such approach is the whole parasite vaccine approach which is showing tremendous progress in field trials (Greenwood, 2017; Hollingdale & Sedegah, 2017).

1.7.2 Whole parasite vaccines

The whole parasite vaccine approach has attracted renewed interest in an effort to address the limitations of sub-unit vaccines. This approach in the context of malaria, utilises the whole parasite, which could be: killed, irradiated, genetically, or
chemically attenuated. Many of the vaccine candidates for malaria that have been
developed using this approach are still at the pre-clinical testing stage but are
showing sufficient promise to enable progression to clinical trials. The major
limitations for whole parasite vaccines include: possible reversion to virulence,
especially for the irradiated and genetically-attenuated parasites and the fact that
live-attenuated vaccines are not suitable for immunocompromised individuals (Hill,
2011). However, there has been tremendous progress with the irradiated
sporozoites approach with several clinical trials including some in malaria endemic
countries being successfully conducted to date (Hollingdale & Sedegah, 2017;
Sissoko et al., 2017).

1.7.2.1 Radiation-attenuated vaccines

Attenuation using this approach is achieved by exposing the sporozoites to an
adequate dose of radiation, which still permits invasion of hepatocytes but does not
allow development through to blood-stage (Vaughan et al., 2010). Thus, the parasite
remains viable and is able to infect liver cells. It is believed that attenuation is
achieved by the introduction of random mutations and breaks within the parasite
DNA by radiation (Vaughan et al., 2010). Initial studies using irradiated P. berghei
sporozoites showed that a blood-stage challenge infection was circumvented in the
immunised mice (Nussenzweig et al., 1967). This approach has now been tested
successfully in a series of studies involving human volunteers inoculated with
irradiated P. falciparum sporozoites. The volunteers were effectively protected
from a subsequent wild-type challenge infection (Seder et al., 2013; Ishizuka et al.,
2016; Epstein et al., 2017; Lyke et al., 2017; Sissoko et al., 2017).

Irradiation has also been shown to attenuate both murine and human erythrocytic
stage Plasmodium parasites (Waki et al., 1982; Waki et al., 1983; Waki, et al., 1986;
Oakley et al., 2013). More recently, immunisation of mice with radiation attenuated
*P. berghei* was shown to protect mice from severe disease and experimental cerebral
malaria (ECM). In this study, parasite specific immune responses mediated by
antibodies as well as splenic IFN-γ production correlated with protection against
ECM and severe disease (Gerald et al., 2011).

**1.7.2.2 Genetically-attenuated parasite vaccines**

Studies in rodent models have shown that inoculation with live, attenuated
sporozoites that have had critical genes knocked out, induces long lasting protective
immune responses mediated by antibodies and CD8+ T cells (Jobe et al., 2007). Genetic attenuation is achieved by deletion of genes that are essential for parasite
development and survival during the liver stage of the parasite life-cycle. These
include: Upregulated in Infectious Sporozoites (*UIS*s; *UIS3* and *UIS4*), *P52*, *P36*,
sporozoite asparagine-rich protein 1 (*SAP1*) and genes that encode for a bacterial-
like type II fatty acid synthesis pathway (*FAS II*). Rodent parasites deficient in one
or a combination of these genes were able to infect hepatocytes with developmental
arrest at different stages before establishment of a blood-stage infection (Mueller et
al., 2005; Vaughan et al., 2009; Vaughan et al., 2010). The main limitation of this
approach is the possibility that some single gene deletions may not result in
complete attenuation and result into breakthrough blood-stage infections (Annoura
et al., 2012). Indeed, a recent first-in-human clinical trial involving genetically-
attenuated *P. falciparum* sporozoites generated by deletion of two pre-erythrocytic
stage expressed genes (*P52* and *P36*), further emphasizes the need for multiple gene
deletions and optimal selection of genes essential for pre-erythrocytic development
of the malaria parasite (Spring et al., 2013). Mikolajczak and others created a *P.
falciparum* genetically-attenuated knock-out parasite (Pf GAP3KO) that had
deletions of 3 genes, \textit{P52}, \textit{P36} and \textit{SAP1} which are critical for pre-erythrocytic development. In a humanized mouse model, this Pf GAP3KO demonstrated complete arrest of pre-erythrocytic development and no blood-stage infection was observed (Mikolajczak et al., 2014). When tested in human volunteers, Pf GAP3KO showed complete attenuation following a single dose immunisation and functional anti-sporozoite antibodies were detected in volunteer sera (Kublin et al., 2017).

1.7.2.3 Chemically-attenuated parasite vaccines

Preclinical studies have demonstrated that seco-cyclopropyl pyrrolo indole analogs, drugs capable of alkylating parasite DNA are able to attenuate both sporozoite and blood-stage rodent \textit{Plasmodium} parasites, inducing a potent cross-species immune response (Purcell, Wong et al., 2008; Yanow et al., 2008; Good et al., 2013; Raja et al., 2016). Centanamycin-attenuated sporozoites have been shown to elicit an immune response mediated mainly by antibodies and CD8$^+$ T cells (Purcell et al., 2008) while immunisation with whole blood-stage chemically attenuated parasites strongly protected mice against a wild-type challenge infection and resulted in a long-lived strain transcending antibody and CD4$^+$ T cell response (Good et al., 2013; Raja et al., 2016). These drugs have genotoxic potential; hence the need to pursue this approach with alternative drugs such as gold compounds that utilise different mechanisms of parasite attenuation.

1.7.2.4 Gold compounds and their anti-malarial properties

Chrysotherapy, defined as the use of gold compounds or gold-based drugs for the treatment of ailments, has been utilised for therapeutic purposes for many decades (Higby, 1982; Fricker, 1996; Shaw, 1999). However, despite their rich therapeutic history, gold compounds have only been used clinically for the effective treatment of rheumatoid arthritis. The limited use of gold compounds was attributed mainly
to toxicity and poor chemical stability (Shaw, 1999; Nobili et al., 2010). With increased understanding of the chemistry and mechanisms of action, more stable and less toxic compounds have been developed, hence the renewed interest as potential anti-cancer and anti-microbial agents (Okada et al., 1993; Milacic, 2008; Navarro, 2009; Nobili et al., 2010; Berners-Price & Filipovska, 2011; Lewis et al., 2011). The molecular mechanisms of action of gold compounds have been described mainly based on their interaction with biological targets such as thiols, selenols, protein and enzymes (Crooke & Snyder, 1986; Nobili et al., 2010). The best described target of the gold compounds has been the thioredoxin system which is present in all living cells (Holmgren, 1985). This system is composed of Thioredoxin (Trx), a protein with active cysteine residues, thioredoxin reductase (TrxR) (a selenoenzyme) capable of reducing several substrates, as well as the electron donor NADPH (Fig. 1.4) (Holmgren, 1985; Arner & Holmgren, 2006; Nobili et al., 2010). The Trx system is required to maintain a reducing environment for DNA synthesis, defence against oxidative stress and reactive oxygen species (ROS) and in the activation of transcription factors involved in the regulation of cell growth and cell survival (Jun-Qin, 1995; Nobili et al., 2010). Expression levels of thioredoxin and thioredoxin reductase are augmented in cancer, inflammation and HIV infection and have been linked to disease aggressiveness, making this system an interesting target for new drugs (Lillig et al., 2001; Arner & Holmgren, 2006; Holmgren, 2007; Holmgren & Lu, 2010).

In Plasmodium, the selenoproteome to which the thioredoxin system belongs is highly conserved across species (Lobanov et al., 2006). The thioredoxin system has been well characterised as a possible drug target for P. falciparum (Fig. 1.4). Malaria parasites are prone to oxidative stress caused by reactive oxygen and nitrogen species within the human host as well as reactive oxygen species in the midgut of
the mosquito (Becker et al., 2004; Molina-Cruz et al., 2008; Jortzik & Becker, 2012). *Plasmodium falciparum* thioredoxin reductase (PfTrxR) is thus an important enzyme in the maintenance of a balanced redox environment for parasite survival within the human host (Krnajski et al., 2002). PfTrxR counteracts oxidative stress by directly detoxifying oxidants such as hydrogen peroxide, tert-butylhydroperoxide, cumene hydroperoxides, S-nitrosoglutathione but also by maintaining the antioxidant capacity of ascorbate, lipoic acid, and lipoamide (Jortzik & Becker, 2012) (Fig. 1.4). Reduced PfTrx also utilises peroxiredoxins to detoxify peroxides as well as interacting with several target proteins involved in protein folding, DNA synthesis, haemoglobin catabolism, and signal transduction (Jortzik & Becker, 2012) (Fig. 1.4). Therefore, chemical compounds with the ability to inhibit PfTrx are believed to be potential anti-malarial drug candidates as well as parasite attenuating agents for whole parasite vaccine development. Gold compounds have been shown to be potent inhibitors of PfTrxR and have been shown to rapidly accumulate in red blood cells, the host cells of the malaria parasite during blood-stage infection (Roberts & Shaw, 1998; Berners-Price & Filipovska, 2011).
Sanella et al (2008), demonstrated that PfTrxR is inhibited by the gold-based drug auranofin (AF) \textit{in vitro}. In addition, computational modelling studies have shown that AF binds to PfTrxR indicating that it is a key drug target to inhibit \textit{P. falciparum} growth (Caroli et al., 2012).

Sodium aurothiomalate, another gold drug used in the treatment of rheumatoid arthritis, has also been shown to delay the onset of breakthrough parasitaemia and subsequent lethal course of murine malaria. In this model, sodium aurothiomalate was demonstrated to induce eryptosis, leading to early recognition of parasitized red cells (pRBCs) by macrophages and their subsequent phagocytosis and rapid clearance from blood circulation (Alesutan et al., 2010).

A gold (I) phosphine compound, [Au(d2pype)2]Cl, synthesized by introducing 2-pyridyl groups in order to reduce the high non-selective thiol activity and hence
toxicity demonstrated by auranofin and its analogs (Berners-Price et al., 1986). This compound ([Au(d2pype)2]Cl) demonstrated intermediate lipophilicity, increased selectivity towards tumor cells and reduced host toxicity while maintaining anti-tumor activity (McKeage et al., 2000; Berners-Price & Filipovska, 2011). However, the anti-malarial activity of [Au(d2pype)2]Cl has not been demonstrated. Therefore, the anti-malarial activity of gold drugs can thus be further exploited to investigate potential parasite attenuation properties to facilitate the development of whole blood-stage vaccines.

1.7.3 Whole killed parasites vaccines

Studies in mice have demonstrated that low doses of killed blood-stage *P. chabaudi* parasites adjuvanted in CpG and Alum are capable of inducing a protective CD4+ T cell response (Su et al., 2003; Pinzon-Charry et al., 2010). Vaccine efficacy was reliant on the use of potent adjuvants in the vaccine formulation. Progression of this approach into clinical trials has been restricted due to lack of a suitable human-compatible adjuvant, however, with new potent adjuvants (Didierlaurent et al., 2016) and alternative antigen delivery systems such as liposomes (Giddam et al., 2016), there is renewed interest in this approach.

1.8 Vaccine adjuvants and antigen delivery systems

In the development of vaccines, the ability to initiate innate immune responses and subsequent potentiation of robust downstream adaptive immune responses need to be considered. This is achieved naturally with live-attenuated vaccines while inactivated and sub-unit vaccines require delivery systems and or adjuvants for efficient presentation to the immune system and additional stimulation to enhance potency (Perrie et al., 2016). To address this, careful selection of adjuvants and
delivery systems needs to be considered early in the vaccine development process. Only a handful of adjuvants such as aluminium based salts (Alum), MF59 (an oil-in-water emulsion consisting of squalene, Tween 80 and Span 85), Adjuvant System 02 (AS02) (monophosphoryl lipid A, QS-21 and squalene emulsion), Adjuvant System 03 (AS03) (a squalene, Tween 90 and α-Tocopherol oil-in-water emulsion), Adjuvant System 04 (AS04) (aluminium hydroxide and monophosphoryl lipid A (MPLA)) and virosomes have been licenced for use in human vaccines (Brito & O'Hagan, 2014). Therefore, there is an unmet need for development of new adjuvants suitable for clinical use.

Liposomes present an attractive adjuvant and antigen delivery platform for malaria vaccine development. Originated by Gregoriadis and Allison (Allison & Gregoriadis, 1974), liposomes are self-assembling phospholipid vesicles capable of incorporating and protecting antigens from degradation as well as facilitating antigen delivery to professional antigen presenting cells (APCs). Given their versatility and plasticity, modification of the physicochemical factors of liposome formulations can enhance the adjuvanticity and antigen delivery capacity (Perrie et al., 2016).

The adjuvanticity of liposomes can be enhanced by the choice of phospholipid used in the formulation. Long chain lipids tend to form rigid ordered bilayer structures while those with shorter tails tend to form fluid and disordered vesicles. Immunisation of animals with liposomes formulated with long chain lipids such as dimethyldioctadecylammonium bromide (DDAB) distearyol derivative of L-α-phosphatidyl choline (DSPC) resulted in stronger antigen-specific responses when compared to animals that received liposomes formulated with shorter chain lipids (Mazumdar et al., 2005). Furthermore, charged liposomes formulated with saturated acyl chain cationic lipids (with a quaternary ammonium head group) have
been shown to promote binding of antigen at the site of injection stimulating interaction with APCs to elicit a robust adaptive immune response (Christensen et al., 2012). In contrast, highly fluid unsaturated acyl chain liposomes that are rapidly cleared from the injection site resulting in lower activation of APCs (Christensen et al., 2012). Additionally, cationic liposomes have been shown to promote higher levels of antigen at the injection site, resulting in a depot-effect whereby the liposome antigen is retained in the tissue for an extended period of time allowing continuous attraction of APCs and subsequent induction of robust cell-mediated immune responses compared to anionic and neutral liposomes that are rapidly cleared from the injection site resulting in lower activation of APCs and subsequent adaptive immune responses (Henriksen-Lacey et al., 2010; Henriksen-Lacey et al., 2010b; Perrie et al., 2016).

The particle size of liposomes has also been shown to impact adjuvanticity and direct the development of the resulting cell-mediated immune response. Studies have shown that the immune response induced following administration of small sized liposome vesicles was skewed towards Th2 while larger vesicles induced a Th1 response characterised by augmented IFN-γ and IgG2a production (Mann et al., 2009). The differences in the profiles of the induced immune response of large verses small vesicles could be due to differences in antigen processing and trafficking to lymph nodes. Large sized vesicles were shown to be more efficiently phagocytosed and processed by macrophages compared to smaller vesicles (Brewer et al., 2004). Additionally, trafficking of liposome particles to lymph nodes has been shown to be size dependent with small sized vesicles freely draining to and specifically targeting lymph node-resident cells while large sized vesicles require dendritic cells for trafficking from the injection site to lymph nodes (Manolova et al., 2008). More recently, using cationic liposomes, large sized vesicles demonstrated
enhanced splenocyte proliferative responses and reduced IL-10 production compared to small sized liposomes (Henriksen-Lacey et al., 2011).

Given their structural versatility and plasticity, liposomes can be customized to incorporate additional lipophilic immunomodulators within or attached to the lipid bilayer to enhance adjuvanticity. Such immunomodulators are crucial in the activation of the cells of innate immune system via pattern recognition receptors (PRRs) which recognize pathogen-associated molecular patterns (PAMPs) on the surface of pathogens subsequently activating the adaptive immune system. The activation of innate immune cells such as dendritic cells and macrophages requires the use of Toll-like receptor (TLR) and NOD-like receptor (NLR) type PRRs to direct a robust immune response (Janeway, 2001). Therefore, the use of synthetic PRR agonists has been predicted to be critical in the formulation of liposome vaccine adjuvants (Gnjatic et al., 2010; Perrie et al., 2016).

Liposome formulations can be customized by incorporating PRR agonists which mediate activation and maturation of APCs which facilitates the uptake and processing of liposome associated antigens resulting in potent cell-mediated immune responses (Perrie et al., 2016). The most widely used PRR agonist MPLA, a TLR-4 agonist, has been used in licenced vaccine formulations Fendrix (hepatitis B) (Tong et al., 2005) and Cervarix (human papillomavirus) (Monie et al., 2008). A synthetic analogue of MPLA, 3′-O-desacyl-4′-monophosphoryl lipid A formulated with Quillaja saponaria Molina, fraction 21 (QS-21) saponin has been included in liposome-based GSK Adjuvant System 01 (AS01) and has been tested in human studies for the malaria vaccine RTS,S (Mosquirix) (RTS S Clinical Trials Partnership, 2015) as well as a shingles sub-unit vaccine HZ/su (Lal et al., 2015). Similarly, liposomes can be tagged with sugars such as mannose to target them to lectin like molecules on APCs to facilitate phagocytic uptake thereby promoting MHC class II
involvement and, via cross presentation, MHC class I. This targeting of liposomes to different uptake pathways may aid direct the resulting immune response towards a mixed Th1/Th2 response (Perrie et al., 2016).

Collectively, it is evident that to achieve an optimal liposome formulation capable of promoting efficient antigen delivery and inducing effective immune responses, careful selection of vesicle physicochemical characteristics as well as immunostimulatory molecules needs to be considered. Indeed, the versatility and plasticity of liposomes facilitates tailoring of the desired immune responses and this can be exploited for the development of a whole parasite blood-stage malaria vaccine.

1.9 Scope of thesis

The data presented here describes novel advances to the development of a whole parasite blood-stage malaria vaccine. The whole parasite approach is believed to circumvent the limitations of sub-unit vaccines such as antigenic polymorphism. Firstly, the collection, culturing and preliminary drug susceptibility characterisation of *Plasmodium* isolates from human volunteers living in a malaria endemic country, is examined. Individuals living in endemic regions are exposed to many different species/strains of the malaria parasite, hence the need to establish a parasite cell banks of field *Plasmodium* parasite species/strains. These clinical isolates are valuable reagents for preliminary evaluation of malaria vaccine candidate prior to deployment for further testing in endemic countries.

Studies in our laboratory have shown that DNA-binding drugs can efficiently attenuate blood-stage parasites and immunisation with the attenuated parasites induced long-lived strain transcending protective immunity in mice (Good et al., 2013; Raja et al., 2016). However, DNA-binding drugs are potentially genotoxic and
hence the need to screen new attenuating compounds, utilising different mechanisms of action to further facilitate vaccine development using this approach. Herein, the anti-malarial activity of two gold (I) phosphine compounds auranofin and \([\text{Au}(\text{d}2\text{pype})_2]\text{Cl}\), were examined to inform their potential use as parasite-attenuating agents and drugs.

The development and clinical evaluation of live-attenuated blood-stage vaccines described above presents a number of challenges including but not limited to regulatory and safety considerations on the use of blood products in volunteers as well as manufacturing, storage and delivery considerations especially in malaria endemic areas (Stanisic & Good, 2015). To address some of these challenges, the utility of a novel liposome-based antigen delivery platform which is easy to prepare and can be readily freeze-dried is examined as a possible alternative to the live-attenuated whole parasite approach.

Results from the studies described in this thesis will inform future use of gold compounds as parasite attenuating agents as well as anti-malarial drugs. Additionally, these studies will advance our understanding of the immune responses elicited following immunisation with liposomes containing inactivated whole parasite antigens and inform the design of future vaccine formulations for use in clinical trials to add to the toolbox of malaria prevention strategies.
Chapter 2: Materials and methods
2.1 Materials

2.1.1 Mice

Inbred Bagg Albino (BALB/c) (H-2^d) congenic severe combined immunodeficient (SCID) (H-2^a) and C57BL/6 (H-2^b) female mice aged 4 - 6 weeks used in the studies described in this thesis, were purchased from the Animal Resource Centre (ARC) (Canning Vale, Western Australia). All animals were housed under special pathogen free, Physical Containment level 2 (PC2) conditions in the Institute for Glycomics animal facility.

2.1.2 Malaria parasites

*Plasmodium chabaudi* AS and *P. yoelii* 17X cloned lines were used in the animal experiments. These were obtained from QIMR Berghofer Medical Research Institute, stocks originally provided by Prof. Richard Carter from the University of Edinburgh, United Kingdom. The cloned lines were maintained by serial passage in inbred mice.

Clinical *P. falciparum* isolates from Uganda (UGMCB-009, UGMCB-013), *P. falciparum* strains 3D7 and 7G8 laboratory lines (Stanisic et al., 2015) and *P. knowlesi* A1H.1 (Moon et al., 2013) were used to test the *in vitro* anti-malarial activity of [Au(d2pype)$_2$]Cl on human malaria parasites.

2.1.3 Drugs for parasite attenuation and *in vitro* drug susceptibility studies

[Au(d2pype)$_2$]Cl·3H$_2$O was synthesized by a modification of the published procedure using [AuCl(SMe)$_2$] as precursor and 1,2-bis(di-2-pyridylphosphino)ethane (d2pype) obtained from Strem Chemicals Inc. (Berners-Price, Bowen, Hambley, & Healy, 1999). Earlier studies have shown that the 1:2 adduct of gold (I) chloride with d2pype exists as a dimer in the solid state but in dilute solution is
predominantly monomeric [Au(d2pype)2]Cl (Berners-Price et al., 1999). Auranofin and chloroquine diphosphate were purchased from Sigma-Aldrich. Riamet, a combination of artemether and lumefantrine, was obtained from Novartis Pharmaceuticals. Gold compounds were dissolved in methanol, chloroquine diphosphate in H2O and Riamet in dimethyl sulfoxide (DMSO) to make 2mM stock solutions. Prior to use, drugs were diluted in RPMI-1640 media (Gibco, Invitrogen Corporation) supplemented with L-glutamine, HEPES, 10% heat inactivated human serum and gentamicin (Gibco) for in vitro assays with *P. falciparum*, and *P. knowlesi* parasites.

### 2.1.4 Reagents

All reagents and chemicals used in the studies described in this thesis were of analytical or tissue culture grade.

#### 2.1.4.1 Phosphate Buffer solution

To prepare 1 L of 10x phosphate buffer solution (PBS), 85 g of sodium chloride, 14.8 g of disodium phosphate and 4.3 g of monopotassium phosphate were dissolved in 1 L of milliQ water and pH was adjusted to pH 7.4. Before use, 10x PBS was diluted 1 in 10 in milliQ water and filter sterilised (under positive pressure) using membrane filtration (Corning). PBS was stored at room temperature.

#### 2.1.4.2 Complete media

Complete medium used in experiments involving rodent parasites was prepared using Roswell Park Memorial Institute (RPMI) medium supplemented with L-Glutamine (Gibco, Invitrogen Corporation), 10% heat-activated foetal bovine serum (FBS) (Bovogen), (Gibco, Invitrogen Corporation), 1% penicillin- streptomycin and 0.1% 2-mercaptoethanol (Gibco, Invitrogen Corporation). Complete medium used to culture human malaria parasites was prepared in RPMI medium supplemented
with L-Glutamine, 25 mM HEPES (Gibco, Invitrogen Corporation), 10% heat inactivated human serum (Australian Red Cross Blood Service) and gentamicin (Gibco, Invitrogen Corporation).

2.1.4.3 ELISA reagents

Coating buffer was prepared by dissolving 3.03 g of sodium carbonate and 6.0 g of sodium bicarbonate in 1 L of distilled water and the pH was adjusted to 9.6. Wash buffer was prepared by adding 200 mL of 10x PBS, 1 mL of Tween-20 to 1800 mL of distilled water. Blocking buffer was prepared by dissolving 10 g of skim milk powder in 100 mL wash buffer. The secondary antibody used was biotinylated anti mouse-IgG (Invitrogen) (diluted 1:3000 in blocking buffer). The substrate used was tetramethylbenzidine (TMB) (OptEIA™ BD Biosciences). To 54 mL of 98% sulfuric acid 1 L of distilled water was added to make 1M stop solution.

2.1.4.4 Gey's lysis buffer

Gey's lysis buffer was used to lyse red blood cells from harvested spleens. The buffer was prepared using 3 stock solutions. Stock A was prepared by dissolving 35 g ammonium chloride, 1.85 g potassium chloride, 1.5 g sodium dihydrogen phosphate dodecahydrate, 0.12 g monopotassium phosphate, 5 g glucose and 50 mg of phenol red in 1 L of distilled water. Stock B was prepared by dissolving 0.42 g magnesium chloride hexahydrate, 0.14 g magnesium sulphate heptahydrate and 0.34 g calcium chloride in 100 mL of distilled water while stock C was prepared by dissolving 2.25 g sodium bicarbonate in 100 mL of distilled water. All stock solutions were stored at 4°C until required. Gey's lysis buffer was prepared by adding 20 parts of stock A, 5 parts of stock B and 5 parts of stock C in 70 parts of distilled water. The mixture was filter sterilized by membrane (0.22 µM) filtration and stored at 4°C until required.
2.1.4.5 ACK lysis buffer

ACK lysis buffer was used to lyse red blood cells in peripheral blood samples collected for assessment of induction of antigen experienced T cells. ACK lysis buffer was prepared by dissolving 8.29 g of ammonium chloride, 1 g of potassium bicarbonate and 37.2 mg of EDTA disodium magnesium in 1 L of distilled water and pH adjusted to 7.4. The mixture was filter sterilized by membrane (0.22 µM) filtration and stored at room temperature until required.

2.1.4.6 MACs buffer

MACS buffer was prepared by dissolving 5 g of bovine serum albumin (BSA) (Sigma-Aldrich) using 1 L of PBS supplemented with 4 mL of 0.5 M UltraPure™ Ethylene diamine tetra acetic acid (EDTA) (Gibco). The mixture was filter sterilized by membrane (0.22 µM) filtration and stored at 4°C until required.

2.1.5 Other chemicals, reagents and kits

Appendix 1 lists all chemicals, reagents and kits together with their respective vendors/manufacturers used in the studies described in this thesis.

2.1.6 Disposable products

Appendix 1 lists all disposable consumables together with their respective vendors/manufacturers used in the studies described in this thesis.

2.2 Study methodologies involving human volunteers

2.2.1 Human ethics Statement

Volunteers were recruited from individuals who presented to health centres located in Entebbe, Uganda, with a confirmed diagnosis of malaria between January and March 2016. Individuals willing to participate were invited for eligibility screening at the Uganda Virus Research Institute-International AIDS Vaccine Initiative (UVRI-
IAVI) HIV Vaccine Program research clinic based at Uganda Virus Research Institute in Entebbe. Prior to conducting any study procedures, informed consent was obtained from each volunteer. Volunteers that met the eligibility criteria were asked to donate blood as a source of malaria parasites. The blood was leukodepleted, cryopreserved and shipped to the Institute for Glycomics, Griffith University. This work was approved by the Uganda Virus Research Institute Research and Ethics Committee (GC/127/15/12/535), Uganda National Council for Science and Technology (HS1962) and the Griffith University Human Research Ethics Committee (GU: 2016/031).

2.2.2 Study procedures

Prior to carrying out any study procedures, informed consent was sought from all volunteers. Next, a full medical history and physical examination was conducted together with ascertainment of concomitant medications used for any chronic conditions at the time of screening. Additionally, consistent with the Uganda national guidelines, all volunteers received Human Immunodeficiency Virus (HIV) counselling.

For screening, 10 mL of blood was collected for malaria testing by microscopy, full blood count, blood grouping (Cypress Diagnostics™) and rapid screening for HIV1/2 (Determine™, Stat-Pak™, Uni-Gold™), syphilis (ABON™, Biotec™ RPR), hepatitis B (SD BIOLINE HBsAg™) and hepatitis C (SD BIOLINE HCV™).

Following confirmation of eligibility according to the inclusion and exclusion criteria (Appendix 2), an additional 200 mL of blood was collected as a source of malaria parasites and retrospective viral nucleic acid (HIV, hepatitis B and C) and serological (Human T cell lymphotropic virus (HTLV) 1 and II) testing.
2.2.3 Cryopreservation of malaria parasites

One hundred and eighty millilitres of blood were collected in a Leukotrap Whole Blood bag system (Haemonetics™) from eligible volunteers as a source of malaria parasites. This blood bag system is capable of collecting and depleting leukocytes from whole blood. Following leucocyte depletion, the blood was centrifuged at 400 g for 10 min, supernatant discarded and red blood cell pellet volume estimated. The blood was then mixed with 2 times the pellet volume of the cryopreservative Glycerolyte 57 (Baxter Healthcare), aliquoted into 2-mL cryovials and transferred to a Controlled Rate Freezer (Planer). The freezing program was set to -1°C/min to -50°C and then -10°C/min to -120°C. At the end of the freezing cycle, the vials were immediately transferred to a cryobox and stored in a vapour-phase liquid nitrogen tank prior to shipment to Griffith University for culture adaptation.

