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Fauzia Khagai Musasia, M.Sc. Biochemistry

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Antibody mediated clearance of ring-infected erythrocytes as a mechanism of protective immunity against *Plasmodium falciparum* malaria

Referees: Prof. Dr. Michael Lanzer

Prof. Faith Osier

Declaration

I hereby declare that this thesis is my own work and has not been submitted for examination or award of degree in any other institution of learning. I also declare that the experiments for the presented work were conducted using the indicated resources between April 2017 and September 2019 in the laboratory of Prof. Faith Osier at the parasitology unit of the centre of infectious diseases at the Ruperto Carola University in Heidelberg.

Heidelberg, 23rd December 2019.

Fauzia Khagai Musasia

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Dedication

I dedicate this thesis to the memory of Bibi, Asha Mohammed, whose inspiring words forever ignite a fire inside me that burns brighter than the fire around me.

Summary

Malaria is still a disease of global health significance. Its causative agent, *Plasmodium*, has a complex lifecycle alternating between the female *Anopheles* mosquito and the vertebrate host. The blood stage of the infection is thought to cause the clinical symptoms of the disease and is also targeted by the humoral immune response of the host. Natural acquired immunity to malaria is largely mediated by immunoglobulins and is reflected both by a lower prevalence of infection with age and lower rates of disease. Previous studies have generally focussed on understanding the protective role of naturally acquired functional antibodies targeting the mature infected erythrocyte (mIE) and highly immunogenic merozoite. However, the ring-infected erythrocyte (rIE), has hardly been explored to date in the context of naturally acquired protective immunity. As it is the stage of the infection that is predominantly found in the peripheral circulation, its immunological significance is uncertain. This thesis was aimed at contributing to this knowledge gap by investigating the role and significance of rIEs in protective immunity.

Firstly, to investigate the immunogenicity of rIEs, I tested whether they were recognised by malaria-immune antibodies using immunofluorescence and flow cytometry. I confirmed that malaria-immune antibodies do bind to rIEs using both laboratory-adapted (lab) and field isolates. This binding was strongly correlated with that directed against mIEs and merozoites, both of which have been shown to be important targets of protective immunity. Malaria-immune antibody binding to rIEs had no direct inhibitory effect on parasite survival and maturation. In separate assays, I tested whether rIEs were retained in the spleen independently of antibody using a microsphiltration assay. As has been previously reported, rIEs were retained in the spleen and I demonstrated for the first time that the retention rate was comparable between lab and field parasite isolates of the same age, suggesting that this may be a mechanism of parasite clearance that contributes to the low parasite densities observed in malaria endemic areas.

Secondly, to investigate the likely physiological relevance of antibody binding to rIEs during *P. falciparum* infection, I tested whether the *in vitro* opsonic phagocytosis of rIEs predicted the outcome of infection in a controlled human malaria infection (CHMI) study. I established the flow cytometry based opsonic phagocytosis assay (OPA) of rIEs in our laboratory. I showed for the first time, to the best of my knowledge, that opsonic phagocytosis of rIEs was significantly associated with the outcome of infection

(lower parasite densities and the need for treatment) in the CHMI study. Interestingly, phagocytosis of uninfected erythrocytes (uEs) in the ring culture was higher in untreated as compared to the treated individuals, suggesting a potential contribution to anaemia, as others have reported. In addition, I developed competition OPAs and used stage-specific spent media, to demonstrate that merozoite specific antibodies mediated the observed opsonic phagocytosis of the ring culture cells and uEs incubated in spent media respectively.

Finally, to investigate the specific parasite antigens on the surface of ring culture cells and uEs incubated in spent media, I employed surface trypsinization, followed by mass spectrometry of supernatants. This is the first surface proteomics study to be conducted on rIEs and in this single study I was able to identify parasite proteins on the surface of rIEs and uEs that had previously been identified singly, in a piecemeal fashion (EBA140, EBA175, RAP1, RAP2, RhopH1, RhopH2, RhopH3 and MCP1), and ENO which I showed for the first time. These proteins have been speculated to be transferred by the merozoite to the surface of the newly invaded erythrocyte during invasion and have also been shown to be correlates of protective immunity, with the exception of MCP1, whose role in parasite clearance and protective immunity has not yet been explored.

This thesis shows for the first time that antibody mediated clearance of rIEs *in vitro*, is correlated with the outcome of *P. falciparum* infection in a CHMI study and was mediated by anti-merozoite antibodies. This study also demonstrates that there are shared parasite targets between merozoites and rIEs. I conclude that rIEs are not immunologically inert and are targeted by antibodies leading to parasite clearance through mechanisms such as phagocytosis. This activity was significantly associated with lower parasite densities and resistance against clinical symptoms in a human malaria challenge study suggesting that it contributes to protective immunity. The enhanced phagocytosis of uEs in protected individuals suggest a potential detrimental impact of anaemia that requires further investigation.

Zusammenfassung

Malaria ist nach wie vor eine Krankheit von globaler gesundheitlicher Bedeutung. Der Malaria-Erreger *Plasmodium* hat einen komplexen Lebenszyklus, der zwischen der weiblichen Anopheles-Mücke und dem Wirbeltierwirt alterniert. Die klinischen Symptome der Krankheit werden durch das Blutstadium des Malariaparasiten verursacht und auch von der humoralen Immunantwort des Wirts beeinflusst. Die natürlich erworbene Immunität gegen Malaria wird größtenteils durch Immunglobuline vermittelt und spiegelt sich sowohl in einer geringeren Prävalenz von Infektionen mit dem Alter als auch in geringeren Krankheitsraten wider. Frühere Studien konzentrierten sich im Allgemeinen auf das Verständnis der Schutzfunktion von natürlich erworbenen funktionellen Antikörpern gegen die reifen, infizierten Erythrozyten und die hoch immunogenen Merozoiten. Der ringinfizierte Erythrozyt (rIE) ist jedoch im Zusammenhang mit einer natürlich erworbenen, schützenden Immunität, bisher kaum erforscht worden. Da sich dieses Infektionsstadium überwiegend im peripheren Kreislauf befindet, ist seine immunologische Bedeutung ungewiss. Ziel dieser Arbeit war es, einen Beitrag zur Schließung dieser Wissenslücke zu leisten, indem die Rolle und Bedeutung von rIEs bei der protektiven Immunität untersucht wurde.

Um die Immunogenität von rIEs zu untersuchen, habe ich zunächst mithilfe von Immunfluoreszenz und Durchflusszytometrie getestet, ob rIEs von Malaria-spezifischen Antikörpern erkannt werden. Ich konnte bestätigen, dass Malaria-spezifische Antikörper an rIEs binden, was sowohl für laborangepasste Parasiten als auch für Feldisolate gezeigt werden konnte. Diese Antikörperbindung korrelierte stark mit der Bindung an reife, infizierte Erythrozyten (mIEs) und Merozoiten, von denen bekannt ist, dass sie wichtige Ziele der schützenden Immunität sind. Die Bindung von Malaria-spezifischen Antikörpern an rIEs hatte keine direkt hemmende Wirkung auf das Überleben und die Reifung der Parasiten. In separaten Assays testete ich mithilfe eines „Mikrosphiltrationstests“, ob rIEs unabhängig von Antikörpern in der Milz zurückgehalten werden. Wie bereits zuvor berichtet, wurden rIEs in der Milz zurückgehalten, und ich konnte zum ersten Mal nachweisen, dass die Retentionsrate „gleichalter“ Ringstadien zwischen Labor- und Feldisolaten vergleichbar war, was vermuten lässt, dass dieser Mechanismus der Eliminierung von Parasiten einen Beitrag zu den in Endemiegebieten beobachtbaren. niedrigen Parasitendichten leistet.

Zweitens testete ich, um die physiologische Relevanz der Antikörperbindung an rIEs während einer *P. falciparum*-Infektion zu untersuchen, ob die antikörperabhängige *in vitro* Phagozytose („opsonic phagocytosis“) von rIEs das Ergebnis einer kontrollierten humanen Malaria-Infektion (CHMI) vorhersagen konnte. Hierzu habe ich in unserem Labor den auf Durchflusszytometrie basierten „Opsonic Phagocytosis Assay“ (OPA) von rIEs etabliert. Ich habe in der CHMI-Studie, nach meinem Wissen, zum ersten Mal gezeigt, dass die antikörperabhängige Phagozytose von rIEs signifikant mit dem „Outcome“ der Infektion (geringere Parasitendichten und Behandlungsbedarf) assoziiert ist. Interessanterweise war die Phagozytose nicht infizierter Erythrozyten (uEs) aus einer Ringstadien-Kultur in der Gruppe der nicht-behandelten (geschützten) Personen höher, was auf einen möglichen Beitrag zur Anämie hindeutet, wie bereits in anderen Studien berichtet wurde. Darüber hinaus entwickelte ich kompetitive OPAs und verwendete stadienspezifische, für die Parasitenkultur zuvor gebrauchte Medien („used media“), um zu zeigen, dass merozoitenspezifische Antikörper die beobachtete antikörperabhängige Phagozytose, der in verbrauchten Medien inkubierten Ringstadien und uEs, vermittelten.

Um die spezifischen Parasiten-Antigene auf der Oberfläche von Ringstadien und in verbrauchten Medien inkubierten uEs zu untersuchen, setzte ich eine Oberflächentrypsinierung ein, gefolgt von einer massenspektrometrischen Analyse der Überstände. Dies ist die erste Studie, die das Oberflächen-Proteom von rIEs untersuchte. In dieser Einzelstudie konnte ich Parasitenproteine auf der Oberfläche von rIEs und uEs identifizieren, die zuvor einzeln in anderen Studien identifiziert wurden (EBA140, EBA175, RAP1, RAP2, RhopH1, RhopH2, RhopH3 und MCP1), wobei ich das Protein Enolase (ENO) zum ersten Mal in diesem Zusammenhang detektieren konnte. Es wurde bereits zuvor spekuliert, dass diese Proteine vom Merozoiten während der Invasion auf die Oberfläche des neu infizierten Erythrozyten übertragen werden, und es wurde auch gezeigt, dass diese Antigene mit einer schützenden Immunantwort korrelieren, mit Ausnahme von MCP1, dessen Rolle bei der Beseitigung von Parasiten und der schützenden Immunität noch nicht erforscht wurde.

Diese Arbeit zeigt zum ersten Mal, dass die antikörpervermittelte Eliminierung von rIEs *in vitro* mit dem Ausgang einer *P. falciparum*-Infektion in einer CHMI-Studie korreliert und durch Anti-Merozoit-Antikörper vermittelt wird. Diese Studie zeigt auch, dass es gemeinsame Parasitenantigene zwischen Merozoiten und rIEs gibt. Ich schließe

daraus, dass rIEs nicht immunologisch inert sind und von Antikörpern angegriffen werden, die durch Mechanismen wie Phagozytose zur Beseitigung von Parasiten führen. Diese Aktivität war in einer humanen Malaria-Expositionsstudie signifikant mit einer geringeren Parasitendichte und Resistenz gegen klinische Symptome verbunden, was darauf hindeutet, dass sie zur schützenden Immunität beiträgt. Die verstärkte Phagozytose von uEs bei geschützten Personen deutet auf eine mögliche, nachteilige Verursachung von Anämie hin, was weiterer Untersuchungen bedarf.

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List of abbreviations

2°Ab	Secondary antibody
AMAI	Apical membrane antigen 1
APC	Allophycocyanin
ATP	Adenosine triphosphate
CHMI	Controlled human malaria infection
CLAG3.1	Cytoadherence linked asexual protein
CR1	Complement receptor 1
CSP	Circumsporozoite protein
DDT	Dichlorodiphenyltrichloroethane
DHA	Dihydroartemisinin
DHE	Dihydroethidium
DMSO	Dimethyl sulphoxide
EBA140	Erythrocyte binding antigen 140
EBA175	Erythrocyte binding antigen 175
EBLs	Erythrocyte binding-like proteins
ELISA	Enzyme-Linked Immunosorbent Assay
FH	Complement factor H
FITC	Fluorescein Isothiocyanate
G6PD	Glucose-6-phosphate dehydrogenase
GPI	Glycosylphosphatidylinositol
HbC	Haemoglobin C
HbE	Haemoglobin E
HbS	Sickle cell haemoglobin
HCl	Hydrochloric acid
HLA	Human leukocyte antigen complex
HRP	Horseradish peroxidase
IEs	Infected erythrocytes
IgG	Immunoglobulin G
LFQ	Label free quantification
MACS	Magnetic-activated cell sorting
m/z	Mass-to-size ration

MCP1	Merozoite capping protein 1
mIEs	Mature infected erythrocytes
MSMS	mass spectrometry
MSP1	Merozoite surface protein 1
MSP2	Merozoite surface protein 2
MSP3	Merozoite surface protein 3
MT	MitoTracker
NADP	Nicotinamide adenine dinucleotide phosphate
NPP	New permeability pathways
OPD	O- phenylenediamine dihydrochloride
PAMPs	Pathogen associated molecular patters
PBS	Potassium Buffered Saline
PFA	Paraformaldehyde
PfEMP1	*P. falciparum* erythrocyte membrane protein 1
PfRhs	*P. falciparum* reticulocyte-binding homologs
PK	Pyruvate kinase
Plasmodb	Plasmodium database
PRRs	Pattern recognition receptors
qPCR	Quantitative Polymerase Chain Reaction
RAP1	Rhoptry-associated protein 1
RAP2	Rhoptry-associated protein 2
RhopH1	High molecular weight rhoptry protein 1
RhopH2	High molecular weight rhoptry protein 2
RhopH3	High molecular weight rhoptry protein 3
RIFIN	Repetitive interspersed family
rIEs	Ring-infected erythrocytes
RON2	Rhoptry neck protein 2
RSP2	Ring-surface protein 2
SG	SYBR Green I
STEVOR	Sub-telomeric variable open reading frame
SURFIN	Surface-associated interspersed
TEP1	Thioester-containing protein 1
uEs	Uninfected erythrocytes

v/v	Volume to volume
w/v	Weight to volume
mM	Milli Molar
µg	Micro gram
µl	Micro Liter
ml	Milli Liter
IU	International Units

Chapter 1

Introduction

1.1. Malaria: epidemiology and burden of disease

Malaria is a vector-borne parasitic disease transmitted in about 91 tropical countries globally. As shown in Figure 1.1, it has been eradicated in temperate countries (Ashley et al., 2018). The absolute number of people at risk of malaria globally has increased between the 20th and 21st century, from 0.9 to 3 billion. However, when one considers the proportion of people at risk, this has actually decreased from 70% to 48% during the same time period (Hay et al., 2004). As it stands, the estimated global burden of malaria in 2018 was 228 million cases and 405 000 related deaths with Africa accounting for the largest share, 93% and 94% respectively (WHO, 2019).

The global burden of malaria has substantially declined since the beginning of the 21st century due to implementation of long-lasting insecticide treated bed nets and artemisinin-based combination therapies (Ashley et al., 2018). However, there is a challenge in maintaining their effectiveness due to increasing drug and insecticide resistance (Hemingway et al., 2016), and thus the need to develop a highly efficacious vaccine to complement the existing malaria eradication strategies (Healer et al., 2017).

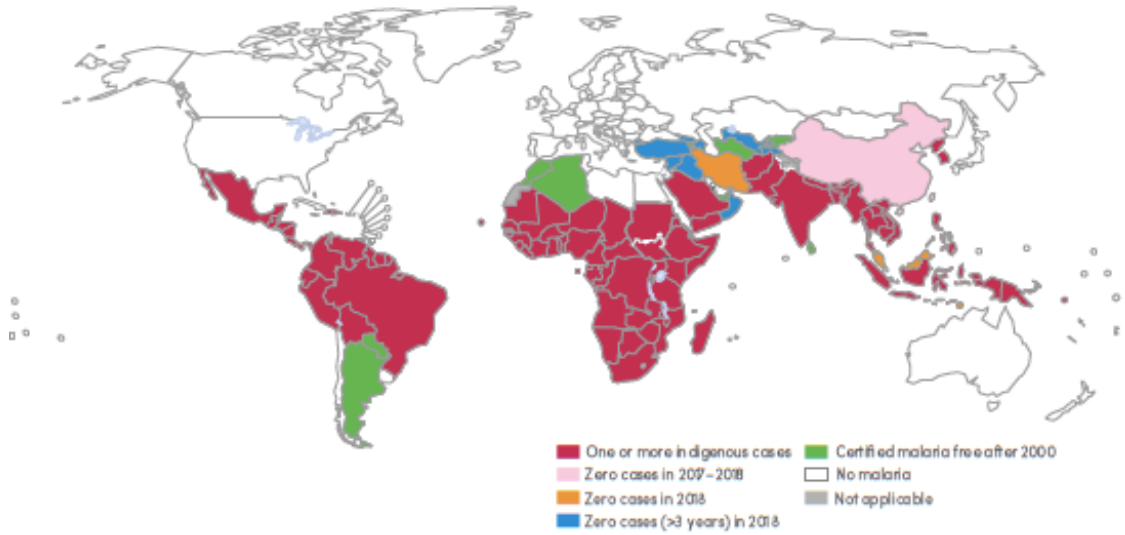

Figure 1. 1: Map showing countries with indigenous cases in 2000 and their status by 2018. Countries with zero indigenous cases over at least the past 3 successive years are considered to be malaria free. In 2018, China and El Salvador reported the second year of zero indigenous cases, and Iran, Malaysia and Timor-Leste for the first time reported zero indigenous cases. The incidence rate of malaria has globally declined between 2010 and 2018, from 71 to 57 cases per 1000 population at risk. However, the progress has stagnated in the period between 2015-2018. In figures, the estimated worldwide malaria cases were 228 million in 2018, which was fewer than the recorded 251 million cases in 2010, but consistent with 231 million cases reported in 2017. Similarly, malaria related deaths estimated globally were 405 000 in 2018, 416 000 in 2017 and 585 000 in 2010. Figure adapted from WHO 2019.

Malaria is caused by a protozoan parasite of the *Plasmodium* genus. Over 120 *Plasmodium* species infecting mammals, reptiles and birds, but only six *Plasmodium* species are known to infect humans: *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale curtisi*, *P. ovale wallikeri* and *P. knowlesi* (Ashley et al., 2018). The most predominant species worldwide include *P. falciparum* which the most dominant and fatal malaria parasite, and *P. vivax* which is the dominant parasite accountable for majority of malaria cases in America (WHO, 2018). *P. malariae* and *P. ovale* have a global distribution with low incidence whereas *P. knowlesi* is a zoonotic parasite found only in South East Asia where it is reported to cause severe malaria (Ashley et al., 2018).

1.2. Malaria pathogenesis

1.2.1. The *Plasmodium* life cycle

The *Plasmodium* species have a complex life cycle that alternates between an infected female *Anopheles* mosquitoes and the vertebrate hosts as shown in Figure 1.2 (Cowman et al., 2016). *Plasmodium* sporozoites are injected into the host dermis during a blood meal, move by gliding motility to traverse different cells from the site of bite to encounter and invade a blood vessel (Amino et al., 2006). The sporozoites that remain in the skin are drained into the lymphatic system, where an immune response is generated by the host leading to their destruction (Vanderberg and Frevert, 2004). From the dermal vasculature, sporozoites are passively transferred in the blood stream to the liver where they switch form a migratory mode to an invasive mode (Coppi et al., 2007). The sporozoites access the liver by crossing the sinusoidal barrier which consists of fenestrated endothelial cells and macrophage like Kupffer cells (Tavares et al., 2013). The interaction of the sporozoite and the liver is made possible by masking of some epitopes of the key surface antigen, circumsporozoite protein (CSP), in order to evade the immune system (Herrera et al., 2015).

Once hepatocyte infection is established, the sporozoite gets enclosed within a parasitophorous vacuole membrane and develops asexually in the liver for 2-10 days to form tens of thousands of merozoites which are packed in vesicles known as merosomes (Vaughan et al., 2012). The liver stage infection is asymptomatic and thus cannot be detect during natural human infections. In *P. falciparum* infection, this liver stage lasts for 6-7 days while in *P. vivax* infection it lasts for 8-12 days with a dormancy phase which accounts for periodic relapse of vivax malaria (Mo and McGugan, 2018).

The hepatocyte-derived merosomes are thought to limit the presentation of phosphatidyl serine on the host membrane, thus ensuring their protection from the host immune system and successful migration into the blood stream (Sturm et al., 2006).

Once the merosomes are released into the hepatic circulation, the free merozoites invade the erythrocytes and develop in their own compartment enclosed by the parasitophorous vacuole membrane. The *P. falciparum* parasite thrives in this niche by modifying the host cell through trafficking numerous parasite proteins (Gruring et al., 2011). The newly invaded erythrocyte, ring stage, rapidly grows by utilizing haemoglobin and develops to trophozoite containing hemozoin in the food vacuole. Finally, the parasite enters into asexual reproductive state, schizogony, during which it undergoes cell division and generates up to 32 merozoites that invade new erythrocytes after host cell rupture (Cowman et al., 2016). During *P. falciparum* infection, only the ring stages are found in circulation whereas the trophozoite and schizont infected erythrocytes sequester in capillaries of different organs via parasite proteins on the host cell surface (Gruring et al., 2011). The continuous asexual development every 48 hours is responsible for the clinical symptoms of malaria (Aly et al., 2009).

During cycles of schizogony in the bloodstream, a small subset of the asexual parasites undergoes a developmental switch that initiates commitment to sexual growth to form male and female gametocytes. The immature *P. falciparum* gametocytes develop in tissues, preferably the bone marrow, for 6-8 days and are present in the human blood circulation after maturity for uptake by a feeding mosquito (Joice et al., 2014). Consequently, each male and female gametocyte taken up during a blood meal differentiates into eight flagellate male gametes and an immotile extracellular female gamete respectively. The differentiation is due to an increase in pH as a result of exposure of the infected blood in air (Nijhout and Carter, 1978) and the presence of xanthurenic acid in the mosquito midgut (Arai et al., 2001). The male microgamete fertilizes a female macrogamete to form a diploid zygote which matures into an invasive form, ookinete, in the mosquito midgut. The ookinete starts to traverse the midgut epithelial cell layer from the apical side and egresses from the basal end to reach the basal lamina, where the surviving ookinetes become sessile and transform into oocysts (Matuschewski, 2006). The oocysts undergo sporogony, during which they asexually replicate extracellularly to form motile sporozoites which are then

released into the haemocoel upon oocyst rapture (Klug and Frischknecht, 2017). The midgut sporozoites are passively dispersed through the haemolymph until they reach and actively invade the salivary gland, where they remain in the ducts awaiting transmission during a blood meal (Aly et al., 2009).

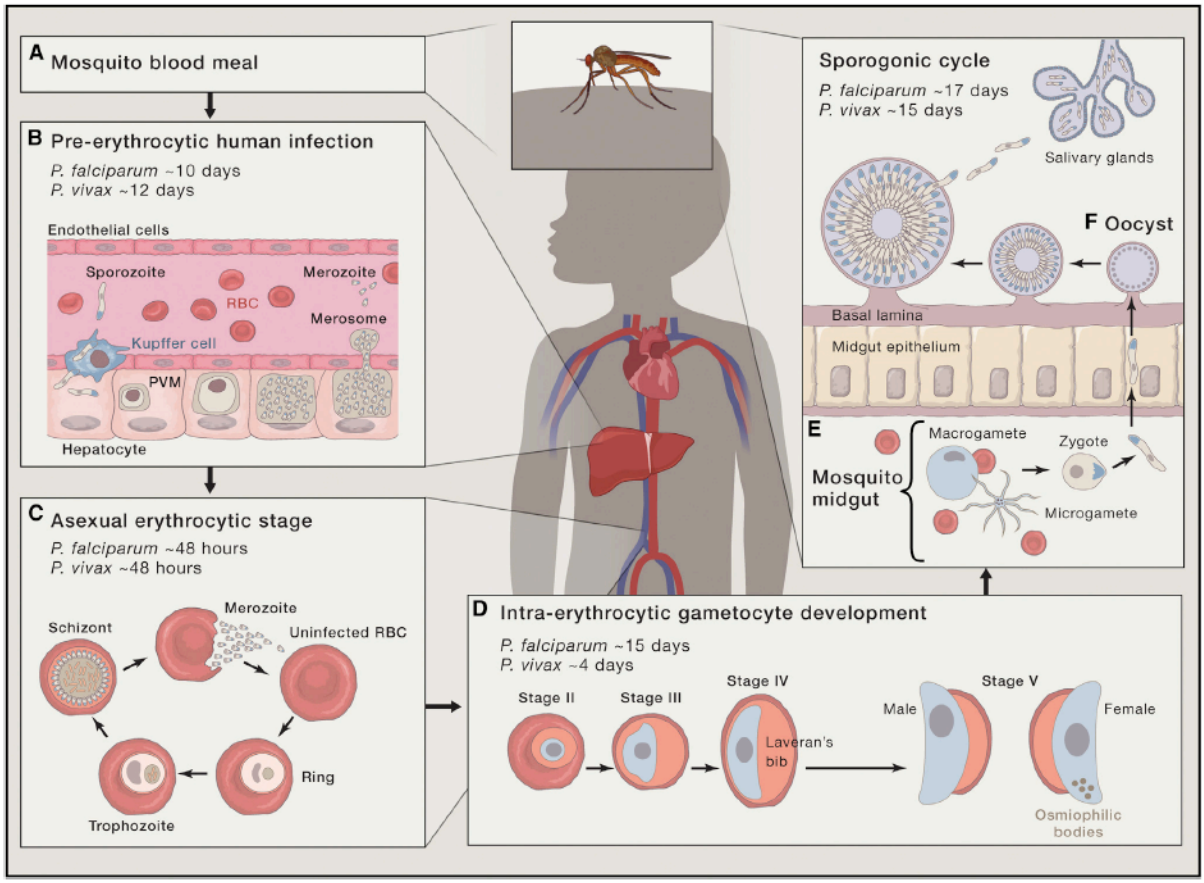

Figure 1. 2: The *Plasmodium* life cycle. The *Plasmodium* life cycle is complex and switches between the vertebrate host (human) and the vector (female *Anopheles* mosquito). (A) The mosquito infects the human host with sporozoites during a blood meal. (B) The sporozoites migrate from the site of bite to the liver establishing a pre-erythrocytic latent infection. (C) The merozoites released from the liver enter into the blood circulation and invade erythrocytes establishing the asexual erythrocytic stage responsible for the clinical symptoms of malaria. (D) A proportion of the asexual blood stages undergo sexual development to form gametocytes, which are taken up by mosquito during a blood meal. (E) The male and female gamete fuse to form a zygote which matures to a motile form, ookinete, that traverses the midgut and transform into oocysts. The oocyst undergoes asexual reproduction, sporogony, to form sporozoites that migrate and invade the salivary glands awaiting transmission during a blood meal. Figure is adapted from Cowman et al., 2016.