2.2.4 Drug sensitivity and in vitro culture of clinical isolates of P. falciparum

The standard tritiated (³[H]) hypoxanthine uptake assay was utilised to assess the drug sensitivity of clinical isolates of Plasmodium (Desjardins et al., 1979). Prior to addition of the drugs, P. falciparum cultures were synchronized using D-sorbitol selection (Lambros & Vanderberg, 1979). Parasite cultures were sub-cultured to 2% haematocrit, 1% parasitaemia for P. falciparum and were exposed to 10 serial dilutions of compounds starting at 2 µM in 96 well flat-bottom cell culture plates under standard culture conditions (37°C, 5% CO2, 5% O2, 90% N2) for 24 h. ³[H]-hypoxanthine (0.5 µCi/well) was added at this point and plates were incubated for an additional 24 h. Plates were frozen overnight at -80°C, and later thawed for harvesting onto 1450 MicroBeta glass fibre filters (Wallac, USA). The filters were air dried and ³[H]-hypoxanthine uptake was measured using a Microbeta2 Microplate Counter (Perkin Elmer) to obtain radioactivity corrected counts per minute (CCPM)
values. Log dose concentration curves were generated from normalized CCPM and IC50 values were obtained using nonlinear regression analysis in GraphPad Prism Software.

2.3 **General study methodologies involving mice**

2.3.1 **Animal ethics statement**

All animal procedures were performed in accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes 8th edition (2013) and approved by the Griffith University Animal Ethics Committee. Experiments with gold compounds were conducted under approval numbers GLY/14/14/AEC and GLY/16/15/AEC while experiments to examine immunogenicity of liposome formulations were conducted under approval numbers GLY/07/16/AEC, GLY/10/16/AEC and GLY/15/17/AEC.

2.3.2 **Malaria parasite challenge and monitoring of vaccinated animals**

To determine the protective efficacy of gold compound-treated parasites (*Chapter 4*) or liposome vaccine formulations (*Chapters 5 and 6*), mice were rested for 4 weeks following the last vaccine dose and then challenged intravenously with 10⁵ *P. chabaudi* AS or Py17X pRBCs. The parasitaemia of challenged mice was monitored by collecting thin blood smears, which were stained with Giemsa for microscopic examination. Haemoglobin levels (HemoCue® 201+ Analyser), weights and clinical scores of challenged mice were recorded. Animals that showed severe clinical symptoms based on the clinical scoring criteria (Appendix 3) were euthanased by CO₂ inhalation.
2.3.3 Microscopic examination of blood films

Thin blood films were obtained by snipping the end of the mouse tail with scissors and a drop of blood was smeared onto labelled glass slides. The thin films were fixed with 100% methanol, air-dried and stained in 10% Giemsa (Merck) for 15 min. Microscopic examination was performed at 100 X magnification (oil immersion) counting at least 300 red blood cells for high parasitaemia and at least 20 fields for low parasitaemia (<1%) and negative films. Parasitaemia was calculated using the formula below:

\[
\% \text{ Parasitaemia} = \left( \frac{\text{Number of pRBCs}}{\text{Total RBCs counted}} \right) \times 100
\]

2.4 Methodologies utilised in studies to investigate anti-malarial activity of gold (I) phosphine compounds as potential drugs and malaria parasite-attenuating agents

2.4.1 Attenuation of rodent malaria parasites and inoculation

*Plasmodium chabaudi* AS infected blood was obtained by cardiac puncture of mice and blood was collected into heparinized tubes. Gold (I) phosphine compounds were diluted to 20 μM working stock solutions and were added to parasitized red cells (pRBCs) to obtain a final concentration of 1 or 2 μM in serum free RPMI-1640. pRBCs were incubated in a vented flask at 37 °C, 5% CO₂ for 80 mins, 2 h or 3 h, with gentle rotation of the flask every 30 min. Following incubation, the pRBCs were washed three times with RPMI-1640, cell counts were obtained using a haemocytometer and an immunizing dose was calculated. BALB/c mice were inoculated intravenously with 1 or 3 doses of 10⁶ gold compound-treated pRBCs. Control mice received gold compound-treated normal red blood cells (nRBCs) or
pRBCs treated with methanol (vehicle controls), diluted in serum-free RPMI-1640. Thin blood smears were collected every 2nd day post inoculation, stained with Giemsa and examined by microscopy to assess the infectivity of gold compound treated pRBCs.

2.4.2 DNA extraction and quantitative real time PCR

DNA extraction and qPCR were performed as described previously (Good et al., 2013). Briefly, samples for DNA extraction were obtained by collecting 20 µL of blood into 180 µL of 0.9% NaCl at different time points post inoculation. The samples were centrifuged at 11,000 g for 5 min and the red cell pellet stored at -80°C for DNA extraction. Using the High Pure PCR Template Preparation Kit (Roche), Genomic DNA (gDNA) was extracted by suspending the red cell pellet in 0.9% NaCl and then eluted in pre-warmed elution buffer. qPCR was performed using the Bio-Rad CFX96 Real-Time PCR System (Bio-Rad). A 68-base pair region of the 18S ribosomal RNA (18S rRNA) gene of *P. chabaudi* AS (GenBank DQ241815.1) was selected as the amplification target. DNA standards were prepared from *P. chabaudi* AS pRBCs to generate a standard curve for the PCR reaction and analyzed in a linear regression model using Bio-Rad CFX Manager 2.1 software (Bio-Rad). The standard curve, controls and test samples were all analyzed in triplicate. An assay was considered valid when the efficiency (E) was between 90% and 100% and an R² value less than 1.

2.4.3 In vivo anti-malarial activity of gold compounds on *P. chabaudi* AS

The *in vivo* anti-parasitic activity of gold compounds on *P. chabaudi* AS was assessed using the Peters 4-day test which measures the inhibitory effects following 4 days of drug administration (Peters, 1975). BALB/c mice were infected intravenously with 1 x 10⁶ *P. chabaudi* AS pRBCs. Gold drugs were dissolved in methanol, diluted
further in distilled water and an oral dosage of 3.7, 7.3, 10.9 and 14.7 µMol/kg for [Au(d2pype)2]Cl or 11.8 µMol/kg for auranofin was administered approximately 2 h post infection and then daily for 3 consecutive days. Thin blood smears were collected every 2nd day following inoculation with pRBCs, stained with Giemsa and examined by microscopy to assess the anti-malarial activity of gold compounds following 4 days of oral treatment.

2.5 Methodologies utilised in studies to investigate the immunogenicity and protective efficacy of liposomes containing inactivated Py17X blood-stage antigens

2.5.1 Collection and storage of P. yoelii 17X parasites for liposome preparation

Mice were infected intraperitoneally with P. yoelii 17X (Py17X) parasites. At approximately 30-40% parasitaemia, blood was collected by cardiac puncture, washed in PBS and a cell count was performed by trypan blue (0.1% w/v in PBS) exclusion using a haemocytometer. The concentration of sample in parasitized red blood cells per millilitre (pRBC/mL) loaded onto the counting chamber was calculated as:

\[
\text{Total pRBCs/mL} = \frac{\text{RBC count} \times \text{Dilution factor} (10^4) \times \text{Parasitaemia}}{100}
\]

The pRBCs were then stored at a concentration of 2 x 10⁸ at -80°C until required.

2.5.2 Depletion of red cell membrane antigen from Py17X parasites

Red cell membrane antigen was depleted from the parasite extract using the EasySep Mouse Streptavidin RapidSpheres™ Isolation Kit (STEMCELL™)
Materials and methods

Technologies) according to the manufacturer's instructions. Briefly, the pRBC suspension was subjected to 6 freeze-thaw cycles and re-suspended in PBS. The biotinylated TER-119 antibody (STEMCELL™ Technologies, Clone TER-119, Biotin) and rat serum were added and incubated at room temperature for 20 min. Next, Streptavidin-coated RapidSpheres™ (STEMCELL™ Technologies) were added and the suspension was incubated for 5 min. Immunomagnetic separation was performed by placing the tube containing the sample into a magnetic field (STEMCELL™ Technologies) and incubating for an additional 5 min at room temperature. The enriched parasite antigen suspension was poured off in one continuous motion into a new tube and aliquoted at the desired concentration based on parasite equivalence or protein estimation using the Pierce™ BCA Protein Assay Kit (Thermo Scientific™).

To confirm the removal of red cell membrane antigens and presence of parasite antigen following magnetic purification, 200 μL of the parasite extract was centrifuged for 10 min at 16,000 g. The pellet was subsequently stained with anti-TER-119 PE (eBioscience) and bisbenzimide hoechst (Sigma-Aldrich) for 20 min at room temperature and then washed in MACs buffer. Samples were acquired on a BD LSR Fortessa (BD Biosciences) and data analyzed using FlowJo software version 10.0 (Tree Star).

2.5.3 Preparation of Py17X parasite without depletion of red cell membrane antigen

Py17X parasite antigen was prepared as described previously (Giddam et al., 2016). Briefly, Py17X pRBCs were centrifuged at 400 g for 10 min, re-suspended in distilled water and incubated for 5 min at room temperature. The pRBCs were subsequently washed twice by centrifuging at 16,000 g for 10 min, suspended in cold PBS and
subjected to 6 freeze-thaw cycles. The resulting pellet was aliquoted at the desired concentration based on parasite equivalence or protein estimation using the Pierce™ Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Scientific™).

2.5.4 Protein estimation of parasite antigen

The parasite antigen protein concentration was determined using the Pierce™ BCA Protein Assay Kit (Thermo Scientific™) according to the manufacturer’s instructions. The concentration of the parasite extract was calculated from a standard curve.

2.5.5 Formulation of liposomes containing parasite antigen extract

Liposome formulations were prepared using the thin film hydration method described previously (Giddam et al., 2016; Szoka & Papahadjopoulos, 1980). Liposomes consisted of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC, Avanti polar lipids), dimethyldioctadecylammonium bromide (DDAB, Sigma-Aldrich) and cholesterol in the ratio of 5:2:1. Where indicated, 10 µg of a mannosylated lipid core peptide (also referred to as F3 from hereonin), 10 µg of Monophosphoryl 3-Deacyl Lipid (PHAD) (Avanti polar lipids) and 30 µg of the P. yoelii circumsporozoite (CS) peptide consisting of 4 repeats of glutamine-glycine-proline-glycine-alanine-proline (QGPGAP)₄ were added to the formulation. Both F3 and (QGPGAP)₄ had a lysine-serine-serine linker and a di-palmitic acid tail for anchoring into the liposome membrane. F3 was dissolved in methanol while all the other lipids mentioned above were dissolved in chloroform (9:1, v/v). The solvents were evaporated using a rotary evaporator (Heidolph™) under vacuum, forming a thin film at the base of the flask. Hydration was performed at 50°C using parasite antigen or PBS for empty liposomes. The average particle size (µM) and size
distribution of liposomes were measured using the Mastersizer 2000 (Malvern Instruments, England, UK).

2.5.6 Immunisations

2.5.6.1 BALB/c study

BALB/c mice (n=10 per group) were immunised subcutaneously on day 0, 14, and 28 with liposome formulations in a volume of 200 μL. The liposomes contained 2 x 10⁷ pRBC equivalent or 100 μg of parasite antigen determined using the Pierce™ BCA Protein Assay Kit (Thermo Scientific™). The parasite antigen was either depleted of red blood cell membranes as described in section 2.5.2 or not depleted of red blood cell membrane as described in section 2.5.3. Each liposome dose administered per mouse contained 500 μg of DPPC, 200 μg of DDAB, 100 μg of cholesterol and where indicated, 10 μg of F3 or PHAD or 30 μg of (QGPGAP)₄. Control mice were immunised with 200 μL of parasite extract without liposomes or liposomes without parasite antigen hydrated with PBS.

2.5.6.2 C57BL/6 study

C57BL/6 mice (n=10 per group) were immunised subcutaneously on day 0, 14, 28 and 35 with liposomes containing 10⁶ or 10⁷ pRBC equivalent red cell membrane-depleted antigens (section 2.5.2) or with red cell membranes (section 2.5.3) in a volume of 200 μL. Each liposome dose administered per mouse contained 500 μg of DPPC, 200 μg of DDAB, 100 μg of cholesterol and where indicated, 10 μg of F3 or PHAD. The additional dose administered on day 35 contained half the phospholipids, F3 or PHAD and parasite antigen. Control mice were immunised with 200 μL of liposomes without parasite antigen in PBS.
2.6 Methodologies utilised in studies to investigate immunogenicity and protective efficacy of liposomes containing inactivated *P. falciparum* blood-stage antigens

2.6.1 Culture of *P. falciparum* parasitized red blood cells

Frozen vials containing *P. falciparum* parasitized red blood cells (pRBCs) in glycerolyte solution (Baxter) were thawed and cultured as described previously (Stanisic et al., 2015). Briefly, a frozen vial containing 1 mL of pRBCs was immersed in a 37°C water bath until just thawed. In a class II biosafety cabinet, the vial was ethanol sterilized, dried and the contents transferred to a tube. One millilitre of pre-warmed 12% sodium chloride (NaCl) was added drop-wise with gentle shaking and the mixture incubated for 5 min at room temperature. Following the incubation, 10 mL of 1.6% NaCl was added drop-wise and the suspension centrifuged at 433 g for 5 min. The supernatant was discarded and 10 mL of 0.9% NaCl was added drop-wise to the pellet with gentle shaking. The tube was centrifuged again at 433 g for 5 min, supernatant discarded and the packed cell volume (PCV) estimated. The cell pellet was transferred to a tissue culture dish in complete medium (RPMI-1640 media (Gibco, Invitrogen Corporation) supplemented with L-glutamine, 25mM HEPES, 10% heat-inactivated human serum (Australian Red Cross Blood Service) and gentamicin (Gibco). Normal human red blood cells (Australian Red Cross Blood Service) were then added, adjusting the haematocrit to 5 %. The parasites were then cultured as described previously (Trager & Jensen, 1976) under standard culture conditions (5 % O₂, 5 % CO₂ and 90 % N₂) at 37°C. Parasite cultures were monitored regularly by thin blood films. These were stained with Giemsa and observed by microscopy to ascertain the parasitaemia and life-cycle stage. Mature parasite forms (trophozoites and schizonts) were used for preparation of whole
parasite antigen in liposomes and for \textit{in vitro} stimulation in splenocyte proliferation assays.

2.6.2  \textbf{Purification of trophozoites and schizonts}

The MACS® Vario system (Miltenyi Biotec) was utilised to purify mature stages from parasite cultures. Parasitized red blood cells were transferred from culture dishes to 50 mL tubes and centrifuged at 433 g for 5 min. To each 10-mL pellet volume, 40 mL of incomplete medium (RPMI-1640 medium supplemented with L-glutamine, 25 mM HEPES and gentamicin) was added. MACS CS columns (Miltenyi Biotec) were assembled according to the manufacturer’s instructions and equilibrated with 2 column volumes of pre-warmed incomplete medium. Using a sterile transfer pipette, the parasite culture suspension was loaded onto the column and allowed to flow through at a slow rate (approximately one drop per second). While still attached to the magnetic field, the column was washed using 30 mL of pre-warmed incomplete medium. The column was then detached from the magnetic field and washed with 15 mL of pre-warmed complete medium to elute the purified mature parasite stages. The eluent was centrifuged at 433 g for 5 min, supernatant discarded, and thin film prepared to establish the purity by microscopy. The purity was determined using the following calculation:

\[
\text{[Total infected RBC/ Total RBC] \times 100}
\]

The pellet was resuspended in pre-warmed complete medium and parasites counted by trypan blue exclusion using a haemocytometer. The total number of parasitized red blood cells (pRBCs) was calculated using the following formula, were RBC count being the number of RBCs in the big square (containing 25 small squares) of the haemocytometer.

\[
\text{Total pRBC count/mL} = \text{RBC count} \times \text{Dilution factor (10}^4\text{)} \times \text{Purity}
\]
The pRBCs were then stored at a desired concentration at -80°C until required.

2.6.3 Depletion red cell membranes from *P. falciparum* trophozoites

Red cell membranes were depleted from the *P. falciparum* trophozoites at >95% purity (as described in section 2.6.2) using the EasySep™ Human Glycophorin A Depletion Kit (STEMCELL™ Technologies) according to the manufacturer’s instructions. Briefly, the pRBC suspension was subjected to 6 freeze-thaw cycles and resuspended in PBS. The EasySep™ Human Glycophorin A depletion cocktail (STEMCELL™ Technologies) was added and incubated at room temperature for 15 min. Next, EasySep™ Magnetic positive selection nanoparticles (STEMCELL™ Technologies) were added and incubated for 10 min. Immunomagnetic separation was then performed by placing the tube containing the pRBC cell suspension into a magnetic field (STEMCELL™ Technologies) and incubating for an additional 10 min at room temperature. The enriched parasite antigen suspension was poured off in one continuous motion into a new tube and aliquoted at desired concentration based on parasite equivalence. To confirm the removal of red cell membranes and the presence of parasite antigen following magnetic purification, 200 μL of the parasite extract was centrifuged for 10 min at 16,000 g. The pellet was subsequently stained with anti- Glycophorin A FITC (Clone HI264, BioLegend) and bisbenzimide hoechst (Sigma-Aldrich) for 20 min at room temperature then washed in MACS buffer. Samples were acquired on a BD LSR Fortessa (BD Biosciences) and data analyzed using FlowJo software version 10.0 (Tree Star).
2.6.4 Preparation of *P. falciparum* parasite antigen without depletion of red cell membrane antigen

*Plasmodium falciparum*-infected red cells at the desired concentration based on parasite equivalence were subjected to 6 freeze-thaw cycles and resuspended in an appropriate volume of PBS ready for use in the liposome formulation.

2.6.5 Formulation of liposomes containing *P. falciparum* antigens

Liposomes were prepared using the thin film hydration method described previously (Giddam et al., 2016; Szoka & Papahadjopoulos, 1980). Liposomes consisted of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC, Avanti polar lipids), dimethyldioctadecylammonium bromide (DDAB, Sigma-Aldrich) and cholesterol in the ratio of 5:2:1. Where indicated, 10 μg of mannosylated lipid core peptide (referred to as F3 from hereon) or 10 μg of PHAD (Avanti polar lipids) were added to the formulation. F3 was attached to a lysine-serine-serine (KSS) spacer and a di-palmitic acid tail for anchoring into the liposome membrane. F3 was dissolved in methanol while all the other lipids mentioned above were dissolved in chloroform (9:1, v/v). The solvents were evaporated using a rotary evaporator (Heidolph™) under vacuum forming a thin film at the base of the flask. Hydration was performed at 50°C using parasite antigen or PBS for empty liposomes.

For preparation of lyophilized liposomes, hydration was performed using PBS 20mM (pH 7.2-7.4) containing 10% trehalose (w/w) and parasite antigen. The hydrated liposomes were aliquoted into scintillation glass vials and snap-frozen on dry ice then dissolved in acetone for 10 min. With the caps loosened, the frozen vials were transferred to a 500-mL freeze-dryer jar which was then connected to the freeze-dryer at -40°C and 0.1 millibar vacuum for 18 - 20 h. Following freeze-drying, the vials were tightly capped, sealed with parafilm and stored at 4°C until required.
Materials and methods

for immunisation. Prior to immunisation, lyophilized liposomes were rehydrated in 1 x PBS. The average particle size (µM) and size distribution of liposomes were measured using the Mastersizer 2000 (Malvern Instruments, England, UK).

2.6.6 Immunisations

BALB/c mice were immunised intravenously or subcutaneously on day 0, 14, and 28 with freshly prepared or lyophilized liposomes in a volume of 200 µL. The liposomes contained of $10^6$ or $10^7$ pRBC equivalent parasite antigens. The parasite extract was either depleted (section 2.6.3) or not depleted of red cell membrane antigen as described in section 2.6.4. Each liposome dose administered per mouse contained 500 µg of DPPC, 200 µg of DDAB, 100 µg of cholesterol and 10 µg of F3 or PHAD was also added. Control mice were immunised with liposomes without parasite antigen in a volume of 200 µL.

2.6.7 Serum binding assay

Sera (50 µL, 1/10 dilution) from immunised mice were incubated with human RBCs (0.2% haematocrit) for 30 min at room temperature. Cells were then incubated with 50 µL of goat anti-mouse IgG AlexaFluor-488 (1:500 dilution) (Invitrogen) for an additional 30 min. The cells were washed twice between each step with 1% FCS/PBS. Samples were then acquired on a BD LSR Fortessa (BD Biosciences) and data analyzed using FlowJo software version 10.0 (Tree Star).

2.6.8 In vivo T cell depletion

Mice were immunised following the 3-dose prime-boost regimen with a primary immunisation on day 0 followed by 2 boosts administered 2 weeks apart. Four weeks after the last immunisation, T cells were depleted with intraperitoneal injections of anti-CD4 (0.25 mg; GK1.5, Bio X Cell), anti-CD8 β (0.25 mg; clone 53.5.8, Bio X Cell), a combination of anti-CD4 and anti-CD8, or control rat IgG (0.25 mg;
Sigma-Aldrich). Antibodies were administered 2 days prior to challenge on day 0 and then every 4 days post-challenge on days 4, 8, 12 and 16. To establish successful T cell depletion, spleens were harvested from unchallenged mice on days 1 and 9 post-challenge and filtered through a 70 µM cell strainer using a sterile syringe plunger into a 50-mL tube. The harvested spleen cells were washed in complete RPMI medium and the red blood cells were lysed using Gey's lysis buffer for 5 min. Following this, spleen cells were washed with complete RPMI medium at 400 g for 5 min and resuspended in 10 mL of complete medium. One hundred microliters of cell suspension were then transferred to a 96-well U-bottomed plate and cells washed in 100 µL MACS buffer by centrifuging at 400 g for 5 min. The supernatant was discarded and 50 µL of anti-Mouse CD16/CD32 (BD Pharmingen™) was added to the pellet to block non-specific binding of antibodies. The plate was incubated on ice for 10 min and washed as described above in MACS buffer. A cocktail of antibodies containing anti-CD3 Molecular complex V450 (Clone 17A2, BD Horizon™), anti-CD4 V500 (Clone RM4-5, BD Horizon™) and anti-CD8 PerCP-Cy5.5 (Clone 53.6.7, BD Pharmingen™) was added to the cells and they were incubated in the dark on ice for 20 min. The cells were washed twice in MACS buffer and transferred into FACS tubes (Falcon) for acquisition on a BD LSR Fortessa (BD Biosciences). The data was analyzed using FlowJo software version 10.0 (Tree Star).

2.7 General methodologies utilised to examine the immunogenicity of liposome formulations in mice

2.7.1 Production of Py17X crude antigen for ELISA

To obtain crude parasite antigen, mice were infected with Py17X pRBCs. At peak parasitaemia (>40%), blood was collected by cardiac puncture, centrifuged twice in PBS at 1500 rpm for 10 min, supernatant was discarded and the PCV estimated. The
pRBCs were incubated with 0.01% saponin (Sigma-Aldrich) (1 mL of saponin per 0.2 mL PCV) at 37°C for 20 min, with mixing at 5 min intervals. The cell suspension was washed twice in PBS and the resulting pellet was sonicated for 1 min at 90% output on ice using VibraCell™ (Sonics®). The sonication was repeated 5 times and the cell suspension subsequently centrifuged at 10,000 rpm for 10 min. The supernatant was collected and dialyzed using a 3,500 MW cassette (Thermo Scientific) overnight in PBS at 4°C. The protein concentration of the dialyzed sample was then estimated using the Pierce™ BCA protein assay kit (Thermo Scientific). The same batch of crude antigen was utilized for all experiments.

2.7.2 Production of *P. falciparum* 7G8 crude antigen for ELISA

To obtain crude antigen, parasites were cultured as described in section 2.2.4 until a high parasitaemia (5-10%) was achieved. The blood culture was then collected into 50 mL tubes and centrifuged at 2,500 rpm for 10 min, supernatant was discarded and the packed cell volume (PCV) estimated. The pRBCs were incubated in twice the PCV of 0.15% saponin /RPMI HEPES (Sigma-Aldrich) for 20 min on ice. The cell suspension was washed twice in cold PBS by centrifuging at 3,000 rpm for 10 min, supernatant was discarded, the sample was resuspended in cold PBS and subjected to 3 freeze-thaw cycles. The sample was centrifuged at 11,000 g for 5 min, resuspended in PBS and sonicated for 1 min at 30% output on ice using VibraCell™ (Sonics®). The sonication was repeated 5 times and the cell suspension subsequently centrifuged at 11,000 rpm for 10 min. The supernatant was collected and dialyzed using a 3,500 MW cassette (Thermo Scientific) overnight in PBS at 4°C. The protein concentration of the dialyzed sample was then estimated using the Pierce™ BCA protein assay kit (Thermo Scientific). The same batch of crude antigen was utilized for all experiments.
2.7.3 Production of control sera for ELISA

To obtain hyperimmune sera, which were used as a positive control in ELISAs, mice were infected intraperitoneally with Py17X pRBCs. At peak parasitaemia, they were treated with 2 mg pyrimethamine for 4 days and left for 3 weeks before the next infection. Sera were collected from these mice after 3 rounds of infection and drug cure and was considered 'Hyperimmune'. Negative control sera were collected from naive uninfected mice.

2.7.4 Detection of serum antibodies by ELISA

For detection of antibodies in vaccinated mice, 96-well flat-bottomed immunoplates (MaxiSorp Nunc) were coated with 10 μg/mL of Py17X crude antigen or 5 μg/mL of C(QPGAP)₄ peptide or 5 μg/ml P. falciparum 7G8 crude antigen in bicarbonate coating buffer (pH 9.6) overnight at 4°C. Plates were subsequently blocked with blocking buffer (10% skim milk in PBS/0.05% Tween-20 (Chem-supply) for 2 h at room temperature. Wells were washed twice (0.05% Tween-20 in PBS), 100 μL of serially diluted sera was dispensed into each well and incubated for 2 h at 37°C. The plates were then washed 5 times and biotinylated anti mouse-IgG (Invitrogen) (diluted 1:3000 in blocking buffer) was added and incubated for 1 h at room temperature in the dark. Wells were washed 5 times to remove unbound antibodies, tetramethylbenzidine (TMB) (OptEIA™ BD Biosciences) was added and plates incubated at room temperature for 30 min. 1 N sulphuric acid was added to each well to stop the reaction. Absorbance was determined at a wavelength of 450 nm using a xMark™ (BIO-RAD) microplate spectrophotometer and parasite-specific antibody titres were calculated as three standard deviations above naïve control sera.
2.7.5 Investigation of peripheral blood antigen-experienced T cells

Circulating antigen-experienced T cells were assessed according to previously published protocols (Butler et al., 2012; Butler et al., 2011). Five to ten drops of blood were collected via the submandibular vein into an eppendorf tube containing 1 mL of 5 mM EDTA (Invitrogen) in PBS and centrifuged at 400 g for 5 min. The supernatant was discarded, and 2 red blood cell lysis steps were performed by resuspending the cell pellet in ACK lysis buffer (150 mM NH₄Cl, 1 mM KHCO₃, 0.1 mM MgNa₂ EDTA) and incubating for 5 min at room temperature. Samples were centrifuged between these lysis steps. The cell pellet was resuspended in 100 µL of MACS buffer (PBS [pH 7.2] without Ca++ and Mg++, 0.5% w/v bovine serum albumin (BSA), 2 mM EDTA) and transferred into a 96 well U-bottom plate. The plate was centrifuged at 400 g for 5 min, supernatants discarded and 50 µL of anti-mouse CD16/CD32 (BD Pharmingen™) was added to block non-specific binding of antibodies. Plates were incubated on ice for 10 min and washed as described above. A cocktail of antibodies containing anti-CD3 molecular complex V450 (Clone 17A2, BD Horizon™), anti-CD4 V500 (Clone RM4-5, BD Horizon™), anti-CD8 PerCP-Cy5.5 (Clone 53.6.7, BD Pharmingen™), anti-CD11a FITC (Clone 2D7, BD Pharmingen™) and anti-CD49d PE (Clone 9C10 (MFR4.B), BD Pharmingen™) was added and the samples were incubated in the dark on ice for 20 min. The cells were washed twice in MACS buffer and transferred into FACS tubes (Falcon) for acquisition on a BD LSR Fortessa (BD Biosciences). The data were analyzed using FlowJo software version 10.0 (Tree Star).

2.7.6 Assessment of spleen cell proliferation and cytokine analysis

Spleens were extracted from mice into complete RPMI medium (RPMI 1640, supplemented with 1% L-glutamine, 10% foetal bovine serum, 0.1% 2-
Materials and methods

 mercaptoethanol and 1% penicillin-streptomycin) and filtered through a 70 µM cell strainer using a sterile syringe plunger. The harvested spleen cells were washed in complete RPMI medium and the RBCs were lysed using Gey’s lysis buffer. Following lysis, spleen cells were washed with complete RPMI medium at 400 g for 5 min and resuspended at $5 \times 10^6$ cells/mL in complete medium. Spleen cells were subsequently seeded into 96-well U-bottomed plates (5 × 10⁵ cells/well) and cultured for 72 h at 37°C and 5% CO₂ in the presence of complete medium (negative control), naïve mouse RBCs (5 × 10⁶ nRBC/mL) (nRBCs, negative control), Py17X pRBCs (5 × 10⁶ pRBC/mL), normal human RBCs (5 × 10⁶ nRBC/mL), purified *P. falciparum* pRBCs (5 × 10⁶ pRBC/mL) or concanavalin A (10μg/mL) (Con A, positive control) (Sigma-Aldrich) in triplicate. After 54 h, culture supernatants were harvested for cytokine analysis. To assess proliferation, splenocytes were pulsed with 1 μCi of 3[H]-thymidine (PerkinElmer)/well and cultured for an additional 18 h. Plates were then frozen at -80°C, and later thawed for harvesting onto 1450 MicroBeta glass fibre filters (Wallac, USA). The filters were air dried and ³[H]-thymidine incorporation measured using a Microbeta2 Microplate Counter (Perkin Elmer) to obtain radioactivity corrected counts per minute (CCPM) values. Soluble cytokine analysis was performed using the Th1/Th2/Th17 CBA kit (BD Biosciences) with a slight modification of the manufacturer’s instructions as described previously (Raja et al., 2016). Ten microliters of culture supernatants from pooled triplicate wells was incubated with a master mix containing 2 µL of each capture bead and an equal volume of PE detection reagent in a 96 well V-bottom plate (Sarstedt) for 2 h at room temperature in the dark. Plates were washed by centrifugation at 800 g for 5 min, resuspended in wash buffer and transferred into FACS tubes for acquisition of on a BD LSR Fortessa flow cytometer (BD Biosciences).
The acquired data were analyzed using FCAP Array software version 1.0.1 (BD Biosciences).

2.8 Statistical data analysis

All statistical analyses were conducted using GraphPad Prism software version 6 (GraphPad Software, Inc., CA). IC$_{50}$ values were determined using a nonlinear regression analysis. All the data were expressed as arithmetic mean ± standard error of the mean (SEM) unless stated otherwise. Data were analyzed using unpaired Mann-Whitney $U$ test or unpaired $t$ test to compare study groups and controls. A $P$ value of $<0.05$ was considered significant for all statistical analyses.
Chapter 3: Collection of *P. falciparum* isolates from human volunteers with malaria
3.0 Introduction

Controlled human malaria infection (CHMI) studies are critical in the preliminary evaluation of vaccine candidates prior to deployment for further testing in large-scale field-based studies due to the significant costs involved. CHMI initiated via mosquito bite or direct venous inoculation of infected sporozoites has been successfully used in the evaluation of pre-erythrocytic vaccine candidates (Roestenberg et al., 2009; Roestenberg et al., 2011; Seder et al., 2013; Lyke et al., 2017). However, blood-stage CHMI studies to assess the efficacy of blood-stage vaccines have been restricted to a handful of trials and volunteers mainly due to regulatory issues and availability of the inoculum. Most of the published studies (Lawrence et al., 2000; Pombo et al., 2002; Duncan et al., 2011; Payne et al., 2016) have depended on the single inoculum obtained over 20 years ago (Cheng et al., 1997). Given that the inoculum is finite, there is need to prepare new inoculum by obtaining parasites from malaria infected volunteers as described by Cheng et al., 1997 or by culturing parasites in vitro and subsequently cryopreserving aliquots of the cultured parasites. Our laboratory has recently developed 2 cultured blood-stage malaria cell banks, derived from *P. falciparum* isolates NF54 and 7G8 in a Good Manufacturing Practice (GMP)-compliant suite (Stanisic et al., 2015) and these parasite banks have been utilised in experimental controlled human malaria infection studies in malaria naïve individuals.

In this chapter, blood was collected as a source of malaria parasites from volunteers living in an endemic country, Uganda, with the ultimate aim of establishing parasite cell banks for use in CHMI studies as well as whole parasite malaria vaccine development.
In the laboratory, these parasites can be culture-adapted and used in immunological assays to assess the breadth of immune responses induced by vaccine candidates in pre-clinical and early phase clinical trials. Additionally, following culture adaptation, these parasites can be developed into Good Manufacturing Practices (GMP)-grade malaria cell banks (Stanisic et al., 2015) and used in CHMI studies. Data from such studies are critical in the optimisation of vaccine design and regimen prior to testing in expensive large-scale field trials. The ultimate test of vaccine efficacy is the ability to protect against infection from multiple species/strains of Plasmodium. An ideal whole parasite vaccine could therefore be formulated by combining circulating parasite isolates from regions of high malaria endemicity. Since individuals living in endemic regions are likely to be exposed to many different malaria parasite species/strains, these collected clinical isolates can thus be used in preparation of candidate whole parasite vaccines.

3.1 Results

3.1.1 Volunteer recruitment and baseline characteristics

A total of 14 volunteers were recruited and screened from patients presenting at Uganda Virus Research Institute (UVRI) study clinic, Our Lady of Consolata Kisubi hospital and Katabi military hospital all located in Wakiso district, Uganda. Of these, 8 volunteers met the eligibility criteria (Appendix 2) and were enrolled. The main reason for exclusion was a negative blood smear except for one female volunteer who had a positive hepatitis C rapid diagnostic test. She was referred for further testing at the National referral hospital. Prior to enrolment, data on baseline blood parameters, parasitaemia and parasite species were collected, and these are
summarized in Table 3.1. All enrolled volunteers were confirmed to have a *P. falciparum* malaria infection.

### Table 3.1. Characteristics of enrolled volunteers.

<table>
<thead>
<tr>
<th>Volunteer ID</th>
<th>Blood group</th>
<th>Haemoglobin (g/dL)</th>
<th>WBC count per μL (x10^3)</th>
<th>Parasites per 200 WBC</th>
<th>Parasites per μL*</th>
</tr>
</thead>
<tbody>
<tr>
<td>UGMCB-004</td>
<td>O RhD+</td>
<td>13.2</td>
<td>4.3</td>
<td>10</td>
<td>215</td>
</tr>
<tr>
<td>UGMCB-007</td>
<td>O RhD+</td>
<td>14.7</td>
<td>4.3</td>
<td>52</td>
<td>1,118</td>
</tr>
<tr>
<td>UGMCB-009</td>
<td>O RhD+</td>
<td>15.9</td>
<td>6.5</td>
<td>198</td>
<td>6,435</td>
</tr>
<tr>
<td>UGMCB-010</td>
<td>O RhD+</td>
<td>14.7</td>
<td>4.1</td>
<td>86</td>
<td>1,763</td>
</tr>
<tr>
<td>UGMCB-011</td>
<td>O RhD+</td>
<td>13.2</td>
<td>4.3</td>
<td>190</td>
<td>4,085</td>
</tr>
<tr>
<td>UGMCB-012</td>
<td>B RhD-</td>
<td>16.3</td>
<td>3.5</td>
<td>87</td>
<td>1,523</td>
</tr>
<tr>
<td>UGMCB-013</td>
<td>O RhD+</td>
<td>15.3</td>
<td>4.3</td>
<td>248</td>
<td>5,332</td>
</tr>
<tr>
<td>UGMCB-014</td>
<td>A RhD+</td>
<td>11.3</td>
<td>7.2</td>
<td>1660</td>
<td>59,760</td>
</tr>
</tbody>
</table>

*Parasitaemia was determined by microscopy and results were reported as number of pRBCs per 200 white blood cells (WBC) counted. Parasites per μL was then determined as a product of the total WBC per μL (x10^3).*

Following blood draw, there were no adverse events reported and volunteers received anti-malarial treatment according to Ministry of Health malaria treatment guidelines. All volunteers except one (UGMCB-007) reported for the follow-up visit 7 days later in good health and no adverse events were recorded.

#### 3.1.2 *In vitro* culture and drug susceptibility of clinical isolates

The collected blood was processed as described in *Chapter 2 section 2.2.3*, cryopreserved and shipped to Griffith University, Institute for Glycomics for culture adaptation. Blood from volunteers UGMCB-009 and UGMCB-013 was cultured *in*
vitro under standard culture conditions described in Chapter 2 section 2.6.1. Fig. 3.1 shows representative images of healthy cultured parasites obtained from volunteer blood.

![Giemsa-stained cultured parasites obtained from volunteer blood. (A) Ring stages and (B) late stages (trophozoites, schizonts and gametocytes).](image)

The standard tritiated ($^3$H) hypoxanthine uptake assay (Desjardins et al., 1979) was utilised to assess the drug susceptibility of the clinical isolates (Chapter 2 section 2.2.4). Based on established ex vivo resistance IC$_{50}$ cut off values of $\geq 100$ nM for chloroquine (Le Bras & Ringwald, 1990) and $\geq 150$ nM for lumefantrine a component of Riamet (Basco et al., 1998), clinical isolates UGMCB-009 and UGMCB-013 were susceptible to chloroquine [23.4 nM (22.0 – 24.9) and 49.2 nM (26.4 - 91.9)] and Riamet (artemether/lumefantrine) [8.1 nM (7.9 – 8.3) and 7.54 nM (5.6 - 10.1)] (Fig. 3.2 and Table 3.2). These IC$_{50}$ values were comparable to those obtained for reference laboratory-adapted strains, chloroquine-sensitive 3D7 [14.4 nM (14.0 - 14.8)] and 7G8 [6.4 nM (4.5 - 9.4)], which were used as controls for chloroquine and Riamet respectively (Fig. 3.2 and Table 3.2).
Collection of *P. falciparum* clinical isolates

Table 3.2. *In vitro* anti-malarial activities (IC$_{50}$ nM) of chloroquine and Riamet against *P. falciparum* strains UGMCB-009, UGMCB-013, 3D7 and 7G8.

<table>
<thead>
<tr>
<th>Drug</th>
<th><em>P. falciparum</em> UGMCB-009</th>
<th><em>P. falciparum</em> UGMCB-013</th>
<th><em>P. falciparum</em> 3D7</th>
<th><em>P. falciparum</em> 7G8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroquine</td>
<td>23.4 (22.0 - 24.9)</td>
<td>49.2 (26.4 - 91.9)</td>
<td>14.4 (14.0 - 14.8)</td>
<td>ND</td>
</tr>
<tr>
<td>Riamet</td>
<td>8.1 (7.9 - 8.3)</td>
<td>7.54 (5.6 - 10.1)</td>
<td>ND</td>
<td>6.4 (4.5 - 9.4)</td>
</tr>
</tbody>
</table>

* ND: not determined

Figure 3.2 Log dose response curves of clinical isolates to chloroquine and Riamet. (A) UGMCB-009, (B) UGMCB-013, (C) chloroquine-sensitive *P. falciparum* 3D7 and (D) *P. falciparum* 7G8. Drugs were added to synchronized ring-stage parasites and incubated for 24 h. $^3$H-hypoxanthine was added, and plates were incubated for an additional 24 h. Drug susceptibility was measured as inhibition of $^3$H-hypoxanthine incorporation into parasite DNA. Data are expressed as mean ± SEM.
3.3 Discussion

The data presented here has shown that parasites collected from human volunteers (UGMCM-009 and UGMCM-013) can be cultured in vitro, and preliminary drug susceptibility characterisation indicates that they are sensitive to chloroquine and Riamet. Additional drug sensitivity studies of these isolates to gold(I) phosphine compounds auranofin and [Au(d2pype)2]Cl, have been performed and are described in Chapter 4 of this thesis. These compounds were tested as potential parasite attenuating agents for malaria vaccine and drug development. However, further characterisation on a larger panel of established anti-malarial drugs will be required especially if these isolates are to be released for use in CHMI studies. These data will guide the selection of the most appropriate prophylactic drug following infection with these isolates in future CHMI studies.

Following successful culture, mature parasite forms (trophozoites, schizonts) of UGMCM-009 were purified as described in Chapter 2, section 2.6.2 and used to examine the breadth of strain-specific immune responses following immunisation with mannosylated liposomes in Chapter 5. Given that the lack of strain/species-transcending immunity presents a major bottleneck to malaria vaccine efficacy, these clinical isolates will be valuable reagents for preliminary evaluation of malaria vaccine candidates prior to deployment for further testing in endemic countries. Additionally, these parasite isolates will be important in the development of future P. falciparum strain-specific whole parasite vaccines.
Chapter 4: Gold (I) phosphine compounds as parasite attenuating agents for malaria vaccine and drug development
Statement of contribution to co-authored published paper

This chapter includes a co-authored paper titled “Gold (I) phosphine compounds as parasite attenuating agents for malaria vaccine and drug development”.

The bibliographic details of the published co-authored paper, including all authors, are:


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My contribution to the paper involved:

Designing and performing all experiments, collection of clinical study data and volunteer recruitment, data analysis and interpretation and writing the original manuscript.

(Signed)

Aloysious Ssemaganda

(Date) 22/02/2018

Signature redacted

(Countersigned)

Corresponding author/Supervisor: Prof. Michael F. Good

(Date) 22/01/15

Signature redacted
Preface

This chapter, is a manuscript published in *Metallomics, 2018, DOI: 10.1039/C7MT00311K*. Herein, the utility of gold(I) phosphines, which act by inhibiting inflammatory pathways as well as redox enzymes, was examined as potential parasite attenuating agents for malaria vaccine and drug development.
Gold (I) phosphine compounds as parasite attenuating agents for malaria vaccine and drug development†


Here, the anti-malarial activity of two gold(I) phosphine compounds auranofin and [Au(d2pype)]Cl (where d2pype is 1,2-bis(di-2-pyridylphosphino)ethane), were examined to inform their use as potential drugs and malaria parasite-attenuating agents. In vitro, the gold compounds were active against Plasmodium falciparum and P. knowlesi as well as the rodent parasite P. chabaudi AS. Attenuation of the parasite was observed when mice were inoculated with P. chabaudi AS infected red blood cells treated in vitro with [Au(d2pype)]Cl (1 or 2 µM) or auranofin (2 µM) for 2 or 3 h. Quantitative PCR data showed persistence of low levels of parasite DNA up to 8 days post inoculation. In some experiments, there was microscopically detectable parasitaemia following inoculation which subsequently cleared. Following 1 or 3 doses of gold compound-treated parasitized red blood cells (pRBCs), protection was not observed when these mice were subsequently challenged with wild type P. chabaudi AS. In experiments where microscopically detectable parasitaemia were observed following in vivo inoculation, mice were subsequently fully protected against a challenge infection with wildtype parasites. In an infect-and-treat rodent model, the gold compounds were unable to inhibit P. chabaudi AS growth in vivo when administered orally. Gold compounds act via the inhibition of antioxidant systems which are critical in the pathogen’s survival from attack by the host oxidants. In vitro, they directly inhibit the parasite thoredoxin reductase, hence the observed suppressive activity. On the other hand, in vivo, the gold compounds may not be readily available for absorption and thus pharmacokinetic studies will be required to further examine drug bioavailability following administration. With structural differences in redox mechanisms of P. falciparum and the human host being identified, gold compounds can be better designed to more efficiently target and selectively inhibit the parasite.

Significance to metallomics
The unique chemistry of gold(I) offers the potential for the design of potent inhibitors of thiol:seleno-containing targets in diseases including cancer and parasitic infections. Previously we have shown that by bidentate phosphine ligand design, selectivity for cancer cells over normal cells is achieved by a mechanism involving inhibition of thiodreoxidin reductase. Here we demonstrate that [Au(d2pype)]Cl exhibits more potent anti-malarial activity compared to auranofin against a variety of Plasmodium parasites including P. falciparum clinical isolates and chloroquine resistant strains. The unusual blood-stage Plasmodium parasite attenuating properties are exploited in a novel strategy for malaria vaccine development, as an alternative to the use of DNA-binding drugs in a clinical trial currently underway.

☆☆ Contributed equally to this work.

RSC Product Identification Number: 76

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Metalomic
Gold (I) phosphine compounds as parasite attenuating agents

Introduction

Malaria remains a serious health problem with transmission still occurring in over 90 countries. The emergence of resistance to the currently available drugs has led to an urgent need for new anti-malarial drugs, as well as the development of a safe and affordable vaccine.

The use of gold complexes for treatment of ailments, (termed as “chrysotherapy”), has a rich therapeutic history, but gold compounds have only been used clinically for the effective treatment of rheumatoid arthritis. The molecular mechanisms of action of gold compounds have been mainly elucidated based on their interaction with biological targets such as thiol and selenol containing proteins and enzymes. Auranothen and other gold-based compounds are known to be active in a variety of parasitic diseases by inhibition of enzymes identified as drug targets, for example thioredoxin glutathione reductase in schistosomiasis and trypanothione reductase in leishmaniasis.

In Plasmodium parasites, the causative agents of malaria, the selenoproteome is highly conserved and has been well characterized as an attractive drug target. Malaria parasites are prone to oxidative stress from reactive oxygen and nitrogen species in the midgut of the mosquito and within the human host. As a survival mechanism, malaria parasites have developed an antioxidant network composed of the thioredoxin and glutathione systems to maintain a balanced reduced environment. Specifically, Plasmodium falciparum thioredoxin reductase (PfTrxR) counteracts oxidative stress by directly detoxifying oxidants such as hydrogen peroxide but also by maintaining the antioxidant capacity of ascorbate, lipid acid and lipoprotein. Additionally, reduced PfTrxR also utilizes peroxiredoxins to detoxify peroxides. Therefore, chemical compounds with the ability to inhibit PfTrxR could function as potential drug candidates or parasite-attenuating agents for whole parasite vaccine development.

Several studies have reported gold compounds to be potent inhibitors of thioredoxin reductase and have observed rapid accumulation within red blood cells, the host cell of erythrocytic-stage malaria parasites. Sanella et al., observed that PfTrxR is inhibited by the gold-based drug, auranothen, in vitro. Additionally, computational modelling studies have shown that auranothen binds to PfTrxR indicating that it is a key drug target to inhibit P. falciparum growth.

The limited clinical use of gold compounds has been attributed mainly to toxicity and poor chemical stability, in particular as a result of their ability to bind indiscriminately to protein thiols. However, with increased understanding of the molecular mechanisms of action of more stable and less toxic gold compounds have been developed, hence the renewed interest as anti-cancer and anti-microbial agents.

In previous work we have shown that, when compared to the parent bis-chelated Au(i) phosphine compound, analogs in which the phenyl substituents are replaced with 2-pyridyl groups exhibit intermediate lipophilicity, increased selectivity towards tumour cells and reduced host toxicity. Moreover, selectivity for cancer cells over normal cells is achieved by a mechanism involving inhibition of thioredoxin reductase. However, the anti-malarial activity of these compounds has not yet been investigated.

A novel strategy for vaccine development has recently been undertaken utilizing DNA-binding drugs to attenuate blood-stage Plasmodium parasites. Following inoculation of mice, protective species transcribing immune responses were observed, underpinning the utility of this approach. In addition, clinical trials using P. falciparum-attenuated with tafarnamycin A (TEA) are currently underway. However, DNA-binding drugs irreversibly alkylation DNA and are potentially genotoxic. Therefore, there is a need to screen new attenuating compounds, utilising different mechanisms of action to further facilitate vaccine development using this approach.

In this study, both auranothen and the bis-chelated Au(i) 2-pyridyl phosphine complex, [Au(d2pyppy)2]Cl (Fig. 1) were investigated as parasite attenuating agents and potential anti-malarial drugs.

Materials and methods

Drugs

[Au(d2pyppy)2]Cl·3H2O was synthesized by a modification of the published procedure using [AuCl(SMe)2] as precursor and 1,2-bis(di-2-pyridylphosphino)ethane (d2pyppy) obtained from Strem Chemicals Inc. Earlier studies have shown that the 1:2 adduct of gold(i) chloride with d2pyppy exists as a dimer in the solid state but in dilute solution is predominantly monomeric [Au(d2pyppy)2]Cl. Auranofin and chlororoquine diphosphate were purchased from Sigma-Aldrich. Riemet, a combination of artemether and lumefantrine, was obtained from Novartis Pharmaceuticals. Gold compounds were dissolved in methanol, chlororoquine diphosphate in water and Riemet in dimethyl sulfoxide (DMSO) to make 2 mM stock solutions. Prior to use, drugs were diluted in RPMI-1640 media (Gilbo, Invitrogen Corporation).
supplemented with L-glutamine, HEPES, 10% heat inactivated human serum and gentamicin (Gibco) for in vitro assays with *P. falciparum* and *P. knowlesi* parasites.

Collection clinical isolates and human ethics approvals

Volunteers UGMCB-009 and UGMCB-013 were recruited from individuals who presented to health centres located in Entebbe, Uganda, with a confirmed diagnosis of malaria between January and March 2016. Individuals willing to participate were invited for eligibility screening at the UVRI-IAVI HIV Vaccine Program research clinic based at Uganda Virus Research Institute in Entebbe. Prior to conducting any study procedures, informed consent was obtained from each volunteer. Volunteers that met the eligibility criteria were asked to donate blood as a source of malaria parasites. The blood was leukodepleted, cryopreserved and shipped to the Institute for Glycomics, Griffith University. This work was approved by the Uganda Virus Research Institute Research and Ethics Committee (GC/127/15/12/535), Uganda National Council for Science and Technology (HS1962) and the Griffith University Human Research Ethics Committee (GU: 2016/031).

Malaria parasites

Clinical *P. falciparum* isolates from Uganda (UGMCB-009, UGMCB-013), *P. falciparum* strains 3D7 and 7G8 laboratory lines and *P. knowlesi* A1H1.1 were used to test the in vitro anti-malarial activity of [Au(Ⅱ)pypp]Cl on human malaria parasites. All animal experiments were performed using *P. chabaudi* AS mouse adapted parasites.

Animals and ethics statement

All animal experiments were performed in accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes 8th edition (2013) and approved by the Griffith University Animal Ethics Committee. Four to six-week old female BALB/c (H-²) and BALB/c congenic SCID (H-²) mice were purchased from the Animal Resource Centre (Willaton, Western Australia) and housed in a pathogen-free environment in the Griffith University Animal Facility. Following challenge with *P. chabaudi* AS, animals were monitored using an observation criteria and clinical scorecard. Animals exhibiting severe distress such as decreased activity, impaired movement and poor grooming were euthanized according to Griffith University animal facility procedures.

In vitro anti-malarial activity of gold compounds on *P. falciparum* and *P. knowlesi*

The standard tritiated ([H] hypoxanthine uptake assay was utilized to assess the anti-malarial activity of the gold compounds. Prior to addition of the drugs, *P. falciparum* cultures were synchronized using α-sorbitol selection. As *P. knowlesi* manifests an asynchronous 24 hour cycle, synchronization was not attempted. Parasite cultures were sub-cultured to 2% haematocrit, 1% parasitemia for *P. falciparum* and 0.25% parasitemia for *P. knowlesi* A1H1.1 and were exposed to 10 serial dilutions of compounds starting at 2 μM in 96 well flat-bottom cell culture plates under standard culture conditions (37 °C, 5% CO₂, 5% O₂, 90% N₂) for 24 h. [H]Hypoxanthine (0.5 μCi per well) was added and plates were incubated for an additional 24 h. Plates were frozen overnight at -80 °C, and later thawed for harvesting onto 1450 MicroBeta glass fibre filters (Wallac, USA). The filters were air dried and [H]Hypoxanthine uptake was measured using a Microbeta2 Microplate Counter (Perkin Elmer) to obtain radioactivity counts per minute (CPM) values. Log dose concentration curves were generated from normalized counts (CPM) and IC₅₀ values were obtained using non-linear regression analysis in GraphPad Prism Software.

Attenuation of rodent malaria parasites and inoculation

*Plasmodium chabaudi* AS infected blood was obtained by cardiac puncture of mice and collection of blood into heparinised tubes. Gold compounds were diluted to 20 μM working stock solutions and were added to parasitized red cells (pRBCs) to obtain a final concentration of 1 or 2 μM in serum free RPMI-1640. They were incubated in a ventilated flask at 37 °C, 5% CO₂ for 60 min, 2 h or 3 h, with gentle rotation of the flask every 30 min. Following incubation, the pRBCs were washed three times with RPMI-1640, cell counts were obtained using a haemocytometer and an immunizing dose was calculated. BALB/c mice were inoculated intravenously with 1 or 3 doses of 1 × 10⁷ gold compound-treated pRBCs. Control mice received gold compound-treated normal red blood cells or pRBCs treated with methanol (vehicle controls), diluted in serum-free RPMI-1640. Thin blood smears were collected every 2nd day post inoculation, stained with Giemsa and examined by microscopy to assess the infectivity of gold compound treated pRBCs.

Malaria parasite challenge infection of mice

To determine the protective efficacy of the treated parasites, mice that did not develop parasitemia following inoculation with 1 or 3 doses of gold compound-treated pRBCs were rested for 4 weeks. *P. chabaudi* AS pRBCs were collected by cardiac puncture from an infected BALB/c mouse and a challenge inoculum was prepared in PBS. 1 × 10⁷ pRBCs were administered to each mouse intravenously. The parasitemia of challenged mice was monitored by collecting thin blood smears, which were stained with Giemsa for microscopic examination. All animals that showed severe clinical symptoms were euthanized by CO₂ inhalation.

DNA extraction and quantitative real time PCR

DNA extraction and qPCR were performed as described previously. Briefly, samples for DNA extraction were obtained by collecting 20 μL of blood into 180 μL of 0.9% NaCl at different time points post inoculation. The samples were centrifuged at 11,000 g for 5 min and the red cell pellet stored at -80 °C for DNA extraction. Using the High Pure PCR Template Preparation Kit (Roche), Genomic DNA (gDNA) was extracted by suspending the red cell pellet in 0.9% NaCl and eluted in pre-warmed elution buffer.
qPCR was performed using the Bio-Rad CFX96 Real-Time PCR System (Bio-Rad). A 68-base pair region of the 18S rRNA (18S rRNA) gene of P. chabaudi AS (GenBank DQ418181.1) was selected as the amplification target.

DNA standards were prepared from P. chabaudi AS pRBCs to generate a standard curve for the PCR reaction and analyzed in a linear regression model using Bio-Rad CFX Manager 2.1 software (Bio-Rad). The standard curve, controls and test samples were all analyzed in triplicate. An assay was considered valid when the efficiency (E) was between 90% and 100% and an R² value less than 1.

In vitro anti-malarial activity of gold compounds on P. chabaudi AS

The in vitro anti-parasitic activity of gold compounds on P. chabaudi AS was assessed using the Peters 4 day test which measures the inhibitory effects following 4 days of drug administration. BALB/c mice were infected intravenously with 1 × 10⁶ P. chabaudi AS infected red cells. Gold drugs were dissolved in methanol, diluted further in distilled water and an oral dosage of 3.7, 7.3, 10.9 and 14.7 μmol kg⁻¹ for [Au(d2pyppe)₂]Cl or 11.8 μmol kg⁻¹ for auranofin which was administered approximately 2 h post infection and then daily for 3 consecutive days. Thin blood smears were collected every 2nd day from the day of infection, stained with Giemsa and examined by microscopy to assess the anti-malarial activity of gold compounds following 4 days of oral treatment.

Statistical data analysis

All statistical analyses were conducted using GraphPad Prism software version 6 (GraphPad Software, Inc., CA). IC₅₀ values were determined using a nonlinear regression analysis. Data of parasitemia curves was expressed as arithmetic mean ± standard error of the mean (SEM).

Results

In vitro anti-malarial activity of gold compounds on P. falciparum and P. knowlesi

Both [Au(d2pyppe)₂]Cl and auranofin were active against the chloroquine-sensitive P. falciparum 3D7, chloroquine-resistant P. falciparum 7G8, P. falciparum clinical isolates UGMCB-009 and UGMCB-013, and P. knowlesi (Table 1 and Fig. S1, ESI†). Chloroquine and Riamet⁴¹ were used as controls. [Au(d2pyppe)₂]Cl demonstrated higher anti-malarial activity compared to auranofin with a 1.2-fold difference against 3D7, 7-fold difference against 7G8, 2.5-fold difference against UGMCB-009, 17.5-fold difference against UGMCB-013 and a 13-fold difference against P. knowlesi A1H.1. Strikingly, [Au(d2pyppe)₂]Cl was as active as chloroquine against P. knowlesi with IC₅₀ values of 3.5 nM and 4.6 nM, respectively.

Attenuation of P. chabaudi AS infected red blood cells with gold compounds

Having observed anti-malarial activity against P. falciparum and P. knowlesi A1H.1, we further assessed the attenuating effects of the gold(II) phosphate compounds on P. chabaudi AS parasites following short term in vitro culture and subsequent inoculation into naïve BALB/c mice. All mice that received pRBCs treated for 80 min developed patent parasitemia and were euthanized based on clinical scores. However, compared to the vehicle controls, there was a 2 to 4 day delay in the onset of parasitemia observed in mice that received P. chabaudi AS pRBCs treated with 1 μM or 2 μM of the gold compounds (Fig. 2A and D).

BALB/c mice that were inoculated with pRBCs treated for 2 h with [Au(d2pyppe)₂]Cl (2 μM) or auranofin (2 μM) usually did not develop parasitemia (Fig. 2B and E). This effect was also observed in mice inoculated with P. chabaudi AS pRBCs treated with [Au(d2pyppe)₂]Cl (1 or 2 μM) or auranofin (2 μM) for 3 h (Fig. 2C and F). Clinical scores were also recorded for mice inoculated with gold compound treated pRBCs. None of the mice that received pRBCs treated with 1 μM or 2 μM [Au(d2pyppe)₂]Cl or 2 μM auranofin for 3 h exhibited clinical symptoms compared to the vehicle controls, indicating that parasite growth was inhibited following short term in vitro culture (Fig. S2, ESI†).