1.2.2. Erythrocyte invasion during *P. falciparum* infection

The interaction between merozoites and erythrocytes is a multi-step sequence of events as shown in Figure 1.3. It begins with the pre-invasion step that involves a robust interaction between the merozoite ligands, such as erythrocyte binding-like proteins (EBLs) and *P. falciparum* reticulocyte-binding homologs (PfRhs), and their erythrocyte receptors, namely glycophorin A, B, C and complement receptor 1 (CR1)

respectively. This interaction results in an actomyosin motor driven deformation of the host cell (Weiss et al., 2015). After deformation of the erythrocyte, the merozoite re-orientates by means of a PfRh5 complex. Its apical end touches the basigin receptor of the erythrocyte membrane (Reddy et al., 2015, Chen et al., 2011, Crosnier et al., 2011). The PfRh5-basigin interaction is associated with influx of calcium ions into the host cell (Weiss et al., 2015). Subsequently, the merozoite and erythrocyte form an irreversible attachment, tight junction, mediated by secretion of the rhoptry neck protein 2 (RON2). RON2 is deposited on the host erythrocyte membrane, bound by apical membrane antigen 1 (AMA1) and translocated on to the merozoite surface (Riglar et al., 2011). After the tight junction formation, the merozoite is propelled into the erythrocyte by force generated by the actomyosin motor of the parasite (Besteiro et al., 2011). The active invasion is culminated by the fusion of membranes at the posterior end of the merozoite sealing the parasite within the parasitophorous vacuole and erythrocyte. Following successful invasion, the erythrocytes appear to shrink and exhibit spiky protrusions, echinocytosis, due to influx of calcium ions into the erythrocyte during the previous PfRh5-basigin interaction (Weiss et al., 2015).

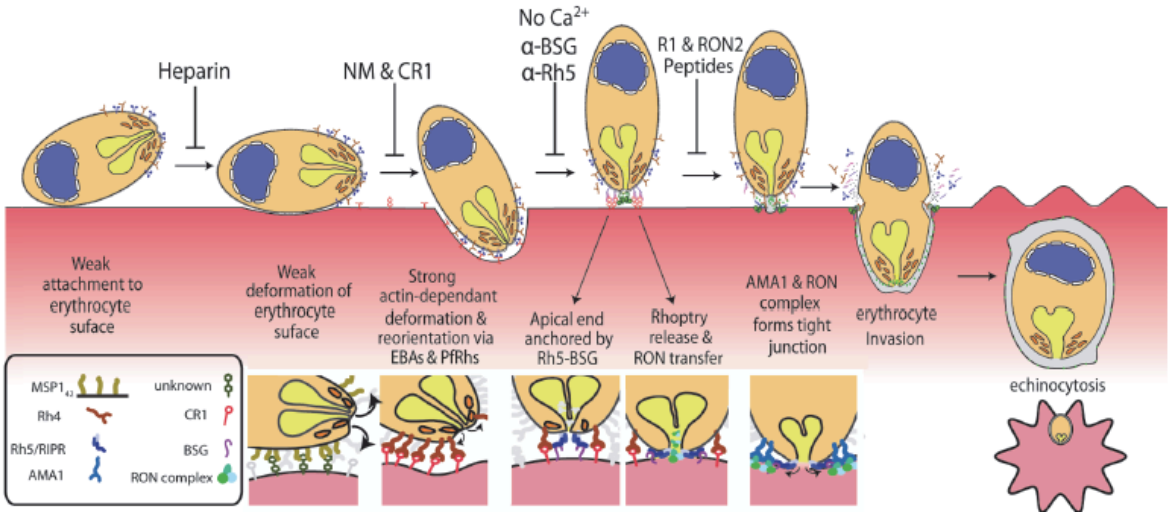

Figure 1. 3: Model showing sequence of interactions during *P. falciparum* invasion of an erythrocyte. The merozoite attaches itself weakly to the erythrocyte surface with the help of its surface proteins leading to a weak deformation of the erythrocyte surface. Merozoite proteins secreted from the microneme and rhoptry (PfEBAs and PfRhs respectively) interact robustly with their receptors on the erythrocyte surface leading to an actomyosin motor driven deformation of the host cell. Thereafter, the PfRh5 ligand secreted form the rhoptry interacts with basigin receptor on the host cell forming a tight junction which marks the commitment of merozoites to invade the erythrocyte. The merozoite is propelled into the erythrocyte by force generated by the actomyosin motor of the parasite and invasion is culminated by the sealing of the parasite within the parasitophorous vacuole and erythrocyte. The evidence of successful invasion is demonstrated by echinocytosis. Figure is adapted from Weiss et al., 2015.

1.2.3. Clinical features of human malaria

The clinical symptoms of malaria are experienced during the erythrocytic stage of the *Plasmodium* life cycle. They occur simultaneously with the rupture of infected erythrocytes (IEs), which involves the release of parasite and erythrocyte debris, such as hemozoin and glycophosphatidylinositol, leading to activation of peripheral blood mononuclear cells and release of cytokines (Mawson, 2013). Additionally, decreased oxygen delivery to tissues caused by low haemoglobin levels leads to metabolic acidosis which is a principle pathophysiological feature during severe malaria (Miller et al., 2002).

The majority of malaria cases present as non-specific febrile illnesses with symptoms, such as malaise, headache, diarrhoea, abdominal pain and muscle aches, which are easily terminated by antimalarial treatment and/or host responses. With the exception of naïve individuals or in cases of poor drug compliance or lack of treatment, relatively few malaria infections progress to severe life-threatening disease (Milner, 2018, Mackintosh et al., 2004). Severe malaria is predominantly caused by *P. falciparum* parasite proteins, primarily *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), found on the surface of mature IEs. These proteins mediate adherence of the IEs to the endothelial lining of the microvasculature and organs, such as placenta, brain, liver, kidney and lungs. The gravity of the disease depends on the organ involved, namely placental malaria, coma, liver, kidney and respiratory failure respectively (Aird et al., 2014). Moreover, these host cell modifications aggravate microvascular obstruction by mediating the binding of the infected erythrocytes to uninfected cells, erythrocyte rosetting, and reduction of the infected erythrocyte deformability as shown in Figure 1.4 (Ashley et al., 2018).

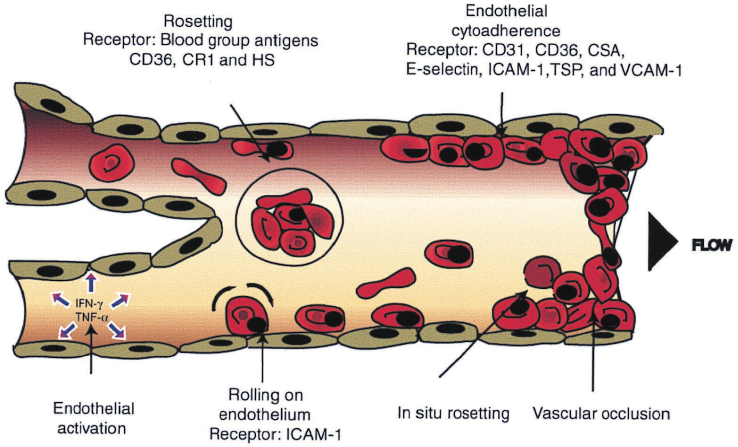

Figure 1. 4: Molecular aspects of severe malaria. The binding of *P. falciparum* mature infected erythrocytes to endothelial lining and to uninfected erythrocytes, rosetting, contributes to occlusion of blood flow and resultant severe disease. Figure is adapted from Chen et al., 2000.

1.2.4. Diagnosis of human malaria

In malaria endemic areas, malaria is primarily diagnosed clinically, based on the patients' signs and symptoms, but this is challenging as there are non-specific (Tangpukdee et al., 2009). On the other hand, the laboratory diagnosis of malaria is effective and reliable because it involves the identification of the malaria parasites or parasite products in patient blood. Multiple techniques exist including, microscopic diagnosis by the staining of thin and thick peripheral blood smears as shown in Figure 1.5, concentration techniques e.g. quantitative buffy coat (Bhandari et al., 2008), rapid diagnostic tests e.g. OptiMAL (Tagbor et al., 2008), and molecular diagnostic methods, such as polymerase chain reaction (Holland and Kiechle, 2005). Early and accurate diagnosis of malaria is a key component of the global malaria control strategy and reduce the misuse of antimalarials, mortality and morbidity (WHO, 2005).

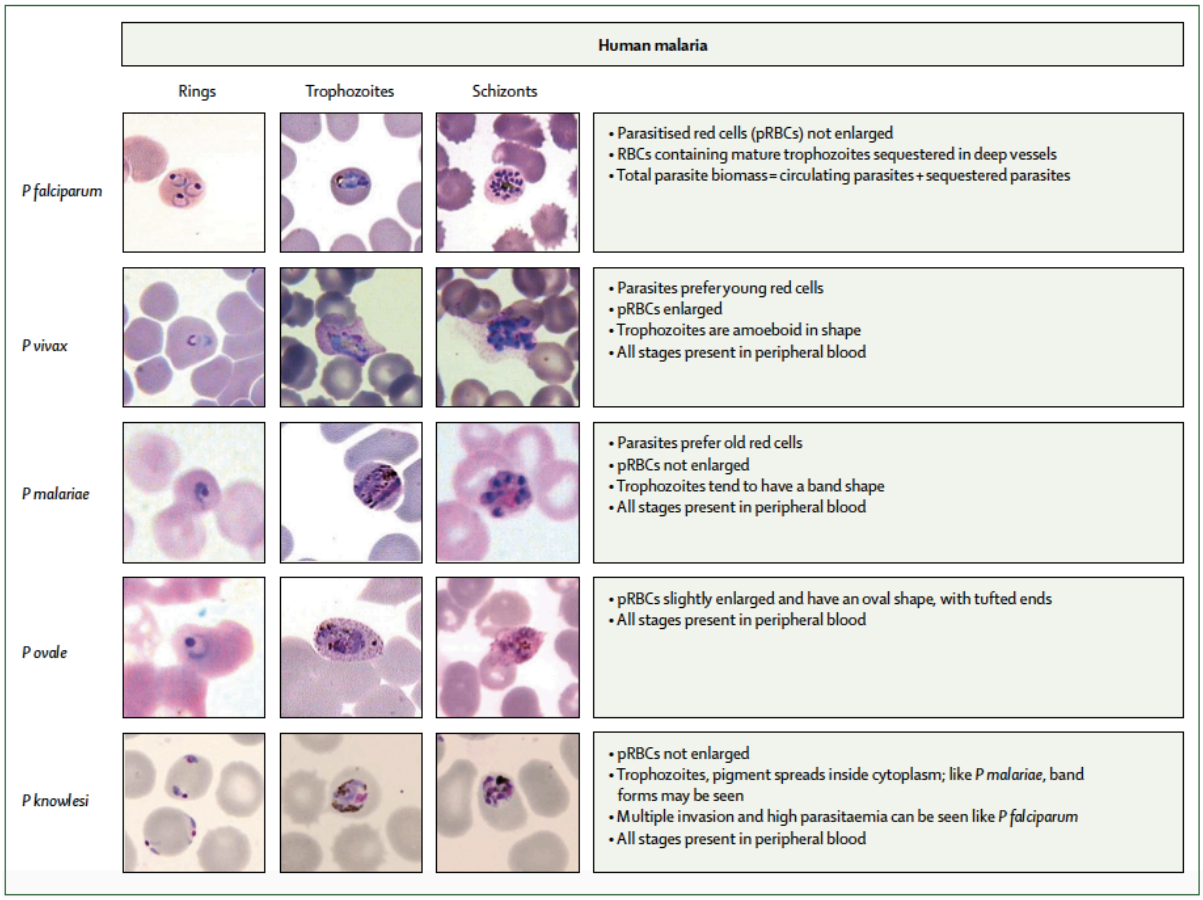

Figure 1. 5: Thin blood smears showing microscopic appearance of the human malarias. All parasite stages are detectable in peripheral blood except in the case of *P. falciparum* infection, during which mature trophozoites sequester in the microvasculature. Figure is adapted from Ashley et al., 2018

1.3. Genetic resistance to malaria

1.3.1. Hemoglobinopathies

Thalassemia

Thalassemia refers to the alteration in the synthesis of the globin chains of the haemoglobin molecule (Vento et al., 2006). An abnormality in the synthesis of the α-globin and ß-globin leads to α-thalassemia and ß-thalassemia respectively (Richer and Chudley, 2005). α -thalassemia is the predominant genetic disorder found in the human population and is caused by decreased synthesis of α-globin due to deletion of the duplicated α-globin genes (Allen et al., 1997), whereas ß-thalassemia is caused by decreased synthesis of ß-globin owing to more than 180 different mutations within a single ß-globin gene (Yuthavong and Wilairat, 1993). α- and ß-thalassemia have been proposed to confer protection against clinical disease and severe forms of *P. falciparum* infection in malaria endemic areas (Williams, 2006). This cause has been linked to *in vitro* recognition of the parasitized α- and ß- thalassaemic erythrocytes by malaria-immune sera leading to significantly higher phagocytosis compared to normal infected erythrocytes (Yuthavong and Wilairat, 1993, Luzzi et al., 1991). Furthermore, α-thalassemia has been associated with deficiency of a rosetting erythrocyte receptor, complement receptor 1 (CR1), leading to enhanced parasite clearance and protection from severe malaria during *P. falciparum* infection (Cockburn et al., 2004).

Sickle cell anaemia (HbS)

The heterozygous sickle cell haemoglobin (HbAS) is the first recognized cause for genetic resistance to malaria in humans (Allison, 1954). It is caused by a single nucleotide polymorphism in position 6 of the ß-globin gene where alanine is substituted with thymidine (GAG to GTG) leading to replacement of the hydrophilic glutamic acid with a hydrophobic valine (E6V). This causes HbS polymerization upon deoxygenation and alteration of the erythrocyte shape (Hedrick, 2011, Lopez et al., 2010). The HbAS has been shown to confer protection against severe malaria using various underlying mechanisms, such as: (i) inhibition of intra-erythrocytic parasite growth due to HbS polymerization when oxygen levels drop below 5% (Roberts and Williams, 2003, Akide-Ndunge et al., 2003), (ii) enhanced phagocytosis of ring-infected sickle erythrocytes compared to ring-infected normal erythrocytes (Ayi et al., 2004), (iii) acquisition of cross reactive antibodies recognizing and responding to different variant surface antigens on the parasitized erythrocytes (Cabrera et al.,

2005), (iv) reduced surface expression of PfEMP1 leading to reduced cytoadherence and pathogenicity of the disease (Cholera et al., 2008), and (v) redox imbalance mediated remodelling of the host actin, which is required for vesicular trafficking of parasite adhesins, leading to reduced cytoadherence of parasitized erythrocytes and increased parasite clearance (Cyrklaff et al., 2016).

Haemoglobin C (HbC)

HbC originates from a single nucleotide polymorphism in position 6 of the ß-globin chain leading to the substitution of a glutamic acid residue with a lysine residue (E6K) and is most prevalent in Western Africa (Fairhurst et al., 2005, Modiano et al., 2001). Unlike other hemoglobinopathies, HbC doesn't follow the Haldane's hypothesis of balanced polymorphism, where the homozygous form (HbC) and not the heterozygous form has been shown to correlate with reduced *P. falciparum* infection and density (Lopez et al., 2010). The association of HbC with protection has been explained by various mechanisms, such as: (i) inability of parasites to proliferate *in vitro* in HbC erythrocytes (Friedman et al., 1979) due to their abnormal pressure which interferes with rupture of schizont-infected erythrocytes and release of infective merozoites (Olson and Nagel, 1986). These lead to a reduction intra-erythrocytic parasites in individuals with mild or severe malaria infection (Rihet et al., 2004), and (ii) display of abnormal PfEMP1 which causes reduction of PfEMP1 mediated cytoadherence leading to enhancement of parasite clearance (Fairhurst et al., 2005).

Haemoglobin E (HbE)

HbE originates from a single nucleotide polymorphism in position 26 of the ß-globin chain leading to the substitution of a glutamic acid residue with a lysine residue (E26K) and is the most prevalent hemoglobinopathy in Southeast Asia (Deng et al., 2018, Ohashi et al., 2004). The heterozygous form, HbAE, has been shown to ameliorate the course of acute *P. falciparum* malaria using various strategies, such as reduced merozoite invasion into the HbAE erythrocyte (Roberts and Williams, 2003, Chotivanich et al., 2002), reduced parasite growth of HbAE infected erythrocytes (Nagel et al., 1981), and enhanced phagocytosis of the parasitized erythrocytes by monocytes (Bunyaratvej et al., 1986). Furthermore, HbAE has been shown to protect against acute *P. vivax* infection (Deng et al., 2018).

1.3.2. Erythrocyte polymorphisms

Ovalocytosis

The disease originates from a structural polymorphism in the major transmembrane protein of the erythrocyte, band 3, attributable to a deletion of codons 400-408 of band 3 causing a loss of 9 amino acids (Williams, 2006, Jarolim et al., 1991). The ovalocytic phenotype has been shown to confer protection against malaria by *in vitro* resistance to invasion in a range of malaria parasite strains (Kidson et al., 1981). This is thought to be due to the increased membrane rigidity and reduced deformability of the ovalocytes (Mohandas et al., 1984). Additionally, ovalocytosis confers protection against cerebral malaria (Genton et al., 1995) but exacerbates malaria anaemia (Allen et al., 1999). Interestingly, ovalocytes have an increased binding to CD36 *in vitro* (Cortes et al., 2005) which may seem contradictory to their protective role in cerebral malaria. This can be explained by the fact that CD36 are found in high levels in non-vital organs, such as skin and muscles (Turner et al., 1994), but in low levels in brain endothelial cells (Silamut et al., 1999).

Duffy antigen

The antigen is postulated to be an obligative invasion receptor on erythrocytes for *P. vivax* merozoites and a chemokine receptor also known as Duffy antigen receptor for chemokines (DARC) (Neote et al., 1994). It is a 35-43 kDa transmembrane protein which spans the erythrocyte 7 times (Lopez et al., 2010, Hadley et al., 1984). The lack of DARC expression is mediated by a point mutation in position 46 of the GATA box of the DARC promoter where an aromatic tyrosine is substituted by a sulphur containing cysteine (T46C) (Michon et al., 2001). The duffy null phenotype has been shown to confer complete resistance to *P. vivax* infection since individuals lacking the duffy antigen are refractory to invasion by *P. vivax* merozoites (Michon et al., 2001, Hamblin and Di Rienzo, 2000). This duffy negativity is predominant in West Africa (Culleton and Ferreira, 2012) and Papua New Guinea (Zimmerman et al., 1999) and this explains why *P. vivax* malaria is unusual in these geographical regions (Cooke et al., 2004).

Dantu antigen

Dantu signifies a serologically distinct blood group that results from the loss of glycophorin B (GYPB) and gain of two glycophorin B-A (GYPB-A) hybrid genes resulting to a novel sialoglycoprotein. The protein contains the extracellular domain of GYPB fused with the intracellular domain of GYPA, and is present in Sub-Saharan

Africa but absent in West African (Kariuki et al., 2018, Leffler et al., 2017). This variant has been associated with reduced risk for severe malaria (Leffler et al., 2017), probably due to impaired merozoite invasion caused by increased tension of the Dantu erythrocytes (Kariuki et al., 2018).

1.3.3. Enzymopathies

Glucose-6-phosphate dehydrogenase (G6PD) deficiency

Glucose-6-phosphate dehydrogenase (G6PD) is a cytoplasmic enzyme that catalyses the first and rate limiting step in the hexose monophosphate pathway leading to synthesis of pentose phosphate (Ruwende and Hill, 1998). It also catalyses conversion of nicotinamide adenine dinucleotide phosphate (NADP) to its reduced form (NADPH) in the pentose phosphate pathway leading to protection of erythrocytes from oxidative damage (Frank, 2005). The G6PD deficiency is one of the most common enzymopathies in human population affecting over 400 million people worldwide as shown in Figure 1.6. The disease results from single nucleotide polymorphisms that lead to reduced enzyme activity and not complete absence (Ruwende et al., 1995). The common deficiency allele in Africa is G6DP A^- resulting from two mutations, an asparagine to aspartate substitution in position 376 and a valine to methionine substitution in position 202. These mutations are associated with a significant reduction in the threat of severe malaria in both G6DP female heterozygotes and male hemizygotes (Ruwende and Hill, 1998). The G6PD deficiency has been shown to confer resistance to *P. falciparum* infection through various underlying mechanisms, such as impaired *in vitro* growth of *P. falciparum* parasites in G6PD-deficient erythrocytes (Roth et al., 1983) and phagocytosis of G6PD-deficient ring-infected erythrocytes (rIEs) by human adherent monocytes (Cappadoro et al., 1998).

Pyruvate kinase (PK) deficiency

Pyruvate kinase is a glycolytic enzyme which catalyses the irreversible conversion of phosphoenolpyruvate to pyruvate. This is a paramount step in anaerobic glycolysis and production of Adenosine triphosphate (ATP) in erythrocytes since they lack mitochondria (Durand and Coetzer, 2008). PK deficiency is caused by over 180 point mutations in the PK gene leading to clinical manifestations in homozygote and heterozygote carriers of the abnormal allele (Durand and Coetzer, 2008). The

deficiency has been shown to be protective against malaria in mice (Min-Oo et al., 2003) and in humans (Durand and Coetzer, 2008). The mutant PK alleles in malaria endemic areas have been proposed to confer resistance to *P. falciparum* infection by phagocytosis of PK deficient rIEs and impaired invasion of homozygous PK deficient erythrocytes (Ayi et al., 2008), probably due to increased membrane rigidity (Min-Oo et al., 2003).

1.3.4. Immunogenic variants

HLA-Bw53/HLA-DRB1*1302-DQB1*0501

The human major histocompatibility complex (MHC) is a set of polymorphic genes coding for proteins which allow essential cellular interactions required for development of an immune response. It is also known as human leukocyte antigen complex (HLA) (Babbitt et al., 1985). The complex is subdivided into various classes, namely class I and II which encode for membrane bound molecules capable of activating T-cells to provoke or enhance an immune response and class III which codes for soluble complement cascade proteins, heat shock proteins, and some cytokines (Lopez et al., 2010). Two malaria protective HLA alleles have been identified, namely class I HLA antigen (HLA-Bw53) which restricts cytotoxic T lymphocyte recognition of a conserved nonamer peptide of the parasite liver stage specific antigen 1 (Hill et al., 1992) leading to protection against severe anaemia and cerebral malaria (Wilkinson and Pasvol, 1997, Hill et al., 1991), and class II HLA haplotype (DRB1*1302-DQB1*0501) which is associated with reduced susceptibility to severe anaemia (Wilkinson and Pasvol, 1997).

Complement receptor 1 (CR1) polymorphism

The complement receptor 1 (CR1) is a 190-280 kDa complement-regulatory protein localized on the erythrocyte membrane and is also known as CD35 (Stoute, 2005, Thathy et al., 2005). It is the erythrocyte receptor which the PfEMP1 parasite ligand binds to form rosettes. Rosettes form when late stage infected erythrocytes bind to uninfected erythrocytes *in vitro*. The process is proposed to exacerbate disease pathogenesis (Rowe et al., 1997). The CR1 encoding gene is speculated to undergo unequal gene crossover leading to formation of polymorphic variants that have been related to different levels of CR1 expression on erythrocytes (Lopez et al., 2010). Two CR1 African allele variants resulting from absence of two Knops blood group antigens,

Swain-langley (Sl2) and McCoy (McCb), have been correlated with low CR1 copy number on erythrocyte and associated with protection from cerebral malaria, however not malaria anaemia in Kenyan children (Stoute, 2005, Thathy et al., 2005). On the contrary, the CR1 knop blood group alleles have been reported to have no association with severe malaria in The Gambia (Zimmerman et al., 2003).

Nitric oxide synthase 2 (NOS2) promoter polymorphism

The nitric oxide synthase 2 (NOS2) enzyme produces nitric oxide which is a mediator of immunity to malaria (Lopez et al., 2010). Nitric oxide production can be modulated by single nucleotide polymorphisms in the promoter of the inducible NOS2 gene (Hobbs et al., 2002), such as −1173 C/T and - 969 G/C which are significantly associated with protection from symptomatic malaria and antimalarial resistance in children respectively (Kun et al., 1998).

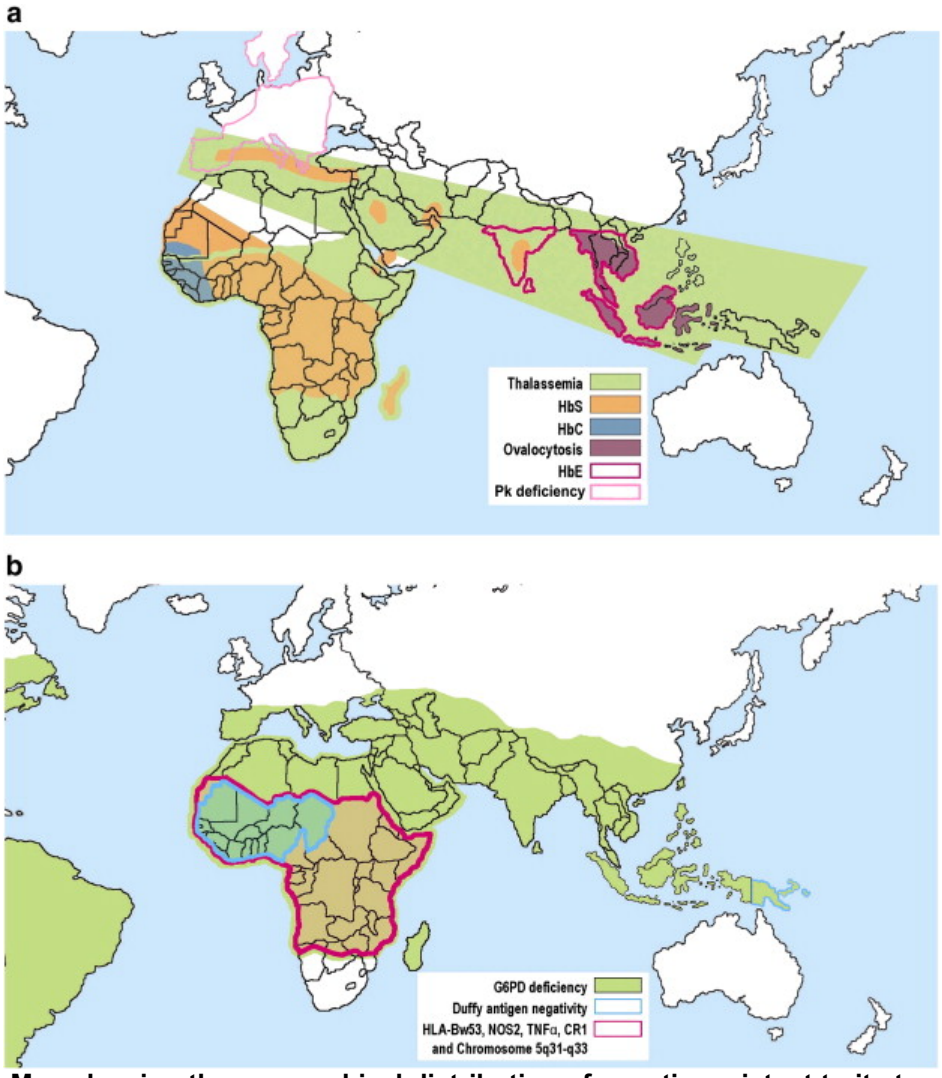

Figure 1. 6: Map showing the geographical distribution of genetic resistant traits to malaria. a) Thalassemia and b) Glucose 6 phosphate dehydrogenase (G6PD) deficiency are the most predominant traits. Figure is adapted from Lopez et al., 2010.