Immune-compromised SCID mice inoculated with pRBCs treated with [Au(d2pyppe)₂]Cl (1 or 2 μM) or auranofin (2 μM) for 3 h did not develop a blood-stage infection as determined by microscopy (data not shown). This observation indicates that the parasites were attenuated following 3 h of drug treatment in vitro. Vehicle control mice (i.e. mice injected with P. chabaudi AS pRBCs treated with the same concentration of methanol used to dissolve the gold compounds) rapidly developed a blood-stage infection following inoculation indicating that methanol alone was unable to inhibit parasite growth (Fig. 2).

However, in some experiments, partial attenuation was observed in BALB/c mice inoculated with pRBCs treated with gold compounds for 3 h. These mice developed low level parasitemia as observed by microscopy, and subsequently self-cured (Fig. 3).

Table 1 In vitro anti-malarial activities (IC₅₀ nM) of [Au(d2pyppe)₂]Cl, auranofin, chloroquine and Riamet against P. falciparum strains and P. knowlesi A1H.1

<table>
<thead>
<tr>
<th>Compound</th>
<th>P. falciparum 3D7</th>
<th>P. falciparum 7G8</th>
<th>P. falciparum UGMCB-009</th>
<th>P. falciparum UGMCB-013</th>
<th>P. knowlesi A1H.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Au(d2pyppe)₂]Cl</td>
<td>51.3 (49.4-53.3)</td>
<td>42.2 (37.9-47.0)</td>
<td>39.0 (37.6-40.4)</td>
<td>17.57 (13.53-22.82)</td>
<td>5.5 (2.0-15.2)</td>
</tr>
<tr>
<td>Auranofin</td>
<td>66.6 (55.1-66.6)</td>
<td>299.3 (279.1-321.5)</td>
<td>90.0 (86.0-114.5)</td>
<td>308.0 (293.7-509.4)</td>
<td>72.3 (26.4-175.9)</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>14.4 (14.0-14.8)</td>
<td>ND</td>
<td>23.4 (22.0-24.8)</td>
<td>49.22 (36.36-61.89)</td>
<td>4.6 (2.6-8.3)</td>
</tr>
<tr>
<td>Riamet</td>
<td>ND</td>
<td>6.4 (4.5-9.4)</td>
<td>8.1 (7.9-8.3)</td>
<td>7.54 (5.63-10.08)</td>
<td>ND</td>
</tr>
</tbody>
</table>
Gold (I) phosphine compounds as parasite attenuating agents

Fig. 2. Parasitemia by microscopy in BALB/c mice (n = 5 per group) inoculated with 1 x 10⁷ gold compound-treated P. chabaudi AS pRBCs. pRBCs were incubated for (A and D) 80 minutes, (B and E) 2 hours and (C and F) 3 hours. ++++ indicate that mice succumbed to the infection. Data are expressed as mean ± SEM.

Fig. 3. Parasitemia by microscopy in BALB/c mice (n = 5) following (A) inoculation with 1 µM [Au(d2pype)₂]Cl-treated P. chabaudi AS pRBCs and (B) challenged with 1 x 10⁵ P. chabaudi AS, 4 weeks post-inoculation. pRBCs were incubated for 5 hours. Arrows indicate mice that developed patent parasitemia and were protected following challenge.

Persistence of gold compound-treated P. chabaudi AS pRBCs in vivo

Blood samples were collected from BALB/c mice in which parasites remained undetectable by microscopy to estimate the parasite burden using qPCR based on the 18S rRNA gene of Plasmodium.

There was a decline in parasite DNA on day 1 in mice inoculated with pRBCs treated for 80 min with auranozin or [Au(d2pype)₂]Cl, before steadily increasing from day 3 with the progression of the infection (Fig. 4A and C). Parasitemia (as determined by measuring parasite DNA) in mice that received pRBCs treated for 3 h with 1 µM or 2 µM [Au(d2pype)₂]Cl declined from 1152 ± 413 and 1247 ± 1816 pRBC per ml 6 h post inoculation to 18 ± 18.11 and 70 ± 46 pRBC per ml, respectively on day 3. It remained at low levels in these mice on day 6 (16.3 ± 16.3 pRBC per ml and 32.4 ± 32.4 pRBC per ml for 1 µM and 2 µM [Au(d2pype)₂]Cl dose, respectively) before becoming undetectable by day 8 (Fig. 4B). Conversely, parasitemia in mice injected with pRBCs treated with 1 µM auranozin for 3 h decreased from 2073 ± 1337 pRBC per ml 6 h post injection to 51 ± 25 pRBC per ml on day 3 and steadily increased by day 5 to 3283 ± 1798 pRBC per ml, progressing into patent parasitemia by day 8. The mice had
Gold(I) phosphine compounds as parasite attenuating agents

to be euthanased based on clinical scores (Fig. 4D). However, in mice that received pRBCs treated with 2 μM auranofin for 3 h, there was a decline in DNA levels by day 3, persisting at low levels up to day 8 (40.5 ± 40.5) before becoming undetectable by day 10 (Fig. 4D). All vehicle control mice had a rapid increase in the amount of parasite DNA detected as the infection progressed (Fig. 4). These data are consistent with results obtained from microscopy (Fig. 2).

Collectively, the data show that *P. chabaudi* parasites treated in vitro with gold compounds are attenuated to varying degrees depending in part on the concentration of drug used to attenuate and the duration of treatment.

Protective efficacy of drug-treated *P. chabaudi* AS pRBCs

We then examined whether mice inoculated with pRBCs treated with the gold(II) phosphine compounds developed protective immunity against a *P. chabaudi* wild-type challenge infection as previously observed with pRBCs attenuated using DNA binding drugs.55

Mice were rested for 4 weeks post inoculation with gold compound-treated pRBCs. They were challenged intravenously with 1 × 10^6 *P. chabaudi* AS parasites and monitored every 2nd day by microscopy for the appearance of parasitemia. We observed that all mice in which the gold compounds had completely inhibited the parasites in vitro, were not subsequently immune. Following challenge, these mice developed patent parasitemia by day 4 and were euthanased by day 8 due to their clinical symptoms (Fig. 5). Following the observation that parasite DNA persisted up to 8 days in mice that did not develop patent parasitemia, we hypothesized that administration of booster inoculations might be critical for induction of protective immunity. However, protection was not observed when mice were given 3 inoculations of gold compound-treated pRBCs and were subsequently challenged with a wild type *P. chabaudi* AS infection (Fig. S3, ESI†). However, as mentioned above, some mice which had been inoculated with the gold compound-treated parasites developed a low grade microscopically patent parasitemia. When these mice were then challenged with un-treated parasites, they were immune, with no mice developing microscopically patent parasitemia (Fig. 3).

**In vitro anti-malarial activity of gold complexes on *P. chabaudi* AS**

As the gold(II) phosphine compounds demonstrated *in vitro* anti-malarial activity against *P. chabaudi* AS, we next examined their *in vivo* activity. Mice were infected intravenously with 1 × 10^6 *P. chabaudi* AS pRBCs and subsequently received gold compounds by oral gavage approximately 2 h following infection (day 0) and daily for 3 consecutive days. Mice received 3.7, 7.3, 10.9 or 14.7 μmol kg^-1 of [Au(d2pyype)_2]Cl or 11.8 μmol kg^-1 of auranofin. No difference was observed in parasite growth when mice received 3.7 or 7.3 μmol kg^-1 of [Au(d2pyype)_2]Cl and 11.8 μmol kg^-1 of auranofin compared to vehicle controls (Fig. 6A, B and D). A high dose of 14.7 μmol kg^-1 was found to be toxic and mice had to be euthanased approximately 4 h
following drug administration. However, there was a significant difference in parasite growth amongst mice that received 14.7 μmol kg⁻¹ of [Au(d2pypp)]Cl compared to vehicle controls (Fig. 6C). The lack of a more significant in vivo effect may be due to pharmacokinetic factors relating to drug availability.

Discussion

With approximately 200 million new malaria infections being reported annually and the increasing emergence of drug resistance, the search for new drugs and the development of a malaria vaccine remains of utmost importance.

Previously, DNA alkylating compounds have been shown to attenuate both sporozoite and blood-stage rodent Plasmodium parasites and injection of these attenuated parasites can induce protective immune responses. These compounds are however potentially genotoxic hence the need to screen and identify new compounds, utilising different mechanisms of action to further facilitate the development of a malaria vaccine. Thioridoxin reductase (TrxR) represents an attractive target as structural differences have been observed in the C-terminal reduct centre of the different TrxR enzymes in the P. falciparum malaria system (malaria parasite, human host and insect vector) that may be exploited in the design of selective inhibitors. Gold compounds (particularly auranofin) have been shown to be amongst the most effective TrxR inhibitors and have demonstrated anti-malarial activity through induction of apoptosis of parasitized red blood cells. The established clinical use of auranofin makes it an attractive candidate for an attenuating agent, however a disadvantage is that, as a linear Au(i) two-coordinate complex, it reacts readily and non-selectively with protein thiol.

In extensive previous work, we have shown that by attention to ligand design in bis-chelated Au(i) complexes with bidentate 2-pyridyl phosphines, the facile ligand exchange reactions with thiols is overcome. More selective targeting of selenol- and thiol-containing reduct ruling proteins is achieved for the four-coordinate Au(i) complexes with bidentate 2-pyridyl complexes, which undergo ligand exchange via a ring opening mechanism. We have previously demonstrated that [Au(d2pypp)]Cl (where d2pypp is 1,3-bis(2-pyridyl-phosphino)propane) selectively induces apoptosis in breast cancer cells but not in normal breast cells, correlating with more pronounced inhibition of intracellular thioridoxin reductase in the cancer cells. In this study, we investigated the related compound [Au(d2pypp)]Cl (the difference being a 5- rather than 6-membered chelate ring). An important consideration in the biological evaluation of metal-based compounds is whether they are stable in solution and especially under the testing conditions and previous studies have verified that [Au(d2pypp)]Cl remains intact after prolonged incubation in either blood plasma or cell culture media.

SanneUa and colleagues have previously shown that auranofin is active against P. falciparum 3D7 in a P. falciparum lactate dehydrogenase (pLDH) assay. In this current study, we used the gold standard tritiated hypoxanthine assay and showed that [Au(d2pypp)]Cl has greater anti-malarial activity in vitro than auranofin against human P. falciparum strains, 3D7, 7G8, clinical P. falciparum isolates UGMCB-009, UGMCB-013 and P. knowlesi AHH. The greater activity is consistent with our previous finding that selective targeting of intracellular thioridoxin reductase is achieved by use of chelated phosphine ligands, which introduce considerable activation barriers to ligand exchange compared to auranofin.

We further assessed the activity of gold compounds on rodent P. chabaudi AS in short term in vitro culture. To test attenuation, gold compound-treated rBCs were inoculated into naive BALB/c mice. Mice inoculated with P. chabaudi AS rBCs treated for 80 min all developed patent parasitaemia indicating incomplete attenuation. However, when compared to the vehicle controls, a 2–4 day delay in the development of parasitaemia was observed which could be attributed to partial attenuation of the gold compound-treated rBCs. Enhanced attenuation was observed when mice were inoculated with rBCs treated with 2 μM [Au(d2pypp)]Cl or auranofin for 2 or 3 hours (Fig. 2).
Gold (I) phosphine compounds as parasite attenuating agents

Fig. 6  Parasitemia and area under the curve (AUC) in BALB/c mice (n = 5 per group) infected with 1 × 10^5 P. chabaudi AS pRBCs and were subsequently treated with (A) 3.7 μmol kg^-1 [Au(d2pyppc)_2]Cl, (B) 7.3 μmol kg^-1 [Au(d2pyppc)_2]Cl, (C) 10.9 μmol kg^-1 [Au(d2pyppc)_2]Cl and (D) 11.8 μmol kg^-1 auranofoil for 4 days with drug administration starting on the day of infection. *++++* indicate that mice succumbed to the infection. Arrows represent time points for drug administration. Data are expressed as mean ± SEM. AUC comparisons were performed using the Mann-Whitney test. Data were considered significant (*) at P < 0.05 and not significant (ns) at P ≥ 0.05.

In addition to monitoring parasitemia by microscopy, a real-time quantitative PCR (RT-qPCR) was performed to monitor the persistence of parasite DNA in mice injected with gold compound treated P. chabaudi AS pRBCs. In all mice in which parasitemia was observed by microscopy (Fig. 2), a decline in parasite DNA levels was observed on day 1 followed by a steady increase by day 5 (Fig. 4). The reduction in parasite DNA on day 1 may be due to the initial clearance of pRBCs from the bloodstream by immune cells or the spleen, followed by a steady rise in DNA levels reflecting parasite growth due to incomplete attenuation.

Mice injected with pRBCs treated for 2 or 3 h with 2 μM gold compounds did not develop microscopically patent parasitemia but carried low levels of parasite DNA for up to day 6 ([Au(d2pyppc)_2]Cl treated) and day 8 (auranofoil treated) becoming undetectable by day 8 and 10, respectively. No attenuation was observed amongst mice that received vehicle treated pRBCs and hence the steady increase from day 1 to day 7 when these mice were euthanased (Fig. 4). However, some mice inoculated with pRBCs treated with gold compounds for 3 h developed low level microscopically patent parasitemia.
which subsequently cleared indicating partial attenuation (Fig. 3).

To ascertain whether inoculation of [Au(d2pype)2]Cl or auranofin treated P. chabaudi AS pRBCs could induce a protective immune response, mice that did not develop microscopically patent parasitemia were challenged with wild type P. chabaudi AS. Protection was not observed as all mice succumbed to infection (Fig. 5). Following the observation that parasite DNA was persistent up to 8 days in mice that did not develop parasitemia, we hypothesized that administration of booster inoculations might be critical for induction of a protective immunity. However, no protection was observed when mice were given 3 inoculations of $1 \times 10^7$ [Au(d2pype)2]Cl or auranofin treated pRBCs and subsequently challenged with a wild type P. chabaudi AS (Fig. S3, ESI†). Previous studies using DNA-binding drugs as parasite attenuating agents have shown that parasite DNA could still be detected 110 days post-inoculation. This could possibly explain the lack of protective immunity observed in the current study since parasite DNA did not persist long enough (undetectable by day 10) (Fig. 4) to induce an immune response. However, amongst mice that had a low-grade parasitemia following inoculation with gold compound-treated pRBCs, protection was observed when challenged with a wildtype infection (Fig. 3) indicating that exposure to partially attenuated parasites is critical for the induction of protective immunity.

Having observed in vitro activity against P. chabaudi AS, a pilot study was conducted to assess whether the gold compounds could inhibit parasite growth in vivo. When administered orally, there was no difference in parasite growth observed amongst mice that received 3.7, 7.3 μmol kg$^{-1}$ of [Au(d2pype)2]Cl or 11.7 μmol kg$^{-1}$ of auranofin compared to vehicle controls (Fig. 6A, B and D). On the other hand, a significant difference in parasite growth was observed amongst mice that received 10.9 μmol kg$^{-1}$ of [Au(d2pype)2]Cl compared to vehicle controls (Fig. 6C) indicating that a higher dose might be required. However, [Au(d2pype)2]Cl was toxic in mice that received 14.7 μmol kg$^{-1}$. Higher doses were not attempted for auranofin. In vivo, the drug may not available for absorption in the gastrointestinal tract when administered orally. Thus, comprehensive pharmacokinetic studies will be required in small animal models to further examine drug bioavailability following administration. When an alternative route of administration – intraperitoneal – was tested with auranofin, there was no anti-malarial effect observed (data not shown). Since no activity was observed with auranofin, intraperitoneal administration was not attempted with [Au(d2pype)2]Cl. However, in a previous study of the evaluation of the in vivo anti-tumour activity of [Au(d2pype)2]Cl in colon 38 tumours in mice, tumour growth delay was optimal following daily intraperitoneal dosing (10 days) at a dose of 4 μmol kg$^{-1}$ day$^{-1}$ and this correlated with highest drug concentrations in plasma and tumour tissues.30

Conclusion

The bis-chelated Au(i) complex [Au(d2pype)2]Cl was previously investigated as an anticancer agent and selected from a series of related four-coordinate Au(i) diphosphines in which hydrophilic-balance was fine-tuned to optimize antitumour activity versus host toxicity. The antitumour mechanism was attributed to uptake into mitochondria and selective inhibition of thioridoxin reductase in tumour cells. The same Au(i) phosphine compound is shown here to exhibit more potent anti-malarial activity compared to auranofin against a variety of P. falciparum strains including clinical isolates and chloroquine resistant strains. In an infect-and-treat rodent model, the compounds were unable to inhibit P. chabaudi AS growth in vivo when administered orally, and pharmacokinetic studies will be required to further examine bioavailability following administration. Despite the extensive investigations of gold compounds as anti-cancer agents in recent years there is little pharmacokinetic data on Au(i) compounds in animals.7

Both auranofin and [Au(d2pype)2]Cl exhibit promising parasite attenuating properties in vitro, which could be exploited in the development of a chemically attenuated blood-stage malaria vaccine. Such an approach offers significant advantages over the direct use of gold compounds as anti-malarial drugs, requiring injection of only the attenuated parasites, so that pharmacokinetic and toxicity issues are avoided. In this study, partial in vivo attenuation of malaria parasites prior to inoculation in mice was critical for the induction of protective immune responses, as complete attenuation, by preventing all parasite growth, did not allow for immunity to be induced. Future studies will be required to further optimize the reproducibility of achieving partial attenuation.

Overall, we conclude that with structural differences in C terminal cysteine motifs of the parasite thioridoxin reductase and the human host being identified,10–12 gold(i) compounds can be designed to more efficiently target and selectively inhibit the parasite. However, the major problem to solve for the development of gold-based anti-malarial drugs is related to drug delivery and bioavailability in vivo.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

The authors gratefully acknowledge the study participants who donated blood samples for the collection of clinical P. falciparum isolates. We thank Dr Tom Pell for the synthesis of [Au(d2pype)2]Cl. Aloysious Saemaganda was supported by Griffith University (Griffith University International Postgraduate Research Scholarship and Griffith University Postgraduate Research Scholarship). This study was funded by a National Health & Medical Research Council (NHMRC) Program Grant (1037304) and an NHMRC Fellowship awarded to Michael F. Good.
Gold (I) phosphine compounds as parasite attenuating agents

References

Gold (I) phosphine compounds as parasite attenuating agents


Supporting information

**Fig. S1** Log dose response curves of [Au(d2pype)₂]Cl and auranofin, chloroquine and Riamet® for (A) Chloroquine sensitive *P. falciparum* strain 3D7 (B) Chloroquine resistant *P. falciparum* strain 7G8 (C) field isolate *P. falciparum* UGMCB-009 (D) field isolate *P. falciparum* UGMCB-013 and (E) *P. knowlesi*. Data is expressed as mean ± SEM.
**Fig. S2** Clinical monitoring of BALB/c mice (n=5 per group) inoculated with 1x10⁶ gold compound treated *P. chabaudi* AS pRBCs. Parasites were incubated for 80 minutes (A, D), 2 hours (B, E) and 3 hours (C, F). ++++ indicate that mice succumbed to the infection. Data is expressed as mean ± SEM.

**Fig. S3** Parasitaemia (A) and clinical scores (B) in BALB/c mice (n=5 per group) following 3 inoculations with [Au(d2pype)₂]Cl or auranofin treated *P. chabaudi* AS pRBCs and challenge with 1x10⁵ *P. chabaudi* AS. ++++ indicate that mice succumbed to the infection. Data is expressed as mean ± SEM.
Chapter 5: Immunogenicity and protective efficacy of liposomes containing inactivated *P. falciparum* blood-stage antigens
5.0 Introduction

Malaria vaccine development has focused primarily on the sub-unit approach in which a restricted number of parasite antigens are formulated with delivery systems and adjuvants to enhance immune responses against specific antigen epitopes. However, this strategy has shown limited success in field studies as the vaccines are moderately efficacious and immunity is short-lived. This, in part, could be attributed to antigenic diversity of the protein sub-units in the vaccines as well as failure to maintain parasite-specific antibody responses (Patarroyo et al., 1988; Genton et al., 2002; Ogutu et al., 2009; Sagara et al., 2009; Sirima et al., 2011; Thera et al., 2011; Ewer et al., 2013; Olotu et al., 2016).

To address these shortcomings, there is renewed interest in the whole parasite vaccine approach where substantial progress has been made with liver-stage vaccines. Irradiated sporozoites (PfSPZ) and chemoattenuated sporozoite (PfSPZ-cVac) vaccines have demonstrated robust sterile immunity in controlled human malaria infection studies (CHMI) (Seder et al., 2013; Ishizuka et al., 2016; Epstein et al., 2017; Lyke et al., 2017; Mordmuller et al., 2017) as well as natural infection studies (Sissoko et al., 2017). Whole blood-stage malaria vaccines including chemically attenuated (Good et al., 2013; Raja et al., 2016) and inactivated parasites in adjuvant (Pinzon-Charry et al., 2010; Z. Su et al., 2003) have also been tested in rodent models. Despite being promising candidates, the use of live-attenuated whole parasite blood-stage vaccines in clinical trials is met with challenges relating to the use of blood products in some individuals, potential induction of anti-red blood cell antibodies in vaccinees as well as storage, delivery and cold-chain maintenance issues especially in malaria endemic regions (Stanisic & Good, 2015).
The alternative approach to circumvent some of these challenges is to use whole inactivated parasite antigens. However, this would require potent adjuvants to deliver the antigens and potentiate a robust immune response. Progress with this approach in human studies has been restricted due to the limited number of suitable human-compatible adjuvants. However, with new potent liposome adjuvants and antigen delivery systems such as AS01, currently being used in the RTS, S clinical trials, there is renewed interest in whole inactivated parasite vaccines.

Liposomes present an attractive adjuvant and antigen delivery platform for the development of a whole parasite malaria vaccine. Given their versatility and plasticity, liposomes can be tagged with sugars such as mannose to target them to lectin like molecules on APCs to facilitate phagocytic uptake thereby promoting MHC class II involvement and, via cross presentation, MHC class I. This targeting of liposomes to different uptake pathways may aid direct the resulting immune response towards a balanced Th1/Th2 response (Perrie et al., 2016). Liposomes containing mannose (also referred to as mannosylated hereonin) have been shown to increase the intracellular targeting of antigens to professional APCs and exhibited potent antibody and cell-mediated immune responses (Copland et al., 2003; Vyas, Goyal, & Khatri, 2010). In the context of malaria, our laboratory has recently demonstrated that mannosylated liposomes encapsulating whole rodent malaria parasites (P. chabaudi and P. yoelii) protected mice from a homologous wild-type challenge infection (Giddam et al., 2016). However, these studies lacked extensive immunological evaluations which are addressed in this thesis chapters 5 and 6.

Antigenic variation between malaria species presents a major bottleneck to vaccine development. Therefore, it is necessary to assess in pre-clinical studies whether or not a selected parasite strain is immunogenic against heterologous species/strains. In the context of whole parasite vaccines, this issue of immunogenicity was further
Liposomes containing inactivated *P. falciparum* blood-stage antigens emphasized in a recent study in Mali where an irradiated sporozoite vaccine (PfSPZ) previously reported to be highly protective against *P. falciparum* in controlled human malaria infection in malaria-naive individuals (Ishizuka et al., 2016; Epstein et al., 2017; Lyke et al., 2017), demonstrated moderate efficacy against seasonal malaria infections in highly endemic areas (Sissoko et al., 2017).

The aim of this chapter is to establish a heterologous challenge model to examine the immunogenicity and protective efficacy of mannosylated liposomes containing human parasite (*P. falciparum*) antigen. Furthermore, a novel immunomagnetic method for depletion of red cell membranes from the whole parasite antigen is assessed and liposomes were lyophilized in order to address some of the challenges to whole parasite blood-stage vaccine development namely; potential for inducing anti-red blood cell antibodies in the vaccinated host and storage as well as delivery respectively.

5.1 Results

5.1.1 Depletion of red cell membranes from *P. falciparum* parasite antigen

One of the considerations for the development of whole parasite blood-stage vaccines is the potential for inducing anti-red blood cell antibodies in vaccinees. Here, using magnetic beads and antibodies to Glycophorin A (GlyA), a major glycoprotein of the human erythrocyte membrane, red blood cell membranes were depleted from *P. falciparum* antigens. Using flow cytometry, median fluorescent intensity (MFI) measurements of particulate parasite antigen indicated a 64.4 to 92% red cell membrane depletion following immunomagnetic purification (Table 5.1 and Fig. 5.1A). To confirm the presence of parasite DNA, the extract was stained with bisbenzimide hoechst. Data from three independent experiments showed that the proportions of parasite DNA observed pre-and post-purification were varied.
indicating that parasite antigen is lost during the immunomagnetic processing (Table 5.2 and Fig. 5.1B).

**Table 5.1. Estimation of red blood cell membrane depletion.** *P. falciparum* antigen was stained with anti-GlyA FITC pre- and post-immunomagnetic purification. The MFI was used to estimate percentage depletion of red cell membranes.

<table>
<thead>
<tr>
<th></th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Post-magnetic purification (MFI)</td>
<td>740</td>
<td>150</td>
<td>180</td>
</tr>
<tr>
<td>Pre-magnetic purification (MFI)</td>
<td>2077</td>
<td>1083</td>
<td>2257</td>
</tr>
<tr>
<td>% Depletion</td>
<td>64.4</td>
<td>86.1</td>
<td>92</td>
</tr>
</tbody>
</table>

**Table 5.2. Confirmation of parasite DNA.** *P. falciparum* antigen was stained with bisbenzimide hoechst pre- and post-immunomagnetic purification. The MFI was used to confirm the presence of parasite DNA.

<table>
<thead>
<tr>
<th></th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Post-magnetic purification (MFI)</td>
<td>5435</td>
<td>3126</td>
<td>1196</td>
</tr>
<tr>
<td>Pre-magnetic purification (MFI)</td>
<td>25458</td>
<td>4215</td>
<td>6972</td>
</tr>
<tr>
<td>% DNA loss</td>
<td>78.7</td>
<td>25.8</td>
<td>82.8</td>
</tr>
</tbody>
</table>
Liposomes containing inactivated *P. falciparum* blood-stage antigens

Next, the induction of anti-human red blood cell (RBC) antibodies was assessed following immunisation of mice with mannosylated liposomes containing $10^7$ pRBC equivalent *P. falciparum* antigens. Serum (1:10 dilution) was collected four weeks after the third immunisation, incubated with normal human RBCs and stained with goat anti-mouse IgG AlexaFluor-488 (*Chapter 2 section 2.6.7*). Flow cytometric analysis revealed a significant reduction in induction of anti-human RBC antibodies in mice immunised with freshly prepared mannosylated liposomes compared to control mice that received three intraperitoneal injections of normal human RBCs ($P<0.05$) (**Fig. 5.2**). Collectively, these data support the use of the immunomagnetic

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**Figure 5.1. Immunomagnetic depletion of red cell membranes from *P. falciparum* antigen.** Representative overlay plots from three independent experiments showing differential staining patterns pre- and post-immunomagnetic depletion. (A) Anti-GlyA FITC and (B) bisbenzimide hoechst staining for red cell membrane antigen and parasite DNA respectively.
method to deplete red cell membranes from parasite antigen and partly address the potential for induction of anti-red cell membrane antibodies in vaccinees.

Figure 5.2. Analysis of serum antibody binding to human red blood cells by flow cytometry. Serum (1:10 dilution) was collected from mice immunised with F3 liposomes containing $10^7$ *P. falciparum* antigen depleted of red cell membranes, empty liposomes or normal human red blood cells (nRBCs), incubated with human nRBCs and stained with goat anti-mouse IgG AlexaFlour-488. (A) Mean fluorescence intensity (MFI) of anti-mouse IgG AlexaFlour-488 labelling are shown for each group of mice. (B) Flow cytometry overlay plots of MFI data of individual mice. Data is expressed as mean ± standard error of the mean (SEM). **** P<0.05.

5.1.2 Characterisation of liposome formulations

Liposomes were formulated using cationic lipids with a mannosylated lipid core peptide (also referred to as F3 from hereonin) anchored into the liposome membrane by two palmitic acid molecules (*Chapter 2 section 2.6.5*). Analysis of particle size, measured as volume-weighted diameter, indicated that freshly prepared and lyophilized liposomes were approximately 32.2 and 65.3 µM diameter size with span values of 2.3 and 2.2 respectively (*Table 5.3*). Formation of intact spherical multilamellar liposome vesicles was confirmed by confocal microscopy (*Fig. 5.3*). The data indicated that lyophilized liposomes were approximately twice
the size of freshly prepared liposomes (Table 5.3). However, confocal microscopy revealed that this could be due to the aggregation of lyophilized liposomes following reconstitution (Fig. 5.3).

Table 5.3. Volume-weighted particle size and size distribution of mannosylated liposomes containing P. falciparum antigen.

<table>
<thead>
<tr>
<th>Liposome formulation</th>
<th>Size (µM)</th>
<th>Span</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freshly prepared F3 + parasite antigen</td>
<td>32.3</td>
<td>2.3</td>
</tr>
<tr>
<td>Lyophilized F3 + parasite antigen</td>
<td>65.3</td>
<td>2.2</td>
</tr>
</tbody>
</table>

5.1.3 Investigation into the route of administration

The route of administration is critical for efficient delivery of liposome-based antigens to immunological sites for processing and subsequent induction of protective immune responses. Here, independent experiments were carried out to examine the protective efficacy of freshly prepared liposomes administered intravenously via the tail vein and subcutaneously on the flanks. BALB/c mice were immunised in a three-dose prime-boost regimen with liposomes with or without F3
Liposomes containing inactivated *P. falciparum* blood-stage antigens

(also referred to as unmannosylated hereonin) containing $10^6$ pRBC equivalent *P. falciparum* antigens on days 0, 14 and 28 (Fig. 5.4). Control mice received $10^6$ pRBC equivalent *P. falciparum* antigens in PBS or F3 liposomes without parasite antigen. Four weeks after the third immunisation, mice were challenged with heterologous *Py17X* pRBCs and parasitaemia was monitored every second day by collecting thin blood smears, which were then stained with Giemsa for microscopic examination.

![Figure 5.4. Immunisation schedule in BALB/c mice immunised with mannosylated liposomes containing red cell membrane-depleted *P. falciparum* antigens.](image)

Mice immunised subcutaneously with F3 liposomes had a lower peak parasitaemia ($32.9 \pm 2.6\%$) compared to mice that received liposomes without F3 (also referred to as unmannosylated hereonin) ($45.4 \pm 5.3\%$; P<0.002) and control mice that received empty liposomes ($53 \pm 7.3\%$; P<0.002) (Fig. 5.5 C). All mice immunised intravenously exhibited a mean high peak parasitaemia of $56.7 \pm 7.4\%$ and had to be euthanased (Fig. 5.5 A). Survival analysis at the end of a 30-day monitoring period showed 100% survival amongst mice immunised subcutaneously with F3 liposomes (Fig. 5.5 D) while no survival was observed amongst mice immunised intravenously with F3 liposomes (Fig. 5.5 B). Additionally, no survival was observed amongst mice immunised subcutaneously or intravenously with unmannosylated liposomes, liposomes without parasite antigen (also referred to as...
empty liposomes) or parasite antigen alone (Fig. 5.5 B and D) indicating that F3 is important in the induction of protective immunity. On the basis of these efficacy data, the subcutaneous route of administration was preferred for subsequent experiments.

Figure 5.5. Monitoring of parasitaemia and survival following challenge with Py17X pRBCs. (A) Parasitaemia and (B) survival in mice immunised intravenously; (C) parasitaemia and (D) survival in mice immunised subcutaneously. Data are expressed as mean ± SEM (n=7 per group). †Indicates the number of mice that were euthanased.

5.1.4 Effect of parasite antigen on the immunogenicity and protective efficacy of liposomes

Previous studies using whole inactivated and live blood-stage parasites showed that an optimal parasite dose is required for the induction of effective immune responses (Elliott et al., 2005; Pinzon-Charry et al., 2010; Pombo et al., 2002) and that high parasite loads during blood-stage infection resulted in abrogation of protective
Liposomes containing inactivated *P. falciparum* blood-stage antigens

immunity (Xu et al., 2002). Having shown that F3 liposomes containing $10^6$ pRBC equivalent of *P. falciparum* antigen administered subcutaneously protected mice against a heterologous challenge with Py17X pRBCs, albeit with the development of high parasitaemias (Fig. 5.5 C and D), the effect of parasite antigen dose on the immunogenicity and efficacy of mannosylated liposomes was then examined. Here, BALB/c mice were immunised according to the three-dose prime-boost regimen illustrated in Fig. 5.4 with liposomes containing $10^6$ or $10^7$ pRBC equivalent of red cell membrane-depleted *P. falciparum* 7G8 antigen.

5.1.4.1 **Assessment of humoral immune responses**

The induction of parasite-specific antibodies was assessed seven days after the third immunisation. Compared to control mice that received empty liposomes, Py17X-specific IgG responses were significantly elevated in mice immunised with F3 liposomes containing $10^7$ pRBC equivalent of red cell membrane-depleted antigen (P<0.001) as well as in mice immunised with unmannosylated liposomes containing $10^6$ (P<0.002) and $10^7$ (P<0.001) pRBC equivalent of red cell membrane-depleted antigen (Fig. 5.6 A).

*P. falciparum* 7G8-specific antibody responses were significantly elevated in all mice immunised with liposomes containing $10^7$ pRBC equivalent of *P. falciparum* antigens (P<0.001) (Fig. 5.6 B). However, since the *P. falciparum* 7G8 crude antigen used to coat plates in these experiments was not depleted of red cell membranes, the antibody response observed here could be a combination of *P. falciparum* 7G8-specific responses and residual anti-human red cell membrane antibody responses. The induction of anti-human red cell membrane responses could be due to incomplete depletion of red cell membranes as observed in Fig. 5.2. Despite this
caveat, the data suggests an antigen dose-dependent increase in parasite-specific antibody responses.

5.1.4.2 Assessment of antigen-experienced CD4+ and CD8+ T cell responses

Blood samples were collected for assessment of antigen-experienced T cells using early activation markers CD49dhiCD11ahi for CD4+ and CD8loCD11ahi for CD8+ cells seven days after the third immunisation (as described in chapter 2 section 2.7.5). Fig. 5.7 illustrates the gating strategy used to interrogate antigen-experienced T cells.
Liposomes containing inactivated *P. falciparum* blood-stage antigens

Compared to mice immunised with empty liposomes, antigen-experienced CD4+ T cells were significantly upregulated amongst mice immunised with liposomes with F3 and unmannosylated liposomes containing red cell membrane-depleted parasite antigens (*P*<0.002) (Fig. 5.8 A) while activated CD8+ T cells were only elevated in mice immunised with F3 liposomes containing $10^6$ pRBC equivalent of *P. falciparum* antigens (*P*<0.002) (Fig. 5.8 B).

Figure 5.7. Gating strategy to identify antigen-experienced T cells following immunisation. Activated CD4+ T cells were characterised as CD3+CD49dhiCD11ahi while CD8+ were CD3+CD8loCD11ahi.
5.1.4.3 Assessment of spleen cell proliferation and soluble cytokine responses

Approximately four weeks after the third immunisation, mice from each group were sacrificed for spleen extraction and assessment of proliferative responses as well as cytokine analysis in culture supernatants. Following in vitro stimulation, significant Py17X and P. falciparum 7G8-specific splenocyte proliferative responses were observed in mice immunised with F3 liposomes containing red cell membrane-depleted parasite antigens (equivalent to $10^6$ and $10^7$ pRBC) and with unmannosylated liposomes containing $10^6$ pRBCs equivalent of red cell membrane-depleted parasite antigens compared to corresponding mouse nRBCs or human nRBCs responses for each group (P<0.033) (Fig. 5.9). However, spleen cells extracted from mice immunised with unmannosylated liposomes $10^7$ pRBC
equivalent of red cell membrane-depleted parasite antigens and empty liposomes did not proliferate in response to *Py17X* and *P. falciparum* 7G8 (Fig. 5.9).

After 54 h of *in vitro* stimulation, culture supernatants were collected for soluble cytokine analysis. All parasite-specific responses were compared to corresponding mouse nRBCs or human nRBCs responses for each group. *P. falciparum* 7G8 and *Py17X*-specific IL-2 responses were significantly elevated following immunisation of mice with F3 liposomes containing 10^6 and 10^7 pRBC equivalent *P. falciparum* antigens as well as unmannosylated liposomes containing 10^6 pRBC equivalent *P. falciparum* antigens (P<0.001) (Fig. 5.10 A). Additionally, significant *P. falciparum* 7G8-specific IFN-γ and TNF responses (P<0.033) as well as *Py17X*-specific TNF responses were observed in mice immunised with F3 liposomes containing 10^7

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**Figure 5.9.** Splenocyte proliferative responses in BALB/c mice immunised with liposomes containing red cell membrane-depleted *P. falciparum* antigens. Spleen cells were incubated with *Py17X* parasitized red blood cells (pRBCs), purified *P. falciparum* 7G8 trophozoites, mouse normal red blood cells (nRBCs), human nRBCs, culture media or Concanavalin A (ConA). After 54 h, the cells were pulsed with tritiated thymidine and incubated for an additional 18 h. The proliferative response measured as counts per minute (CPM) was expressed as mean ± SEM (n=3 mice per group). Data were analysed using unpaired t test to compare proliferative responses following *Py17X* pRBC/ *P. falciparum* 7G8 pRBC stimulation to mouse nRBC/human nRBC responses for each liposome formulation. *P<0.033, **P<0.01, ***P<0.002, ****P<0.001.
pRBC equivalent *P. falciparum* antigens and unmannosylated liposomes containing $10^6$ pRBC equivalent *P. falciparum* antigens (P<0.033) (Fig. 5.10 B and C).
Liposomes containing inactivated *P. falciparum* blood-stage antigens

Figure 5.10. Th1 cytokine production in BALB/c mice immunised with liposomes containing red cell membrane-depleted *P. falciparum* antigens. Spleen cells were incubated with Py17X pRBCs, *P. falciparum* 7G8 pRBCs, mouse nRBCs, human nRBCs, culture media or ConA. After 54 h, culture supernatants were collected for assessment of (A) IL-2 (B) IFN-γ and (C) TNF cytokine responses. The cytokine production response (pg/ml) was expressed as mean ± SEM (n=3 mice per group). Data were analysed using unpaired t test to compare cytokine production responses following Py17X pRBC/ *P. falciparum* 7G8 pRBC stimulation to mouse nRBC/human nRBC responses for each liposome formulation. *P<0.033, ***P<0.001.
P. falciparum 7G8-specific IL-4, IL-6 and IL-10 responses were significantly enhanced in mice immunised with F3 liposomes containing $10^6$ and $10^7$ pRBC equivalent P. falciparum antigens as well as unmannosylated liposomes containing $10^6$ pRBC equivalent P. falciparum antigens (P<0.033) (Fig. 5.11 A, B and C). Furthermore, immunisation with F3 liposomes resulted in significant upregulation of Py17X-specific IL-4, IL-6 and IL-10 responses (P<0.033, P<0.001 and P<0.033 respectively) (Fig. 5.11 A, B and C). However, no parasite-specific Th1/Th2 cytokine responses were observed following immunisation with empty liposomes (Fig. 5.10 and 5.11).
Liposomes containing inactivated *P. falciparum* blood-stage antigens

Figure 5.11. Th2 cytokine production responses in BALB/c mice immunised with liposomes containing red cell membrane-depleted *P. falciparum* antigens. Spleen cells were incubated with Py17X pRBCs, *P. falciparum* pRBCs, mouse nRBCs, human nRBCs, culture media or ConA. After 54 h, culture supernatants were collected for assessment of (A) IL-4 (B) IL-6 and (C) IL-10 cytokine responses. The cytokine production response in pg/ml was expressed as mean ± SEM (n=3 mice per group). Data were analysed using unpaired t test to compare cytokine production responses following Py17X pRBC/ *P. falciparum* 7G8 pRBC stimulation to mouse nRBC/human nRBC responses for each liposome formulation. *P<0.033, **P<0.002, ***P<0.001.
Liposomes containing inactivated *P. falciparum* blood-stage antigens

Taken together, these cytokine data suggest that immunisation with F3 liposomes containing red cell membrane-depleted *P. falciparum* 7G8 antigens results in the induction of a Th1/Th2 parasite specific responses.

5.1.4.4 **Assessment of protective efficacy of liposomes containing *P. falciparum* antigens**

Immunised mice were rested for four weeks after the second boost prior to a heterologous challenge infection with $10^5$ Py17X pRBCs and parasitaemia was monitored every second day by collecting thin blood smears, which were then stained with Giemsa for microscopic examination. BALB/c mice immunised with F3 liposomes containing a parasite equivalent of $10^7$ were strongly protected against a heterologous Py17X infection with a low peak parasitaemia of $6.84 \pm 0.48$ % by day 6 and 100 % survival compared to control mice that received empty liposomes (peak parasitaemia $32.6 \pm 5.7$ % and 0% survival by day 10) ($P<0.001$) (Fig. 5.12 A and B, Table 5.4). Mice immunised with F3 liposomes containing $10^6$ pRBC equivalent *P. falciparum* antigens were moderately protected from a challenge infection with peak parasitaemia of $22.3 \pm 1.8$ % by day 10 and 71.4 % survival (Fig. 5.12 A and B, Table 5.4). Conversely, mice immunised with liposomes without F3 containing $10^6$ or $10^7$ pRBC equivalent *P. falciparum* antigens were not protected and demonstrated peak parasitaemias of $27.5 \pm 2.2$ % and $30.9 \pm 4.3$ % respectively similar to control mice that received empty liposomes ($32.6 \pm 5.7$ %) ($P>0.999$) (Fig. 5.12 A).
Liposomes containing inactivated *P. falciparum* blood-stage antigens

Clinical scores and haemoglobin levels (a marker for anaemia) were recorded to monitor disease severity. Mice immunised with F3 liposomes containing 10^6 and 10^7 pRBC equivalent *P. falciparum* antigens displayed better overall clinical outcomes with scores of 0.8 ± 0.2 and 0.2 ± 0.08 respectively compared to mice that received unmannosylated liposomes containing 10^6 and 10^7 pRBC equivalent *P. falciparum* antigens (1.2 ± 0.3 and 2 ± 0.4 respectively) and empty liposomes (1.4 ± 0.6) (Fig. 5.13 A). Furthermore, 8 days post-challenge when some mice were first

### Table 5.4. Protection of BALB/c mice against challenge with *Py17X* pRBCs following immunisation with liposomes containing *P. falciparum* antigens.

<table>
<thead>
<tr>
<th>Liposome formulation</th>
<th>No. protected/No. challenged</th>
<th>% protection</th>
</tr>
</thead>
<tbody>
<tr>
<td>F3 + Liposomes + <em>P. falciparum</em> antigen (10^6)</td>
<td>5/7</td>
<td>71.4</td>
</tr>
<tr>
<td>F3 + Liposomes + <em>P. falciparum</em> antigen (10^7)</td>
<td>7/7</td>
<td>100</td>
</tr>
<tr>
<td>Liposomes + <em>P. falciparum</em> antigen (10^6)</td>
<td>1/7</td>
<td>14.3</td>
</tr>
<tr>
<td>Liposomes + <em>P. falciparum</em> antigen (10^7)</td>
<td>0/7</td>
<td>0</td>
</tr>
<tr>
<td>F3 + Liposomes in PBS</td>
<td>0/7</td>
<td>0</td>
</tr>
</tbody>
</table>

Clinical scores and haemoglobin levels (a marker for anaemia) were recorded to monitor disease severity. Mice immunised with F3 liposomes containing 10^6 and 10^7 pRBC equivalent *P. falciparum* antigens displayed better overall clinical outcomes with scores of 0.8 ± 0.2 and 0.2 ± 0.08 respectively compared to mice that received unmannosylated liposomes containing 10^6 and 10^7 pRBC equivalent *P. falciparum* antigens (1.2 ± 0.3 and 2 ± 0.4 respectively) and empty liposomes (1.4 ± 0.6) (Fig. 5.13 A). Furthermore, 8 days post-challenge when some mice were first
Liposomes containing inactivated *P. falciparum* blood-stage antigens euthanased, mice immunised with F3 liposomes containing $10^7$ pRBC equivalent *P. falciparum* antigens were less anaemic with haemoglobin levels of $105.6 \pm 3.1$ g/L compared to mice immunised with F3 liposomes containing $10^6$ pRBC equivalent *P. falciparum* antigens ($69.4 \pm 2.4$ g/L), unmannosylated liposomes containing $10^6$ and $10^7$ pRBC equivalent *P. falciparum* antigens ($77.3 \pm 3.6$ g/L and $75.6 \pm 3.9$ g/L respectively) as well as empty liposomes ($73.2 \pm 3.7$ g/L) (**Fig. 5.13 B**).

**Figure 5.13. Monitoring of disease severity following challenge of immunised BALB/c mice with *Py17X* pRBCs.** (A) Clinical scores and (B) Haemoglobin (g/L) following heterologous challenge of immunised BALB/c mice with *Py17X* pRBCs. Data were expressed as mean ± SEM (n=7 mice per group). †indicates the number of mice that were euthanased.

Taken together, immunisation with F3 liposomes containing $10^7$ pRBC equivalent *P. falciparum* antigens strongly protects mice against clinical disease compared to mice the received unmannosylated and empty liposomes.

**5.1.4.5 Investigation into the correlates of protective immunity**

Based on the immunogenicity data, the correlates of protective immunity were next interrogated. It was found that *Py17X*- and *P. falciparum* 7G8-specific antibody responses amongst challenged mice did not correlate with protection ($P>0.999$) (**Fig. 5.14 A and B**). Similarly, there was no significant difference observed in
peripheral blood CD4$^+$ and CD8$^+$ T cell activation in protected mice and mice that succumbed to infection (Fig. 5.14 C and D).

**Figure 5.14.** Immune correlates of protection following a Py17X challenge infection. (A) Py17X-specific antibody responses, (B) *P. falciparum* 7G8-specific antibody responses, (C) activated CD4$^+$ and (D) CD8$^+$ T cells in mice that were protected compared to those that succumbed to a wildtype Py17X challenge infection. Data were analysed using Mann-Whitney rank correlation test. ns: non-significant.

On the basis of immunogenicity and protective efficacy data, subsequent experiments in this chapter were performed with F3 liposomes containing $10^7$ pRBC equivalent of *P. falciparum* antigens.
5.1.5 Investigation into the immunogenicity and efficacy of mannosylated liposomes containing parasite antigen with and without depletion of red cell membranes

Having established an optimal parasite antigen dose required for the induction of protective immune responses, the impact of red cell membrane depletion from parasite antigen on the immunogenicity and protective efficacy of mannosylated liposomes was then examined. Here, liposomes were formulated with F3 and parasite antigen extract with or without depletion of red cell membranes as described in Chapter 2, sections 2.6.3 or 2.6.4 respectively. BALB/c mice were immunised according to the 3-dose prime-boost regimen illustrated in Fig. 5.4 and immunogenicity and protective efficacy examined.

5.1.5.1 Assessment of humoral immune responses

Compared to control mice immunised with empty liposomes, mice immunised with F3 liposomes containing parasite antigen with or without depletion of red cell membranes showed were significantly elevated Py17X and P. falciparum 7G8-specific IgG responses seven days after the third immunisation (P<0.001) (Fig. 5.15 A and B). However, there was no significant difference in parasite-specific antibody responses amongst mice immunised with F3 liposomes containing parasite antigen with red cell membranes compared to mice that received F3 liposomes containing red cell membrane-depleted parasite antigen (Fig. 5.15 A and B).
5.1.5.2 Assessment of antigen-experienced CD4+ and CD8+ T cell responses

Antigen experienced peripheral blood CD4+ T cells were significantly upregulated in mice immunised with F3 liposomes containing parasite antigen with or without depletion of red cell membranes (P<0.001) seven days after the third immunisation (Fig. 5.16 A). Conversely, there was no significant CD8+ T cell activation observed in these mice (P>0.999) (Fig. 5.16 B). Additionally, there was no significant difference in T cell activation in mice immunised with F3 liposomes containing parasite antigen with red cell membranes compared to mice that received F3 liposomes containing red cell membrane-depleted parasite antigen (P>0.999) (Fig. 5.16 A and B).
Liposomes containing inactivated *P. falciparum* blood-stage antigens

5.1.5.3 Assessment of spleen cell proliferation and soluble cytokine responses

Approximately four weeks after the third immunisation, mice from each group were sacrificed for spleen extraction and assessment of proliferative responses as well as soluble cytokine analyses. In this experiment, the induction of additional species (*P. knowlesi*) and strain (UGMCM-009, described in Chapter 3)-transcending immune responses were examined. All parasite-specific responses were compared to corresponding mouse nRBCs or human nRBCs responses for each group.

Immunisation with F3 liposomes containing parasite antigen with or without depletion of red cell membranes significantly enhanced *Py*17X, *P. knowlesi*, *P. falciparum* 7G8 and UGMCM-009-specific splenocyte proliferative responses (P<0.002) (Fig. 5.17). However, there was no difference in parasite-specific proliferative responses in mice immunised with F3 liposomes containing parasite...
antigen with red cell membranes compared to mice that received F3 liposomes containing red cell membrane-depleted parasite antigen (P>0.999) (Fig. 5.17).

Cytokine analysis in culture supernatants revealed significant upregulation of *Py17X*, *P. knowlesi*, *P. falciparum* UGMCB-009 and 7G8-specific IL-2 and IFN-γ production responses in mice immunised with F3 liposomes containing parasite antigen with or without depletion of red cell membranes (P<0.033) (Fig. 5.18 A and B). On the other hand, *Py17X*, UGMCB-009 and 7G8-specific TNF production responses were significantly enhanced amongst mice immunised with F3 liposomes containing parasite extract without red cell membranes (Fig. 5.18 C) (P<0.033) while *P. knowlesi*-specific TNF production responses were elevated in mice immunised with F3 liposomes containing parasite antigen depleted of red cell membranes (P<0.033) (Fig. 5.18 C).
Figure 5.18. Th1 cytokine production responses in BALB/c mice immunised with mannosylated liposomes containing *P. falciparum* 7G8 antigens with and without red cell membranes. Spleen cells were incubated with *Py17X* pRBCs, *P. falciparum* 7G8 or UGMCB-009 lysate, *P. knowlesi* lysate, mouse nRBCs, human nRBCs, culture media or ConA. After 54 h, culture supernatants were collected for assessment of (A) IL-2 (B) IFN-γ and (C) TNF cytokine responses. The cytokine production response (pg/ml) was expressed as mean ± SEM (n=3 mice per group). Data were analysed using unpaired t test to compare *Py17X*-/*P. falciparum* 7G8-/*UGMCB-009*-/*P. knowlesi*-specific cytokine production responses to mouse nRBC/human nRBC responses for each liposome formulation. *P<0.033, **P<0.002, ***P<0.001.
IL-4, IL-6 and IL-10 cytokine production in response to *in vitro* stimulation with Py17X pRBCs, *P. knowlesi*, *P. falciparum* UGMCB-009 and 7G8 pRBC lysates was significantly elevated in mice immunised with F3 liposomes containing parasite antigen with and without depletion of red cell membranes compared to responses following stimulation with mouse nRBCs or human nRBCs for each group (*P*<0.033) (*Fig. 5.19 A, B and C*).

However, there was no significant difference in cytokine production responses amongst mice immunised with F3 liposomes containing parasite antigen with red cell membranes compared to mice that received F3 liposomes containing red cell membrane-depleted parasite antigen (*Fig. 5.18 and 5.19*).
Liposomes containing inactivated *P. falciparum* blood-stage antigens

**Figure 5.19.** Th2 cytokine production responses in BALB/c mice immunised with mannosylated liposomes containing *P. falciparum* 7G8 antigens with and without red cell membranes. Spleen cells were incubated with *Py17X pRBCs*, *P. falciparum* 7G8 or UGMCB-009 lysate, *P. knowlesi* lysate, mouse nRBCs, human nRBCs, culture media or ConA. After 54 h, culture supernatants were collected for assessment of (A) IL-4, (B) IL-6 and (C) IL-10 cytokine responses. The cytokine production response (pg/ml) was expressed as mean ± SEM (n=3 mice per group). Data were analysed using unpaired t test to compare *Py17X*/7G8*/UGMCB-009*/*P. knowlesi*-specific cytokine production responses to mouse nRBC/human nRBC responses for each liposome formulation. *P<0.033, **P<0.002, ***P<0.001.
Together, the data suggest that depletion of red cell membranes from parasite antigen does not affect the immunogenicity and is consistent with the results described in section 5.1.4.3, immunisation F3 liposomes containing *P. falciparum* antigens induces a Th1/*P. falciparum* cytokine response.

5.1.5.4 **Assessment of the protective efficacy of mannosylated liposomes containing parasite antigen with and without red cell membranes**

To assess the impact red cell membrane depletion on the efficacy of mannosylated liposomes, mice were challenged four weeks after the third immunisation with *Py17X* pRBCs, and parasitaemia monitored every second day by microscopic examination Giemsa-stained blood smears. BALB/c mice immunised with F3 liposomes containing parasite antigen with red cell membranes showed a lower peak parasitaemia of 30.5 ± 1.6 % compared to 35.6 ± 3.3 % (P>0.999) in mice that received F3 liposomes containing red cell membrane-depleted parasite antigen and 44.6 ± 0.8 % (P>0.999) in control mice immunised with empty liposomes (**Fig. 5.20 A**). Area under the curve analysis on day 8 post-challenge showed no significant difference in the control of parasite growth in mice immunised with F3 liposomes containing a parasite antigen with or without red cell membrane depletion compared to control mice immunised with empty liposomes (P>0.999) (**Fig. 5.20 B**). Similarly, there was no significant difference in parasitaemia AUC amongst mice immunised with F3 liposomes containing parasite antigen with red cell membranes compared to mice immunised with F3 liposomes containing red cell membrane-depleted parasite antigen (P>0.999) (**Fig. 5.20 B**). All mice had to be euthanased on day 10 due to poor clinical outcomes (**Fig. 5.20 C**).
Liposomes containing inactivated *P. falciparum* blood-stage antigens

5.1.6 Investigation into the immunogenicity and protective efficacy of lyophilized mannosylated liposomes

Storage and preservation of vaccines are some of the major logistical challenges encountered in the delivery of vaccines especially in malaria endemic areas (Stanisic & Good, 2015). To address this, mannosylated liposomes were lyophilized as previously described with trehalose as a lyoprotectant (Zaman et al., 2016), stored at 4 °C and re-suspended in PBS prior to immunisation. Mannosylated lyophilized liposomes were formulated with a parasite equivalent of 10^7 red cell membrane-depleted antigen. Additionally, a freshly prepared liposome formulation containing a parasite equivalent of 10^7 red cell membrane-depleted antigen was also prepared.
as a comparator. BALB/c mice were immunised thrice as per the prime-boost regimen illustrated in Fig. 5.4 and immunogenicity as well as protective efficacy examined.

5.1.6.1 Assessment of humoral immune responses

Py17X and *P. falciparum* 7G8-specific IgG responses were significantly elevated in mice immunised with freshly prepared and lyophilized mannosylated liposomes compared to mice immunised with empty liposomes (P<0.002) (Fig. 5.21 A and B). Additionally, mice immunised with lyophilized liposomes had a significantly higher Py17X-specific antibody response compared to mice that received freshly prepared F3 liposomes (P<0.002) (Fig. 5.21 A). Conversely, there was no difference in *P. falciparum* 7G8-specific antibody responses amongst mice immunised with lyophilized F3 liposomes compared to mice that received freshly prepared F3 liposomes (P>0.999) (Fig. 5.21 B).
Antigen experienced peripheral blood CD4+ T cells were significantly upregulated in mice immunised with lyophilized F3 and freshly prepared liposomes seven days after the third immunisation (P<0.033) (Fig. 5.22 A). Conversely, there was no significant CD8+ T cell activation observed in these mice (Fig. 5.22 B).
Assessment of spleen cell proliferation and soluble cytokine responses

Approximately four weeks after the third immunisation, mice from each group were sacrificed for spleen extraction and assessment of proliferative responses as well as soluble cytokine production analyses. All parasite-specific splenocyte proliferative and cytokine production responses were compared to corresponding mouse nRBCs or human nRBCs responses for each group. Similar to immunisation with freshly prepared liposomes, lyophilized F3 liposomes significantly enhanced species (Py17X and P. knowlesi) and strain (UGMCB-009)-transcending splenocyte proliferative responses in BALB/c mice (P<0.033) (Fig. 5.23).
Cytokine analysis of culture supernatants collected following 54 h of *in vitro* culture indicated differential expression of Th1 and Th2 cytokine production responses amongst immunised mice with lyophilized and freshly prepared F3 liposomes. *P. knowlesi*, *P. falciparum* UGMCB-009 and 7G8-specific IL-2 production responses were significantly elevated amongst mice immunised with lyophilized and freshly prepared F3 liposomes while *Py17X*-specific IL-2 production responses were only upregulated amongst mice immunised with freshly prepared liposomes (P<0.002) (Fig. 5.24 A). Immunisation with lyophilized and freshly prepared liposomes resulted in significant *Py17X*, *P. knowlesi*, *P. falciparum* UGMCB-009 and 7G8-specific IFN-γ production responses (P<0.033) (Fig. 5.24 B) while *P. falciparum* UGMCB-009-specific TNF responses were only observed amongst mice immunised with freshly prepared F3 liposomes (P<0.002) (Fig. 5.24 C).
Figure 5.24. Th1 cytokine production responses in BALB/c mice immunised with fresh and lyophilized liposomes containing P. falciparum 7G8 antigens. Spleen cells were incubated with Py17X pRBCs, P. falciparum 7G8 or UGMCB-009 lysate, P. knowlesi lysate, mouse nRBCs, human nRBCs, culture media or ConA. After 54 h, culture supernatants were collected for assessment of (A) IL-2 (B) IFN-γ and (C) TNF cytokine production responses. The cytokine production response (pg/ml) was expressed as mean ± SEM (n=3 mice per group). Data were analysed using unpaired t test to compare Py17X- / P. falciparum 7G8-/UGMCB-009- / P. knowlesi-specific cytokine responses to mouse nRBC/human nRBC responses for each liposome formulation. *P<0.033, **P<0.002, ***P<0.001.
In response to *Py17X* and *P. falciparum* 7G8 pRBC stimulation, IL-4 production was restricted to mice immunised with freshly prepared liposomes (P<0.033) (Fig. 5.25 A) while *P. falciparum* UGMCB-009 and *P. knowlesi*-specific IL-4 production responses were observed in mice immunised with lyophilized and freshly prepared liposomes (P<0.033) (Fig. 5.25 A). *Py17X, P. knowlesi, P. falciparum* UGMCB-009 and 7G8- specific IL-6 and IL-10 production responses were restricted to mice immunised with freshly prepared F3 liposomes (P<0.033) (Fig. 5.25 B and C). However, there was no significant difference in cytokine production responses amongst mice immunised with lyophilized liposomes compared to mice that received freshly prepared liposomes (Fig. 5.18 and 5.19).
Liposomes containing inactivated *P. falciparum* blood-stage antigens

Figure 5.25. Th2 cytokine production responses in BALB/c mice immunised with fresh and lyophilized liposomes containing *P. falciparum* 7G8 antigens. Spleen cells were incubated with Py17X pRBCs, *P. falciparum* 7G8 or UGMCB-009 lysate, *P. knowlesi* lysate, mouse nRBCs, human nRBCs, culture media or ConA. After 54 h, culture supernatants were collected for assessment of (A) IL-4 (B) IL-6 and (C) IL-10 cytokine production responses. The cytokine production response (pg/ml) was expressed as mean ± SEM (n=3 mice per group). Data were analysed using unpaired t test to compare Py17X/ *P. falciparum* 7G8-/UGMCB-009-/ *P. knowlesi*-specific cytokine responses to mouse nRBC/human nRBC responses for each liposome formulation. *P<0.033, **P<0.002, ***P<0.001.
Collectively, the data indicates that immunisation with lyophilized liposomes results in the induction of a Th1 dominant cytokine response, while freshly prepared liposomes induce a mixed Th1/Th2 cytokine response, consistent with previous experiments (Section 5.1.4.3 and 5.1.5.3).