1.4. Immunity

1.4.1. Immune response to *P. falciparum* in *Anopheles* mosquitoes

The *P. falciparum* parasite faces various hurdles before it can successfully establish infection in the *Anopheles* mosquito vector (Demarta-Gatsi et al., 2017). The mosquito uses various physical barriers and elicits cellular and humoral innate immune responses to protect itself against the parasite. The physical barriers are the first line of defence and includes the midgut peritrophic membrane (Osta et al., 2004), the formation of capsule around the parasite by mosquito melanin (Crompton et al., 2014), and microbiota which stimulate basal innate immune responses against the parasite (Clayton et al., 2014). Additionally, the cellular innate immune response in mosquitoes is mediated primarily by haemocytes which are involved in phagocytosis, melanisation, agglutination, secretion of antimicrobial peptides, nodule formation, an and the production of reactive oxygen species (Smith et al., 2016, Clayton et al., 2014, Molina-Cruz et al., 2008, Osta et al., 2004). Humoral innate immune responses in mosquitoes involves the complement C3-like, also known as thioester-containing protein 1 (TEP1), which binds to the surface of the ookinete and marks them for killing (Clayton et al., 2014).

It is important to note that mosquitoes rely entirely upon the innate immune system since it lacks an adaptive immune response (Dennison et al., 2015). However, mosquitoes can obtain transmission blocking antibodies from the host during a blood meal. These have been shown to reduce infection in the mosquito by: (i) preventing gamete fusion by targeting the HAP2 male sterility gene (Blagborough and Sinden, 2009), (ii) inducing complement killing of gamete or ookinetes (Volohonsky et al., 2010), and (iii) averting ookinete motility, penetration of the midgut wall and formation of oocyst by targeting the Pbs21, P25 and P28 ookinete surface antigens (Baton and Ranford-Cartwright, 2005, Ranawaka et al., 1994). These transmission blocking vaccines can be produced by vaccination but unfortunately the humoral immune response cannot be enhanced by repeated infections because the gamete and ookinete are not present in the mammalian host during infection.

1.4.2. Immune response to *P. falciparum* in humans

1.4.2.1. Innate immunity

Innate immunity is the first line of defence in humans against pathogens and consists primarily of pattern recognition receptors (PRRs) (Mellouk et al., 1994). In case of malaria infection, the PRRs identify pathogen associated molecular patters (PAMPs), such as hemozoin, glycosylphosphatidylinositol (GPI) anchors and immunostimulatory nuclei (Gazzinelli et al., 2014). The recognition of PAMPs leads to the induction of the type 1 interferon pathway and the subsequent production of cytokines - IFNγ, IL-6, and TNFα that recruit leukocytes through induction of the inducible nitric oxide synthase enzyme (Goh et al., 2019, Bliss et al., 2018, Mellouk et al., 1994). The recruited cellular innate immune cells include: (i) CD8α Dendritic cells, which are triggered just a few hours after an infective mosquito bite and directly take up the sporozoite before priming the CD8 T cells in the draining lymph nodes of the skin (Radtke et al., 2015, Chakravarty et al., 2007), (ii) NK cells, which are abundant in the hepatocytes and directly inhibit parasite development and also prime CD4 T cells to bridge the gap between innate and acquired immunity (Ing and Stevenson, 2009), and (iii) NKT and γδ T cells, which are innate-like T cells involved in killing late stage liver parasites and priming adaptive T cell responses respectively (Zaidi et al., 2017).

1.4.2.2. Natural acquired immunity

Natural acquired immunity refers to the protective immunity an individual achieves after being naturally exposed to a pathogen. Robert Koch observed that natural immunity to malaria in endemic areas is acquired after years of repeated exposure to the *Plasmodium* parasite but is less efficient and achieves only partial protection (Ly and Hansen, 2019). In high transmission areas, children suffer few symptomatic infections and later develop immunity against severe malaria but remain at risk of uncomplicated malaria as shown in Figure 1.7 (Griffin et al., 2015). Following years of repeated infections, individuals gradually develop clinical immunity with age and are able to reduce parasite burdens (Ndungu et al., 2015). However, this form of protection does not culminate in sterile immunity that prevents re-infection (Tran et al., 2013). Adults continue to experience asymptomatic infections with low parasite densities which remain a possible source for human-to-mosquito transmissions (Okell et al., 2012). On the other hand, in low transmission areas both children and adolescents

are susceptible to severe malaria and adults continue to experience mild malaria and asymptomatic malaria as shown in Figure 1.7.

Although natural acquired immunity requires both cellular and humoral immune responses, natural acquired clinical immunity to malaria targets parasites in the blood stages (Ly and Hansen, 2019) and is governed by antibodies (Cohen et al., 1961). The clinical immunity may depend on the cumulative acquisition of a breath of antibodies that respond to a diverse range of parasite antigens (Murungi et al., 2013) and has been shown to increase with age in malaria endemic areas (Crompton et al., 2010). The slow acquisition of immunity to malaria in endemic areas has been attributed to antigen variation and substantial decline in *Plasmodium* specific antibody responses after reduced parasite exposure (Weiss et al., 2010).

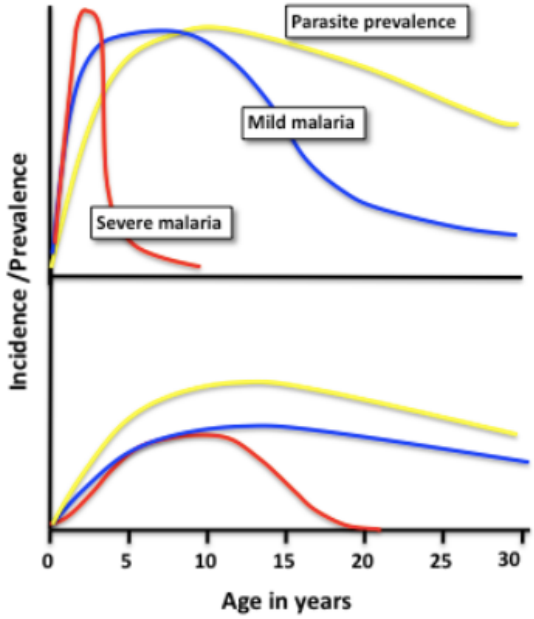

Figure 1. 7: A typical age pattern for incidence of severe and mild malaria and prevalence of asymptomatic malaria infection. The upper and lower graphs represent malaria infection in areas of high and low transmission respectively. Figure is adapted from Kinyanjui et al., 2012.

1.4.2.3. Adaptive immune response to skin and liver stages

At the skin stage, antibodies protect the host by limiting pre-erythrocytic stage infection and development. More specifically, they do so by inhibiting sporozoite motility in the dermis (Vanderberg and Frevert, 2004) and mediating cytotoxicity against sporozoites in the host skin (Aliprandini et al., 2018). Despite the immune response at the skin, some sporozoites manage to enter the blood stream, where the circulating antibodies prevent hepatocyte invasion (Nudelman et al., 1989), and opsonize the sporozoites facilitating their phagocytosis by monocytes and macrophages in the liver and spleen

(Schwenk et al., 2011). The neutralizing capacity of circulating antibodies is more at the site of inoculation than in the blood circulation (Flores-Garcia et al., 2018).

Once the sporozoite is inside the liver, it can be attacked by CD4 and CD8 T cells (Renia et al., 1991) since the hepatocytes express both MHC class I and class II molecules that can be loaded with parasite antigens (Pichugin et al., 2016, Renia and Goh, 2016). CD8 T cytotoxic cells are the principal effector cells providing protection against malaria (Goh et al., 2019). The significance of CD8 T cells in protective immunity was first shown in mice vaccinated with irradiated sporozoites (Schofield et al., 1987). Cytotoxic T cells mediate protection against murine malaria in mice by killing the parasites directly or indirectly through lysis of infected hepatocytes or IFNγ-mediated protection respectively (Goh et al., 2019). Moreover, CD8 T cells have been associated with protection from severe malaria in humans (Hill et al., 1992).

On the other hand, the role of CD4 T cells in malaria is not well understood, but it is clear that they are activated to amplify responses against the parasite by driving B cell germinal responses and assisting CD8 T cell activation (Goh et al., 2019). Additionally, antibodies also act in the liver stage by inhibiting sporozoite development inside the hepatocytes (Nudelman et al., 1989) and binding to neo-antigens expressed on the surface of infected hepatocytes. The binding induces parasite death by involving Kupffer cells or NK cells (Renia et al., 1990). Furthermore, it is important to note that, in addition to high levels of antibodies, the quality of antibodies is also imperative. Various *in vitro* assays have been established to examine the functionality of antibodies induced after vaccination with pre-erythrocytic stage vaccines (Goh et al., 2019). These pre-erythrocytic stage antibody functional assays include; gliding motility assay (Behet et al., 2014), sporozoite traversal and invasion inhibition assay (Kaushansky et al., 2012) and liver stage development inhibition assay (Zou et al., 2013, Mazier et al., 1985).

1.4.2.4. Adaptive immune response to asexual blood stages

Adaptive immunity against blood stage parasites is primarily conferred by humoral immune response due to the absence of antigen processing mechanism in erythrocytes (Yazdani et al., 2006). It is more complex than adaptive immunity against the liver stage since it targets different asexual forms of the parasite that present different antigens (Renia and Goh, 2016).

The mature intraerythrocytic parasites express several variant surface antigens (Frech and Chen, 2013) encoded by multigene families, namely: (i) the *var* (Su et al., 1995), (ii) sub-telomeric variable open reading frame (*stevor*) (Cheng et al., 1998), (iii) surface-associated interspersed (*surf*) (Winter et al., 2005) and (iv) repetitive interspersed family (*rifins*) for *P. falciparum* (Kyes et al., 1999) or the *Plasmodium* interspersed repeats (*pir*) gene family for *P. vivax* (*vir*) and *P. knowlesi* (*kir*) (Janssen et al., 2004). These genes encode for antigens which enhance disease by several adhesive phenomena, such as cytoadherence to endothelial lining of tissues (David et al., 1983), rosette formation (Udomsangpetch et al., 1989) and agglutination of infected erythrocytes by bridging platelets (Pain et al., 2001). Antibodies targeting these variant surface antigens, primarily the PfEMP1 coded by the *var* gene family, have been shown to limit progression of the blood stage *in vitro* by preventing sequestration and rosette formation (Chan et al., 2014), mediating phagocytosis of infected erythrocytes (Celada et al., 1982), facilitating agglutination of infected erythrocytes (Renia and Goh, 2016) and promoting antibody dependent cellular cytotoxicity (Arora et al., 2018). Furthermore, broad reactive antibodies present in African individuals have undergone somatic hypermutation and thus contain insertion of a collagen binding protein, LAIR1, which enhances their binding to infected erythrocytes via the *rifin* family (Pieper et al., 2017, Tan et al., 2016).

Merozoite specific antibodies impede the progression of the erythrocytic stage and the following has been demonstrated *in vitro* using various mechanisms, such as: (i) prevention of merozoite egress from schizont-infected erythrocytes by targeting SERA5 which are papain like proteins located in the parasitophorous vacuole (Collins et al., 2017a), (ii) inhibition of erythrocyte invasion by either directly targeting *P. falciparum* reticulocyte binding homologue 2 (PfRh2) (Reiling et al., 2010), apical membrane antigen 1 (AMA1) (Dutta et al., 2007), region II of erythrocyte binding antigen 175 (EBA175), or in conjunction with complement factors via merozoite surface proteins 1 and 2 (MSP1 and MSP2) (Boyle et al., 2015), (iii) agglutination of released merozoites (Miller et al., 1975), (iv) promotion of phagocytosis (Khusmith et al., 1982) by targeting merozoite surface protein 2 and 3 (MSP2 and MSP3) (Osier et al., 2014), (v) generation of reactive oxygen species in a neutrophil-based antibody dependent respiratory burst (ADRB) assay (Joos et al., 2010), (vi) facilitation of monocyte mediated clearance of intraerythrocytic parasites in an antibody-dependent cellular inhibition (ADCI) assay (Bouharoun-Tayoun et al., 1990), where antibodies

bind to the merozoite surface antigens and stimulate phagocytes to release cytokines, which in turn activate the phagocytes to produce mediators leading to killing of surrounding intraerythrocytic parasites (Bouharoun-Tayoun et al., 1995), and (vii) activation of NK cells which leads to lysis of merozoites through degranulation and production of IFNγ in an antibody-dependent cellular cytotoxicity (ADCC) assay (Odera, unpublished).

Toxins released by the parasite could also be targets of the immune system (Renia and Goh, 2016). These toxins contribute to disease pathogenicity by inducing inflammation and they include hemozoin which is a by-product of haem degradation (Boura et al., 2013), GPI moieties present in many merozoite proteins (Renia and Goh, 2016), a TatD-like DNase which counteracts activated neutrophil cells (Chang et al., 2016), a tyrosine-tRNA synthase, which is a housekeeping protein translation enzyme (Bhatt et al., 2011), and lipids extracted from *P. vivax* schizont-infected erythrocytes (Wijesekera et al., 1996). The ability of anti-toxin antibodies to protect from disease has been shown experimentally by a mouse model using synthetic glycans that mimic GPI (Schofield et al., 2002).

Despite the lack of MHC antigens on the surface of infected erythrocytes, T cells are also significant immune effectors against the blood stage. B cell maturation and multiplication depend on cytokines released by CD4 T helper cells for optimal production of parasite specific antibodies (Langhorne et al., 2008). Interestingly, CD8 T cells, which were once thought to have minimal significance in blood stage immunity, have been shown to inhibit blood stage infection in mice (Podoba and Stevenson, 1991). More specifically, the perforin and IFNγ-secreting CD8 T cells are key in preventing chronic murine blood stage malaria infection in mice (Imai et al., 2010).

1.4.2.5. Adaptive immune response to sexual blood stages

Similar to the asexual blood stages, the sexual parasite forms in the blood are also targets of the immune system. The sexual forms, gametocytes, are targeted by antibodies which induce their complement-mediated elimination in the host blood (Margos et al., 2001, Read et al., 1994). These gametocyte-specific specific antibodies rely on CD4 T helper cells for optimal production.

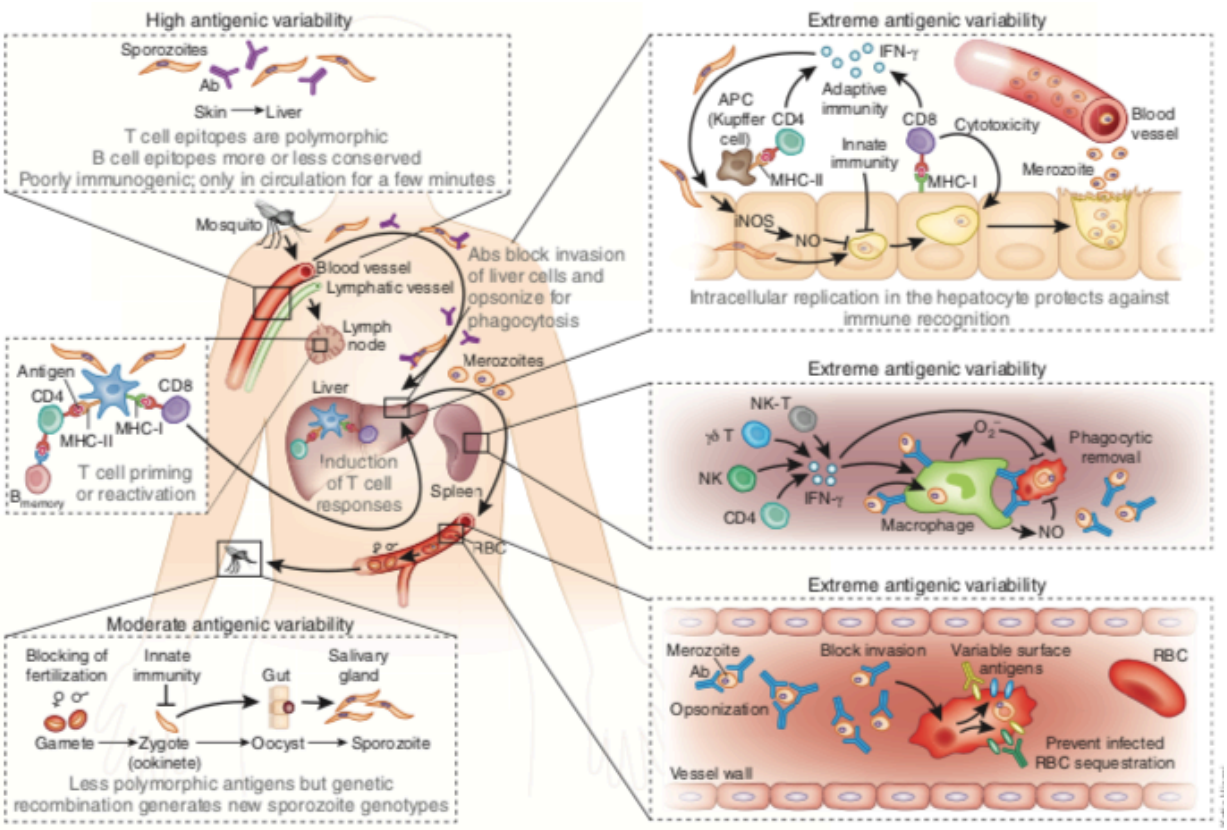

Figure 1. 8: Immune responses that control the different parasite stages in the human. At the skin, the sporozoites are drained into the lymph nodes, where they activate T and B cells. Antibodies (Ab) prevent sporozoite invasion in the liver. When in the liver, IFN-γ–producing CD4+ and CD8+ T cells prevent parasite development into merozoites inside the hepatocyte. Despite the immune response, some merozoites emerge from the liver and invade the erythrocytes. Merozoite-specific antibodies agglutinate merozoites, prevent invasion and opsonise merozoites to mediate Fc effector cellular functions. Antibodies targeting variant surface antigens prevent cytoadherence and rosette formation, enhance agglutination and opsonise infected erythrocytes to mediate Fc effector cellular functions. Gametocyte specific antibodies fix complement that lyse parasite in the human host. In the vector, innate mosquito immunity and transmission blocking antibodies from the human host prevent gamete fusion, ookinete development and oocyst rupture to release sporozoites. Figure is adapted from Riley et al., 2013.

1.4.3. Immune evasion of *P. falciparum*

1.4.3.1. Immune evasion mechanisms in *Anopheles* mosquitoes

The parasite evades the vector's innate immune response primarily by the help of the Pfs47 gene which is a female gamete-specific protein. The Pfs47 prevents activation of the TEP1 complement-like lysis by suppressing the nitration response in the midgut (Molina-Cruz et al., 2013). Additionally, the host complement factor H (FH) obtained from the blood meal also prevents complement attack. More specifically, the *P. falciparum* glideosome-associated protein 50 (PfGAP50), which is a FH receptor, binds the host FH leading to the inactivation of complement protein C3b which

subsequently protects the extracellular gamete from complement-mediated lysis (Simon et al., 2013).

1.4.3.2. Immune evasion mechanisms of liver stage in humans

The sporozoites must evade the resident macrophages of the liver, Kupffer cells, in order to establish the liver stage infection. Once the sporozoites come in contact with the Kupffer cells, they are able to manipulate the macrophage functions and immunosuppress the microenvironment in various ways, such as: (i) inhibition of reactive oxygen species formation by binding to the Kupffer cells surface proteins and proteoglycans via the CSP (Usynin et al., 2007), (ii) downregulation of inflammatory Th1 cytokines (TNFα and IL-6) and upregulation of anti-inflammatory Th2 cytokines (IL-10) (Ikarashi et al., 2013), (iii) induction of T cell tolerance by stimulating apoptosis of Kupffer cells and reducing expression of MHC class I molecules (Casares and Richie, 2009, Steers et al., 2005) and finally (iv) upregulation of the host haem oxygenase 1 which modulates the host inflammatory response thereby enhancing development of intrahepatic parasites (Pamplona et al., 2007). Additionally, the sporozoites interfere with the mammalian target of rapamycin (mTOR) pathway, which is the central regulator of cell metabolism, leading to altered levels of proteins involved in cell growth, survival and proliferation (Hanson et al., 2013). After successful establishment of the liver infection, the parasite stays within a parasitophorous vacuole which physically separates itself from the host cytoplasm preventing lysosomal degradation (Gomes et al., 2016). For successful establishment of the blood stage infection, the merozoites leave the hepatocytes covered in membranes derived from the host, merosomes, which protect them from the phagocytic cells of the liver (Sturm et al., 2006).

1.4.3.3. Immune evasion mechanisms of intraerythrocytic stages in humans

The intraerythrocytic stage is the most primitive survival strategy of the parasite, where it avoids direct contact with the host immune system. Moreover, the parasites' decision to invade the non-nucleated erythrocytes is favourable because red blood cells do not express MHC class I molecules on their surface and thus avoid recognition by CD8 T cells (Bowen and Walker, 2005). Additionally, the mature intraerythrocytic parasites sequester from the immune system and avoid splenic clearance by expressing

adhesive parasite proteins on their surface which mediates their adherence to endothelial lining of tissues (Belachew, 2018, Gomes et al., 2016). Clustering with uninfected erythrocytes to form rosettes is an additional escape strategy (Moll et al., 2015). These adhesive proteins are expressed by multigene families and thus undergo antigen variation making it difficult for them to be recognised by the host immune system. More specifically, each parasite carries approximately 150-200 *rif*, 50-60 *var* and 30-35 *stevor* gene copies per genome which encode for RIFIN, STEVOR and PfEMP1 antigens respectively (Mwakalinga et al., 2012, Kraemer and Smith, 2006). Furthermore, sequestration of the intracellular parasite can also be mediated by host platelets which enhance parasite agglutinating with uninfected erythrocytes (Gomes et al., 2016), and non-specific IgM which recognises PfEMP1 and promote rosette formation (Dennison et al., 2015, Ghumra et al., 2008). Interestingly, hemozoin hinders phagocytic functions of the immune system since monocytes containing hemozoin can only take up few infected erythrocytes (Belachew, 2018).

1.4.3.4. Immune evasion mechanisms by merozoites in humans

The merozoite sheds and secretes erythrocyte binding like proteins, such as MSPs, PfEBA, PfRHs and PfAMA1, which bind to their respective erythrocyte receptors for successful invasion (Weiss et al., 2015). These erythrocyte invasion ligands show a high degree of polymorphisms because they exist in numerous alleles in the genome. This enables the merozoite to evade the host immune system thereby establishing the blood stage infection and disease progression (Holder et al., 1999). Moreover, invasion ligands such as the EBA and RH families have redundant functions that evolved to promote antigenic diversity and immune evasion (Souza-Silva et al., 2014). Furthermore, merozoites protect themselves from complement-mediated lysis by binding to the CCP5 module of the complement regulator FH via a transmembrane protein, Pf92, which inactivates the complement component C3b thereby preventing lysis (Kennedy et al., 2016).

1.4.3.5. Immune evasion mechanism of gametocytes in humans

The developing stage 1-1V gametocytes are absent in the peripheral circulation during malaria because they sequester in the spleen and bone marrow to avoid splenic clearance (Joice et al., 2014). This unique maturation process has made it difficult to

investigate the underlying molecular mechanism of *P. falciparum* gametocytes using rodent models. However, certain adhesive parasite proteins have been observed on the infected erythrocyte surface of the developing gametocytes and they include: PfEMP1, which mediates adhesive interactions with host endothelial receptors and STEVOR, which is implicated with enhance deformability (Sanyal et al., 2012).

1.5. Control strategies for *P. falciparum* malaria

1.5.1. Chemoprophylaxis

1.5.1.1. Introduction to malaria treatments

The design of antimalarial drugs is based on the major metabolic differences between the *Plasmodium* parasite and the mammalian host (Alam et al., 2009). Interestingly, the apicoplast and mitochondria of the parasite have a separate prokaryotic-like genome which defines the specificity of the drug targets and prevent attack of the eukaryotes (Yadav et al., 2019). These metabolic differences include: (i) the apicoplast mevalonate-independent isoprenoid biosynthesis pathway, where the parasite utilizes 1-deoxy-d-xylulose-5- phosphate (DOXP) as a precursor molecule while the mammalian host depends on mevalonate for isoprenoid synthesis (Goodman and McFadden, 2013, McFadden and Roos, 1999); (ii) the parasite ubiquinone/coenzyme Q in the mitochondrial electron transport chain is different from the mammalian ubiquinone and plays an important role in ATP synthesis (Ellis, 1994); (iii) the shikimate pathway, absent in mammals, responsible for production of precursor chorismate required for synthesis of ubiquinone, aromatic amino acids, folate and vitamin K (McConkey, 1999); and (iv) the enzymes used in purine and pyrimidine synthesis during nucleic acid metabolism differ between the parasite and the humans (Heikkila et al., 2007, Keough et al., 1999).

Despite the numerous attempts to design antimalarial treatments, drug resistance remains a recurring issue. The parasite is able to develop resistance using various mechanisms, such as: (i) direct catalysis of the drug leading to detoxification; (ii) a stress response, which mitigates the toxicity induced by the drug; (iii) mutations and/or amplifications of the gene coding the target enzyme(s) and transporter(s) which pump and remove the drug out of the parasite (Ross and Fidock, 2019, Haldar et al., 2018). Moreover, resistance of most antimalarial drugs emerged from Southeast Asia and this is due to various factors: (i) low malaria transmission intensity causing reduced

frequency of human exposure to malarial parasites and subsequently leading to low immunity against malaria; (ii) the monoclonal nature of *P. falciparum* infections which encourages relatively unfit mutations to disseminate because of lack of competition; and finally (iii) prevalence of hemoglobinopathies and G6PD deficiency causing alteration of the erythrocytes' redox state and possibly influencing the efficiency of drugs which act by increasing oxidative stress (Ross and Fidock, 2019, Blasco et al., 2017). Currently, initiatives are in place to develop new and effective antimalarial molecules by combining previously effective drugs, identifying new drugs for the validated targets and novel targets for malaria treatment (Yadav et al., 2019).

1.5.1.2. Classification of antimalarial drugs

Aryl-amino alcohols

Aryl-amino alcohols are organic substances that contain both aryl and amino functional groups and include quinine, lumefantrine and mefloquine. Importantly, quinine was discovered from cinchona tree bark in Peru and was the first antimalarial drug to be employed in Europe in the 1630s until early twentieth century (Blasco et al., 2017, Achan et al., 2011). Aryl-amino alcohols are speculated to be involved in haemoglobin transportation from the cytosol and haem detoxification in the digestive vacuole but the exact mechanism is unknown (Tse et al., 2019, Yadav et al., 2019). Resistance to quinine is associated with amplification of *P. falciparum* chloroquine resistance transporter (*pfcrt*) gene whereas resistance to lumefantrine and mefloquine are associated with amplification of the *P. falciparum* multidrug resistant protein 1 (*pfmdr1*) transporter gene (Blasco et al., 2017).