**5.1.6.4 Protective efficacy of lyophilized mannosylated liposomes**

To assess the impact of lyophilization on the efficacy of mannosylated liposomes, mice were challenged four weeks after the third immunisation with Py17X pRBCs, and parasitaemia monitored every 2nd day by microscopic examination of Giemsa-stained blood smears. BALB/c mice immunised with freshly prepared F3 liposomes showed no difference in parasitaemia from the mice that received lyophilized liposomes (27.9 ± 1.4 % vs 30.5 ± 2.4 %, respectively; P>0.999) and neither did the control mice immunised with empty liposomes (38.7 ± 2.7 %; P>0.999). Parasitaemia AUC analysis on day 12 post-challenge when control mice had to be euthanased due to poor clinical outcomes (Fig. 5.26 C) showed no difference in the control of parasite growth in mice immunised with freshly prepared and lyophilized liposomes compared to control mice immunised with empty liposomes (P>0.999) (Fig. 5.26 B). All liposome immunised mice had to be euthanased on day 14 due to poor clinical outcomes (Fig. 5.26 C).
5.1.7 Immune mechanisms of protection following immunisation with mannosylated liposomes

5.1.7.1 Role of T cells

Having demonstrated that mannosylated liposomes containing *P. falciparum* antigens were highly immunogenic and, in some instances, protected mice against a heterologous *Py17X* challenge infection (Fig. 5.5 C and 5.12), the role of T cells in the induction of protective immunity was then assessed. Here, BALB/c mice were immunised following the established 3-dose prime boost regimen with freshly prepared F3 liposomes containing $10^7$ pRBC equivalent *P. falciparum* 7G8 antigens on days 0, 14 and 28 (Fig. 5.4). Two days prior to challenge on day 0, T cells were...
Liposomes containing inactivated *P. falciparum* blood-stage antigens depleted with intraperitoneal injections of anti-CD4 (Clone GK1.5, Bio-x-cell), anti-CD8 β (Clone 53.5.8, Bio X Cell) antibodies with further injections every four days on days 4, 8, 12 and 16 post challenge. Flow cytometric analysis of spleens harvested from immunised unchallenged mice (n=2) on days 1 and 9 post-challenge showed that administration of antibodies efficiently depleted CD4 T cells (approximately 98%) and CD8 T cells (approximately 95%) (Fig. 5.27, Table 5.5).

**Figure 5.27. Gating strategy to assess T cell depletion in immunised mice.** Splenocytes were harvested from spleens of immunised mice and stained with fluorescein-conjugated T cell marker specific antibodies CD3, CD4 and CD8. Lymphocytes were first identified based on (A) size and granularity and confirmed by (B) CD3 staining. Representative flow cytometry plots of stained splenocytes from immunised mice that received (C) anti-CD4 depletion antibodies, (D) anti-CD8 depletion antibodies and (E) control rat IgG.
**Table 5.5. Monitoring CD4\(^+\) and CD8\(^+\) T cell depletion.** Splenocytes were harvested from spleens of immunised mice following administration of T cell depletion antibodies. T cell proportions in control mice which received rat IgG were used to estimate % depletion.

<table>
<thead>
<tr>
<th>Days post-challenge</th>
<th>Rat IgG</th>
<th>Rat IgG</th>
<th>CD4 depleted</th>
<th>CD8 depleted</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>% CD4(^+) T cells</td>
<td>% CD8(^+) T cells</td>
<td>% CD4(^+) T cells (% depletion)</td>
<td>% CD8(^+) T cells (% depletion)</td>
</tr>
<tr>
<td>1</td>
<td>24.2</td>
<td>70.0</td>
<td>0.32 (98.6)</td>
<td>3.06 (95.7)</td>
</tr>
<tr>
<td>9</td>
<td>16.8</td>
<td>76.6</td>
<td>0.31 (98)</td>
<td>3.69 (95.1)</td>
</tr>
</tbody>
</table>

Following challenge, immunised BALB/c mice that received rat IgG injections showed no difference in mean peak parasitaemia (24.7 ± 10.1%) compared to mice depleted of CD4\(^+\) T cells (42.5 ± 6.6%; P>0.103) and CD8\(^+\) T cells (45.6 ± 8.4%; P>0.103) ([Fig. 5.28 A](#)). Additionally, assessment of disease severity showed that there was no difference in overall clinical scores amongst mice which received rat IgG injections (0.5 ± 0.2) compared to mice depleted of CD4\(^+\) T cells (1.7 ± 0.5; P>0.999) and CD8\(^+\) T cells (1.9 ± 0.6; P>0.999) ([Fig. 5.28 B](#)). Survival analysis revealed that immunised mice that received rat IgG injections had a 40% survival compared to 0% in mice that received depletion antibodies. Although there was no significant difference in peak parasitaemia and clinical scores was observed in mice that received depletion antibodies compared to those that received rat IgG, survival data suggests that both CD4\(^+\) and CD8\(^+\) T cells might be crucial in the induction of protective immune responses following immunisation of mice with F3 liposomes containing *P. falciparum* antigens.
Liposomes containing inactivated P. falciparum blood-stage antigens

Figure 5.28. Monitoring of parasitaemia, clinical scores and survival following challenge with $10^5$ Py17X pRBCs. (A) Parasitaemia, (B) clinical scores and (C) survival in BALB/c mice (n=5 per group) immunised with mannosylated liposomes and subsequently injected with T cell depletion antibodies or rat IgG prior to and following challenge with $10^5$ Py17X pRBCs. Data are expressed as mean ± SEM. † Indicates the number of mice that were euthanased.

5.2 Discussion

The development of live-attenuated blood-stage malaria vaccines has recently shown tremendous promise in pre-clinical studies (Good et al., 2013; Raja et al., 2016). However, the utility of these vaccines in human studies is met with several challenges relating to the use of blood products in some individuals, potential induction of anti-red blood cell antibodies in vaccinees as well as storage, delivery and cold-chain maintenance limitations especially in malaria endemic regions (Stanisic & Good, 2015). The use of whole inactivated parasite antigens therefore provides a plausible alternative to the live-attenuated approach. Indeed, studies
Liposomes containing inactivated *P. falciparum* blood-stage antigens have shown that inactivated blood-stage parasites in adjuvant elicit robust immune responses and protect mice against wild-type malaria challenge infection (Pinzon-Charry et al., 2010; Su et al., 2003). However, such studies have been restricted to pre-clinical studies due to the limited number of suitable human-compatible adjuvants. Recently our laboratory developed a novel liposome-based whole parasite vaccine and showed that mannosylated liposomes containing rodent parasites were efficiently taken up by professional antigen presenting cells and mice immunised with these liposomes were protected against homologous challenge infection (Giddam et al., 2016). However, the studies of immunological evaluations were limited. Therefore, to further develop this approach, in this chapter, the immunogenicity and protective efficacy of mannosylated liposomes containing the human parasite, *P. falciparum* was examined in a mouse model. Additionally, some of the challenges to development of whole parasite blood-stage vaccines such as (i) the potential for inducing anti-red blood cell antibodies in vaccinees using a novel immunomagnetic method and (ii) storage and delivery limitations of vaccines by evaluating lyophilized liposomes were addressed.

Firstly, to address the induction of anti-red blood cell antibodies, it was shown that using a novel immunomagnetic method, red cell membranes were efficiently depleted from parasite antigen (**Fig. 5.1, Table. 5.1**). However, there was variability in particulate parasite DNA with significant reduction post-purification in some experiments (**Fig. 5.1, Table. 5.2**) indicating that antigen was being lost along with red cell membranes during the immunomagnetic process. This in effect, could affect the parasite antigen dose in the vaccine formulation. Following this observation, adjustments were made in the amount of parasite antigen pre-purification to account for the loss in parasite DNA post purification. Furthermore, it was shown that using this immunomagnetic method, the induction of anti-red blood cell
Liposomes containing inactivated *P. falciparum* blood-stage antigens antibodies is significantly reduced in sera from mice immunised with liposomes containing red cell membrane-depleted parasite antigen compared to mice that received injections of normal hRBCs (Fig. 5.2). The data strongly support the use of this method to deplete red cell membranes from parasite antigen for use in liposome formulations although additional optimisation will be required to further minimize and if possible completely eliminate the induction of anti-red blood cell antibodies as well as loss in parasite DNA during the immunomagnetic purification.

Secondly, size characterisation of freshly prepared and lyophilized liposomes was carried out. It was shown that the particle sizes were approximately 32.2 and 65.3 µM with span values of 2.3 and 2.2 respectively (Table 5.3) and the formation of intact multilamellar vesicles was confirmed by confocal microscopy (Fig. 5.3). The volume-weighted size measurements of lyophilized liposomes showed that they were approximately twice the size of freshly prepared liposomes (Table 5.3). However, confocal microscopy revealed that this could be due to aggregation following reconstitution (Fig. 5.3), suggesting that vortexing of lyophilized liposomes is required prior to immunisation and ensure homogeneity of liposome vaccine doses. Additionally, previous studies have shown that large liposome particles (in µM range) create an antigen depot at the site of injection to leading to prolonged release of antigen to the immune system. This in turn enhances antigen uptake by dendritic cells and subsequent transit to draining lymph nodes where an effective immune response is induced (Bachmann & Jennings, 2010; Henriksen-Lacey et al., 2011; Watson et al., 2012). However, in the studies presented in this chapter, the effect of size and homogeneity of liposome formulations was not investigated, and further studies will be required to examine the effect of size on the subsequent immune response generated following immunisation.
Liposomes containing inactivated *P. falciparum* blood-stage antigens

The route of administration is critical for efficient delivery of liposome-based vaccine antigens to immunological sites for processing and subsequent induction of protective immune responses. In this chapter, it was shown that subcutaneous but not intravenous immunisation of mice with F3 liposomes containing *P. falciparum* 7G8 antigen protected mice against a challenge infection (Fig. 5.5). Studies have shown that subcutaneous administration of liposomes results in antigen depot formation at the site of injection leading to prolonged release of parasite antigen, facilitating enhanced uptake by APCs and subsequent potentiation of an effective immune response (Bachmann & Jennings, 2010; Henriksen-Lacey et al., 2011; Watson et al., 2012). In our studies, intravenous administration may have resulted in the targeted uptake of mannosylated liposomes by the mannose receptors on hepatic sinusoidal endothelial cells resulting in clearance from circulation by liver macrophages (Malovic et al., 2007). This could explain the lack of efficacy when mannosylated liposomes were intravenously administered as they might have been rapidly cleared from the blood-stream affecting the delivery of parasite antigen to the cells of the immune system. Therefore, in all subsequent experiments described in this thesis, liposomes were administered subcutaneously.

Previous studies using whole inactivated and live blood-stage parasites showed that an optimal parasite dose is required for the induction of robust immune responses (Elliott et al., 2005; Pinzon-Charry et al., 2010; Pombo et al., 2002). Therefore, the impact of parasite antigen dose on the immunogenicity and protective efficacy of mannosylated liposomes was next examined. Robust heterologous *Py17X*-specific antibody responses were induced in mice immunised with F3 liposomes containing $10^7$ pRBC equivalent of parasite antigens, unmannosylated liposomes containing $10^7$ pRBC equivalent of parasite antigens and to a lesser extent amongst mice immunised with unmannosylated liposomes containing $10^6$ pRBC equivalent
Liposomes containing inactivated \textit{P. falciparum} blood-stage antigens (Fig. 5.6 A). Homologous \textit{P. falciparum} 7G8-specific antibody responses on the other hand, were only observed in mice immunised with mannosylated and unmannosylated liposomes containing $10^7$ pRBC equivalent parasite antigens (Fig. 5.6 B). However, since the \textit{P. falciparum} 7G8 crude antigen used to coat plates in these experiments was not depleted of red cell membranes, the antibody response observed could have been a combination of \textit{P. falciparum} 7G8 and anti-human red cell membrane-specific antibody responses. The induction of anti-human red cell membrane responses is due to incomplete depletion of red cell membranes as observed in Fig. 5.2. Despite this caveat, the data suggest an antigen dose-dependent parasite-specific antibody response. Data from subsequent experiments confirmed that indeed mannosylated liposomes containing a parasite equivalent of $10^7$ induced robust homologous and heterologous antibody responses and that these responses were not altered by depletion of red cell membranes (Fig. 5.15 A and B) or lyophilization (Fig. 5.21 A and B). However, these parasite-specific antibody responses did not correlate with protection in challenged mice (Fig. 5.14 A and B). Therefore, future experiments such as \textit{in vitro} growth inhibition assays, serum transfer studies and B cell knock-out animal studies will be required to elucidate the role of parasite-specific antibodies in the induction of protective immunity.

Studies in mice have shown that parasite-specific T cells are critical in the control of blood-stage malaria infection are also abundant in both lymphoid and non-lymphoid organs (Muxel et al., 2011; Villegas-Mendez et al., 2015). Therefore, the induction of antigen-experienced T cell was next examined. Assessment of peripheral blood antigen-experienced CD4$^+$ and CD8$^+$ T cell activation seven days after the third immunisation revealed that CD4$^+$ T cells were enhanced in all mice immunised with liposomes containing \textit{P. falciparum} 7G8 antigen (Fig. 5.8 A) while
activated CD8+ T cells were significantly elevated only in mice that received F3 liposomes containing $10^6$ pRBC equivalent parasite antigens (Fig. 5.8 B). Subsequent experiments indeed confirmed that antigen-experienced CD4+ T cells are upregulated following immunisation with mannosylated liposomes containing $10^7$ pRBC equivalent parasite antigens (Fig. 5.16 A and 5.22 A). However, peripheral blood T cell activation did not correlate with protection in challenged mice (Fig. 5.14 C and D). Therefore, assessment of non-circulating antigen-experienced T cells in other immunological sites such as spleen and other lymphoid organs will be crucial in elucidating the role of activated T cells in future protective efficacy studies.

The spleen plays an important role in immunity to malaria infection (Engwerda et al., 2005; Kumar et al., 1989; Winkel & Good, 1991). Therefore, in vitro splenocyte proliferative responses were investigated four weeks after the last immunisation. Robust homologous (P. falciparum 7G8) and heterologous (Py17X) splenocyte proliferative responses were observed amongst mice immunised with mannosylated liposomes containing $10^6$ and $10^7$ pRBC equivalent parasite antigens (Fig. 5.9). However, following immunisation with unmannosylated liposomes, splenocyte proliferative responses were only observed in mice that received a lower parasite antigen dose ($10^6$) (Fig. 5.9). This antigen dose-dependent response is consistent with studies which showed that exposure to higher parasite doses resulted in the depletion of effector cellular responses (Xu et al., 2002) and indicates that in our studies, the presence of F3 in the liposome formulations might be critical in the maintenance of these responses. In subsequent experiments, it was shown that splenocyte proliferative responses were not altered by depletion of red cell membranes from parasite antigen or lyophilization of mannosylated liposomes containing $10^7$ pRBC equivalent parasite antigens (Fig. 5.17 and 5.23).
Liposomes containing inactivated *P. falciparum* blood-stage antigens

Furthermore, it was shown that immunisation with mannosylated liposomes resulted in the induction of robust splenocyte proliferative responses to other *Plasmodium* species (*P. knowlesi*) and strain (*P. falciparum* clinical isolate UGMCB-009) (Fig. 5.17 and 5.23). However, it is yet to be established whether the robust proliferative responses observed correlate with protection. This was not assessed in our studies since the assays were conducted in a separate subset of unchallenged mice. Therefore, future studies will need to be designed to examine the association of proliferative responses and induction of protective immune responses.

Assessment of cytokine production responses revealed that immunisation with mannosylated liposomes containing $10^7$ pRBC equivalent parasite antigens resulted in the induction of a balanced species-transcending Th1/Th2 cytokine response compared to a Th2 dominant response amongst mice immunised with F3 liposomes containing $10^6$ pRBC equivalent parasite antigens (Fig. 5.10 and 5.11). IFN-γ and TNF, critical mediators of immunity to blood-stage malaria (Jacobs et al., 1996a, Jacobs et al., 1996b; Luty et al., 1999; McCall & Sauerwein, 2010; Riley et al., 1992), were observed in mice immunised with F3 liposomes containing $10^7$ pRBC equivalent parasite antigens and unmannosylated liposomes containing $10^6$ pRBC equivalent parasite antigens (Fig. 5.10). Previous studies have shown that a balanced Th1/Th2 cytokine response to blood-stage malaria is crucial in the control of parasite replication, modulation of immune responses and control of pathology (Langhorne et al., 1989; Linke et al., 1996; Perez-Mazliah & Langhorne, 2014; Stevenson & Tam, 1993; Vonderweid et al., 1994). Since mannosylated liposomes containing $10^7$ pRBC equivalent parasite antigens induced a mixed Th1/Th2 response and more importantly IFN-γ and TNF (Fig. 5.10 and 5.11), and following challenge, mice immunised with these liposomes were strongly protected (Fig. 5.12), subsequent experiments were performed with mannosylated liposomes.
containing 10⁷ pRBC equivalent parasite antigens. Furthermore, it was observed that depletion of red cell membranes from parasite antigen did not alter the species/strain-transcending Th1/Th2 cytokine response (Fig. 5.18 and 5.19) further supporting the utility of the immunomagnetic method to deplete red cell membranes from parasite antigen. On the other hand, immunisation of mice with lyophilized liposomes altered the cytokine response to a predominantly Th1 response (Fig. 5.24), indicating that repeat studies will be required to optimise freeze-drying protocols in order to achieve reproducible optimal Th1/Th2 cytokine responses. Similar to proliferative responses, the cytokine responses observed in our studies could not be directly associated with protection following challenge since the assays were conducted in a separate subset of unchallenged mice. Therefore, future studies will need to be designed to critically examine the role of this cytokine milieu in the induction of protective immunity.

The results of efficacy studies presented in this chapter were inconsistent. Initial experiments showed that the efficacy of mannosylated liposomes was dependent on the dose of parasite antigen in the formulation (Fig. 5.5 C and 5.12) and neither humoral nor cellular responses directly correlated with protection (Fig. 5.14). However, in subsequent experiments when mice were immunised with mannosylated liposomes containing 10⁷ pRBC equivalent parasite antigens, no protection was observed following challenge with Py17X pRBCs (Fig. 5.20 and 5.26). Therefore, further liposome formulation optimisation studies will be required to achieve reproducibility of protective efficacy in preparation for transitioning to clinical trials.

Having observed induction of robust humoral and cell-mediated immune responses as well as protection albeit with inconsistent results following immunisation with mannosylated liposomes, mechanisms of immune protection were then examined.
Liposomes containing inactivated *P. falciparum* blood-stage antigens

The data presented in this chapter suggests that T cells might be critical in the induction of protective immune responses since mice that received CD4+ and CD8+ T cell depleting antibodies all succumbed to malaria infection while some control mice that received rat IgG survived (Fig. 5.28). Studies such as *in vitro* growth inhibition assays, immune serum transfer experiments and B cell knock-out animal studies will be required to elucidate the role of humoral immune responses in the induction of protective immunity.

In summary, the studies described herein show that immunisation with mannosylated liposomes containing inactivated *P. falciparum* 7G8 antigen depleted of red cell membranes results in the induction of robust immune responses and in some instances protects mice against a heterologous wild-type infection (Fig. 5.5 C, 5.12 and 5.28). This heterologous challenge infection model strongly supports transition of mannosylated liposome vaccines to human trials. Such a vaccine capable of inducing robust heterologous protective immune responses and controlling parasite growth as demonstrated in some experiments (Fig. 5.5 C, 5.12 and 5.28 A) might be of relevance in malaria endemic areas where semi-immune individuals are exposed to different parasite strains/species and remain asymptomatic with high parasite burdens (>2000 parasites per μl) (Afrane et al., 2014; Elliott et al., 2005; Geiger et al., 2013; Sirima et al., 2011; Williams et al., 2012). Administration of such a vaccine could effectively reduce the parasite densities and prevalence severe clinical disease, in effect reducing malaria transmission in these populations.
Chapter 6: Immunogenicity and protective efficacy of mannosylated liposomes containing inactivated Py17X blood-stage antigens
6.0 Introduction

Studies in our laboratory have previously shown that mannosylated liposomes encapsulating rodent whole parasites (P. chabaudi and P. yoelii) protected mice from a homologous blood-stage challenge infection (Giddam et al., 2016). However, in these studies immunological evaluations were limited. In Chapter 5, it was shown that immunisation of mice with mannosylated liposomes containing red cell membrane-depleted human parasite, P. falciparum antigens, resulted in the induction of robust species-transcending immunity and in some instances protected mice against a wild-type heterologous challenge infection.

In this chapter, a homologous challenge model was developed to examine the immunogenicity and protective efficacy of a multi-stage liposome-based malaria vaccine formulation. It is hypothesised that such a vaccine will elicit potent immune responses against both liver and blood-stage malaria infections. Here, liposomes were formulated with different combinations of F3, the circumsporozoite (CS) repeat peptide derived from the rodent parasite, P. yoelii (QGPGAP)4 and 3D-PHAD, a synthetic analogue of Monophosphoryl Lipid A (MPLA) (also referred to as PHAD hereonin). The repeat peptide (QGPGAP)4, is the immuno-dominant B cell epitope of the P. yoelii circumsporozoite protein (CSP) and antibodies against it have been shown to protect mice from sporozoite challenge (Charoenvit et al., 1990; Ak et al., 1993; Wang et al., 1995). Previous studies using circumsporozoite repeat peptides in liposome formulations have shown that addition of MPLA is critical in the induction of robust humoral and cell-mediated immune responses (Wang et al., 1995; Richards et al., 1998), hence the inclusion of PHAD in our formulations. Furthermore, in this chapter, the immunogenicity of liposomes formulated with PHAD as an additional adjuvant was investigated in a different mouse strain.
Additionally, the utility of the immunomagnetic method, piloted in Chapter 5, to deplete red cell membranes from Py17X antigens was further examined.

6.1 Results

6.1.1 Depletion of red cell membrane antigen from Py17X parasites

Here, antibodies to TER-119, the major glycoprotein of mouse erythrocyte membranes were used to deplete red cell membranes from Py17X antigens (*method described in Chapter 2 section 2.5.2*). Using flow cytometry, median fluorescent intensity (MFI) measurements of particulate parasite antigen showed 77.9 - 98.6% red blood cell membrane depletion following immunomagnetic purification (*Table 6.1 and Fig. 6.1 A*). To confirm presence of parasite DNA, the extract was stained with bisbenzimide hoechst. Similar proportions of parasite DNA were observed pre- and post-purification (*Table 6.2 and Fig. 6.1 B*). These data support further support the utilisation of this method for depletion of red cell membranes from parasite antigen.

| Table 6.1. Estimation of red blood cell membrane depletion. Py17X antigen was stained with anti-TER-119 PE pre- and post-immunomagnetic purification. The MFI was used to estimate percentage depletion of red cell membranes. |
|-------------------------------------------------|-----------------|-----------------|
| Experiment 1                                    | Experiment 2    | Experiment 3    |
| Post-magnetic purification (MFI)                 | 1691            | 1048            | 95.3            |
| Pre-magnetic purification (MFI)                  | 10052           | 4741            | 6963            |
| % Depletion                                     | 83.2%           | 77.9%           | 98.6%           |
Table 6.2. Confirmation of parasite DNA. Py17X antigen was stained with bisbenzimide hoechst pre- and post-immunomagnetic purification. The MFI was used to confirm the presence of parasite DNA in extract.

<table>
<thead>
<tr>
<th></th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Post-magnetic purification (MFI)</td>
<td>11974</td>
<td>11776</td>
<td>1529</td>
</tr>
<tr>
<td>Pre-magnetic purification (MFI)</td>
<td>11940</td>
<td>11930</td>
<td>2007</td>
</tr>
</tbody>
</table>

Figure 6.1. Immunomagnetic depletion of red cell membranes from Py17X antigen. Representative overlay plots from 3 independent experiments showing differential staining patterns before and after immunomagnetic purification. (A) Anti-TER-119 PE and (B) bisbenzimide hoechst were used to stain samples for red cell membrane antigen and parasite DNA respectively.

6.1.2 Liposome size characterisation

Liposomes were formulated using cationic lipids with F3, PHAD or (QGPGAP)_4 anchored into the liposome membrane by two palmitic acid molecules. The liposome formulations were prepared as described in Chapter 2 section 2.5.2. Analysis of particle size, measured as volume-weighted diameter, indicated that particle sizes ranged from 13-28 µM with span values between 1.6 – 3 (Table 6.3).
Table 6.3. Volume-weighted particle size and size distribution of liposome formulations.

<table>
<thead>
<tr>
<th>Liposome formulation</th>
<th>Size (µM)</th>
<th>Span</th>
</tr>
</thead>
<tbody>
<tr>
<td>F3 + PHAD + (QGPGAP)$_4$ + Py17X antigen</td>
<td>17.8</td>
<td>2.0</td>
</tr>
<tr>
<td>F3 + PHAD + Py17X antigen</td>
<td>28.6</td>
<td>2.0</td>
</tr>
<tr>
<td>F3 + (QGPGAP)$_4$ + Py17X antigen</td>
<td>28.6</td>
<td>3.0</td>
</tr>
<tr>
<td>PHAD+ (QGPGAP)$_4$ + Py17X antigen</td>
<td>13.4</td>
<td>1.6</td>
</tr>
<tr>
<td>F3 + PHAD + (QGPGAP)$_4$ in PBS</td>
<td>16.6</td>
<td>2.1</td>
</tr>
</tbody>
</table>

6.1.3 Investigation of the immunogenicity and protective efficacy of liposomes containing Py17X antigens in BALB/c mice

Here, liposomes were formulated with different combinations of F3, PHAD and the circumsporozoite (CS) repeat peptide derived from the rodent parasite, *P. yoelii* (QGPGAP)$_4$. This peptide is the immuno-dominant B cell epitope of the circumsporozoite protein (CSP) and antibodies against it have been shown to protect mice from sporozoite challenge (Charoenvit et al., 1990; Ak et al., 1993; Wang et al., 1995). Liposomes were prepared with red cell membrane-depleted parasite antigen (*Chapter 2 section 2.5.2*) or with red cell membranes in parasite antigen (*Chapter 2 section 2.5.3*). Liposomes were administered subcutaneously in a 3-dose prime-boost regimen with a primary immunisation on day 0 followed by two boosts given two weeks apart (*Fig. 6.2*).
Liposomes containing inactivated Py17X blood-stage antigens

Figure 6.2. Immunisation schedule. BALB/c mice were immunised subcutaneously with liposomes containing Py17X parasite antigen on days 0, 14 and 28. Four weeks after the third immunisation mice were challenged intravenously via the tail vein with $10^5$ Py17X pRBCs.