4-amino quinolines

4-amino quinolones are organic compounds that contain an amino group at the fourth position of the quinoline structure and include chloroquine, amodiaquine, piperaquine and pyronaridine. Importantly, chloroquine was the first line antimalarial drug to be used from 1940s to 1980s because of its safety, efficacy and affordability (Ross and Fidock, 2019). Chloroquine is permeable in its unprotonated form and can freely diffuses into the erythrocyte where it gets protonated in the acidic digestive vacuole thereby becoming impermeable (Chinappi et al., 2010). Consequently, chloroquine accumulates in the digestive vacuole, similar to the other 4-amino quinolines and quinine, where it prevents detoxification of haem by binding to hematin and thus

preventing formation of ß hematin (hemozoin) which is non-toxic and chemically inert (Egan et al., 2002, Robert et al., 2002). Hence, chloroquine action is effective against intraerythrocytic trophozoite stages involved in haemoglobin degradation but ineffective against parasite stages that don't consume haemoglobin, such as liver schizonts and mature gametocytes (Chimanuka et al., 2001). Resistance to chloroquine is due to parasite-mediated drug efflux resulting from point mutations in the *pfcrt* and *pfmdr1* transporters located in the digestive vacuole membrane (Blasco et al., 2017). Additionally, resistance to piperaquine is associated with copy number variation of *plasmepsin 2 and 3* which encode for proteases involved in haemoglobin digestion (Witkowski et al., 2017).

Antifolates

Antifolates are chemical compounds that disrupt folic acid metabolism in the parasite and include pyrimethamine, sulfadoxine and proguanil. Folic acid is converted to dihydropteroate by dihydropteroate synthase (*dhps*) enzyme and then reduced by dihydrofolate reductase (*dhfr*) to form tetrahydrofolate which is essential in DNA synthesis (Hyde, 2005). Antifolate agents used in malaria treatment interrupt folate metabolism in the cytosol by inhibiting the enzymes *dhps* and *dhfr* (Nzila, 2006). Resistance to sulfadoxine is associated with mutation in the *dhps* enzyme whereas resistance to pyrimethamine and proguanil are associated with mutation in the *dhfr* enzyme .(Blasco et al., 2017).

Naphthoquinone

Naphthoquinone is a group of organic compounds that structurally relates to naphthalene. A good example is atovaquone which is a ubiquinone analogue. Atovaquone prevents the binding of ubiquinone to cytochrome b (*cytb*) which disrupts the mitochondrial electron transport chain (Srivastava et al., 1997) and, consequently, leads to the inhibition of the ubiquinone-dependent mitochondrial enzyme dihydroorotate dehydrogenase (DHODH) required for pyrimidine biosynthesis (Blasco et al., 2017). Additionally, the combination of atovaquone and proguanil yields an effective antimalarial drug, malarone, which is used for both treatment and prophylaxis (Radloff et al., 1996). Resistance to atovaquone is associated with a single point mutation in the *cytb* subunit of the cytochrome bc1 complex, which has a significant fitness cost in the sexual stage development in the mosquitoes and thus the resistance is not transmissible in the field (Goodman et al., 2016).

8-amino quinolines

8-amino quinolines are organic compounds that contain amino group at the eighth position of the quinoline structure and include primaquine and tafenoquine. Importantly, primaquine is the only 8-aminoquinoline currently in clinical use (Flannery et al., 2013). Additionally, it is the only available antimalarial drug that prevents relapse in ovale and vivax malaria and the only effective gametocytocidal drug in falciparum malaria (Ashley et al., 2014). The mode of action of 8-amino quinolines is unknown, and no genetic change has been associated with clinical resistance to primaquine (Blasco et al., 2017).

Antibiotics

Antibiotics are antibacterial drugs which are also active in fighting malaria infection and they include clindamycin, doxycycline and tetracycline (Tse et al., 2019). They function by inhibiting protein synthesis in the apicoplast. Resistance is associated with mutations in the ribosomal ribonucleic acid (rRNA) of the apicoplast (Blasco et al., 2017).

Endoperoxides

Endoperoxides are sesquiterpene lactones and the main antimalarial endoperoxide is artemisinin whose active metabolite is dihydroartemisinin (Tilley et al., 2016). Artemisinin, also known as qinghaosu, was first isolated in 1970 from a Chinese sweet wormwood plant, *Artemisia annua*, by a Chinese scientist called Tu Youyou, who was awarded the 2015 Nobel Prize (Tse et al., 2019). Artemisinin can be derived from methyl, hemisuccinate and ethyl ether to form Artemether, Artesunate and Arteether respectively (Ashley et al., 2018).

Artemisinin activation is centred on the reductive scission of the endoperoxidase ring. The cleavage of the endoperoxidase depends on its contact with ferrous-haem, a by-product of haem degradation, during the mature intraerythrocytic parasite stages (Wang et al., 2015), and free ferrous iron during early ring stage (Xie et al., 2016). The mode of action of dihydroartemisinin is by provoking the parasites' stress mechanism using two main ways: (i) protein damage, which is achieved by alkylation of already folded proteins and unfolding of newly synthesised proteins (Mok et al., 2015) and (ii) inhibition of the parasite proteasome function, which leads to accumulation of polyubiquitinated proteins (Bridgford et al., 2018).

Artemisinin resistance is defined as parasite survival above 1% *in vitro* and parasite clearance half-life of more than 5 hours *in vivo* and is associated with *kelch13* mutation

(Ariey et al., 2014). The exact mechanism of *kelch13* mutant remains unknown, but is thought to encourage the parasite survival by antagonizing the dihydroartemisinin activity through various ways, such as: (i) the unfolded protein response, which induces a transcriptional program that alleviates the cellular stress (Tilley et al., 2016); (ii) the ubiquitin-proteasome system, which inhibits the function of the ubiquitin-activating enzyme thereby reducing the level of polyubiquitinated proteins and alleviating the cellular stress (Dogovski et al., 2015); and (iii) dysregulation of the proteostasis control of *P. falciparum* phosphatidylinositol 3 kinase (PfPI3K) by reducing its interaction with dihydroartemisinin, which leads to less PfPI3K ubiquitination and in turn increases production of phosphatidyl 3 phosphate (P13P) signalling molecule (Mbengue et al., 2015).

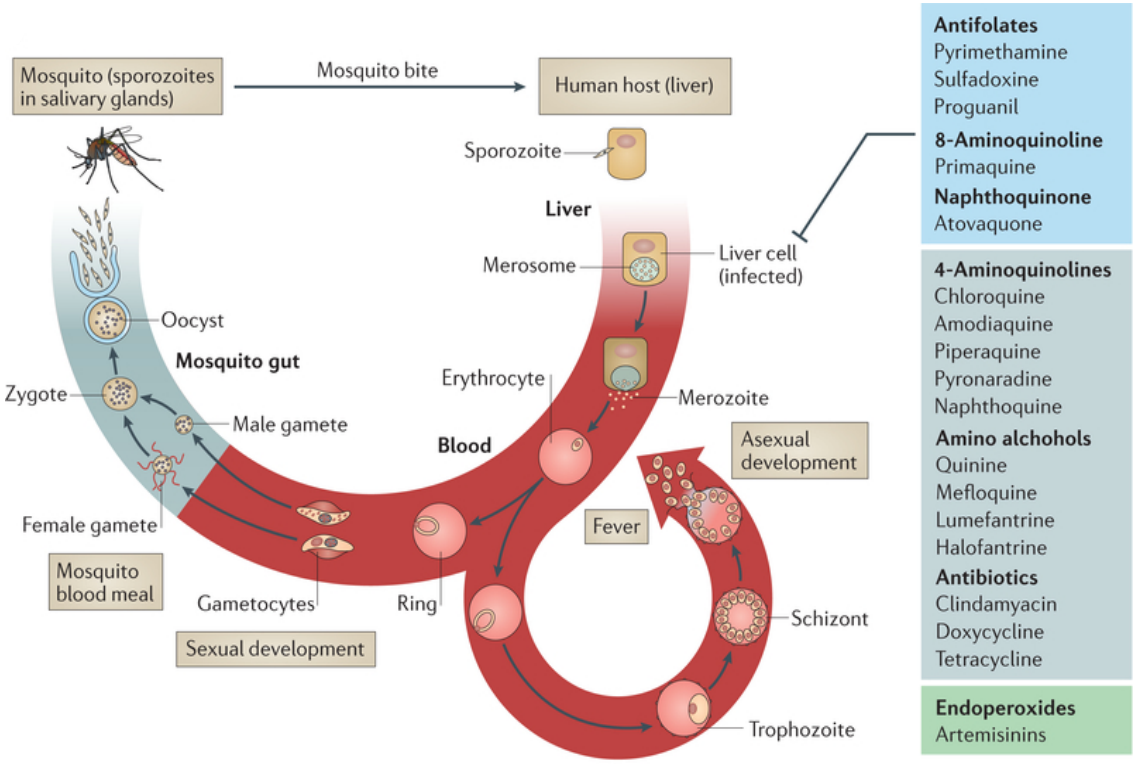

Figure 1. 9: The *P. falciparum* life cycle stages that are targets of the antimalarial drugs. All antimalarial drugs target the asexual trophozoite and schizont blood stage. The antifolates, primaquine and atovaquone also target the liver stage parasites. Artemisinins also target the asexual ring stage and the early sexual blood stages. Figure adapted from Haldar et al., 2018.

1.5.1.3. Future for malaria chemoprophylaxis

Improvement of existing antimalarial drugs

The effect of the rapid acting artemisinin can be enhanced by combination therapy which in turn helps prevent the parasite from developing resistance to artemisinin. Artemisinin-based combination therapies (ACTs) include: (i) the recently approved combination of artemisinin and pyronaridine (West African Network for Clinical Trials of Antimalarial, 2018); (ii) the combination of artesunate with ferroquine, a third generation 4-aminoquinoline, which proves promising in treating falciparum malaria especially in areas that have developed resistance to ACTs (Held et al., 2015); and (iii) the triple combination therapies, such as artemisinin + lumefantrine + amodiaquine and dihydroartemisinin + piperaquine + mefloquine, that show increased efficacy in clinical trials (Ross and Fidock, 2019). In addition, next generation endoperoxides have been developed. One example is OZ439 which shows longer plasma half-life of 23 hours as compared to 30 minutes in the case of dihydroartemisinin (Charman et al., 2011) and further example - tetraoxane which has desirable rapid killing and lifelong properties (O'Neill et al., 2017).

Discovery of drugs with new modes of action

Newly discovered antimalarial drug targets are shown in Figure 1.10. Membrane biosynthesis, where a new choline analogue, G25, targets the phosphatidylcholine and phosphatidylethanolamine plasma fatty acid synthesis pathways and thus inhibit malarial infection *in vitro* and *in vivo* (Roggero et al., 2004). Transporters inhibitors, where an O-3 hexose derivative inhibits uptake of fructose and glucose by targeting the hexose transporter in mouse model (Joet et al., 2003) and a spiroindolone compound KAE609/ciparagamin enhances the cell membrane rigidity which in turn inhibits parasite development and transmission to mosquitoes by targeting the P-type Na+ ATPase PfATP4 (Blasco et al., 2017). Shikimate pathway, where compounds targeting the enzymes involved in the aromatic biosynthetic pathway inhibit progression of malaria infection due to the specificity of the targets and avoidance of off-target effects (McRobert et al., 2005, McConkey, 1999). Plasmodial proteases in the food vacuole, where potent synthetic inhibitors prevent haemoglobin degradation by targeting various aspartic and cysteine proteases such as plasmepsin (Coombs et al., 2001) and falcipain proteins (Lee et al., 2003) respectively. Mitochondrial isoprenoid biosynthesis, where the antibacterial fosmidomycin and its derivative FR-900098 inhibit parasite development by targeting the DOXP reductase (DXR) which is

a key enzyme in the mevalonate-independent isoprenoid biosynthesis pathway (Wiesner et al., 2007, Lell et al., 2003). Mitochondrial redox system, where the 5,8-Dihydroxy-1,4-naphthoquinone and 5-nitro-2-furanacrolein target the thioredoxin reductase thus prevent the intraerythrocytic parasite from overcoming oxidative stress *in vitro* (Yadav et al., 2019) and methylene blue which inhibits glutathione reductase thereby reducing mature gametocyte survival (Siciliano et al., 2017). Nucleic acid metabolism, where DSM265 targets the mitochondrial located plasmodial dihydroorotate dehydrogenase (DHOODH) enzyme which is essential for pyrimidine synthesis (McCarthy et al., 2017). Fatty acid metabolism, where antimicrobials such as thiolactomycin analogues, triclosan and cerulenin target enzymes in the type II fatty acid biosynthesis subunit thereby inhibiting the parasite growth (Yadav et al., 2019, Surolia and Surolia, 2001, Waller et al., 1998).

Moreover, drugs targeting multiple life stages of the parasite have been discovered. These drugs include: an imidazolopiperazine compound, KAF156, which cures uncomplicated malaria by acting against the liver stage, asexual and sexual blood stages (Kuhen et al., 2014) and MMV390048, which is effective against all stages of the parasites' life cycle in a mouse model except from the liver hypnozoites (Paquet et al., 2017).

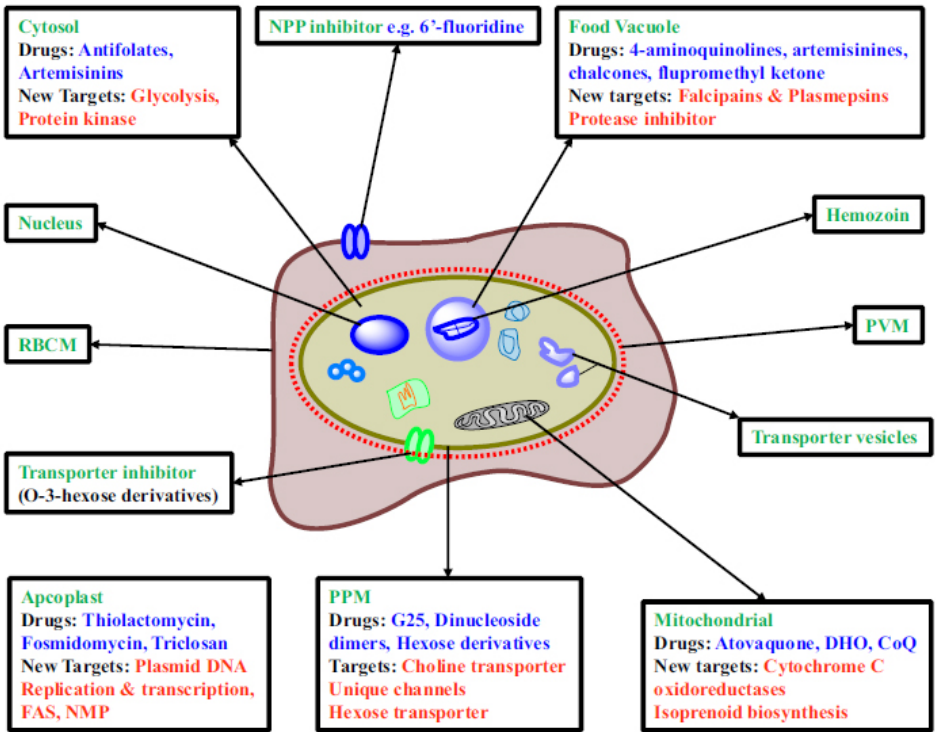

Figure 1. 10: Potential drug targets of *P. falciparum* intraerythrocytic parasite stage. The possible new therapeutic targets within various cellular structures of the parasite. Figure adapted from Yadav et al., 2019.

1.5.2. Vector control

1.5.2.1. Introduction to vector control

The major malaria vectors in Africa, include *Anopheles funestus* and *Anopheles gambiae sensu lato* which is also known as the *Anopheles gambiae* complex (Hemingway, 2014, Halcrow, 1956). The complex consists of the following eight cryptic species; *Anopheles arabiensis*, *Anopheles merus*, *Anopheles melas*, *Anopheles bwambae*, *Anopheles gambiae* which is recognised as *Anopheles sensu stricto* and *Anopheles coluzzi* in the S and M form respectively, and *Anopheles quadriannulatus* which is also known as *Anopheles amharicus* (Coetzee et al., 2013, Sinka et al., 2010). Vector control depends primarily on indoor residual sprays (IRS) and long-lasting insecticide nets (LLINs), which resulted to reduced malaria deaths by a third in the period between 2000 to 2010 (WHO, 2012). However, insecticide resistance is already a major issue in Africa (Hemingway, 2014).

1.5.2.2. Classification of insecticides

Organochlorines and pyrethroids.

The most effective organochlorine was dichlorodiphenyltrichloroethane (DDT). It superseded the use of pyrethrum extract obtained from flowers and was considered the magic bullet in malaria control in 1950s after the second world war (Hargreaves et al., 2000). However, the widespread effective use of organochlorine insecticides for agricultural reasons and house spraying during malaria control led to selection pressure and development of resistant malaria mosquitoes strains in malaria endemic areas (Brown, 1986).

Recently, DDT was replaced by a new generation of highly potent photostable synthetic pyrethroids, which resemble natural pyrethrins obtained from the flowers of pyrethrums, and include deltamethrin, permethrin and alpha-cypermethrin (Chrustek et al., 2018). Additionally, pyrethroids are cheap to produce, easy to formulate and relatively safe to use in close proximity to humans thereby making them the only insecticide recommended for use in LLINs (WHO, 2012). Consequently, the intense use of pyrethroids resulted to emergence of resistance, to the extent that there is currently no country in Africa that has fully pyrethroid susceptible malaria vectors (Hemingway, 2014).

Pyrethroids and DDT have a similar mode of action involving binding to the sodium channels of the nervous system (Lund and Narahashi, 1983). They modify the kinetics of the voltage-sensitive sodium channel causing the channels to close slowly after each potential causing repetitive nerve excitation and ultimately lead to paralysis and death (Bloomquist, 1996). However, knockdown resistance (*kdr*) to DDT and pyrethroids occurs and is caused by mutations in the voltage-gated sodium ion channel gene, which involve a leucine-phenylalanine substitution at position 1014 (L1014F) observed in West Africa (Martinez-Torres et al., 1998) and a leucine-serine substitution at the same codon (L1014S) observed in East Africa (Ranson et al., 2000), thereby leading to insensitivity of the target site. Furthermore, metabolic resistance to pyrethroids is correlated with increased activity of cytochrome P450 enzymes which play a role in metabolism of xenobiotics (Berge et al., 1998).

Organophosphates and carbamates

The malathion organophosphate and bendiocarb carbamate IRS were adopted following the emergence of resistant malaria vector strains to organochlorines. They have short residual activity and are more expensive compared to DDT and pyrethroids (WHO, 2012). Organophosphates and carbamates impede the activity of acetylcholinesterase (AChE) in the malaria vector by phosphorylating and carbamoylating the serine found in the active site of the enzyme respectively (Mutero et al., 1994). Following the inactivation of AChE, the breakdown of acetylcholine neurotransmitter is prevented, thereby resulting in neuromuscular overstimulation and ultimate death of the vector (WHO, 2012). However, resistance to organophosphates and carbamates occurs and is associated with insensitivity of the AChE which is caused by several point mutations in the catalytic subunit of the enzyme (Feyereisen, 1995), such as glycine-serine substitution at position 119 of the *ace-1* gene (Aizoun et al., 2013).

1.5.2.3. Future for vector control strategies

Larval control interventions

Larvicides are control measures that target the mosquito larval habitats. They offer a promising solution because larvae cannot change their behaviour to avoid control intervention and thus could mitigate the observed insecticide resistance (Killeen et al., 2002). Chemical larvicides, such as Paris green (copper acetoarsenite) and heavy

petroleum are cheap, highly effective but cannot be implemented because they are unsafe and highly toxic. Additionally, bacterial larvicides products based on *Bacillus thuringiensis* var. *israelensis* and *Bacillus sphaericus* are promising because they are highly effective and they selectively kill the larvae with insignificant toxicity to non-target organisms (Derua et al., 2019, Walker and Lynch, 2007). Furthermore, plant-based larvicides provide mosquito repellents and substances that are toxic to the mosquito with minimal toxicity to humans, but this research area is still in the preliminary phase (Beier et al., 2018).

Wolbachia

Wolbachia are endosymbionts that are vertically transmitted and maternally inherited in a wide range of arthropods. They have a significant contribution to the mating behaviour of mosquito vectors and thus could be used as a gene drive system for regulating the population of the mosquito vector and ultimately aid in reducing the transmission of the parasite (Wiwatanaratanabutr et al., 2010).

Eave tubes and attractive toxic sugar baits

Eave tube technology was initiated based on the architectural structure of African houses, where a ventilation gap exists between the walls and roof and thus exposing the residents to infective mosquito bites. It is a new way of delivering insecticides by employing plastic tubes containing insecticides coated mesh under the roofline and fitting of a screen to cover the remaining gap, thereby ensuring the houses are protected from mosquitoes (Knols et al., 2016). Additionally, the control of mosquito vectors can be enhanced by the lure and kill approach, where the human residents exploit the instinct of the mosquito to feed on sugar by placing natural attractants such as flower and fruit scents containing toxic substances that will lure the vector and ultimately lead to its death (Beier et al., 2012).

Ivermectin

Ivermectin is an endectocide which acts as a feed through insecticide. It is given as a single oral dose which remains in the human blood stream and can kill mosquitoes during a blood feed (Chaccour and Rabinovich, 2017).

Genetically modified mosquito technique

This refers to the impairment of the *Plasmodium* life cycle inside the mosquito by altering its genetic makeup. It involves the introduction of genetically altered mosquitoes, such as irradiated sterile male mosquitoes (Bouyer and Lefrancois, 2014,

Oliva et al., 2012) and insects carrying a dominant lethal (RIDL) mutation (Bourtzis et al., 2016), which compete with the wild type in order to reduce the mosquito population in nature. This technique is still in its preliminary phases and is highly unlikely to be available in the near future (Beier et al., 2018).

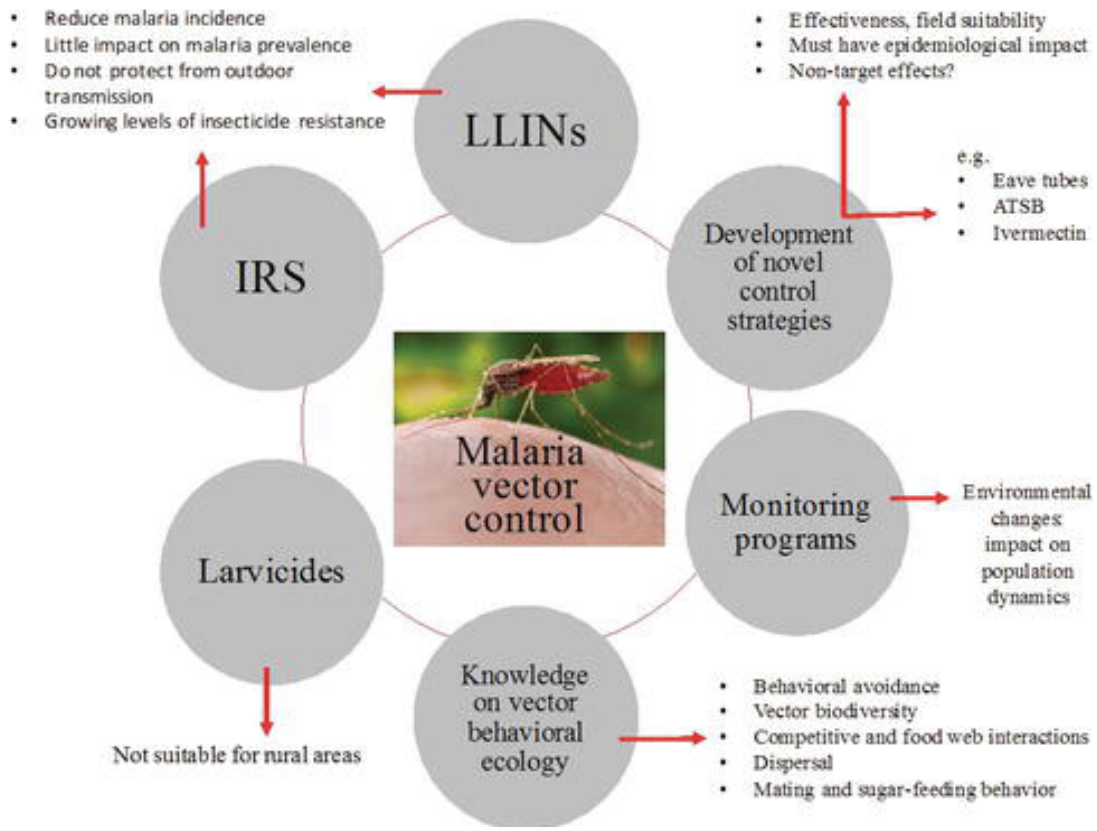

Figure 1. 11: The trends and main challenges of current malaria vector control strategies. Figure adapted from Beier et al., 2018.

1.5.3. Vaccines

1.5.3.1. Introduction to malaria vaccines

The employment of coordinated control interventions such as early diagnosis, combination drug therapies, indoor residual sprays and insecticide treated bed nets has remarkably reduced the global mortality and morbidity of malaria since 2000 (Bhatt et al., 2015). However, there is a challenge in maintaining their effectiveness due to increasing drug and insecticide resistance (Hemingway et al., 2016), and hence the need to develop a highly efficacious vaccine to complement the existing malaria eradication strategies (Healer et al., 2017). The development of antimalarial vaccines is based in part on the following two school of thoughts: (i) the understanding of mechanisms responsible for natural acquired immunity which takes long to develop

after stimulation by continuous parasite exposure in malaria endemic areas (McGregor, 1987) and (ii) the Jenner principle of vaccination which employs a formulation of a harmless target to provoke an immune response in healthy individuals thereby preventing successive infection (Renia and Goh, 2016). The focus in malaria vaccine development has been to prompt the cellular and humoral adaptive immune system to induce an efficacious and sterile response by targeting various points of intervention as shown in Figure 1.12 (Healer et al., 2017).

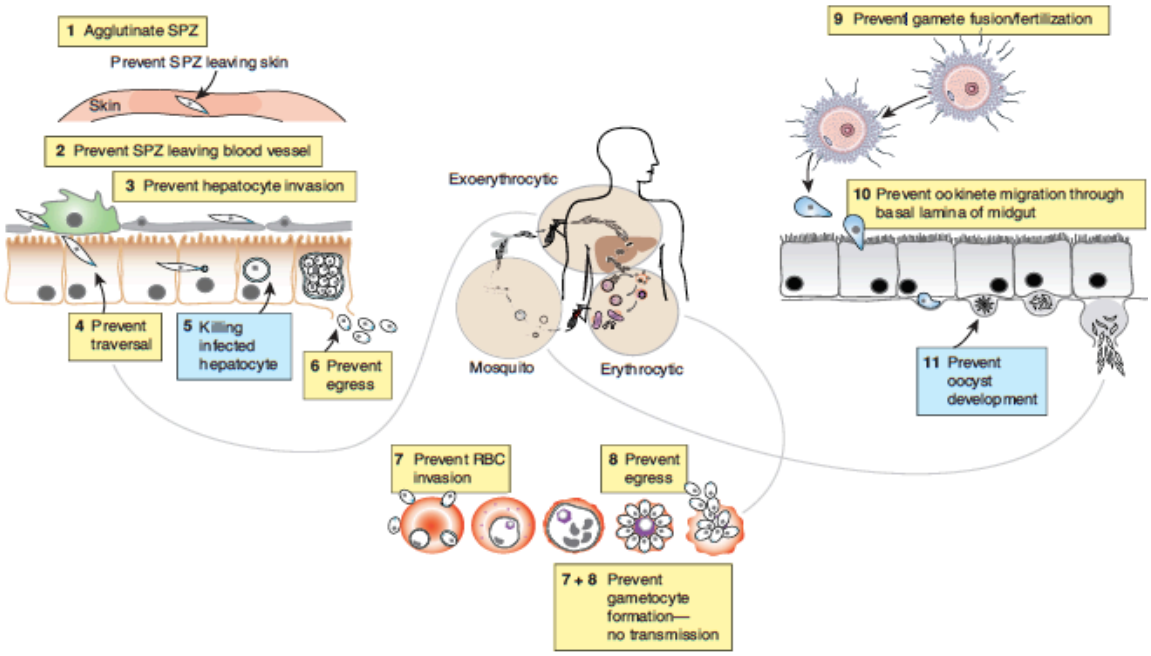

Figure 1. 12: Possible points of intervention for an efficacious malaria vaccine. The susceptible areas to vaccine targets within the *P. falciparum* life cycle that would employ antibody mediated and cell mediated immune mechanisms are coloured in yellow and blue respectively.

A vaccine can be comprised of protein subunits or entire parasites, termed as subunit vaccines or whole parasite vaccines respectively. Subunit vaccines contain selected antigens and are designed to provoke a desired immune response against the pathogen and are usually safe and easy to produce to scale and to administer in the field. On the other hand, whole parasite vaccines are considerably more successful because they contain all parasite antigens and allow the development of different type of immune responses. Nevertheless, many hurdles such as large scale production, delivery and administration in the field persist (Goh et al., 2019).

Malaria vaccine development began more than 100 years ago when the Sergent brothers employed heat inactivated *P. relictum* sporozoites, the most common agent

of avian malaria, to immunize canary birds which developed partial protection from avian malaria (Sergent and Sergent, 1910). This was followed by immunization of domestic fowls by injections of serum and heat inactivated *P. gallinaceum* sporozoites to induce both humoral and cellular responses against malaria (Russell and Mohan, 1942). In order to further boost the immune responses, a Freund's adjuvant containing lanolin-like substances, paraffin oil and killed tubercle bacilli was invented and used to formulate vaccines by combining it with formalin-inactivated blood infected with *P. lophurae* (Freund et al., 1945a) and *P. knowlesi* (Freund et al., 1945b) which resulted to considerable resistance to homologous malaria infection in avian and monkey respectively. In 1946, the first attempt in humans was conducted, where formalin inactivated *P. vivax* infected blood was used to immunize volunteers but no protection was induced (Heidelberger et al., 1946). Unfortunately, the Freund's adjuvant could not be used to boost immune responses because of its toxic effects in humans. In 1967, irradiated *P. berghei* sporozoites were injected in mice and conferred sterile protective immunity at high dosage (Nussenzweig et al., 1967). In 1973, malaria-naïve human volunteers were vaccinated by irradiated infected *Anopheles* mosquitoes and showed low efficacy when challenged by infectious mosquito bites (Clyde et al., 1973). These preliminaries studies have paved way for malaria vaccine development. Various approaches and technologies have been employed to generate vaccines that have been tested in preclinical and clinical trials, but very few vaccine candidates have demonstrated vaccine efficacy in human as shown in Figure 1.13 (Renia and Goh, 2016).

1.5.3.2. Subunit vaccines

Peptide and recombinant proteins

The most clinically-advanced malaria vaccine is a liver stage subunit vaccine, RTS,S/ASO1, which has shown efficacy in malaria-naïve adults, semi-immune adults and children residing in malaria endemic areas (Rts, 2015, Cohen et al., 2010). The RTS,S vaccine also known as Mosquirix, is based on a recombinant protein and developed by GlaxoSmithKline scientists. It contains 19 copies of the central Arg-Ala-Arg-Pro (NANP) repeats and 210-398 amino acids of the C-terminal domain of the CSP-NF54 *P. falciparum* strain. The CSP domains are genetically fused with hepatitis B virus surface antigen (HBsAg) which is co expressed in genetically engineered *Saccharomyces cerevisiae* yeast cells with three free HBsAgs (Draper et al., 2018,

Gordon et al., 1995). The exact immune mechanisms of RTS,S/AS01 vaccine is not known, however it is thought to elicit antibody responses towards the NANP amino acid repeat region (Oyen et al., 2017, White et al., 2015, Schwenk et al., 2003) and CD4 T cell response against the two CD4 T cell epitopes found in the C-terminal region (Kazmin et al., 2017).

A major limitation with RTS,S is the inability to maintain high antibody titres (White et al., 2015), even after being administered with AS01 adjuvant which is the leading formulation for provoking high antibody titres in humans. Moreover, the RTS,S vaccine lacks a principal immunogenic epitope found in the junction of the N terminal part and the central repeat regions of the CSP protein, which has been shown to elicit effective antibody responses in humans vaccinated with whole irradiated sporozoites and can confer sterile protection in mice during passive transfer studies (Kisalu et al., 2018, Tan et al., 2018). Hence, several initiatives have already been executed to redesign the RTS,S vaccine to attain maximum vaccine efficacy, such as improving antibody responses to the central repeat by removing the four HBsAg and displaying a higher proportion of CSP, R21 vaccine, (Collins et al., 2017b) and using nearly the full length CSP containing the N terminal and novel adjuvants: FMP013 malaria protein (Genito et al., 2017).

Blood stage antigens are often polymorphic and show limited efficacy when tested as single peptides vaccines. This has led to the development of several blood stage multi-protein vaccines, such as: (i) PfAMA1-DiCo which aimed to cover diversity by containing three recombinant AMA1 variants. It has been tested in European and African adults inducing antibodies with strain transcending reactivity (Sirima et al., 2017); (ii) PfAMA1-RON2L complex which contains AMA1 with its peptide ligand RON2 and has shown protection in *Aotus* monkeys inducing antibodies with high neutralising activity compared to AMA1 alone (Srinivasan et al., 2017); and (iii) VAR2CSA which consists of subunits against pregnancy-associated malaria and is the first placental vaccine (Pehrson et al., 2017).

The challenge of long-standing polymorphism was consoled by the identification of the highly conserved PfRh5 which forms a significant interaction with basigin erythrocyte receptor during invasion (Crosnier et al., 2011). The PfRh5 has low natural immunogenicity and has been shown to induce antibodies conferring strain transcending protection in non-human primates that correlate to *in vitro* growth inhibition activity (Douglas et al., 2015, Draper et al., 2015). It can also confer *in vivo*

protection in humanized mouse model during passive transfer studies (Foquet et al., 2018). Additionally, several multi-protein complexes containing PfRh5 have been revealed which may improve the vaccine potency, such as PfRH5-PfRipr-PfCyRPA-basigin complex which is important for Ca^{2+} release and tight junction formation (Volz et al., 2016), PfRh5-PfP113 which anchors the N terminal of Rh5 to the merozoite surface (Galaway et al., 2017), PfRhopH3-cyclophilin B-basigin-PfRh5 multi-protein complex (Prakash et al., 2017) and PfRh5-PfRAP2 (Zhang et al., 2018) which aid in erythrocyte invasion. Furthermore, screening of novel merozoite antigens by reverse vaccinology have identified PfRAMA which elicit production of antibodies that synergize with anti-PfRh5 and anti-PfCyRPA (Bustamante et al., 2017). Apart from polymorphism and redundant erythrocyte invasion pathways, the blood stage vaccines development faces the hurdle of identifying blood target(s) that will produce long lasting, high quantity of functional antibodies (Draper et al., 2018).

Transmission blocking vaccines have been developed to aid direct individual protection and arrest malaria transmission. Some of the subunit transmission blocking vaccines include: (i) the ookinete surface protein Pfs25/ISA 51, which was the first to progress clinically. It induced transmission blocking immunity in human that blocked parasite infectivity to mosquitoes but was reactogenic due to the formulation with Montanide ISA 51 (Wu et al., 2008). Hence, the delivery platform and efficacy was improved by employing EPA which is an exoprotein A of *Pseudomonas aeruginosa* (Shimp et al., 2013) and even IMX313 molecular adjuvant (Li et al., 2016); (ii) the female gamete-specific protein Pfs47, which is important in mediating evasion of the mosquito immune system (Molina-Cruz et al., 2013); (iii) the sexual stage protein Pfs230, which is expressed in gametocytes (MacDonald et al., 2016); and (iv) the gamete membrane fusion protein PfHAP2, which induces antibodies that confer strain transcending transmission blocking immunity (Angrisano et al., 2017). One major hurdle being faced by the transmission blocking vaccines is that the human humoral immune response cannot be enhanced by repeated infections because the gamete and ookinete stages are not present in the mammalian host during infection.

DNA vaccines

The insertion of DNA encoding an antigen to stimulate an immune response was first utilized as a vaccine delivery system in protection against influenza (Ulmer et al., 1993). This approach was taken in malaria vaccine development, where a CSP-based DNA vaccine was developed, that induced antigen specific cytotoxic T cell responses

(Wang et al., 1998b) but failed to induce antigen-specific antibodies during a phase 1 clinical study (Le et al., 2000). A DNA construct encoding multiple genes was also developed (Haddad et al., 2004, Wang et al., 1998a). In order to improve the vaccine efficacy, the malaria DNA vaccines were developed together with DNA constructs encoding GM-CSF cytokine leading to increased immunogenicity and efficacy in mouse models (Weiss et al., 1998) but these didn't confer protection against sporozoite challenge in humans (Richie et al., 2012). Despite DNA-based vaccines being easy to produce, cheap with long-shelf life, they are incapable of inducing good cellular and humoral adaptive responses.

Recombinant vectored vaccines

They are non-pathogenic, live replicating microorganisms engineered to carry immunogenic genes from a pathogen (Bull et al., 2019). In malaria, the viral vector is the most popular delivery system and have shown promising efficacy in animal models. These viral vectors used in malaria so far include: (i) recombinant influenza and vaccinia viruses expressing CD8 T and B cell epitopes of CSP (Rodrigues et al., 1994), (ii) sindbis virus expressing a CD8 T cell epitope of CSP (Tsuji et al., 1998), (iii) Yellow fever virus expressing the B cell epitope of CSP (Bonaldo et al., 2002), (iv) replication-deficient adeno virus (Hollingdale et al., 2017) and (v) cytomegalovirus expressing CSP, AMA1, thrombospondin-related adhesive protein (TRAP) and MSP1 antigens (Hansen et al., 2019).

Moreover, several bacterial vectors have also shown good immunogenicity and confer protection against sporozoite challenge in mice, such as: (i) Bacille-Calmette Guerin (BCG) expressing CSP (Arama et al., 2012), (ii) *Shigella flexneri* 2A strain expressing the cell transversal protein for ookinetes and sporozoites (PfCelTOS) and CSP vaccine candidates (Bergmann-Leitner et al., 2013), and (iii) *Salmonella* expressing CSP (Aggarwal et al., 1990, Sadoff et al., 1988) which is also the first recombinant vectored vaccine to be evaluated in humans and appears safe and immunogenic (Gonzalez et al., 1994). Furthermore, parasites vectors expressing CSP such as *Leishmania enriettii* (Wang et al., 1995) and *Toxoplasma gondii* (Charest et al., 2000, Di Cristina et al., 1999) have conferred partial protection in mice. These delivery systems have been able to induce protection in animal models, and it remains to be seen if they will induce protection in humans.

Prime boost combinations

This refers to the vaccination with a subunit vaccine or DNA construct followed by viral vector(s) containing one or multiple antigens (Hill et al., 2010, Webster et al., 2005). This approach has been used in several malaria vaccine development studies in mouse models (de Camargo et al., 2018, Arama et al., 2012, Stoyanov et al., 2010, Sedegah et al., 1998) but only those that express the TRAP liver antigen and not CSP have demonstrated vaccine efficacy in humans (Bliss et al., 2018, Rampling et al., 2016, Ewer et al., 2015, McConkey et al., 2003). The most advanced prime-boost vaccine in malaria vaccine development is the heterologous ChAd63-MVA ME-TRAP, which contains the simian adenovirus 63 and the modified vaccinia Ankara viral vectors. Each vector expresses the liver stage malaria antigen thrombospondin related adhesion protein fused to a multiple epitope string. Multiple priming with ChAd63 ME-TRAP and boosting with MVA ME-TRAP has been shown to induce potent T cell and antibody responses respectively in phase II clinical trials in humans (Bliss et al., 2018). Another quite advanced prime boost vaccine is the ChAdOx1-MVA LS2 which employs the chimpanzee adenovirus and the modified vaccinia Ankara viral vectors each expressing the liver stage dual antigens (LSA1 and LSAP2) (Draper et al., 2018). These advanced prime boost combinations are still in clinical trials.

1.5.3.3. Whole parasite vaccines

Radiation attenuated

Vaccination with radiation-attenuated sporozoites (RAS) is the most clinically advanced of whole parasite-based vaccines (Richie et al., 2015). This was facilitated by the ability to isolate a purified, aseptic, metabolically active and cryopreserved product of irradiated NF54 sporozoites and deliver into the humans by direct venous inoculation during clinical trials (PfSPZ vaccine) (Seder et al., 2013). This protective immune response induced by PfSPZ vaccine could be mediated by the PfSPZ-specific IFNγ-producing CD8 T cells as demonstrated by studies using animal models (Epstein et al., 2011). Additionally, passive transfer studies in liver-chimeric mouse models prevented liver infection (Ishizuka et al., 2016) and this could be mediated by complement fixing anti-sporozoite IgM (Zenklusen et al., 2018). However, the PfSPZ vaccine faces numerous challenges, such as: (i) the need for a high dose of sporozoites to induce a high efficacy level making it difficult for mass vaccination

(Richie et al., 2015, Seder et al., 2013), (ii) the route of administration providing robust CD8 T cell responses is the direct intravenous injection which requires professional expertise (Epstein et al., 2011), (iii) the induced protective immunity targets polymorphic antigens because sterile protection was attained against homologous challenge (Seder et al., 2013) and reduced efficacy against heterologous challenge (Lyke et al., 2017, Sissoko et al., 2017, Schats et al., 2015), and (iv) the delicate procedure of attenuation since over-irradiated sporozoites do not induce protection (Goh et al., 2019, Vanderberg et al., 1968).

Genetically attenuated parasites (GAP)

This refers to parasite that have been modified to attenuate their growth at a certain stage allowing the host immune system to be exposed to a wide repertoire of parasite antigens. In malaria vaccine studies so far, GAPs are modified to arrest liver development after hepatocyte infection. This modification is achieved by deleting essential genes but does not affect parasite viability in the host, mosquito infectivity and sporozoite production in animal models (Vaughan et al., 2010). Early arrest of liver stage development has been shown to offer long lasting and sterile protection in rodent models and this can be achieved by deleting various genes which play a critical role in completion of liver stage development, such as: (i) UIS3 (Mueller et al., 2005b), (ii) UIS4 (Mueller et al., 2005a) and (iii) P36p (van Dijk et al., 2005) genes. Moreover, two early arresting NF54 *P. falciparum* GAPs have been established by deletion of two (p52–/p36–) (Spring et al., 2013) and three p52–/p36–/sap1 (PfGAP3KO) (Kublin et al., 2017) liver stage genes which were administered by infective mosquito bites and conferred protection with and without breakthrough infections respectively in human subjects. To enhance further the efficacy of GAP vaccines, second generation genetically attenuated parasites (next generation), such as fabb/f- (Butler et al., 2011) and plasmei2-/LISP2- (Vaughan et al., 2018) have been developed and arrest during late stage liver development allowing the host to be exposed to a greater antigen repertoire, thereby conferring superior protection in rodent models. One major hurdle facing the further development of GAP vaccines is the difficulty in generating late arresting *P. falciparum* next generation GAPs.

Chemically attenuated parasites

This is a new approach employed in malaria vaccine development to inactivate whole parasites by use of chemicals either *in vitro* before vaccination or *in vivo* after vaccination with live parasites. Chemically attenuated sporozoites (CAS) were

established by *in vitro* treatment with a DNA alkylation agent, centanamycin, and their vaccination conferred sterile immunity and cross protection against heterologous challenge in mouse model (Purcell et al., 2008). Additionally, vaccination with chemically attenuated whole-parasite blood stage, that was treated *in vitro* using centanamycin and tafuramycin-A chemical attenuating agents, induced strong proliferative responses against homologous and heterologous parasites in mouse models (Raja et al., 2017). These *in* vitro chemically attenuated vaccines are yet to be examined in humans to determine their efficacy. The most studied *in vivo* chemoattenuated approach is the vaccination with live cryopreserved NF54 (chloroquine sensitive strain) sporozoites administered by direct intravenous injection under chloroquine prophylaxis (PfSPZ-CVac) and has shown sterile protection against homologous CHMI (Mordmuller et al., 2017). Although this approach requires about 10 to 100-fold less sporozoites compared to PfSPZ vaccine it is faced with a major hurdle on how to overcome the potential safety issues related to using a live vaccine that requires concurrent treatment to prevent disease.

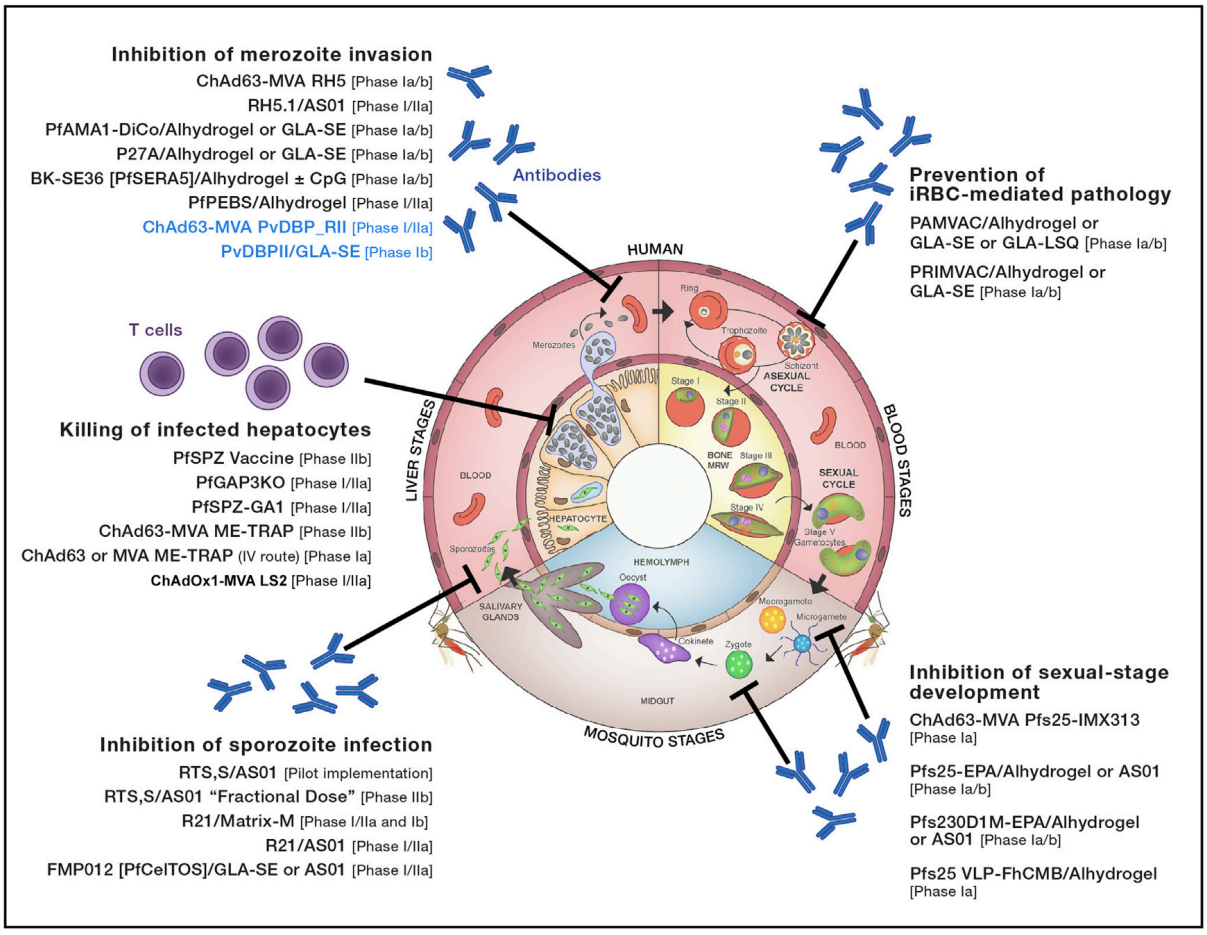

Figure 1. 13: Malaria vaccine candidates in clinical development. The vaccines target different stages of the parasite's life cycle. Figure adapted from Draper et al., 2018.

1.5.3.4. Model systems for malaria vaccines

Mouse model

Mice are the most popular models used to initiate *in vivo* studies examining the development of vaccine candidates because of its affordability and availability. The use of mouse models has pioneered two significant vaccine studies, and these include: (i) the first demonstration that irradiated whole sporozoite vaccine induces sterile protection from infective sporozoites using a *P. berghei* mouse model (Nussenzweig et al., 1967), and (ii) the first evidence that vaccination with live sporozoites under chloroquine treatment offer promising efficacy. This was demonstrated using *P. berghei* and *P. yoelli* mouse models (Belnoue et al., 2004). Moreover, the examination of the efficacy of human malaria antigens has been facilitated in the mouse model (Kisalu et al., 2018, Espinosa et al., 2015, Kastenmuller et al., 2013) by the establishment of transgenic rodent malaria parasites, knock-in parasites, expressing *P.* falciparum (Tewari et al., 2002) or *P. vivax* genes (Espinosa et al., 2013).

The main challenge with the mouse model is the inability to conclusively interpret findings obtained from the mouse model and translate these to the humans because of various reasons, such as: (i) the difference at genomic and proteomic levels between the rodent and human species which is illustrated by the rodent parasite lacking orthologs for more than 730 *P. falciparum* genes (Hall et al., 2005, Carlton et al., 2002), (ii) the difference in the liver stage during rodent and human malaria infection which takes 2-3 days and 7-10 days respectively and formation of hypnozoites during *P. vivax* infection (Vaughan et al., 2012, Krotoski et al., 1982), (iii) the limited MHC genetic repertoire in the genetically homogenous inbred mice which doesn't imitate the large MHC diversity experienced in humans (Goh et al., 2019) and (iv) the laboratory mice are pathogen free and this may bias infection and vaccine studies because the cellular composition of the innate and adaptive system resembles neonates and not human adults (Beura et al., 2016).

Non-human primates

They share a lot more similarities with the humans compared to the mouse model (Beignon et al., 2014). The *Aotus* monkeys can support infection with *P. falciparum* and *P. vivax* (Collins et al., 2006, Herrera et al., 2002). Additionally, the *Aotus* and *Samiri* species are essential in examining *P. vivax* erythrocytic stage vaccines because of their capability to forming hypnozoites (Joyner et al., 2015). Moreover,

simian malaria parasites, for example *P. cynomolgi* and *P. knowlesi*, have been employed for immune and vaccine studies in macaques (Wijayalath et al., 2008, Deans, 1984). Furthermore, vaccination of Toque monkeys with live sporozoites under chloroquine treatment have shown promising vaccine efficacy (Wijayalath et al., 2012). The hurdles facing the use of non-human primates include the high cost of colony maintenance, unavailability and restriction of utilization because of ethical issues (Goh et al., 2019).

Humanized mouse

They are immunodeficient mice which permit long-lasting establishment of components of human immunity by xenotransplantation of human hepatopoietic and/or erythropoietic cells and tissues. The following humanized mice have been used in malaria research: (i) the SCID/Alb-uPA mouse which is a human liver chimeric immunodeficient mouse transgenic for the urokinase plasminogen activator used to study pre erythrocytic stage vaccines (Morosan et al., 2006, Sacci et al., 2006), the (ii) $FAH^{-/-}Rag2^{-/-}IL2R\gamma^{null}$ (FRG huHep) liver chimeric immunodeficient mouse which lacks the fumarylacetoacetate hydrolase and doesn't suffer hepatotoxicity, thereby allowing transplantation of human erythrocytes and supports transition from liver stage to blood stage *P. falciparum* infection (Foquet et al., 2018, Vaughan et al., 2012), (iii) the TK-NOD model which is a NOD mouse (deficient for cytokine IL2Rγ gene) transgenic for thymidine kinase gene which can be engrafted by both human hepatocytes and erythrocytes allowing full *P. falciparum* development in the liver and transition to the blood stage (Soulard et al., 2015, Hasegawa et al., 2011) and (iv) HIS-CD4/B model which possess the human immune system with a functional human CD4 T cells and B cells and are important pre-clinical models used to unravel immune response against malaria by using transgenic rodent malaria parasites expressing certain *P. falciparum* antigens (Huang et al., 2015).

Human volunteers

The establishment of the controlled human malaria infection (CHMI) models have contributed remarkably to malaria research. They involves healthy human volunteers who are exposed to the parasite, either by infective mosquito bites or the direct intravenous route, and monitored for signs and symptoms after which they are treated upon detection of parasites and/or fever (Roestenberg et al., 2009, Dunachie et al., 2006). The drawback of the CHMI study is that it does not reflect the natural field

setting because it is usually performed using one parasite strain and the parasite load is administered at one single dose (Goh et al., 2019).

1.5. Aim of the study

Formative studies have been conducted to shed light on correlates of protective immunity during natural *P. falciparum* infections, with a major focus being on the highly immunogenic mature asexual blood stages. However, ring-infected erythrocytes which are the predominant forms detected in the peripheral circulation, have received very little attention in this context. The overall aim of this study was to investigate whether antibody mediated clearance of rIEs is a mechanism of protective immunity against *P. falciparum* infection. The hypothesis is that, the rIE gets in contact with parasite proteins which are shed and/or secreted by the merozoite during invasion, and thus possibly making the newly invaded erythrocyte a target of the immune system. Several approaches were employed to test the above hypothesis.

Firstly, in chapter three, I investigated the immunogenicity of rIEs, and how this differs between lab and field parasite isolates. I used plasma samples from malaria-immune individuals from a malaria endemic area and tested their recognition of rIEs and compared it to antibody response to mIEs and merozoites. I also tested and compared the splenic retention of rIEs between lab and field parasite isolates. Secondly, in chapter four, I investigated the physiological relevance of the immunogenicity of the rIEs. I established an antibody mediated functional assay for rIEs in our lab and tested whether it correlated to outcome of infection in a CHMI study. Finally, in chapter five, I aimed to identify parasite proteins on the surface of ring-infected erythrocytes. I conducted a surface proteomics approach which involved surface trypsinization of the ring culture cells followed by mass spectrometry analysis of the obtained supernatants.