6.1.3.1 Assessment of humoral immune responses

Seven days after the third immunisation, Py17X-specific IgG titres were significantly elevated in all mice immunised with liposomes containing Py17X antigens compared to control mice that received empty liposomes. Additionally, parasite-specific antibody responses were significantly elevated in mice immunised with liposomes containing PHAD compared to those that received liposomes without PHAD (P<0.033) (Fig. 6.3 A).

Significant (QGPGAP)$_4$-specific IgG titres were observed in mice immunised with F3-PHAD-(QGPGAP)$_4$ liposomes containing parasite antigen with or without red cell membranes and PHAD-(QGPGAP)$_4$ liposomes containing red cell membrane-depleted parasite antigen compared to control mice that received empty liposomes (P<0.033) (Fig. 6.3 B).
Liposomes containing inactivated Py17X blood-stage antigens

6.1.3.2 Assessment of antigen-experienced CD4+ and CD8+ T cell responses

Peripheral blood samples were collected seven days after the first and third immunisation for assessment of antigen-experienced T cells using early activation markers CD49dhiCD11a hi for CD4+ and CD8loCD11a hi for CD8+ (Butler et al., 2012; Butler et al., 2011). Fig. 5.7 in Chapter 5 shows the strategy used to gate for activated CD4+ and CD8+ T cells.

After the primary immunisation, antigen-experienced CD4+ T cells were significantly elevated in mice immunised with F3-PHAD-(QGPGAP)_4 and PHAD-(QGPGAP)_4 liposomes containing red cell membrane-depleted Py17X antigens compared to control mice that received empty liposomes (P<0.002) (Fig. 6.4 A). However, seven days after the third immunisation, significant upregulation of activated CD4+ and CD8+ T cells was observed in all mice immunised with liposomes containing red cell membrane-depleted Py17X antigens compared to the control mice that received empty liposomes (P<0.002) (Fig. 6.4 C and D).
Liposomes containing inactivated Py17X blood-stage antigens

6.1.3.3 Assessment of spleen cell proliferation and soluble cytokine production responses

Approximately four weeks after the third immunisation and prior to challenge, three mice from each group were sacrificed for spleen extraction and assessment of splenocyte proliferative responses as well as soluble cytokine production analyses. All Py17X-specific proliferative and cytokine production responses were compared to the corresponding mouse nRBCs stimulation for each group. Significant splenocyte proliferative responses to Py17X pRBCs were observed in all mice.
immunised with liposomes containing Py17X parasite antigens with or without red cell membrane depletion (P<0.001) (Fig. 6.5).

After 54 h of in vitro splenocyte culture, supernatants were collected for assessment of soluble cytokine production. In response to Py17X pRBC stimulation, IL-2 and TNF production were significantly elevated in mice immunised with F3-PHAD-(QGPGAP)_4, F3-PHAD and PHAD-(QGPGAP)_4 liposomes containing Py17X antigens with or without red cell membrane depletion (P<0.033) (Fig. 6.6 A and C), while, IFN-γ production was only elevated in mice that received F3-PHAD-(QGPGAP)_4 liposomes containing Py17X antigens with red cell membranes (P<0.001) (Fig. 6.6 B).
Liposomes containing inactivated Py17X blood-stage antigens

Figure 6.6. Th1 cytokine production responses in BALB/c mice immunised with liposomes containing Py17X antigen. Spleen cells were incubated with Py17X pRBCs, nRBCs, culture media or ConA. At 54 h, culture supernatants were collected for assessment of (A) IL-2, (B) IFN-γ and (C) TNF cytokine production responses. The cytokine production response in pg/ml was expressed as mean ± SEM (n=3 mice per group). Data were analysed using unpaired test to compare Py17X-specific cytokine responses to nRBC responses for each liposome formulation. *P<0.033, **P<0.002, ***P<0.001.
Liposomes containing inactivated Py17X blood-stage antigens

Immunisation with F3-PHAD-(QGPGAP)$_4$ liposomes containing parasite antigen with or without red cell membrane antigen as well as PHAD-(QGPGAP)$_4$ liposomes resulted in significant upregulation of Py17X specific-IL-4 responses ($P<0.033$) (Fig. 6.7 A). IL-6 production was enhanced in mice immunised with F3-PHAD-(QGPGAP)$_4$, F3-PHAD and PHAD-(QGPGAP)$_4$ liposomes containing Py17X antigens ($P<0.033$) (Fig. 6.7 B) while IL-10 production was significantly elevated in mice immunised with F3-PHAD-(QGPGAP)$_4$ liposomes containing Py17X antigens with red cell membranes and to a lesser extent, in mice immunised with PHAD-(QGPGAP)$_4$ liposomes containing red cell membrane-depleted Py17X antigens ($P<0.033$) (Fig. 6.7 C).
Figure 6.7. Th2 cytokine production responses in BALB/c mice immunised with liposomes containing Py17X antigen. Spleen cells were incubated with Py17X pRBCs, nRBCs, culture media or ConA. At 54 h, culture supernatants were collected for assessment of (A) IL-4, (B) IL-6 and (C) IL-10 cytokine responses. The cytokine production response in pg/ml was expressed as mean ± SEM (n=3 mice per group). Data were analysed using unpaired test to compare Py17X-specific cytokine production responses to nRBC responses for each liposome formulation. *P<0.033, **P<0.002, ***P<0.001.
Liposomes containing inactivated Py17X blood-stage antigens

Together, the data suggest that red cell membranes in this model might be critical in the induction of robust Th1/Th2 cytokine production responses. Additionally, cytokine responses were only observed in mice immunised with liposomes containing PHAD, indicating that PHAD is an essential component in the formulation of these liposomes and might be critical in the induction of robust cytokine responses.

**6.1.3.4 Assessment of the protective efficacy of liposomes containing Py17X antigen in BALB/c mice**

Four weeks after the third immunisation, mice were challenged intravenously with $10^5$ Py17X pRBCs and parasitaemia was monitored every second day by collecting thin blood smears, which were then stained with Giemsa for microscopic examination. Mice immunised with F3-PHAD-(QGPGAP)$_4$ liposomes containing Py17X antigen with red cell membranes displayed lower mean peak parasitaemia (10.3 ± 4.6%) compared to control mice [empty liposomes (46.1 ± 1.3 %, P<0.001), Py17X in PBS (36.2 ± 1.9%; P<0.033)] as well as mice immunised with liposomes containing red cell membrane-depleted Py17X antigen [F3-PHAD-(QGPGAP)$_4$ (23.1 ± 6.2%; P>0.999), PHAD-(QGPGAP)$_4$ (21.5 ± 6.5%; P>0.999), F3-PHAD (25.3 ± 8.8%; P>0.999), F3-(QGPGAP)$_4$ (34.8 ± 2.4%; P>0.999)] (Fig. 6.8 A). Additionally, mice immunised with F3-PHAD-(QGPGAP)$_4$ liposomes containing Py17X antigens with red cell membranes and PHAD-(QGPGAP)$_4$ liposomes significantly controlled parasite growth as measured by parasitaemia AUC at day 10 (P<0.033) (Fig. 6.8 B). Furthermore, survival analysis at the end of the 30-day monitoring period showed that mice immunised with F3-PHAD-(QGPGAP)$_4$ liposomes containing Py17X antigens with red cell membranes, F3-PHAD-(QGPGAP)$_4$, F3-PHAD and PHAD-
Liposomes containing inactivated Py17X blood-stage antigens (QGPGAP)₄ liposomes containing red cell membrane-depleted Py17X antigens had 57.1%, 28.5%, 14.3% and 42.9% survival rate respectively (Fig. 6.8 C, Table 6.4).

Table 6.4. Protection of BALB/c mice against challenge with Py17X pRBCs following immunisation with F3-PHAD liposomes containing Py17X antigen

<table>
<thead>
<tr>
<th>Liposome formulation</th>
<th>No. protected/No. challenged</th>
<th>% protection</th>
</tr>
</thead>
<tbody>
<tr>
<td>F3 + PHAD+(QGPGAP)₄ + Py17X with RBC membranes</td>
<td>4/7</td>
<td>57.1</td>
</tr>
<tr>
<td>F3 + PHAD+(QGPGAP)₄ + Py17X without RBC membranes</td>
<td>2/7</td>
<td>28.5</td>
</tr>
<tr>
<td>F3 + PHAD+ Py17X without RBC membranes</td>
<td>1/7</td>
<td>14.3</td>
</tr>
<tr>
<td>PHAD+(QGPGAP)₄ + Py17X without RBC membranes</td>
<td>3/7</td>
<td>42.9</td>
</tr>
</tbody>
</table>

Figure 6.8. Monitoring of parasitaemia and survival following challenge with Py17X pRBCs of BALB/c mice immunised with liposomes containing Py17X antigen (A) Parasitaemia, (B) parasitaemia area under the curve (AUC) on day 10 post challenge and (C) Kaplan-Meier survival curves up to 30 days post challenge. Data are expressed as mean ± SEM (n=7 mice per group). Data were analyzed using unpaired Mann-Whitney U test to compare vaccinated groups to mice that received liposomes without parasite antigen. *P<0.033, ***P<0.001. †Indicates the number of mice that were euthanased.
Liposomes containing inactivated Py17X blood-stage antigens

Clinical scores and haemoglobin levels (a marker for anaemia) were recorded to monitor disease severity. At the peak of clinical disease on day 12 when some mice had to be euthanased due to poor clinical outcomes, mice immunised with liposome formulations containing Py17X antigen displayed lower mean clinical scores [F3-PHAD-(QGPGAP)₄ liposomes containing parasite antigen with red cell membranes (1.7 ± 0.2), F3-PHAD-(QGPGAP)₄ (2.3 ± 0.3), PHAD-(QGPGAP)₄ (2.5 ± 0.4), F3-PHAD (2.7 ± 0.4), F3-(QGPGAP)₄ (3.1 ± 0.1)] compared to control mice [Empty liposomes (4 ± 0), Py17X in PBS (3.7 ± 0.1) (Fig. 6.9 A). Conversely, there was no observed difference in anaemia amongst liposome immunised and control mice by day 12 (Fig. 6.9 B). However, some mice immunised with F3-PHAD and PHAD liposomes recovered to normal haemoglobin levels by day 21 post-challenge (Fig. 6.9 B).

Figure 6.9. Monitoring of disease severity following challenge with Py17X pRBCs of BALB/c mice immunised with liposomes containing Py17X antigen. (A) Clinical scores and (B) Haemoglobin (g/L) following homologous challenge with Py17X infected red blood cells. Data were expressed as mean ± SEM (n=7 mice per group). †Indicates the number of mice that were euthanased.
Liposomes containing inactivated Py17X blood-stage antigens

These challenge data suggest that immunisation with liposomes containing Py17X antigen is critical in the induction of protective immune responses in BALB/c mice as none of the controls (mice immunised with empty liposomes and Py17X antigen in PBS) were protected against a wild-type challenge infection and recorded the highest parasitaemias and clinical scores (Fig. 6.8 A and C). Additionally, protection was only observed amongst mice immunised with PHAD-containing liposomes, indicating that PHAD is an essential component in the formulation of these liposomes and might be critical in the induction of protective immune responses in mice (Fig. 6.8 A and C).

6.1.3.5 Investigation into the correlates of protective immunity

Based on the immunogenicity data, the correlates of protective immunity were then interrogated. It was found that mice that were protected against a Py17X challenge had significantly higher Py17X-specific antibody responses compared to mice that had to be euthanized due to poor clinical outcomes (P<0.001) (Fig. 6.10 A). However, there was no significant difference observed in peripheral blood CD4+ and CD8+ T cell activation in protected mice and mice that succumbed to infection (P>0.999) (Fig. 6.10 B and C).
Liposomes containing inactivated Py17X blood-stage antigens

Collectively, these data suggest that F3-PHAD and PHAD liposomes containing Py17X antigen with or without red cell membranes are highly immunogenic and protect BALB/c mice against a wild-type Py17X challenge infection possibly via an antibody-mediated immune response. However, immunogenicity and survival were higher in mice immunised with liposomes containing membranes.

Figure 6.10. Correlates of protection following challenge in BALB/c mice immunised with liposomes containing Py17X antigen. (A) Antibody responses, (B) activated CD4+ and (C) CD8+ T cells in mice that were protected compared to those that succumbed to a wildtype Py17X challenge infection. Data were analysed using Mann-Whitney rank correlation test. ***P<0.001, ns: not significant.
6.1.4 Investigation into the immunogenicity and protective efficacy of liposomes containing Py17X antigens in C57BL/6 mice

Based on the data from the BALB/c mouse study (section 6.1.3), a follow-up parasite antigen dose-escalation study was carried out in a different mouse strain, C57BL/6 mice, which displays distinct blood-stage disease manifestations and immune response profiles (Langhorne et al., 2002), with modifications to the liposome formulations. Due to the low antibody responses to the CSP repeat peptide (Fig. 6.3B), in this study the peptide was not included in the formulations. Furthermore, the impact of PHAD on the immunogenicity of liposomes as well as the effect of an additional booster dose was examined. Fig. 6.11 shows the immunisation protocol for the study. The third boost was administered seven days after the third immunisation and consisted of half the amount of lipids and parasite antigen.

![Immunisation protocol](image)

**Figure 6.11. Immunisation schedule for C57BL/6 mouse study.** Mice were immunised subcutaneously with liposomes containing Py17X parasite antigen on days 0, 14, 28 and 35. Four weeks after the fourth immunisation mice were challenged intravenously via the tail vein with $10^5$ Py17X pRBCs.

6.1.4.1 Assessment of humoral immune responses

Py17X–specific IgG responses were significantly elevated in mice immunised with F3, F3-PHAD and PHAD liposomes containing $10^7$ pRBC equivalent red cell
membrane-depleted antigen indicating that a higher parasite antigen dose might be crucial in the induction of robust humoral immune responses (P<0.001) (Fig. 6.12).

![Figure 6.12. Py17X-specific total IgG antibody responses in C57BL/6 immunised with liposomes containing Py17X antigen.](image)

**Figure 6.12.** *Py17X*-specific total IgG antibody responses in *C57BL/6* immunised with liposomes containing *Py17X* antigen. Sera were collected 7 days after fourth immunisation. The antibody response (serum dilution 1:100) represented as OD measured at 450 nm wavelength was expressed as mean ± SEM (n=10 per group). Data were analyzed using unpaired Mann-Whitney U test to compare antibody responses in immunised mice to controls that received empty liposomes. ***P<0.001. HIS: *Py17X* Hyperimmune serum used as positive control.

### 6.1.4.2 Assessment of antigen-experienced CD4+ and CD8+ T cell responses

In *C57BL/6* mice, there was no significant activation of CD4+ or CD8+ T cells after one immunisation (Fig. 6.13 A and B). However, after the fourth immunisation, a significant upregulation of antigen-experienced CD4 and CD8+ T cells was observed in mice vaccinated with F3, F3-PHAD and PHAD liposomes containing 10^7 pRBC equivalent RBC membrane depleted antigens compared to control mice immunised with empty liposomes (P<0.002, P<0.002 and P<0.001 respectively for CD4+ T cells and P<0.001 for CD8+ T cells) (Fig. 6.13 C). Additionally, enhanced activation of antigen-experienced CD8+ T cells was observed in mice immunised with liposomes...
containing $10^7$ pRBC equivalent red cell membrane depleted parasite antigen compared to $10^6$ pRBC equivalent red cell membrane-depleted parasite antigen (P<0.001) (Fig. 6.13 D).

Figure 6.13. Peripheral blood T cell activation in C57BL/6 immunised with liposomes containing Py17X antigen Antigen-experienced (A) CD4+, (B) CD8+ 7 days after the 1st immunisation and (C) CD4+, (D) CD8+ 7 days after the fourth immunisation antigen-experienced CD4+ and CD8+ T cells were characterised as CD49dhiCD11ahi and CD8+CD11ahi respectively. Data are expressed as mean ± SEM (n=10 mice per group). Data were analyzed using unpaired Mann-Whitney U test to compare T cell activation in immunised mice to controls that received empty liposomes. *P<0.033, **P<0.002, ***P<0.001.

6.1.4.3 Assessment of spleen cell proliferation and soluble cytokine production responses

Approximately four weeks after fourth immunisation and prior to challenge, three mice from each group were sacrificed for spleen extraction and assessment of splenocyte proliferative responses as well as soluble cytokine production analysis. All parasite-specific proliferative and cytokine production responses were
Liposomes containing inactivated Py17X blood-stage antigens compared to the corresponding mouse nRBCs or human nRBCs responses for each group. Significant splenocyte proliferative responses to Py17X pRBC stimulation were elevated in all mice immunised with liposomes containing Py17X parasite antigens (P<0.001) (Fig. 6.14 A) while *P. falciparum* 7G8- specific proliferative responses were significantly higher in mice immunised with PHAD only liposomes containing $10^7$ pRBC equivalent red cell membrane depleted parasite antigens (P<0.001) (Fig. 6.14 B).
Liposomes containing inactivated Py17X blood-stage antigens

Cytokine analysis of supernatants collected following 54 h of culture with homologous Py17X pRBCs showed a significant upregulation of IL-2 production in all mice immunised with F3, F3-PHAD and PHAD liposomes containing a red cell membrane-depleted parasite antigen (P<0.001) as well as F3 liposomes containing 10^6 pRBC equivalent with or without red cell membranes depleted (P<0.001) (Fig.

Figure 6.14. Splenocyte proliferative responses in C57BL/6 immunised with liposomes containing Py17X antigen. Spleen cells were incubated with (A) Py17X pRBCs, (B) *P. falciparum* 7G8 pRBCs, mouse nRBCs or culture media and pulsed with tritiated thymidine. The proliferative response represented as ³H thymidine uptake CPM was expressed as mean ± SEM (n=3 mice per group). Data were analysed using unpaired t test to compare Py17X /7G8-specific proliferative responses to mouse nRBC/human nRBC responses for each liposome formulation. ***P<0.001.
Liposomes containing inactivated Py17X blood-stage antigens

**6.15 A.** In contrast, *P. falciparum* 7G8 specific-IL-2 production responses were significantly elevated in mice immunised with F3-PHAD liposomes containing $10^6$ pRBC equivalent red cell membrane-depleted parasite antigens ($P<0.002$) (**Fig. 6.15 A**). In response to homologous and heterologous stimulation, IFN-γ responses were significantly elevated in mice immunised with PHAD only liposomes ($P<0.002$) (**Fig. 6.15 B**) while TNF responses were significantly enhanced amongst mice immunised with F3-PHAD and PHAD liposomes containing red cell membrane-depleted parasite antigens ($P<0.001$) (**Fig. 6.15 C**).
Liposomes containing inactivated Py17X blood-stage antigens

Figure 6.15. Th1 cytokine production responses in C57BL/6 immunised with liposome formulations containing Py17X antigen. Spleen cells were incubated with Py17X pRBCs, *P. falciparum* 7G8 pRBCs, mouse nRBCs or culture media. At 54 hours, culture supernatants were collected for assessment of (A) IL-2, (B) IFN-γ and (C) TNF cytokine responses. The cytokine production response in pg/ml was expressed as mean ± SEM (n=3 mice per group). Data were analysed using unpaired t test to Py17X/7G8-specific cytokine responses to mouse nRBC/human nRBC responses for each liposome formulation. **P<0.002, ***P<0.001.
Immunisation with F3, F3-PHAD and PHAD liposomes containing $10^7$ pRBC equivalent red cell membrane depleted parasite antigens resulted in significant Py17X-specific IL-6 production responses (P<0.033) while *P. falciparum* 7G8-specific IL-6 production responses were elevated in mice immunised with F3-PHAD and PHAD liposomes containing $10^7$ pRBC equivalent red cell membrane depleted parasite antigens (P<0.033) (Fig. 6.16 B). However, no responses of IL-4 and IL-10 production were observed following immunisation of C57BL/6 mice with F3, F3-PHAD and PHAD liposomes (Fig. 6.16 A and C).
Liposomes containing inactivated Py17X blood-stage antigens

Figure 6.16. Th2 cytokine production responses in C57BL/6 immunised with liposome formulations containing Py17X antigen. Spleen cells were incubated with Py17X pRBCs, *P. falciparum* 7G8 pRBCs, mouse nRBCs or culture media. At 54 h, culture supernatants were collected for assessment of (A) IL-4 (B) IL-6 and (C) IL-10 cytokine responses. The cytokine production response in pg/ml was expressed as mean ± SEM (n=3 mice per group). Data were analysed using unpaired t test to compare Py17X /7G8-specific cytokine responses to mouse nRBC/human nRBC responses for each liposome formulation.

*P<0.033, **P<0.002, ***P<0.001.
Collectively, the data suggests that immunisation of C57BL/6 with F3-PHAD and PHAD liposomes containing Py17X antigens results in the induction of a Th1-biased cytokine production response.

6.1.4.4 **Assessment of the protective efficacy of liposomes containing Py17X antigen in C57BL/6 mice**

Four weeks after the last immunisation, mice were challenged intravenously with 10^5 Py17X pRBCs and thin blood films were collected every second day to monitor parasitaemia by microscopy. On day 12 post-challenge, mice immunised with F3-PHAD and PHAD liposomes containing 10^7 pRBC equivalent red cell membrane depleted parasite antigens exhibited lower mean parasitaemias (22.3 ± 7.8% and 24.9 ± 2.8 % respectively) compared to control mice immunised with empty liposomes (49.4 ± 4.7 %) as well as mice immunised with other liposome formulations [F3 (10^6) with red cell membranes (39.3 ± 6.9 %), (F3 (10^6 and 10^7) without red cell membranes (41.1 ± 5.8 % and 37 ± 3.4 %), F3-PHAD (10^6) with or without red cell membranes (39.5 ± 8.2 % or 32.3 ± 3.7 %)] (Fig. 6.17 A). Furthermore, AUC analysis on day 12 showed that mice immunised F3-PHAD and PHAD liposomes containing 10^7 pRBC equivalent red cell membrane depleted parasite antigens significantly controlled parasite growth compared to control mice (P<0.033 and 0.002 respectively) (Fig. 6.17 B).
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Clinical disease severity monitoring on day 12 post challenge indicated that mice immunised with F3-PHAD and PHAD liposomes containing 10⁷ pRBC equivalent red cell membrane depleted parasite antigens overall, displayed lower clinical scores (1.7 ± 0.2 and 1.8 ± 0.1 respectively) compared to control mice (3 ± 0) as well as mice immunised with other liposome formulations [F3 (10⁶) pRBC equivalent parasite antigens with red cell membranes (2.8 ± 0.2), (F3 (10⁶ and 10⁷) pRBC equivalent red cell membrane depleted parasite antigens (3 ± 0 and 2.7 ± 0.2), F3-PHAD (10⁶) pRBC equivalent parasite antigens with or without red cell membranes (2.4 ± 0.2 or 2.7 ± 0.3)] (Fig. 6.18 A). Conversely, there was no observed difference in anaemia amongst liposome immunised and control mice by day 12 (Fig. 6.18 B). All mice developed severe disease and had to be euthanased by day 18 based on clinical scores.

Figure 6.17. Monitoring of parasitaemia following challenge in C57BL/6 immunised with liposome formulations containing Py17X antigen (A) Parasitaemia and (B) Parasitaemia Area Under the Curve (AUC) at day 12 post challenge. Mice were challenged with 10⁵ Py17X infected red blood cells 4 weeks after third booster immunisation. The data were expressed as mean ± SEM (n= 7 mice per group). AUC data were analyzed using unpaired Mann-Whitney U test to compare vaccinated groups to mice that received empty liposomes. *P<0.033, **P<0.002. +Indicates mice that were euthanased.
Liposomes containing inactivated Py17X blood-stage antigens

In summary, these data suggest that immunisation with F3, F3-PHAD and PHAD liposomes containing a parasite equivalent of $10^7$ antigens result in the induction of robust humoral and cell-mediated immune responses but unable to induce protective immunity in C57BL/6 mice following challenge.

6.2 Discussion

In this chapter, the immunogenicity and protective efficacy of whole parasite liposome formulations was examined in 2 mouse strains (BALB/c [H-2d] and C57BL/6 [H-2b]), with different genetic backgrounds and display distinct disease manifestations and immune response profiles (Langhorne et al., 2002; Sellers et al., 2012). Additionally, the utility of the immunomagnetic method, piloted in Chapter 5, to deplete RBC membranes from Py17X antigens was further examined.

Firstly, to address the induction of anti-RBC antibodies in vaccinees, it was shown that using a combination of magnetic beads and antibodies, RBC membranes can be efficiently depleted from parasite antigen without affecting the parasite DNA.

Figure 6.18. Monitoring of disease severity following challenge in C57BL/6 mice immunised with liposomes containing Py17X antigen. (A) Clinical scores and (B) Haemoglobin (g/L) monitoring of disease severity following homologous challenge with Py17X infected red blood cells. Data are expressed as mean ± SEM (n=7 mice per group). † Indicates the number of mice that were euthanased.
Liposomes containing inactivated Py17X blood-stage antigens

content (Fig. 6.1 and Table. 6.1 and 6.2). In this homologous model, the induction of RBC antibodies amongst immunised mice was not examined since liposomes were prepared using pRBCs collected from the same inbred mouse strain.

Secondly, the size of the different liposome formulations was characterised, and it was found that the particle sizes ranged from 13-28 μM (Table. 6.3). Similar to results described in Chapter 5, the effect of size and homogeneity of liposome formulations was not investigated, and further studies will be required to examine the effect of size on the subsequent immune response generated following immunisation.

In BALB/c mice, a multi-stage liposome formulation was attempted with the addition of circumsporozoite (CS) repeat peptide sequences, (QGPGAP)₄, derived from the rodent parasite, P. yoelii. These repeat peptide sequences have been shown to require an antigen delivery platform and T cell helper epitopes for effective immunogenicity in mice (Grillot et al., 1990). It was hypothesized that charged liposomes and parasite antigen can be utilised as carriers and T cell helper epitopes. It was hoped that if strong anti-CSP responses were induced this approach could also be used to limit sporozoite inoculation into hepatocytes. However, low antibody (QGPGAP)₄ titres (<10⁴) were observed in immunised mice and the (QGPGAP)₄-specific antibody response was associated with the presence of PHAD in the liposome formulations (Fig. 6.3 B). Since liposomes containing lipid A have been previously shown to be efficient carriers of P. yoelii circumsporozoite peptides (Wang et al., 1995), the low (QGPGAP)₄ titres to could be due to inefficient T cell help provided by parasite antigen. Wang et al., 1995, utilised T epitopes derived from tetanus toxin to provide T cell help for induction of protective antibody response to (QGPGAP)₄. Additionally, the low antibody response to (QGPGAP)₄ observed could also have been due to the mouse strain (BALB/c [H-2d]) used in the studies
Liposomes containing inactivated Py17X blood-stage antigens described in this chapter as previous studies have shown that antibody responses to repeat peptides sequences of *P. yoelii* CSP are genetically restricted to mice bearing the H-2^b^ haplotype (Grillot et. al 1990). Given that high antibody titres (>10^4^) are required for protection against sporozoite challenge infection (Ak et al., 1993; Charoenvit et al., 1990; Wang et al., 1995), for this to be developed as a viable liver-stage vaccine, the addition of universal T helper epitopes or a conjugated carrier protein such as diphtheria / tetanus toxoid in the liposome formulations as well as use of a responder mouse strain such as C57BL/6 (H-2^b^) needs to be considered in future studies.

Parasite–specific antibody responses were observed in BALB/c mice immunised with liposomes containing Py17X antigens and PHAD indicating that PHAD could be critical in the induction of robust humoral immune responses in these mice (Fig. 6.3 A). In C57BL/6 mice, Py17X–specific IgG responses were significantly elevated in mice immunised with F3, F3-PHAD and PHAD liposomes containing an equivalent of 10^7^ Py17X parasites (Fig. 6.12). Previous studies using F3 liposomes containing an equivalent of 10^6^ Py17X antigens with red cell membranes did not report significant parasite-specific antibody responses in C57BL/6 (Giddam et al., 2016) indicating that the responses reported in the current study are associated with a higher parasite antigen dose and presence of PHAD in the formulations. Following challenge, there was a strong association between elevated parasite–specific antibody titres and protection in BALB/c mice (Fig. 6.10 A). In the C57BL/6 mouse model, groups with significant parasite-specific antibody responses-controlled infection with lower parasitaemia AUC (Fig. 6.17 B). Taken together, these data underpin the relevance of a PHAD (in BALB/c mice) and a high antigen dose (in C57BL/6) for the induction of humoral immune responses and might be critical in the control of infection following challenge. Therefore, future experiments such as
serum transfer studies and B cell knock-out animal studies will be required to elucidate the role of parasite-specific antibodies in the induction of protective immunity.