Chapter 2

Materials

3.1. Laboratory equipment

Autoclave	Systec GmbH, Göttingen
Centrifuge, Megafuge 16R	Heraeus Instruments, Hanau
FACS Contour II	Becton Dickinson, Heidelberg
Freezers -20°C	Liebherr International Deutschland GmbH, Biberbach an der Riss
Freezers -80°C	Thermo Fischer Scientific, Karlsruhe
Fridge	Bosch, Stuttgart
Incubator, Cell star cytoperm 2	Heraeus Instruments, Hanau
Incubator CO_2, BBD6620	Heraeus Instruments, Hanau
Laminar Flow Cell culture hood	Heraeus Instruments, Hanau
Light microscope, Axiolab	Zeiss, Jena
Magnetic stirrer	VWR International GmbH, Darmstadt
Microplate reader	Thermo Fischer Scientific, Karlsruhe
Multi-channel pipettes	Eppendorf AG, Hamburg
pH meter, Five Easy	Mettler-Toledo GmbH, Gießen
Pipetman Gilson P (2,10,20,200,1000)	Gilson International, Bad Camberg
Pipettus* akku	Hirschmann, Labortechnik, Eberstadt
Printer hp LaserJet 1300	Hewlett Packard, Heidelberg
Spinning disc confocal microscope	PerkinElmer, Waltham, USA
Vortex	VWR International GmbH, Darmstadt
Waterbath	Labor Mannheim GmbH, Mannheim

3.2. Soft ware

FlowJo version 10	FlowJo, LLC, Ashland
Gen 5 3.02	Thermo Fischer Scientific, Karlsruhe
GraphPad Prism version 7.0	GraphPad software, San Diego, USA
Inkscape	Freeware
ImageJ	National Institute of Health, Bethesda, USA
Volocity 5.1	PerkinElmer, Waltham, USA

3.3. Consumables

BD Falcon Round bottom tubes 5 ml	Becton Dickinson, Heidelberg
Cell culture flasks	Greiner Bio-One, Frickenhausen
Cover Glass (Haemocytometer)	Neolab, Heidelberg
CS&T beads for research	Becton Dickinson, Heidelberg
CS Columns for MACS	Miltenyi Biotec GmbH, Germany
DMSO Hybrimax	Sigma-Aldrich, Taufkirchen
Eppendorf (1.5 ml, 2 ml) tubes	Neolab, Heidelberg
Falcon (15 ml and 50 ml) flasks	Greiner Bio-One, Frickenhausen
Falcon 96 well plates round bottom	Neolab, Heidelberg
Filter (0.2 µm pore size)	Greiner Bio-One, Frickenhausen
Filter (Acrodisc 1.2 µm pore size)	Pall, Deutschland GmbH, Dreieich
Filter (for dialysis)	Merck Millipore, Darmstadt
Gloves latex	VWR International GmbH, Darmstadt
4HBX Nunc Immunolonplates	Thermo Fischer Scientific, MA, USA
Kimtech wipes	Greiner Bio-One, Frickenhausen
Syringe	Greiner Bio-One, Frickenhausen
Slides	Paul Marienfeld GmbH, Lauda
Pipette tips	Gilson, Heidelberg, Germany
Plastic pipettes	Greiner Bio-One, Frickenhausen

3.4. Chemicals

Albumax I	Thermo Fischer, MA, USA
Bovine Serum Albumin	Sigma-Aldrich, Taufkirchen
BD Perm/Wash (FACS)	Becton Dickinson, Heidelberg
BD Clean (FACS)	Becton Dickinson, Heidelberg
BD Flow (FACS)	Becton Dickinson, Heidelberg
BD FACS Rinse solution (FACS)	Becton Dickinson, Heidelberg
BD FACS Shutdown solution (FACS)	Becton Dickinson, Heidelberg
Cell Trace Far Red fluorescent dye	Thermo Fischer Scientific, MA, USA
Cell Trace Violet fluorescent dye	Thermo Fischer Scientific, MA, USA

Cytochalasin	Sigma-Aldrich, Taufkirchen
D-Glucose	Sigma-Aldrich, Taufkirchen
D-Sorbitol	Sigma-Aldrich, Taufkirchen
Dihydroartemisinin (DHA)	Sigma-Aldrich, Taufkirchen
Dihydroethidium Nucleic Acid stain	Abcam Limited, Cambridge
Dimethyl sulfoxide (DMSO)	Thermo Fischer, MA, USA
Ethanol 100%	VWR International GmbH, Darmstadt
FBS (Fetal Bovine Serum)	Invitrogen, Karlsruhe
Formaldehyde solution	Thermo Fischer Scientific, MA, USA
Gentamycin 10 mg/ml	Sigma-Aldrich, Taufkirchen
Glycerol 99%	Sigma-Aldrich, Taufkirchen
Heparin	Klinik Apo direkt, Heidelberg
Hoechst 33342 (Nucleic Acid stain)	Thermo Fischer, MA, USA
Hypoxanthine	Sigma-Aldrich, Taufkirchen
Hydrochloric acid	Sigma-Aldrich, Taufkirchen
Methanol 100%	Sigma-Aldrich, Taufkirchen
Mito Tracker Deep Red	Thermo Fischer Scientific, MA, USA
o-Phenylenediamine dihydrochloride	Sigma-Aldrich, Taufkirchen
Penicillin-streptomycin	Gibco, Heidelberg
Paraformaldehyde	Alfa Aesar by Thermo Fischer Scientific
RAL 555	RAL Diagnostics, Martillac, France
Potassium Sulphate	Bio repair, Germany
Skimmed milk	Carl Roth, Karlsruhe
Sodium Chloride	Sigma-Aldrich, Taufkirchen
SYBR Green I (Nucleic Acid stain)	Thermo Fischer, MA, USA
Trypan blue	Carl Roth, Karlsruhe
Trypsin inhibitor	Promega, Madison, USA
Trypsin Sequencing grade modified	Promega, Madison, USA
Tween	Merck, Darmstadt

3.5. Kits

Quick Start Bradford Protein Assay	Bio Rad, München
FITC Annexin V Apoptosis Detection Kit	Becton Dickinson, Pharmingen

3.6. Antibodies

Alexa Flour 647 antihuman IgG (IFA)	Biolegend, UK LTD, London
FITC anti-human CD235a (IFA)	Biolegend, UK LTD, London
Mouse anti-human APC (FACS)	Biolegend, UK LTD, London
Rabbit anti-human IgG, polyclonal, HRP	Agilent Technologies Deutschland, Böblingen

3.7. Recombinant proteins

PfAMA1 (3D7 strain, expi293F)	KEMRI Wellcome Trust, Kilifi, Kenya
PfEBA-175 (3D7 strain, expi293F)	KEMRI Wellcome Trust, Kilifi, Kenya
PfMSP-1 (3D7 strain, expi293F)	KEMRI Wellcome Trust, Kilifi, Kenya
PfMSP-2 (3D7 strain, expi293F)	KEMRI Wellcome Trust, Kilifi, Kenya
PfMSP-3 (3D7 strain, expi293F)	KEMRI Wellcome Trust, Kilifi, Kenya
CD45 (3D7 strain, expi293F)	KEMRI Wellcome Trust, Kilifi, Kenya

3.8. Parasite strain and cell lines

P. falciparum 3D7	Center of infectious diseases, Parasitology, Heidelberg
P. falciparum 3D7 (GFP tagged)	MR4, BEI resources, USA
P. falciparum FCR3	Center of infectious diseases, Parasitology, Heidelberg
P. falciparum NF54	KEMRI Wellcome Trust, Kilifi, Kenya
THP-1 monocytes	KEMRI Wellcome Trust, Kilifi, Kenya

3.9. Buffer, solution and media

Common buffers

10x PBS	1.4 M NaCl 27 mM KCl 100 mM Na_2HP0_4 18 mM K_2HP0_4 pH 7.4
1x PBS	100 ml 10x PBS 1 L ddH_20

ELISA Assay

PBST buffer	0.05% Tween 20 in PBS
Blocking buffer	1% skimmed milk powder in PBST
Substrate	o-Phenylenediamine dihydrochloride, OPD, (1 grey + 1 gold) tablet (Sigma-Aldrich) in 20 ml dH_20
Stopping solution	1 M HCl

Microsphiltration

Microsphere suspension	95.5% tin, 3.0% silver and 0.5% copper microspheres in incomplete culture media

Immunofluorescence Assay

Fixing buffer	4% Paraformaldehyde & 0.0075% Glutaraldehyde in PBS
Quenching solution	125 mM Glycine in PBS
Blocking buffer	3% Bovine Serum Albumin (BSA) in PBS

Antibody binding Assay

FACS/Wash buffer	0.5% Bovine Serum Albumin (BSA) in PBS

Ring Survival Assay

Wash solution	RPMI 1640 media with L-Glutamine and 25 mM HEPES
Dihydroartemisinin (DHA) stock	1 mM DHA in DMSO

Phagocytosis Assay

THP1 incomplete medium	RPMI 1640 (500 ml) 2mM L-Glutamine 10mM HEPES 1% Penicillin-streptomycin
Dilution/Wash buffer	0.5% Bovine Serum Albumin (BSA) in PBS
Red cell Lysis buffer	Biolegend, UK LTD, London

Cell culture of *P. falciparum*

Human erythrocytes, blood group o+	Blood bank. Heidelberg
Parasite culture medium	RPMI 1640 (500 ml) L-Glutamine 25 mM HEPES 0.1 mM Hypoxanthine 20 µg/ml Gentamycin 0.25% (v/v) Albumax I
Thawing solutions	1) 12% NaCl in ddH_20 2) 1.6% NaCl in ddH_20 3) 0.9% NaCl & 0.2% Glucose in ddH_20
Freezing solution	56% (v/v) Glycerol 3% (v/v) Sorbitol 0.65% (w/v) NaCl
D-Sorbitol solution	5% (w/v) Sorbitol in ddH_20

Cell culture of THP1 cells

THP1 culture medium	RPMI 1640 (500 ml) 2mM L-Glutamine 10mM HEPES 1% Penicillin-streptomycin 10% Fetal Bovine Serum (FBS)
Thawing solution	50% FBS in incomplete THP1 medium
Freezing solution	10% DMSO in FBS
RPMI 1640 with L-Glutamine & HEPES	Invitrogen, Heidelberg; Germany

Chapter 3

The immunogenicity of *Plasmodium falciparum* ring-infected erythrocytes

3.1. Introduction

Plasmodium species have a complex life cycle that alternates between the female *Anopheles* mosquito and the vertebrate host (Cowman et al., 2016). The blood stage of *P. falciparum* life cycle is responsible for the clinical symptoms of malaria in humans (Yazdani et al., 2006, Neva et al., 1970). In the blood stage, the ring-infected erythrocytes (rIEs) are found primarily in circulation, whereas the mature stages sequester in the microvasculature avoiding clearance by the spleen (White, 2017). The rIEs are therefore the most predominant asexual blood stage detected in the peripheral blood smear during *P. falciparum* malaria diagnosis (White, 2017). However, it is important to note that peripheral blood smear can only account for parasitemia but not total parasite burden, because the sequestered parasites are not enumerated (White et al., 1992). Interestingly, during natural asymptomatic infections, *P. falciparum* rIEs are maintained at low densities and do not manifest the anticipated exponential growth every 48 hours (Anand and Puri, 2005, Farnert et al., 1997).

The availability of the rIEs in circulation during *P. falciparum* malaria may suggest that they are not immunogenic. *P. falciparum* rIEs have been shown to have a unique ability to cytoadhere to the endothelial cells of micro vessels and to placental syncytiotrophoblasts possibly via ring surface proteins: RSP1 and/or RSP2 (Douki et al., 2003, Pouvelle et al., 2000). The rIEs has also been shown to have reduced deformability (Nash et al., 1989) that could be responsible for their splenic retention in experimental studies (Safeukui et al., 2008, Buffet et al., 2006) and clinical infections (Prommano et al., 2005, Lee et al., 1989). Moreover, the detection of antibody binding on rIEs has been reported in peripheral blood smears during acute *P. falciparum* malaria infection in humans (Chotivanich et al., 2002, Angus et al., 1997, Perlmann et al., 1984), and even in the *P. chabaudi* mouse model (Perlmann et al., 1984). In order to shed light on the possible contribution of rIEs to parasite clearance and protective immunity, I began by evaluating the immunogenicity of rIEs using different *P. falciparum* parasite isolates and plasma samples from semi-immune adults from malaria endemic areas.

3.2. Rationale

In this chapter, I sought to investigate the immunogenicity of rIEs, and whether this differs between laboratory (lab) and field isolates. To test this, I used various methods namely; i) an antibody binding detection assay, ii) a ring-stage survival assay and iii) an artificial splenic assay. For the antibody binding assay and the *in-vitro* ring-stage survival assay, I used; a pool of hyper immune plasma from malaria-immune adults, individual plasma samples from malaria exposed adults from Junju sub-location, Kilifi, Kenya and a pool of plasma from malaria-naïve individuals as a negative control. I also used the *P. falciparum* FCR3 laboratory isolate for all the experiments, and a clinical isolate from Kilifi Kenya (P0000072) for the antibody binding assay and the splenic retention assay.

Antibody binding detection assay

I employed the antibody binding assay to investigate whether antibodies from malaria-immune plasma bound to rIEs. The ability of antibody to bind on infected erythrocytes (IEs) has been successfully detected by use of an immunofluorescence assay (IFA) (Awah et al., 2011, Sterkers et al., 2007, Douki et al., 2003) and a flow cytometry assay (Awah et al., 2009, Layez et al., 2005). I therefore expected that malaria-immune antibodies capable of recognising rIEs would be detected by a fluorophore-conjugated secondary antibody specific for the fragment crystallizable region (Fc region).

Merozoite based Enzyme-Linked Immunosorbent Assay (ELISA)

I used the merozoite based ELISA in order to estimate the immune levels of the individuals from Junju sub-location and formed a good basis for comparison with antibody response to rIEs. I used merozoites from the *P. falciparum* NF54 lab isolate, which were conveniently available in our laboratory. I expected that plasma samples from individuals with high and low immune levels would have high and low antibody responses to merozoites respectively.

In-vitro Ring-stage Survival Assay (RSA^{0-3h})

I employed the ring survival assay to investigate the effect of malaria-immune plasma on survival of rIEs. This method has been used to evaluate the susceptibility of *P. falciparum* to artemisinin (Witkowski et al., 2013). The survival of the parasite reflects the effect of the drug on the ring stage, which is the only parasite stage that comes in contact with the treatment. I therefore expected that antibodies with detrimental effect on rIEs would prevent ring survival and maturation.

Artificial splenic assay (Microsphiltration)

I used the microsphiltration method to determine the splenic retention rates of rIEs. This method is based on the filtration of IEs through a mixture of metal microspheres of different sizes that mimic the geometry of the narrow and short inter-endothelial splenic slits and is shown to mimic the *ex-vivo* splenic perfusion system (Deplaine et al., 2011). I expected rIEs with reduced deformability to be retained within the micro-spherical beads.

3.3. Overall objective

To investigate the immunogenicity of *P. falciparum* rIEs.

3.4. Specific objectives

- To investigate the ability of antibody to bind to rIEs and whether this was correlated with antibody responses to other asexual blood stages.
- To find out whether malaria-immune plasma had a direct inhibitory effect on the survival of rIEs.
- To compare splenic retention of rIEs between lab and field parasite isolates.

3.5. Laboratory methods

3.5.1. *P. falciparum* culture and synchronization

The *P. falciparum* FCR3 laboratory isolate and the P0000072 clinical isolate were maintained in *in vitro* culture through infection of human O^+ erythrocytes (with less than 2 weeks of storage at 4°C). Culture media, which consisted of RPMI 1640 media supplemented with L-Glutamine, 25 mM HEPES, 0.25% (v/v) Albumax I, 0.1 mM hypoxanthine, and 20 µg/ml gentamycin, at 2% haematocrit. The cultures were gassed with 5% (v/v) O_2 and 5% (v/v) CO_2 balanced with N_2 at 37°C in a dark incubator. The parasite developmental stages were determined using thin blood smears stained with Giemsa, and subcultured in order to maintain a parasitemia of 8-10%.

Parasite synchronization was done every 46 hours using the D-sorbitol lysis method. Prewarmed 5% D-Sorbitol (10 volumes of culture pellet volume) was added to the pellet in 15 ml conical flask followed by vigorously vortexing for 30 seconds, and then incubated at 37°C for 10 minutes before further vortexing. Trophozoite and schizont infected erythrocytes are mature-infected erythrocytes (mIEs) and are known to be

highly permeable compared to rIEs, and thus take up sorbitol to a higher extent leading to lysis of the mIEs (Radfar et al., 2009). The resultant cultures (with predominantly single and multiple rIEs) were further synchronised by 30 IU of heparin to inhibit parasite invasion until most of the parasites progressed to the mature schizont stage, when culture supernatant was removed by centrifugation at 800 xg for 4 minutes at room temperature. Heparin ensures invasion inhibition because released merozoites will bind to the soluble heparin in culture, preventing the merozoite surface proteins ($MSP1_{42}$ and $MSP1_{33}$) from binding to heparin-like invasion receptors found on the surface of erythrocytes (Boyle et al., 2010). Fresh media was added to the culture to allow merozoite invasion for 10 hours and then synchronized further with 5% D-sorbitol to get rid of the unruptured schizont infected erythrocytes. However, for the study of rIEs and mIEs, the final sorbitol synchronization step was omitted, and the two population were differentiated by flow cytometry gating.

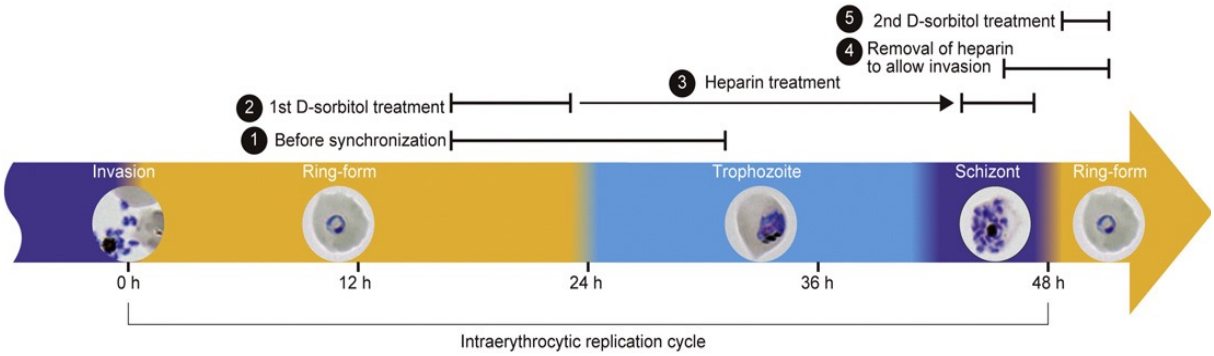

Figure 3. 1: An overview of the synchronization method. 1) The ring form present in the culture is 2) treated with D-sorbitol to selected it before development to trophozoite stage. 3) Heparin is introduced to the culture to inhibit merozoite invasion. 4) Heparin is removed from culture when most of the parasites have progressed to mature schizont stage. 5) The second D-sorbitol treatment stops merozoite invasion and defines the synchronization window. Figure adopted from Koboyashi and Kato 2016.

3.5.2. Antibody binding detection assay

Parasite cultures of approximately 5-10% parasitemia and 4% hematocrit were used in the experiments. For the detection of antibody binding by flow cytometry; 0.5 µl of culture were stained with 1X SYBR Green I (SG) DNA dye for 30 minutes at 37°C followed by opsonization with plasma samples of individuals from Junju sub-location, diluted 1:12.5, for 30 minutes at room temperature and washed thrice with the wash buffer. After the secondary antibody staining with mouse anti-human allophycocyanin (APC) conjugated IgG (diluted 1:50) and washing, the cells were fixed in 0.5%

paraformaldehyde (PFA) on ice for 20 minutes and washed twice with the wash buffer. The cells were then resuspended in 200 µl of the wash buffer. Fluorescence was measured with a FACSCanto II flow cytometer fitted with 488 nm and 640 nm solid state lasers and analysed using FlowJo software, version 10. The erythrocytes were selected according to the forward and side scatter properties using erythrocytes alone as a control. The uninfected erythrocytes (uEs), rIEs and mIEs were easily and clearly distinguished since they displayed different SYBR Green fluorescent intensities on fluorescence dotplots. The erythrocyte bound by antibody from the three different erythrocytes population were defined by a region set for red APC fluorescence. The sample acquisition was set to stop when at least 2,000 events of antibody bound rIEs were collected for each plasma sample.

For the detection of antibody binding by immunofluorescence; 100 µl of packed ring infected erythrocyte cells were suspended in fixation buffer (10 volumes of the cell pellet) and incubated with light shaking at 37°C for 30 minutes. The fixation solution was replaced by 1 ml quenching buffer and incubated at room temperature for 15 minutes. They were then incubated with a pool of hyper immune plasma from malaria-immune adults and plasma samples from malaria-naïve individuals, diluted 1:1000, overnight at 4°C. The cells were washed and incubated with mouse anti-human IgG Alexa Fluor 647-conjugated antibody and mouse anti-human CD235a (Glychophorin A) fluorescein Isothiocyanate (FITC) conjugated monoclonal antibody, diluted 1:500, for 1 hour at room temperature. Glychophorin A is a sialoglycoprotein present in the cell membrane of erythrocytes.

The cells were finally washed two times with wash buffer and finally with buffer containing 1 µg/ml Hoechst dye and mounted on concavalin A coated slides. Concavalin A is a carbohydrate binding protein, lectin, and thus the glycophorin on the erythrocyte membrane would bind and ensure that a monolayer of erythrocytes forms on the slides for optimal microscopic visualization. Pictures were acquired on PerkinElmer UltraVIEW VoX spinning disc confocal with Nikon TiE using a Hamamatsu Ocra Flash 4 camera controlled by Volocity software. Images were visualised with 60X oil-immersion objective lens and processed using the FIJI open-source software.

3.5.3. Merozoite based Enzyme-Linked Immunosorbent Assay (ELISA)

To measure the anti-*P. falciparum* antibody titre of the 37 individuals from Junju sub-location: 100 µl of 5.0 X 10^6 merozoites/ml *P. falciparum* NF54 isolate were coated per well and incubated overnight at 4°C. After washing, the 96 well plates were blocked with casein for 2 hours at 37°C, followed by incubation of each well with 100 µl of plasma samples of the individuals, diluted 1:500, for 2 hours at 37°C. A pool of hyperimmune plasma from malaria-immune adults and plasma samples from malaria-naïve individuals were included in each plate as positive and negative controls respectively. The plates were washed, and each well was incubated with 100 µl horseradish peroxidase (HRP) conjugated goat anti-human IgG antibody, diluted 1:2500, for 1 hour at 37°C. After washing, each well was incubated with 100 µl of O-phenylenediamine dihydrochloride (OPD) substrate for 30 minutes at room temperature. The OPD is a water-soluble chromogenic substrate that yields a yellow colour during degradation of hydrogen peroxide by HRP enzyme. 30 µl of 1M hydrochloric acid (HCl) was added to each well to stop the reaction and produced a very stable orange end solution whose absorbance was read at 490nm using the CYTATION|3 imaging reader and analysed using the Gen5 v3.02 software.

3.5.4. *In-vitro* Ring-stage Survival Assay RSA$^{0\text{-}3h}$

The *P. falciparum* FCR3 isolate was cultured and synchronised as explained in section 3.5.1. Ring stage parasite cultures (0-3 hours synchronization window) of 0.5-1% parasitemia and 2% hematocrit were used in the experiments. A pool of hyperimmune plasma from malaria-immune adults, purified immunoglobulin G (MIG) from malaria-immune adults, and plasma from malaria-naïve and 3 randomly selected malaria exposed individuals from Junju sub-location were used in the experiment. For the ring-stage survival assay: 5 µl of dialysed only or dialysed plus heat-inactivated plasma sample was added to each well, containing 45 µl of ring stage parasite culture. Dialysis and heat-inactivation was aimed to free the plasma from drugs and complement components respectively. A concentration of 700 nM Dihydroartemisinin (DHA) and 1% dimethyl sulfoxide (DMSO) were included in each plate as a positive and negative control respectively. The plates were incubated at standard culture conditions as described in section 3.5.1 for exactly 6 hours. After thorough washing and removal of the treatments, the cells were transferred to new 96 well plates and maintained in

culture for exactly 66 hours, followed by staining with 1X SG / 0.6 µM MitoTracker (MT) deep red for 30 minutes at 37°C. The SG fluoresces upon intercalation of double-stranded deoxyribonucleic acid (DNA) whereas MT stains live mitochondria. Human erythrocytes contain neither nuclei nor mitochondria and thus the SG and MT dyes are specific for the parasites. The cells were washed and then resuspended in 200 µl of the wash buffer. Parasitemia was counted by measuring fluorescence with a FACSCanto II flow cytometer, counted 20,000 events of infected erythrocytes, and the flow cytometry data was analysed using FlowJo version 10 software.

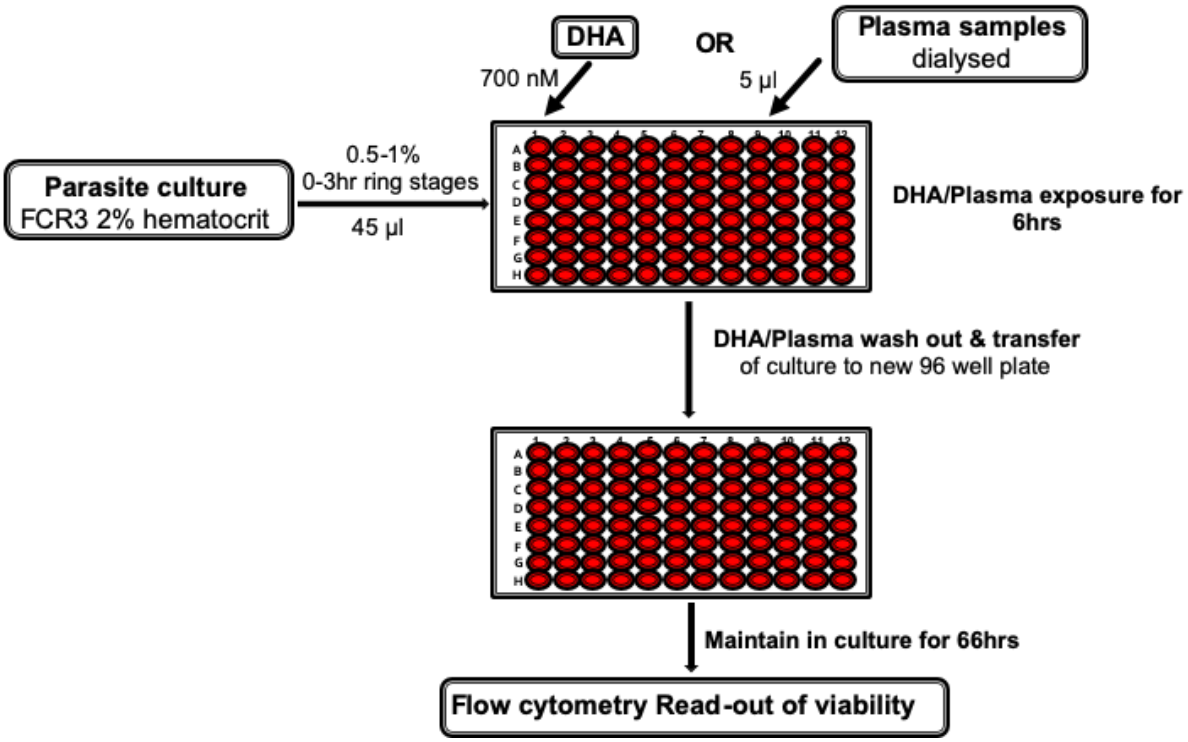

Figure 3. 2: Procedure of *in-vitro* RSA^{0-3h} assay. The *P.* falciparum-FCR3 strain (0-3 hours and 0.5-1% parasitemia) was co-cultured with dialysed plasma samples for 6 hours and then washed and transferred to a new 96 well plate for 66 hours. The parasitemia level and viability was detected and quantified by flow cytometry.

3.5.5. Artificial splenic assay (Microsphiltration)

Calibrated metal microbeads; 96.5% tin, 3.0% silver and 0.5% copper, with 5-15 µm and 15-25 µm diameter dimensions were used in the experiment. For the artificial splenic assay: 600 µl of the bead suspension, composed of 2 g of dry microbeads of each sort suspended in 8 ml complete culture medium, was poured into an inverted 1 ml filtered pipette tip and allowed to settle forming a 5-6 mm thick sphere layer above the filter. After introducing 600 µl of rIEs culture (2% hematocrit and less than 10% parasitemia) upstream from the microbead layer, the cells were perfused through the

bead layer at 60 ml/h flow rate using an electric pump. The bead layer was washed with 6 ml of complete media and the downstream sample for each isolate was retrieved. To obtain the retained IEs: the inverted filter tip was vortexed to allow the beads to release the retained cells which were separated by 3 successive settling steps. Schizont infected erythrocytes and fresh uEs were used as positive and negative control respectively. Thin giemsa-stained smears were used to observe the retained rIEs. The cells in the upstream and downstream were stained with 1X SG for 30 minutes at 37°C in order to measure the parasitemia using flow cytometry, and consequently determine the splenic retention rates. To determine the background retention, the amount of uninfected erythrocyte from the upstream and downstream samples were detected by haemoglobin count whose absorbance was read at 540nm using the CYTATION|3 imaging reader and analysed using the Gen5 v3.02 software.

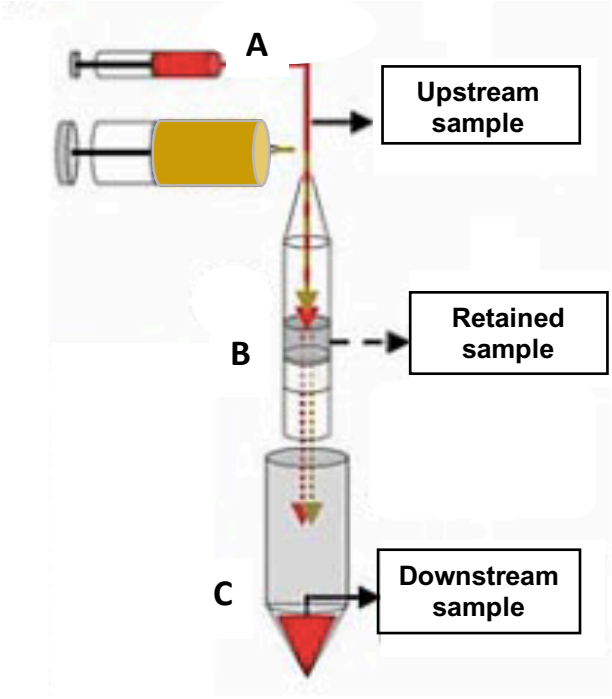

Figure 3. 3: Procedure of microsphiltration assay. (A) The test culture was injected as upstream sample into an inverted 1 ml filtered pipette tip containing 5-6 mm thick micro spherical bead layer above the filter (B). The less deformed erythrocytes were retained within the layer and the more deformable pass through the layer and collected in the conical flask (C) as downstream sample. Figure modified from Deplaine et al., 2011.

3.6. Statistical analysis

All analysis was done using GraphPad PRISM (version 7.0). The Kruskal-Wallis H test was used to analyse the trend between antibody responses and different plasma samples. The Mann-Whitney U test was used to compare; the percentage of antibody

binding to IEs between the malaria-immune and malaria-naïve categories using different parasite isolates, and the antibody responses to merozoites between the malaria-immune and malaria-naïve categories. The Spearman's rank correlation coefficient test was used to measure the linear relation between; antibody binding to rIEs and antibody responses to merozoites, and antibody binding to rIEs and antibody binding to mIEs. The Student's t-test was used to determine whether they were any statistically significant difference between effect of malaria exposed and malaria-naïve plasma on ring survival, and splenic retention of lab and field rIEs isolates.

3.7. Ethics statement

Ethical approval has been granted by the institutional research ethics committee, Kenya Medical Research Institute Scientific and Ethics Review Unit (KEMRI/SERU/ CGMR-C/029/3190) and by the sponsor of the study (University of Oxford) through the Oxford Tropical Research Ethics Committee (OxTREC, 2–16). Informed consent was obtained from participants. It was also approved for the samples to be used in Germany.

3.8. Results

3.8.1. Detection of antibody binding to ring-infected erythrocytes (rIEs)

The ability of antibodies of malaria-immune plasma to bind to rIEs was detected by use of immunofluorescence microscopy and flow cytometry assays as described in the methods above. To ensure that the detection of antibody binding was indeed on the surface of the rIEs, the periphery of the erythrocyte was localized using the major intrinsic membrane protein of the erythrocyte: Glychophorin A, using a FITC-conjugated anti-Glycophorin A antibody. The detection of antibody binding to mIEs using malaria-immune plasma was used as a positive control, whereas the detection of antibody binding to rIEs using secondary antibody (2°Ab) only in the absence of malaria-immune plasma was considered as a negative control for validation of the assay.

There was no visual detection of antibody binding to rIEs following opsonization with malaria-naïve plasma and in the negative control, 2°Ab only. However, we observed a low and modest level of antibody binding to rIEs and mIEs respectively, in the presence of malaria-immune plasma (Figure 3.4-A). Similarly, a negligible proportion

of rIEs were recognised by antibody when tested using malaria-naïve plasma and with the negative control. However, we observed a low and moderate percentage of rIEs and mIEs respectively that were recognised by antibody when using malaria-immune plasma (Figure 3.4-B). The antibody in the test plasma bound to the rIEs and mIEs in a dose dependent manner (Appendix 3.2)

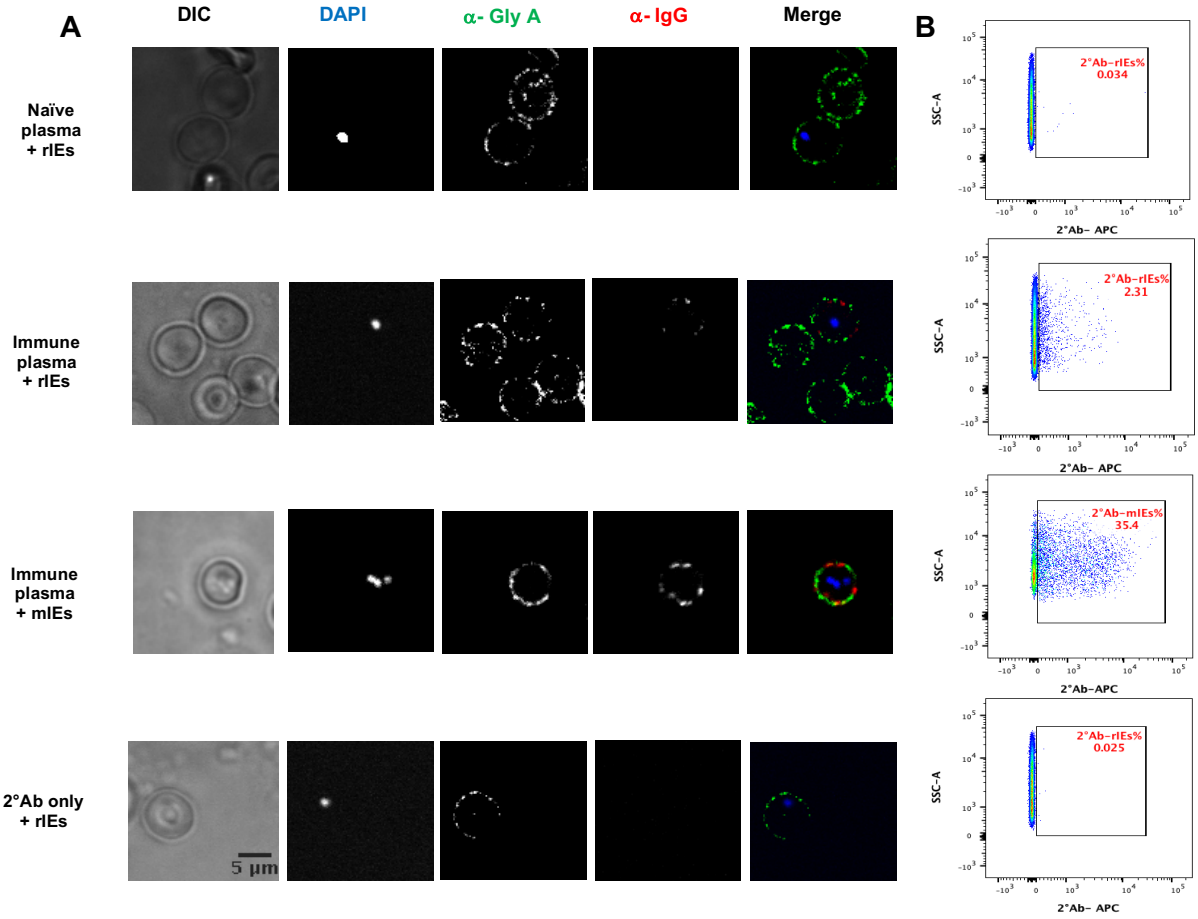

Figure 3. 4: Images showing detection of antibody binding to ring-infected erythrocytes. A) Immunofluorescent assay images show no or low detection of antibody binding to rIEs in the presence of malaria-naïve immune plasma and malaria-immune plasma respectively, modest detection of antibody binding to mIEs in the presence of malaria-immune plasma as a positive control, and no detection of antibody binding with 2°Ab only in absence of immune plasma as a negative control for the assay validation. B) Flow cytometry scatterplots show negligible and low levels of antibody binding to rIEs when using malaria-naïve and malaria-immune plasma respectively, moderate antibody binding to mIEs with malaria-immune plasma and negligible antibody binding to rIEs in case of 2°Ab only in absence of immune plasma as a negative control for the assay validation.

3.8.2. Correlation of antibody binding to ring-infected erythrocytes to antibody responses to other asexual blood stages

Malaria-immune plasma samples of adults from a malaria endemic area, Junju sub-location in Kilifi, Kenya, were used to assess how the antibodies in individual test samples would bind to rIEs, and also this would correlate to antibody binding to mIEs and antibody responses to merozoites. Detection of antibody binding to IEs was conducted using FCR3-lab strain (known to have high binding phenotype) and a lab adapted field strain obtained from Kilifi Kenya and required a consistent flow cytometry gating strategy (Appendix 3.1). Antibody response to merozoites was measured using the NF54 lab strain which was conveniently available in the laboratory.

Antibody binding of all the Junju adult plasma samples to lab and field rIEs isolates was significantly higher ($P<0.0001$) compared to that using malaria-naïve plasma (Figure 3.5-A & Figure 3.6-A respectively). Similar findings were observed when these data were categorized based on top and average response to merozoites (Figure 3.5-B & Figure 3.6-B respectively). It is important to note that detection of secondary antibody binding in absence of plasma was negligible and thus a good control to ensure that the signal to noise ratio is indeed high and thus the detection of antibody binding is specific to the test plasma. Antibody binding to lab rIEs and mIEs showed a strong positive correlation of $r=0.7581$; $P<0.0001$ (Figure 3.5-C), while the correlation was positive but lower between antibody binding to field rIEs and mIEs $r=0.5232$; $P=0.0009$ (Figure 3.6-C). Similarly, antibody responses to merozoites showed a strong positive correlation of $r=0.8462$; $P<0.0001$ and $r=0.6902$ $P<0.0001$ with antibody binding to lab (Figure 3.5-D) and field (Figure 3.6-D) rIEs respectively.

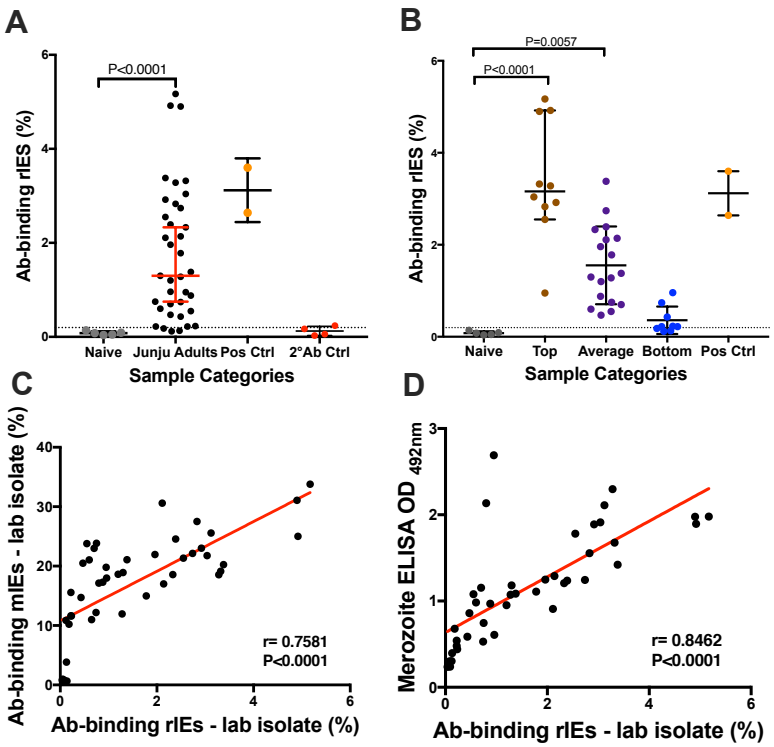

Figure 3. 5: Antibody binding to FCR3-lab isolate ring-infected erythrocytes. A) Detection of antibody binding using all Junju adult's plasma was significantly higher (P<0.0001, Mann Whitney test) compared to malaria-naïve plasma, and detection of secondary antibody binding in absence of plasma was negligible indicating a high signal to noise ratio. B) Detection of antibody binding focussing on the different categories of the Junju adults based on the antibody response to merozoites was significantly higher compared to malaria-naïve plasma (P<0.0001, Kruskal-Wallis test), specifically; the top and average responders (P<0.0001 and P=0.0057 respectively, Dunn's multiple comparison test). C) Antibody binding to rIEs showed a strong positive correlation (spearman r=0.7581; P<0.0001), with antibody binding to mIEs, and D) antibody binding to rIEs showed a strong positive correlation (spearman r=0.8462; P<0.0001), with antibody response to NF54 merozoites. The positive control was a pool of hyper immune plasma samples of Junju adults from Kilifi Kenya. The dotted horizontal line in graphs A and B is the seropositivity mark = {mean of the antibody binding to rIEs using naïve plasma + 3(STDEV)}. Statistics was calculated using GraphPad PRISM version 7.0.

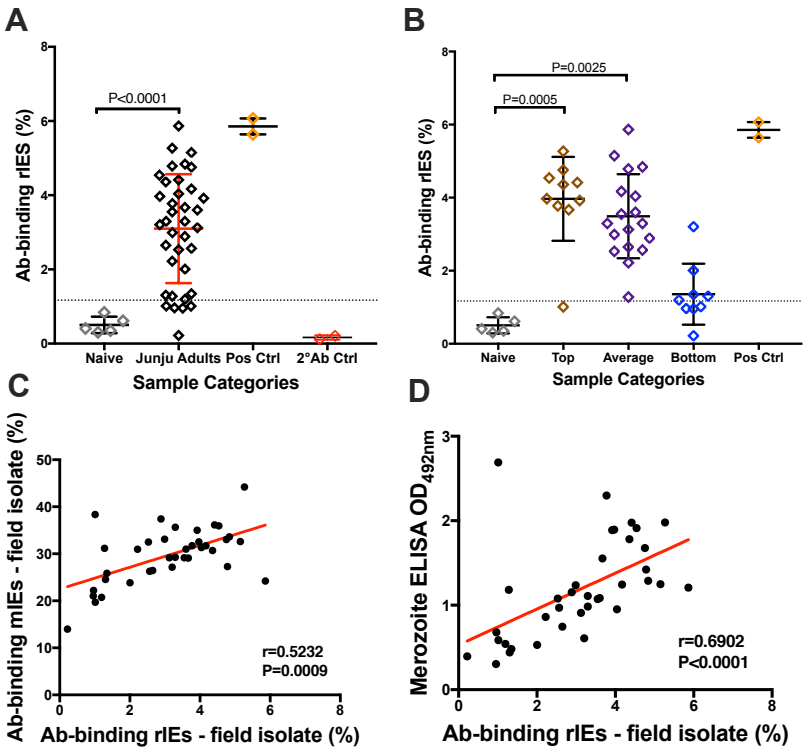

Figure 3. 6: Antibody binding to field isolate ring-infected erythrocytes obtained from Kilifi. A) Detection of antibody binding using all Junju adult's plasma was significantly higher (P<0.0001, Mann Whitney test) compared to malaria-naïve plasma, and detection of secondary antibody binding in absence of plasma was negligible indicating a high signal to noise ratio. B) Detection of antibody binding focussing on the different categories of the Junju adults based on the antibody response to merozoites was significantly higher compared to malaria-naïve plasma (P<0.0001, Kruskal-Wallis test), specifically; the top and average responders (P=0.0005 and P=0.0025 respectively, Dunn's multiple comparison test). C) Antibody binding to rIEs showed a strong positive correlation (spearman r=0.5232; P=0.0009), with antibody binding to mIEs and D) antibody binding to rIEs showed a strong positive correlation (spearman r=0.6902; P<0.0001), with antibody response to NF54 merozoites. The positive control was a pool of hyper immune plasma samples of Junju adults from Kilifi Kenya. The dotted horizontal line in graphs A and B is the seropositivity mark = {mean of the antibody binding to rIEs using naïve plasma + 3(STDEV)}. Statistics was calculated using GraphPad PRISM version 7.0.

The percentage of antibody binding to field rIEs as compared to lab rIEs was significantly higher for all Junju adults, average and bottom responders (P<0.0001, P<0.0001 and P=0.0012 respectively), (Figure 3.7-A). However, there was no significant difference between antibody binding to field and lab rIEs when considering the top responder's category. In general, antibody binding to field and lab rIEs showed a strong positive correlation, r=0.8396; P<0.0001 (Figure 3.7-B).

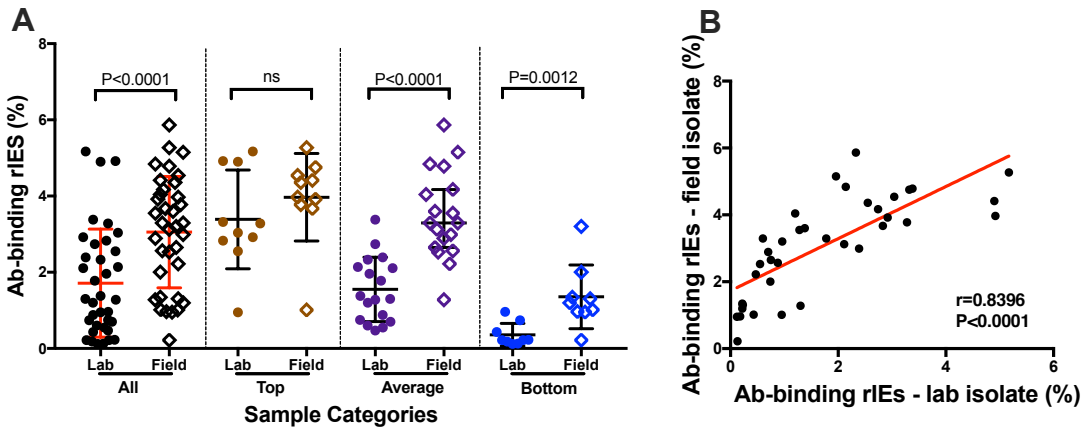

Figure 3. 7: Comparison of antibody binding between laboratory and field isolate ring-infected erythrocytes. A) The percentage of antibody binding to field rIEs was significantly higher focussing for all Junju adults, average and bottom responders (P<0.0001, P<0.0001 and P=0.012 respectively, Mann Whitney test) as compared to antibody binding to lab rIEs. B) Antibody binding to field rIEs showed a strong positive correlation (spearman r=0.8396; P<0.0001), with antibody binding to lab rIEs. Statistics was calculated using GraphPad PRISM version 7.0.

3.8.3. Effect of malaria-immune plasma on ring survival using Dihydroartemisinin (DHA)

The relevant controls for the ring survival assay were important to obtain for meaningful data interpretation. The percentage survival of parasites after exposure to DHA drug was calculated relative to the DMSO exposed parasites {percentage survival = (% viable after DHA exposure/%viable after DMSO exposure) x 100}. The percentage ring survival after exposure to DHA was 3.27% (Figure 3.8).

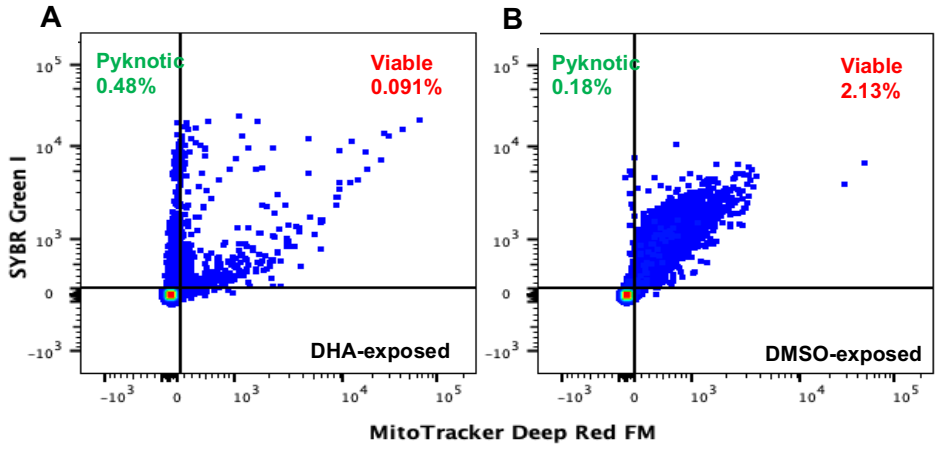

Figure 3. 8: Flow cytometry scatterplots of (A) DHA - and (B) DMSO - exposed *P. falciparum* FCR3 parasites. The percentage of viable and pyknotic parasites in 10,000 events is shown in the upper right and upper left quadrant respectively. The percentage ring survival in this case is 0.091%/2.13% multiplied by 100 = 4.27%.

Exposure of rIEs to dialysed only or dialysed plus heat-inactivated plasma enhanced parasite growth in a similar manner, unlike exposure to DHA which inhibited parasite growth (Appendix 3.7). Dialysis and heat-inactivation was aimed to free the plasma from drugs and complement components respectively. The percentage survival of rIEs after exposure to plasma was calculated relative to the DMSO exposed parasites. The survival rate was comparable when rIEs were exposed to naïve plasma, a pool of hyperimmune serum, purified malaria immunoglobulins and plasma from randomly selected malaria-immune adults from Kilifi, Kenya (Figure 3.9 and Appendix 3.8). The parasite survival rates were similar when exposed to the dialysed only or dialysed and heat-inactivated state of each of the test plasma (Appendix 3.9).

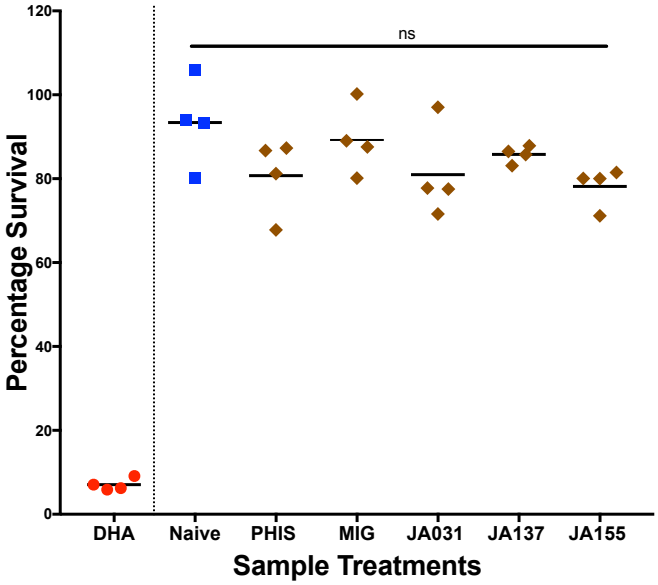

Figure 3. 9: Effect of dialysed immune plasma on ring survival. Percentage survival of rIEs when treated with dihydroartemisinin (DHA, positive control), naïve plasma (negative control), PHIS (pool of hyper immune plasma), MIG (purified malaria immunoglobulins) and randomly selected plasma of malaria-immune adults from Kilifi (JA031, JA137, JA155). The percentage survival rate was comparable when parasites were exposed to naïve plasma, pool of hyper immune serum, purified malaria immunoglobulins and plasma from randomly selected malaria-immune adults from Kilifi, Kenya, (P = 0.1528, one-way ANOVA). n= 4 independent replicates. Statistics was calculated using GraphPad PRISM version 7.0.

3.8.4. Splenic retention of ring-infected erythrocytes of lab and field isolates

The micro beads retained the erythrocytes from the upstream sample that had reduced deformability, whereas the deformable erythrocytes were collected downstream. The parasitemia of the downstream and upstream samples was measured by flow cytometry and the obtained values were used to calculate the retention rates {retention rate = 1 – (parasitemia downstream/parasitemia upstream) x 100}. Retention in the

microbeads was complete at the (40-48) hrs in mIEs stages as expected for the positive control, and partial for the fresh uEs (negative control) which was lower than that of (0-10) hrs rIEs (Figure 3.10). However, there was no significant difference between the (0-10) hrs rIEs retention rates of field isolates adapted in culture and lab isolates (Figure 3.10). The erythrocytes retained within microbeads were retrieved by sedimentation, because the microbeads are denser, leaving the erythrocytes in suspension. The retained fraction was stained by Giemsa to visualise the retained rIEs (Figure 3.10).