With respect to cell-mediated immune responses, immunisation of BALB/c mice with liposomes containing red blood cell membrane-depleted Py17X antigens augmented levels of activated peripheral blood CD4+ and CD8+T cells 7 days after the last immunisation (Fig. 6.4 C and D). Additionally, in C57BL/6 mice, a parasite antigen dose-dependent induction of antigen-experienced T cells was observed. However, T cell activation did not correlate with protection following challenge in BALB/c mice (Fig. 6.10 B and C). Studies in mice have shown that parasite-specific T cells critical in the control of blood-stage malaria infection are also abundant in both lymphoid and non-lymphoid organs (Muxel et al., 2011; Villegas-Mendez et al., 2015). In the studies described herein, antigen-experienced T cells were only examined in peripheral blood. Therefore, assessment of non-circulating antigen-experienced T cells in lymphoid and non-lymphoid organs following immunisation with liposome formulations will be critical in elucidating the protective role of T cells following challenge in future experiments.

In vitro splenocyte proliferative responses were investigated approximately four weeks after the last immunisation. In response to homologous stimulation with Py17X pRBCs, robust proliferative responses were observed in splenocytes from both BALB/c and C57BL/6 mice (Fig. 6.5 and 6.14 A). In the C57BL/6 model, proliferative responses to heterologous P. falciparum pRBCs stimulation were only observed in the spleens of mice immunised with PHAD liposomes containing $10^7$ pRBC equivalent Py17X parasites (Fig. 6.14 B).

Cytokine production analysis of culture supernatants revealed that cytokine responses were primarily observed in mice (BALB/c and C57BL/6) immunised with
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liposome formulations containing PHAD (Fig. 6.6, 6.7, 6.15 and 6.16), further emphasizing the relevance of PHAD in the induction of robust immune responses. In BALB/c mice, immunisation with F3-PHAD liposomes containing parasite antigen with red cell membranes resulted in the induction of higher Th1 (IFN-γ, IL-2, TNF) (Fig. 6.6) and Th2 (IL-4, IL-6, IL-10) (Fig. 6.7) cytokine responses compared to mice immunised with liposomes containing red cell membrane-depleted parasite antigen. Interestingly, significant concentrations of IFN-γ, IL-4 and IL-10 (Fig. 6.6 B, Fig. 6.7 A and C) were observed in mice belonging to liposome immunised groups that had some animals survive a wild-type infection challenge (Fig. 6.8 C and Table 6.4). These data indicate that red cell membranes and PHAD might be critical in the induction of robust cytokine production responses in BALB/c mice. The ability of these liposomes to induce both pro-inflammatory and immunomodulatory cytokine responses to clear the infection and regulate the inflammatory response respectively, might partly explain the protection observed in BALB/c mice. In C57BL/6 mice, the response was restricted to pro-inflammatory cytokine production (IFN-γ, IL-2, TNF and IL-6) (Fig. 6.15 and 6.16) which might explain the failure of these mice to control infection and immune-mediated pathology. Similar to proliferative responses, the cytokine responses observed in our studies could not be directly associated with protection following challenge since the assays were conducted in a separate subset of unchallenged mice. Therefore, future studies will need to be designed to critically examine the role of this cytokine milieu in the induction of protective immunity.

In conclusion, F3-PHAD and PHAD liposomes therefore present an attractive whole parasite antigen delivery platform that might be exploited for the development of a multi-stage malaria vaccine. Further studies will be required to examine the utility
of F3-PHAD and PHAD liposomes in a heterologous challenge model such as that described in *Chapter 5*. 
Chapter 7: General conclusions and future directions
General conclusions and future directions

Introduction

Despite the significant reduction in incidence, morbidity and mortality over the last 6 years (World Health Organisation, 2017), malaria remains a major global health problem. The best hope for ending malaria, particularly in endemic areas, is through the development and administration of a safe, affordable and effective vaccine. The studies described herein have focused on the development of a whole parasite blood-stage malaria vaccine. An effective blood-stage vaccine will be critical in the reduction of clinical disease and malaria transmission. Additionally, such a vaccine will bridge the gap between partially effective liver-stage vaccines such as RTS, S and control measures such as anti-malarial drugs and insecticide-treated bed nets that are threatened by resistance of the parasite and mosquito vector respectively, as well as inappropriate use. Below is a summary of the findings in this thesis as well as an insight into future work that will be required to advance these approaches.

Collection of \textit{P. falciparum} isolates from human volunteers with malaria

In this chapter, clinical isolates of \textit{P. falciparum} from human volunteers living in Uganda were successfully collected. These isolates are valuable reagents for the preliminary evaluation of malaria vaccine candidates prior to deployment for further testing in endemic countries. In the studies presented in this thesis, cryopreserved clinical isolates UGMCB-009 and UGMCB-013 were successfully culture-adapted \textit{in vitro} and used in parasitological (Chapter 4) and immunological assays (Chapter 5). Preliminary anti-malarial drug susceptibility characterisation revealed that these parasites are sensitive to chloroquine and Riamet as well as gold(I) phosphine compounds auranofin and [Au(d2pype)2]Cl. Further characterisation with a larger panel of established anti-malarial drugs will be required, especially if these isolates are to be used in future CHMI studies. These
data will guide selection of the most appropriate prophylactic drug if treatment is required in CHMI studies using these isolates. However, only two of the eight isolates collected were culture-adapted and their drug sensitivity determined. Therefore, to increase access to diverse *P. falciparum* strains for use in laboratory assays and CHMI studies, the remaining clinical isolates will also need to culture-adapted and characterised.

**Chemical attenuation of blood-stage malaria parasites for malaria vaccine development**

Previous studies in our laboratory showed that DNA-binding drugs can effectively attenuate blood-stage parasites and immunisation with these attenuated parasites can induce long-lived strain-transcending protective immunity in mice (Good et al., 2013; Raja et al., 2016). However, DNA-binding drugs are potentially genotoxic and hence there is a need to screen new attenuating compounds, utilising different mechanisms of action to further facilitate vaccine development using this approach. In this thesis, the anti-malarial activity of gold (I) phosphine compounds was exploited to investigate their utility as attenuating agents in vaccine development. These compounds act via the inhibition of antioxidant networks, which are critical for the parasite’s survival from oxidative stress. Complete attenuation of blood-stage rodent parasites was achieved following short-term *in vitro* culture with gold (I) phosphine compounds. Immunisation of mice with completely attenuated parasites did not result in the induction of protective immunity following challenge with a wild-type malaria infection. However, when mice were immunised with partially attenuated parasites, they were protected following challenge indicating that partial *in vitro* attenuation was critical for the induction of protective immune responses. Future studies will therefore be required to optimise the reproducibility of achieving partial attenuation or the dose of completely attenuated pRBCs and
thus enable further development of this approach for an effective blood-stage malaria vaccine.

Furthermore, in this chapter, the anti-parasitic activity of gold (I) phosphine compounds was tested \textit{in vivo}. When administered orally, these compounds were unable to inhibit parasite growth indicating that pharmacokinetic studies will be required to further examine bioavailability following administration. Additionally, with structural differences in redox mechanisms of \textit{P. falciparum} and the human host being identified (Boumis et al., 2012), gold compounds can be better designed to more efficiently target and selectively inhibit the parasite.

**Immunogenicity and protective efficacy of liposome-based whole parasite vaccines**

The development and clinical evaluation of live-attenuated blood-stage vaccines such as the one described in \textit{Chapter 4} of this thesis presents a number of challenges including but not limited to regulatory and safety considerations for the use of blood products in humans as well as manufacturing, storage and delivery especially in malaria endemic areas (Stanisic & Good, 2015). To address some of these challenges, our laboratory has recently developed a novel liposome-based antigen delivery platform as an alternative to the live-attenuated whole parasite approach. Vaccination studies using this platform demonstrated that liposomes formulated with mannose (mannosylated) and containing whole inactivated rodent parasites (\textit{P. chabaudi} and \textit{P. yoelii}) protected mice from a homologous challenge infection (Giddam et al., 2016). However, in these studies immunological evaluations were limited. Therefore, to further develop this approach, in the studies outlined in this thesis, two pre-clinical rodent models were used: (i) a heterologous challenge model to enable the pre-clinical assessment of a \textit{P. falciparum} mannosylated liposome
vaccine and (ii) a homologous challenge model to examine the immunogenicity and protective efficacy of a whole parasite multi-stage *P. yoelii* liposome-based vaccine. Multiple parameters were examined to optimise the liposome formulations and address some challenges to the development of whole parasite blood-stage malaria vaccines and these included: removal of red cell membranes from parasite antigen, parasite antigen dose-ranging and lyophilization of liposome formulations.

To address the possible induction of antibodies against red blood cell antigens, it was shown that using immunomagnetic methods, red cell membranes were efficiently depleted from parasite antigen. Additionally, when mice were immunised with liposomes containing red cell membrane-depleted parasite antigen, induction of antibodies against the surface of the red blood cell was significantly reduced compared with control mice that received injections of intact normal red blood cells. Furthermore, it was shown that depletion of red cell membranes from parasite antigen did not affect the immunogenicity of liposome formulations. These data strongly support the use of this immunomagnetic method to deplete red cell membranes from parasite antigen for use in liposome formulations although additional optimisation of depletion antibodies and incubation times will be required to further minimize and if possible completely eliminate the induction of anti-red blood cell antibodies.

A major logistical challenge encountered in the delivery of vaccines, particularly in malaria endemic areas, is the storage and maintenance of cold-chain. To address this, mannosylated liposomes were lyophilized, stored at 4°C and re-hydrated in PBS prior to immunisation. Interestingly, lyophilization did not affect the induction of humoral immune responses but altered the cytokine response to a predominantly Th1 response compared to a balanced Th1/Th2 response following immunisation with freshly prepared liposomes. It has been shown previously that a balanced
Th1/Th2 cytokine response to blood-stage malaria is crucial in the control of parasite replication, modulation of immune responses and control of pathology (Langhorne et al., 1989; Linke et al., 1996; Perez-Mazliah & Langhorne, 2014; Stevenson & Tam, 1993; Vonderweid et al., 1994). Therefore, repeat studies will be required to further optimise the freeze-drying protocols in order to achieve optimal Th1/Th2 cytokine responses consistently following immunisation with lyophilized liposomes.

In the heterologous challenge model, evaluation of the freshly prepared *P. falciparum* mannosylated liposomes showed that the formulation was highly immunogenic, and in some studies, species-transcending protective immunity was observed following challenge, with no clear correlate of immune protection. Therefore, additional studies need to be conducted to further optimise formulations to attain reproducible protective efficacy following heterologous challenge.

In the homologous challenge model, the utility of a multi-stage liposome formulation capable of inducing protective immune responses to both liver-stage and blood stage malaria was examined. Immunisation with formulations containing the circumsporozoite (CS) repeat peptide (QGPGAP)₄, derived from the rodent parasite, *P. yoelii*, whole Py17X blood-stage antigen and the additional adjuvant, PHAD, resulted in the induction of low (QGPGAP)₄-specific antibody titres (<10⁴). As high antibody titres (>10⁴) are required for protection against liver-stage infection (Ak et al., 1993; Charoenvit et al., 1990; Wang et al., 1995), a sporozoite challenge was not attempted in these studies. The low (QGPGAP)₄-specific antibody responses could have been due to lack of adequate T cell helper epitopes. Furthermore, the low antibody response to (QGPGAP)₄ observed could also have been due to the mouse strain used (BALB/c [H-2ᵈ]) used in the studies described herein as previous studies have shown that antibody responses to repeat peptides sequences of *P. yoelii*
CSP are genetically restricted to mice bearing the H-2\textsuperscript{b} haplotype (Grillot et. al 1990). Therefore, for this to be further developed as a viable multi-stage vaccine, the addition of universal T helper epitopes or a conjugated carrier protein such as diphtheria toxoid in the liposome formulations as well as use of a responder mouse strain such as C57BL/6 (H-2\textsuperscript{b}) needs to be considered in future studies.

However, in these studies, robust parasite-specific antibody responses were observed in mice immunised with liposomes containing PHAD, indicating that PHAD is critical in the induction of robust humoral immune responses. Furthermore, these parasite-specific antibody responses were strongly correlated with protection against a homologous wild-type challenge infection. These data underpin the need for PHAD as an adjuvant to enhance protective immune responses in future whole parasite liposome-based vaccine studies.

Collectively, the whole parasite liposome-based blood-stage vaccine presented in this thesis, which in some studies induced protective immune responses that effectively controlled parasite growth following challenge, could be of relevance in malaria endemic areas where semi-immune individuals are exposed to different parasite strains/species and remain asymptomatic with high parasite burdens. Administration of such a vaccine might result in boosting of pre-existing naturally acquired immune responses, reduction of parasite densities and subsequent reduction in malaria transmission in these populations.

In conclusion, the data presented in this thesis lays the foundation for future studies on the use of gold compounds as parasite attenuating agents as well as anti-malarial drugs. These studies also advance our understanding of the immune responses elicited following immunisation with liposomes containing inactivated whole parasite antigens and will inform the design of future vaccine formulations suitable
for human use. Importantly, the data strongly support transition of whole parasite liposome-based vaccines to human trials.
Appendix 1: Chemicals, reagents, assay kits and consumables

Chemicals and Reagents

0.9 % Saline, Pfizer, NY, USA.

1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), Avanti Polar Lipids, Inc.,
Alabama, USA.

2-Mercaptoethanol, Gibco™, Life technologies, Grand Island, NY, USA.

Auranofin, Sigma-Aldrich, St. Louis, MO, USA.

Biotinylated TER-119 antibody, STEMCELL™ Technologies, Vancouver, BC, Canada.

Bisbenzimide hoechst, Sigma-Aldrich, St. Louis, MO, USA.

Bovine Serum Albumin, Sigma-Aldrich, St. Louis, MO, USA.

CD11a FITC (Clone 2D7, BD Pharmingen™), BD Biosciences, Franklin Lakes, NJ, USA.

CD16/CD32 (BD Pharmingen™), BD Biosciences, Franklin Lakes, NJ, USA.

CD3 Molecular complex V450 (Clone 17A2, BD Horizon™), BD Biosciences, Franklin
Lakes, NJ, USA.

CD4 (GK1.5) Bio X Cell, Lebanon, New Hampshire, USA.

CD4 V500 (Clone RM4-5, BD Horizon™), BD Biosciences, Franklin Lakes, NJ, USA.

CD49d PE (Clone 9C10 (MFR4.B), BD Pharmingen™), BD Biosciences, Franklin
Lakes, NJ, USA.

CD8 PerCP-Cy5.5 (Clone 53.6.7, BD Pharmingen™), BD Biosciences, Franklin Lakes,
NJ, USA.

CD8 β (53.5.8) Bio X Cell, Lebanon, New Hampshire, USA.

Chloroform, anhydrous, ≥99%, Sigma-Aldrich, St. Louis, MO, USA.

Chloroquine diphosphate, Sigma-Aldrich, St. Louis, MO, USA.

Cholesterol Avanti Polar Lipids, Inc., Alabama, USA.

Dimethyl Sulfoxide, Sigma-Aldrich, St. Louis, MO, USA.
Dimethyldioctadecylammonium bromide (DDAB), Sigma-Aldrich, St. Louis, MO, USA
Ethanol, Molecular Biology Grade, Sigma-Aldrich, St. Louis, MO, USA.
Gentamycin, Life Technologies Australia Pty Limited, Victoria, Australia.
Giemsa Staining, Merck KGaA, Darmstadt, Germany.
Glycerollyte 57 Solution, Baxter Healthcare Corporation, Deerfield, IL, USA.
Glycophorin A FITC (Clone HI264) BioLegend Inc., San Diego, CA, USA.
Goat Anti-Mouse IgG-HRP, Invitrogen, Rockford, IL, USA.
Heat inactivated New-born Calf Serum (HI NBCS), Life technologies, Auckland, New Zealand.
Immersion oil, Sigma-Aldrich, St Louis, MO, USA.
L-Glutamine (Gibco ™) Life technologies, Grand Island, NY, USA.
Methanol, anhydrous, 99.8%, Sigma-Aldrich, St. Louis, MO, USA.
Monophosphoryl 3-Deacyl Lipid (PHAD) Avanti Polar Lipids, Inc., Alabama, USA.
Penicillin Streptomycin, Life Technologies, Carlsbad, CA, USA.
(QGPGAP)₄ P. yoelii circumsporozoite (CS) peptide, ChinaPeptides Co., Ltd, Wujiang, Suzhou, Jiangsu, China.
Rat IgG, Sigma-Aldrich, St. Louis, MO, USA.
Riamet® (Artemether and lumefantrine), Novartis Pharmaceuticals Pty, NSW, Australia
RPMI 1640, Gibco ™, Life technologies, Grand Island, NY, USA.
Saponin, Sigma-Aldrich, St. Louis, MO, USA.
Scintillation fluid, Wallac Scintillation Products, Turku, Finland.
TaqMan® Fast Advanced Master mix, Applied Biosystems ™ by Life Technologies,
Tetramethylbenzidine Substrate Reagent Set (OptEIA™), BD Biosciences, Franklin Lakes, NJ, USA.
Trypan Blue Stain (Gibco ™), Life Technologies, Grand Island, NY, USA.
Tween® 20, Merck KGaA, Darmstadt, Germany.

UltraPure™ 0.5M EDTA (Gibco™), Life Technologies, Grand Island, NY, USA.

**Radio-chemicals**

$^3$[H] Thymidine, Perkin Elmer, Waltham, MA, USA.

$^3$[H]-hypoxanthine, Perkin Elmer, Waltham, MA, USA.

**Assay kits**

ABON™ Syphilis Kit, Inverness Medical Innovations Ltd, Hong Kong.

Alere Determine™ HIV1/2, Alere Connected Health Ltd, Yavne, Israel.

BCA Protein Assay Kit (Pierce™), Thermo Scientific, Life technologies, Carlsbad, CA, USA.

Biotec™ RPR, Trinity Biotech, NY, USA.

Blood grouping reagent set, Cypress Diagnostics™, Langdorp, Belgium.

EasySep Mouse Streptavidin RapidSpheres™ Isolation Kit, STEMCELL™ Technologies, Vancouver, BC, Canada.

EasySep™ Human Glycophorin A Depletion Kit, STEMCELL™ Technologies, Vancouver, BC, Canada.

High Pure PCR Template Preparation Kit, ROCHE, Basel, Switzerland.

MACS Column CS, Miltenyi Biotec, Bergisch Gladbach, Germany.

SD BIOLINE HBsAg, Standard Diagnostics Inc., Yongin-si Geonggi-do, Republic of South Korea.

SD BIOLINE HCV, Standard Diagnostics Inc., Yongin-si Geonggi-do, Republic of South Korea.

Stat-Pak™ HIV1/2, CHEMBIO Diagnostic systems, Inc., NY, USA.

Th1/Th2/Th17 CBA kit, BD Biosciences, Franklin Lakes, NJ, USA.

Uni-Gold™ HIV1/2, Trinity Biotech, NY, USA.
Primers and Probes for rodent qPCR

Primer 18SNCB2-F5'-ACTTCCATTAATCAAGAACGAAAGTT-3’, Sigma-Aldrich, St Louis, MO, USA.
Primer 18SNCB2-R5'-TGGTTAAGATTACGATCGGTATCTGA-3’, Sigma-Aldrich, St Louis, MO, USA.
MGB Probe 18SNCB2-P FAM- 5’-AAGGGAGTGAAGCGA3’-MGBNFQ, Applied Biosystems, CA, USA.

Disposable products

3 mL EDTA Collection Tubes, BD Biosciences, Franklin Lakes, NJ, USA.
3 mL Lithium-Heparin Collection Tubes, BD Biosciences, Franklin Lakes, NJ, USA.
250 mL Membrane Filter, Corning, Dallas, TX, USA.
500 mL Membrane Filter, Corning, Dallas, TX, USA.
96 well flat-bottom tissue culture plates, Corning Glass Works, Corning, NY, USA.
Cryogenic tubes, Nalgene Nunc International, NY, USA.
1.5 mL DNA Lo-bind tubes, Eppendorf, Hamburg, Germany.
1.5 mL eppendorf tubes, Eppendorf, Hamburg, Germany.
Flasks (75 cm², 175 cm²), Corning Glass Works, Corning, NY, USA.
Microscope Glass Slides, Livingstone, Rosebery, NSW, Australia.
Nunc-Immuno 96 well MaxiSorp Plates, Thermo Scientific by Life technologies, Carlsbad, CA, USA.
5 mL Polystyrene round-bottom FACS tubes, Becton Dickinson Labware, NJ, USA.
PCR Strip Caps, BioRad, Hercules, CA, USA.
PCR Strip Tubes, BioRad, Hercules, CA, USA.
5 mL Serological Pipettes, Sarstedt, Nümbrecht, Germany.
10 mL Serological Pipettes, Sarstedt, Nümbrecht, Germany.
25 mL Serological Pipettes, Sarstedt, Nümbrecht, Germany.
0.5 mL (27G x 13mm) Syringe, Terumo, Elkton, MD, USA.
1 mL (27G x 13mm) Syringe, Terumo, Elkton, MD, USA.
5, 10, 20 mL Syringes, Terumo, Elkton, MD, USA.
5 mL Syringe, BD Biosciences, Franklin Lakes, NJ, USA.
3.5 mL Transfer Pipette, Sarstedt, Nümbrecht, Germany.
50 mL Tubes, Sarstedt, Nümbrecht, Germany.
15 mL Tubes, Sarstedt, Nümbrecht, Germany.
10 mL Tubes, Sarstedt, Nümbrecht, Germany.
5 mL Tubes, Sarstedt, Nümbrecht, Germany.
U-bottom 96 well plates, Greiner Bio-One GmbH, Kremsmünster, Austria
V-bottom 96 well Plates (Sarstedt) Nümbrecht, Germany.
1450 MicroBeta glass fibre filters Perkin Elmer Life and Analytical Sciences, CT, USA.
Appendix 2: Eligibility criteria

Inclusion criteria

1. Volunteers aged between 18 and 50 years
2. Volunteers must have a laboratory confirmed malaria infection by microscopy.
3. Volunteers must be hemodynamically stable (i.e. blood pressure ≥100/60 mmHg) and pulse ≤120bpm.
4. Volunteers must understand the procedures involved and agree to participate in the study by giving fully informed, written consent.
5. Good peripheral venous access.

Exclusion criteria

1. Clinical signs of severe malaria
2. Significant anaemia (Haemoglobin <10g/dl)
3. Female volunteers must be willing to take a pregnancy test
4. Volunteers should not have had any other illness in the preceding 2 weeks.
5. Presence of current serious chronic/inter-current diseases by medical history such as cardiac or autoimmune disease (HIV or other immunodeficiencies), insulin dependent diabetes, progressive neurological disease, severe malnutrition, acute or progressive hepatic disease, acute or progressive renal disease, psoriasis, rheumatoid arthritis, asthma, epilepsy or obsessive-compulsive disorder, skin carcinoma excluding non-spreadable skin cancers such as basal cell and squamous cell carcinoma.
6. Individuals who have lived in England, Scotland, Wales, Northern Ireland, Channel Islands, Isle of Man or the Falkland Islands for a 6 month between 01/01/1980 and 31/12/1996
7. Individuals who have received a transfusion of blood or blood components at any time since 01/01/1980
8. Individuals born in Central or South America and have received blood components in this area
9. Individuals with a family history of Creutzfeldt-Jakob disease (CJD), Gerstmann-Straussler-Scheinker Syndrome (GSS) or Fatal Familial insomnia (FFI)
10. Individuals wishing to donate blood to the Uganda Blood Transfusion Service within 3 months of blood sampling.

11. Currently receiving anti-malarial treatment or drugs with potential anti-malarial activity such as tetracycline, azithromycin, clindamycin and hydroxychloroquine.

12. A history of drug habituation, or any prior intravenous usage of an illicit substance.

13. Participation in any research study involving significant blood sampling, or blood donation during the previous 3 months.

14. Are known to be positive for HIV, Hepatitis B, Hepatitis C, Human T-cell Lymphotropic Virus I & II (HTLVI & HTLVII), TB or syphilis.

15. Evidence of any condition that, in the opinion of the clinical investigator, might adversely impact on recovery following blood donation.
Appendix 3: Clinical score sheet for the monitoring of animals

This clinical score sheet is for use in Professor Michael Good’s group at the Institute for Glycomics, Griffith University. It will be used in monitoring the health of any mice that are infected with malaria parasites.

<table>
<thead>
<tr>
<th>Clinical Score</th>
<th>Observation</th>
<th>Grade</th>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy (score of 0)</td>
<td>Pallor</td>
<td>N</td>
<td>No pallor: ankles, feet and ears pink in colour</td>
</tr>
<tr>
<td></td>
<td>Posture</td>
<td>N</td>
<td>Gait and movement is smooth (no hunching)</td>
</tr>
<tr>
<td></td>
<td>Activity</td>
<td>N</td>
<td>Very active and running around</td>
</tr>
<tr>
<td></td>
<td>Fur Texture</td>
<td>N</td>
<td>Coat normal, glossy</td>
</tr>
<tr>
<td></td>
<td>Reaction to Stimuli</td>
<td>N</td>
<td>Quick reaction to external stimuli</td>
</tr>
<tr>
<td></td>
<td>Urine</td>
<td>N</td>
<td>Yellow urine (normal)</td>
</tr>
<tr>
<td>Mild (score of 1): If any one or more of the following criteria</td>
<td>Pallor</td>
<td>A</td>
<td>Slight pallor: slightly pale feet, ankles and/or ears</td>
</tr>
<tr>
<td></td>
<td>Posture</td>
<td>A</td>
<td>Slight hunched appearance</td>
</tr>
<tr>
<td></td>
<td>Activity</td>
<td>A</td>
<td>Activity may be slightly decreased</td>
</tr>
<tr>
<td></td>
<td>Fur Texture</td>
<td>A</td>
<td>Slight ruffling of fur</td>
</tr>
<tr>
<td></td>
<td>Reaction to Stimuli</td>
<td>A</td>
<td>Slightly slowed reaction to external stimuli</td>
</tr>
<tr>
<td></td>
<td>Urine</td>
<td>A</td>
<td>Change in urine colour (slightly green or pale)</td>
</tr>
<tr>
<td>Moderate (score of 2): Score of 1 plus any of the following criteria</td>
<td>Pallor</td>
<td>B</td>
<td>Moderate pallor: includes pale white feet, ankles and/or ears</td>
</tr>
<tr>
<td></td>
<td>Posture</td>
<td>B</td>
<td>Moderate hunching notice while at rest</td>
</tr>
<tr>
<td></td>
<td>Activity</td>
<td>B</td>
<td>Activity moderately decreased but normal movement when stimulated</td>
</tr>
<tr>
<td></td>
<td>Fur Texture</td>
<td>B</td>
<td>Moderate ruffling of fur</td>
</tr>
<tr>
<td></td>
<td>Reaction to Stimuli</td>
<td>B</td>
<td>Moderately slowed reaction to external stimuli</td>
</tr>
<tr>
<td></td>
<td>Urine</td>
<td>B</td>
<td>Yellow-green urine</td>
</tr>
<tr>
<td>Moderate – Severe (score of 3): Score of 2 plus any of the following criteria</td>
<td>Pallor</td>
<td>C</td>
<td>Severe pallor: very pale feet, ankles and/or ears</td>
</tr>
<tr>
<td></td>
<td>Posture</td>
<td>C</td>
<td>Moderate hunching, gait and movement affected</td>
</tr>
<tr>
<td></td>
<td>Activity</td>
<td>C</td>
<td>Activity is moderately decreased and movement upon stimulation is slow</td>
</tr>
<tr>
<td></td>
<td>Fur Texture</td>
<td>C</td>
<td>Severe ruffling of fur</td>
</tr>
<tr>
<td></td>
<td>Reaction to Stimuli</td>
<td>C</td>
<td>Reaction to external stimuli is very slow</td>
</tr>
<tr>
<td></td>
<td>Urine</td>
<td>C</td>
<td>Dark yellow-green or orange urine</td>
</tr>
<tr>
<td>Severe (score of 4): Score of 3 plus any of the following criteria</td>
<td>Pallor</td>
<td>D</td>
<td>Extreme pallor: pale feet, ankles and/or ears</td>
</tr>
<tr>
<td></td>
<td>Posture</td>
<td>D</td>
<td>Moderate-extreme hunching - movement impaired</td>
</tr>
<tr>
<td></td>
<td>Activity</td>
<td>D</td>
<td>Activity can be very reduced - If not stationary, movement is severely affected</td>
</tr>
<tr>
<td></td>
<td>Fur Texture</td>
<td>D</td>
<td>Severe ruffling and poor grooming (faeces stuck to fur or white matter near eyes)</td>
</tr>
<tr>
<td></td>
<td>Reaction to Stimuli</td>
<td>D</td>
<td>Little to no reaction (just sits huddled in ball even with stimulation)</td>
</tr>
<tr>
<td></td>
<td>Urine</td>
<td>D</td>
<td>Urine bloody or dark green</td>
</tr>
</tbody>
</table>

Animals are checked daily. Moribund animals, that is, animals with any grade D symptom and therefore a clinical score of four (4), will be immediately euthanased. Animals exhibiting four (4) or more grade C symptoms for two (2) consecutive days will be euthanased.
Bibliography


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