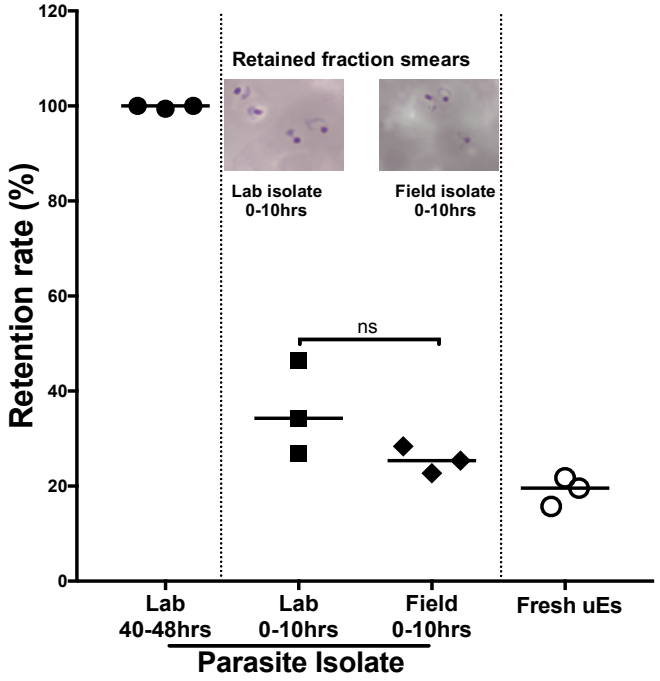

Figure 3. 10: Retention rate of mature-infected erythrocytes and ring-infected erythrocytes in an artificial spleen. Retention rate of 40-48 hrs mIEs (positive control), comparison between (0-10 hrs) rIEs retention of a lab and field isolate determined by flow cytometry, and retention of fresh uEs determined by haemoglobin count using spectrophotometry (negative control). There was no significant difference in the rIEs retention between the lab and field isolate (P= 0.1530, unpaired t-test), but there was a difference between background retention (fresh uEs) and retention of rIEs of the lab isolate (P=0.0469, unpaired t-test). Microscopic images depict Giemsa-stained smears of retained fractions showing rIEs of the lab and field isolate. n= 3 independent replicates. Statistics was calculated using GraphPad PRISM version 7.0.

3.9. Discussion

Malaria-immune antibody binding to the surface of rIEs

Antibody from a pool of hyperimmune plasma was detected on the surface of rIEs using immunofluorescence (Figure 3.4) and this confirmed previous findings which showed membrane staining of rIEs in *in vitro* cultures with *P. falciparum* hyperimmune sera (Perlmann et al., 1984) . This previous observation gave rise to the 'pitting' phenomenon, where immunostaining of peripheral blood smears revealed positive membrane staining of the circulating *P. falciparum* rIEs and uEs when tested with ring-infected surface antigen (RESA) monoclonal antibody and pool of hyperimmune sera (Chotivanich et al., 2000, Perlmann et al., 1984). The positive staining of uEs for malaria antigens was suggested to be an indication of the pitted circulating rIEs and was used to explain the decline of circulating rIEs after treatment with artemisinin (White, 2017, Angus et al., 1997). This supports the idea that the detection of antibody binding using *in vitro* parasite cultures is physiologically relevant.

The percentage of rIEs parasite cultures that were recognised by the malaria-immune plasma were quantified (±6%) and varied depending on the malaria specific antibody titres of the individuals. Individuals with high antibody responses to rIEs also showed high antibody responses to merozoites and mIEs (Figure 3.5 and Figure 3.6 respectively). Despite of the low level of antibody binding to rIEs in both lab and field parasite cultures, the strong positive correlation to antibody response to merozoites and mIEs suggests possibility of shared targets between these stages and may suggest a modest contribution to protective immunity against *P. falciparum* malaria. Individuals with very low anti-merozoite titres had rIEs antibody binding detection similar to the naïve individuals, and this confirms previous observation: sera with low ELISA reactivity to total *P. falciparum* antigens displayed negative membrane staining of peripheral blood smears (Perlmann et al., 1984). These individuals may have lacked repeated or prolonged exposure to *P. falciparum* which is a prerequisite for high antibody titres against the parasite.

Effect of malaria-immune antibodies on survival of rIEs

Antibodies against various essential merozoite surface proteins that are neither cleaved nor shed during invasion; $MSP1_{19}$, MSP2 and MSP4, have been shown to be internalized into the erythrocyte during invasion (Beeson et al., 2016, Boyle et al., 2014). One important question is whether these malaria specific antibodies have a direct effect on the survival of the resultant rIEs after merozoite invasion. In this study,

antibodies obtained from malaria-immune plasma had a negligible effect on early rIEs (Figure 3.9; Appendix 3.7 and Appendix 3.8), as compared to treatment with dihydroartemisinin which is known to have a detrimental effect on rIEs survival in an *in vitro* ring survival assay (Witkowski et al., 2013). This suggests that direct inhibition of rIEs survival by antibodies may not be an important mechanism of parasite clearance. However, it is important to note that antibodies have been shown to inhibit parasite growth, particularly the development of mature forms, and the merozoite invasion of erythrocytes using the growth inhibition assay and invasion inhibition assay respectively (Teo et al., 2016).

Splenic retention of rIEs parasite isolates

Retention of rIEs was evident in the Giemsa-stained smears of the retained fraction (Figure 3.10), and the retention rates confirmed findings from previous studies which demonstrated that rIEs are retained in an artificial spleen albeit at a lower rate than mIEs (Deplaine et al., 2011). In addition, this study also showed that the retention of rIEs was comparable between the lab and field parasite isolates. The retention of rIEs could be explained by its reduced deformability and slight cell membrane stiffening (White, 2017, Nash et al., 1989). Other findings from *ex vivo* perfusion assays, showed that a fraction of rIEs was retained in isolated-perfused human spleens and accumulated upstream from interendothelial sinus wall slits of the open, slow red pulp microcirculation (Safeukui et al., 2008, Buffet et al., 2006). Moreover, pathology studies in fatal malaria demonstrated the accumulation of parasitized erythrocytes of all stages including the rIEs (White, 2017, Prommano et al., 2005). The observed artificial splenic retention of rIEs *in vitro* may have a physiological relevance by contributing to parasite clearance and thus protective immunity as previously hypothesized (Buffet et al., 2006). Of note, the artificial splenic assay in this study was conducted without antibodies, which may further enhance splenic clearance as demonstrated by Lee et al., 1989, where IgG sensitized infected erythrocytes were rapidly removed from circulation in patients with uncomplicated *P. falciparum* malaria. The clearance of IgG labelled rIEs could be facilitated by interaction with other cell types such as macrophages.

Summary

I confirm that malaria-immune antibodies bound to rIEs surface and show that the binding was strongly correlated with antibody response to merozoites and mIEs. I also show that recognition of rIEs by antibodies did not lead to direct inhibition of rIEs survival. I also confirm that rIEs were retained in an artificial spleen, and that the retention was comparable between lab and field isolates. My work confirms and extends previous publication reports and in chapter 4, I investigate the significance of antibody binding to rIEs on the outcome of *P. falciparum* infection.

Chapter 4

The physiological relevance of the immunogenicity of the ring-infected erythrocyte

4.1. Introduction

In malaria endemic populations, young children have the highest risk of severe disease and death (Cowman et al., 2016). Although morbidity and mortality rapidly decrease with age, individuals remain susceptible to uncomplicated malaria as they get older and to asymptomatic infections in adulthood without achieving sterilizing immunity (Langhorne et al., 2008). In endemic areas, immunity to malaria is reflected both by a lower prevalence of infection with age and lower rates of disease (Marsh and Kinyanjui, 2006). Immunity to malaria has been defined as either natural which is the hosts' inherent property or acquired which could be as a result of previous exposure or passive transfer of protective substances to the host (Doolan et al., 2009). Long-standing evidence suggests that natural acquired immunity to *P. falciparum* malaria is largely mediated by immunoglobulin G (IgG) (Sabchareon et al., 1991, Cohen et al., 1961).

Naturally acquired and vaccine-induced functional antibodies targeting various antigens of the *P. falciparum* asexual blood stages have been associated with protection from clinical symptoms and protective immunity respectively (Teo et al., 2016). For example: 1) anti-merozoite surface protein 1_{19} ($MSP1_{19}$) antibodies inhibited merozoite invasion in an invasion inhibition assay (O'Donnell et al., 2001); 2) antibodies against merozoite proteins: erythrocyte binding antigens (Chitnis et al., 2015, Irani et al., 2015), apical membrane antigen 1 (AMA1) (Mugyenyi et al., 2013, Remarque et al., 2012) and rhoptry binding homologue (Tran et al., 2014, Richards et al., 2013) prevented parasite growth in growth-inhibition assays; 3) cytophilic antibodies against merozoite surface protein 2 (MSP2) fixed C1q component in a complement fixation assay (Boyle et al., 2015); 4) merozoite opsonising antibodies targeting merozoite surface proteins interacted with phagocytic cells (Osier et al., 2014); 5) anti-merozoite surface protein 5 (MSP5) antibodies interacted with neutrophils in an antibody dependant respiratory burst assay (Perraut et al., 2014); and 6) antibodies targeting mIEs prevented cytoadherence (Turner et al., 2015, Fried et al., 1998), inhibited rosette formation (Carlson et al., 1990), and arrested schizont rupture (Raj et al., 2014). On the contrary, antibodies targeting rIEs have been shown

to mediate opsonic phagocytosis of rIEs (Chan et al., 2012, Awah et al., 2011, Awah et al., 2009, Celada et al., 1982) but this has not been explored in the context of possible parasite clearance and correlation with protective immunity. I therefore sought to uncover the physiological relevance of opsonic phagocytosis of rIEs on outcome of *P. falciparum* malaria infection.

4.2. Rationale

In this chapter, I investigated whether the antibody binding to rIEs demonstrated in chapter 3 was likely to be physiologically relevant in *P. falciparum* malaria infections. I employed the opsonic phagocytosis assay (OPA) of rIEs and plasma samples from individuals in a controlled malaria infection (CHMI) study in order to investigate whether *in vitro* OPA of rIEs could predict outcome of malaria challenge in the clinical study. The CHMI study site was in Kenya, specifically Kilifi and Nairobi where malaria transmission is moderate and low respectively. The study was conducted over several years (2013, 2016, 2017 and 2018), and involved a total of 189 adults. At screening, participants were included if they were malaria parasite negative, not pregnant, free of immunosuppressive infections, did not have genetic diseases associated with malaria resistance and had not taken systemic antibiotics with known antimalarial activity. In the study design (Figure 4.1), the participants were challenged intravenously by administration of 3,200 sporozoites of NF54 strain obtained from Sanaria (Inc). The samples used for my study were those collected one day before challenge. The parasitemia was monitored by quantitative Polymerase Chain Reaction (qPCR) twice daily, and individuals who had more than 500parasites/µl and/or symptoms of malaria were treated. The symptoms included fever above 37°C, hypotension, tachycardia, chills, rigor, sweating, headache, nausea, vomiting, fatigue, lower back pain, myalgia and arthralgia. Eventually after the end of the study, 21 days post challenge, all the participants were treated (Kapulu et al., 2018).

Several outcomes of the malaria challenge emerged from the analysis of the qPCR parasitemia data and symptoms obtained during the study period. These were defined as follows: 1) treated - 81 individuals who had more than 500 parasites/µl and /or symptoms, 2) slow growth - 51 individuals who had patent parasitemia but less than 500 parasites/µl, 3) cleared - 18 individuals who had patent parasitemia that spontaneously resolved by day 10 and 4) high immune - 39 individuals who did not develop parasitemia during the entire follow up period (Figure 4.2).

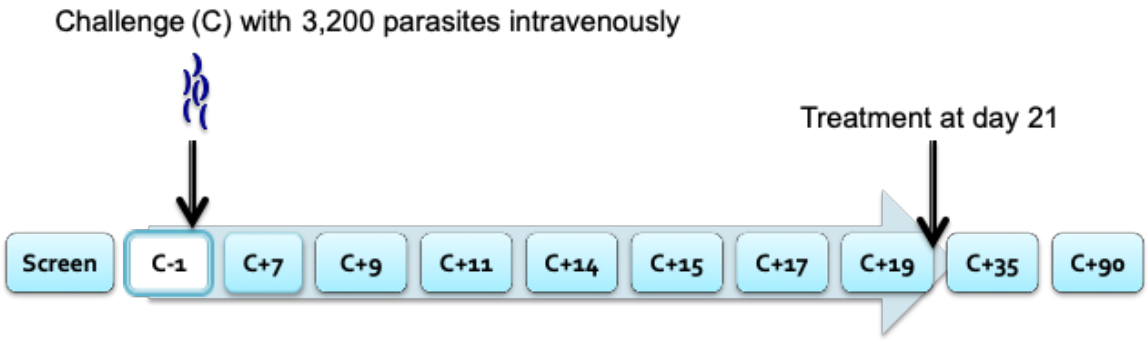

Figure 4. 1: Overview of the Controlled Human Malaria Infection challenge procedure. The CHMI study was conducted in Kenya in 2013, 2016, 2017 and 2018, and involved 189 individuals who met the inclusion criteria: malaria parasite negative, nonpregnant, and free of immunosuppressive infections, genetic diseases that cause malaria resistance and systemic antibiotics of antimalarial activity. The participants were challenged intravenously by 3200 sterile sporozoites and were followed up for parasitemia monitoring by qPCR twice daily, and those with 500 parasites/µl and/or symptoms (>37°C, hypotension, tachycardia, chills, rigor, sweating, headache, nausea, vomiting, fatigue, lower back pain, myalgia and arthralgia) were treated. All individuals were treated at the end of the study. Blood samples collected one day before challenge were used for functional studies in our laboratory.

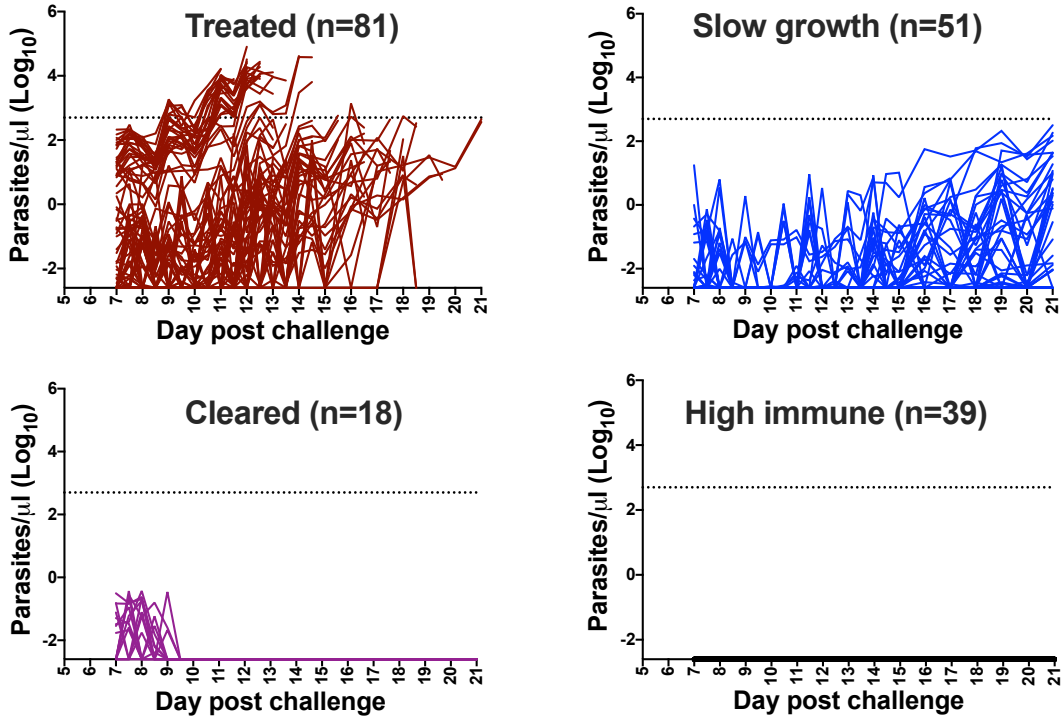

Figure 4. 2: Categories of the Controlled Human Malaria Infection study individuals based on parasitemia patterns during the study. Treated - 81 individuals who had >500 parasites/µl and /or symptoms, slow growth - 51 individuals who had recurrent parasitemia but <500 parasites/µl, cleared - 18 individuals who were cleared parasites by day 10, and high immune - 39 individuals who parasite negative during the entire follow up period.

Opsonic phagocytosis assay (OPA)

Non-opsonic and opsonic phagocytosis of infected erythrocytes has been successfully conducted using human/murine monocytes-derived macrophages (Ayi et al., 2005) and human monocytic THP-1 cell line (Chan et al., 2012) respectively. I established

the OPA of rIEs using the THP-1 cell line in our lab in order to investigate whether the opsonization of rIEs with the CHMI plasma samples could lead to phagocytosis.

Antigen based Enzyme-Linked Immunosorbent Assay (ELISA)

The CHMI plasma samples were pre-incubated with known recombinant merozoite antigens, and then used to compare the effect of the merozoite specific antibody depletion to the opsonic phagocytosis of ring culture cells. The demonstration of the depletion of antigen specific antibodies was detected by ELISA, where antibody responses to the various antigens were analysed. This was important in order to ensure that the effect of using plasma samples pre-incubated with the recombinant antigens in opsonic phagocytosis of ring culture cells could indeed be linked to the depletion of the merozoite specific antibodies.

Competition phagocytosis assay

The comparison of opsonic phagocytosis of ring culture cells between non competed and competed plasma samples was important in order to find out the origin of the antigens responsible for opsonic phagocytosis of the ring culture cells. The merozoite was considered a possible source of the parasite proteins on the ring culture cells because it sheds and secrets various proteins during invasion into the erythrocyte. Some merozoite proteins have been suggested to be transferred to the surface of rIEs and uEs during invasion, for example ring surface protein 2/ rhoptry associated protein 2 (RSP2/RAP2) as demonstrated by Douki et al., 2003.

4.3. Overall objective

To investigate the physiological relevance of the immunogenicity of *P. falciparum* rIEs.

4.4. Specific objectives

- To investigate whether the observed antibody binding to rIEs mediated opsonic phagocytosis by THP-1 cells.
- To find out whether opsonic phagocytosis of rIEs predicted the outcome of infection in a CHMI study.
- To find out the source of the antigens responsible for opsonic phagocytosis of the ring culture cells.

4.5. Laboratory methods

4.5.1. *P. falciparum* culture and synchronization

The *P. falciparum* NF54 laboratory isolate was used for the functional assay because it was the same strain that was intravenously challenged to the individuals who took part in the CHMI study. The parasites were cultured and synchronized as explained in chapter 3 (section 3.5.1), in order to obtain a final ring stage parasite culture (0-10 hours synchronization window) of at least 10% parasitemia.

4.5.2. THP-1 culture

THP-1 is a human leukaemia cell line (cultured from the blood of a one-year old boy who had acute monocytic leukaemia) with Fc receptors and thus has the ability of phagocytose opsonized particles. The non-adherent THP-1 human monocyte cell line was maintained *in vitro* in 150 ml culture flasks with THP-1 culture media, which consists of RPMI 1640 media supplemented with 2 mM L-Glutamine, 10 mM HEPES, 10% (v/v) fetal bovine serum and 1% pen strep (10,000 units/ml penicillin and 10,000 µg/ml streptomycin). The culture was kept in a humidified 37°C incubator which was gassed with 5% (v/v) $C0_2$ and 95% (v/v) air. The cells were counted by a haemocytometer in order to maintain a cell density of between 1 X 10^5 cells/ml to 1 X 10^6 cells/ml.

4.5.3. Opsonic phagocytosis assay (OPA)

The ring stage parasite cultures of at least 10% parasitemia were used in the experiment. For phagocytosis experiments, the ring stage culture was centrifuged at 800g for 4 minutes, and the pellet was resuspended in 1X Potassium Buffered Saline (PBS) containing 5 µg/ml Dihydro ethidium (DHE) and 2 µM Cell Trace™ Violet for 20 minutes at 37°C. The DHE is a reduced form of ethidium bromide (EtBr) which is dehydrogenated inside the cell to form the ethidium cation that intercalates into the DNA, whereas Cell Trace™ Violet is cleaved by intracellular esterases resulting to a highly fluorescent compound that stains the cell cytoplasm. The dual labelling was used to distinguish between uEs and rIEs in the ring culture, which stained positive for the Cell Trace™ Violet only and both dyes respectively. The dual labelled parasites were opsonised with heat inactivated (incubated at 56°C for 30 minutes) plasma samples of the individuals from the CHMI study, diluted 1:12.5, for 30 minutes at room

temperature, and washed twice with wash buffer. A pool of heat inactivated hyperimmune plasma from malaria-immune adults and heat inactivated plasma samples from malaria-naïve individuals were included in each plate as positive and negative controls respectively. The opsonised, dual labelled ring culture (4.0 X 10^6 cells) was added to wells containing 150 µl of 2.0 X 10^6 THP-1 cells and incubated for 4 hours at 37°C in 5% (v/v) CO_2 and 95% (v/v) air. The THP-1 monocytes (effector) to erythrocytes (target) ratio was 1E:200T. The cells were centrifuged at 500g for 4 hours at 4°C to stop the phagocytosis reaction and resuspended in 200 µl of red cell lysis buffer at 37°C for 8 minutes with frequent agitation to lyse the un-phagocytosed erythrocytes before being washed twice with THP-1 culture media. The cells were then fixed in 1X Paraformaldehyde (PFA) at room temperature for 30 minutes and finally resuspended in 200 µl wash buffer for flow cytometric analysis. Fluorescence was measured using FACSCanto II fitted with 405 nm and 488 nm solid state lasers, and analysed using FlowJo software, version 10. The THP-1 cells alone were used as a control to select the THP-1 population using the forward and side scatter properties. The gated events were displayed on violet versus red fluorescence dotplots, where the THP-1 containing phagocytosed uEs erythrocytes (positively labelled with Cell Trace™ Violet) were defined in a region gated for violet fluorescence and those THP-1 containing phagocytosed rIEs (positively labelled with both Cell Trace™ Violet and DHE) were defined by a region gated for double positive violet and red fluorescence. The sample acquisition was set to stop when at least 2,000 events of the THP-1 containing phagocytosed rIEs was collected for each sample.

In experiments to validate the successful establishment of the assay in our lab and investigate whether antibody binding to rIEs mediates opsonic phagocytosis of rIEs, THP-1 monocytes were incubated with dual labelled; fresh uEs cultured in fresh media, fresh uEs cultured in used media (centrifuged at 4000 g for 15 minutes) and ring culture under the following conditions. To increase phagocytosis, labelled erythrocytes were opsonised with heat-inactivated hyperimmune plasma (malaria-immune adults from Kilifi, Kenya) for 30 minutes at room temperature and washed twice before phagocytosis. As a control, labelled erythrocytes were opsonised with heat-inactivated plasma from malaria-naïve individuals in a similar manner, and also non opsonised erythrocytes were employed. To inhibit phagocytosis, THP-1 monocytes were pre-incubated with 5 µM cytochalasin D for at least 1 hour at 37°C

before the addition of the labelled erythrocytes. Cytochalasin D is an actin polymerase inhibitor which would prevent the monocytes from phagocytosis.

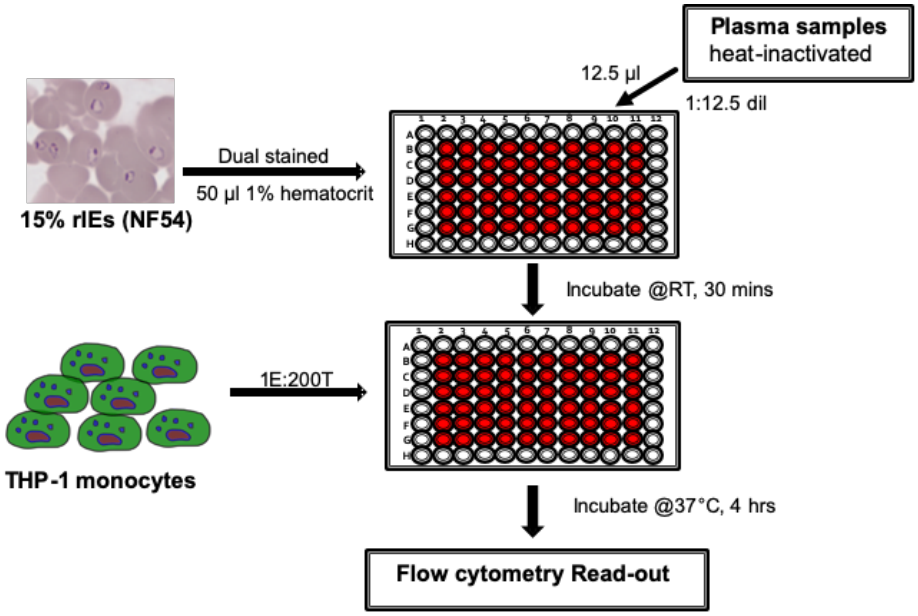

Figure 4. 3: Procedure of opsonic phagocytosis of ring-infected erythrocytes assay. Ring culture of 15% ring-infected erythrocytes was dual stained with DHE - DNA stain and Cell Trace - erythrocyte cytoplasmic dye and opsonised with heat-inactivated plasma samples for 30 minutes before exposure to THP-1 monocytes for 4 hours. The phagocytosis read-out was obtained by flow cytometry which differentially detected and quantified the percentage of THP-1 cells that phagocytised uEs and rIEs because they stained positive for Cell Trace positive only and DHE and Cell Trace dual positive respectively. E: T refers to the effector (THP1 monocytes) to target (erythrocytes) ratio.

4.5.4. Depletion of merozoite specific antibodies

We randomly selected six CHMI study individuals who had varied antibody responses to merozoites: 1) those with high (18A0012 and 16K0036 with optical densities (ODs) of 2.925 and 2.081, respectively), 2) those with medium (17K0005 and 17K0028, ODs 1.831 and 1.333, respectively), and 3) those with low (17K0074 and 17K0084, ODs 0.802 and 0.557, respectively). Each of these randomly selected plasma samples were incubated overnight at 4°C with 100 µg/ml (the concentration was previously optimised in our lab) of recombinant merozoite antigens. The antigens used were Merozoite Surface Protein 1 (MSP1), Merozoite Surface Protein 2 (MSP2), Merozoite Surface Protein 3 (MSP3), Erythrocyte Binding Antigen-175 (EBA-175) and Apical Membrane Antigen 1 (AMA1). We used these antigens because they were available in our laboratory at the time and are also known potential malaria vaccine candidates.

4.5.5. Antigen based Enzyme-Linked Immunosorbent Assay (ELISA)

To investigate the depletion of anti-merozoite specific antibodies from the randomly selected plasma samples of the CHMI study individuals: each recombinant merozoite antigen was coated per well at previously optimized coating concentrations: MSP1, MSP2, MSP3, EBA-175 and AMA1 were coated at 19.2 µg/ml, 0.5 µg/ml, 0.5 µg/ml, 10 µg/ml and 0.5 µg/ml respectively, and incubated overnight at 4°C. After washing, the 96 well plates were blocked with 1% skimmed milk for 5 hours at room temperature, followed by incubation of each well with 100 µl of plasma samples of the randomly selected CHMI study individuals, diluted 1:100, overnight at 4°C. Each recombinant protein was tested for each individual sample before and after competition in order to access the difference between the antigen specific antibody responses in the two conditions. The plates were washed, and each well was incubated with 100 µl horseradish peroxidase (HRP) conjugated goat anti-human IgG antibody, diluted 1:5000, for 3 hours. After washing, each well was incubated with 100 µl of O- phenylenediamine dihydrochloride (OPD) substrate for 30 minutes at room temperature. OPD is a water-soluble chromogenic substrate that yields a yellow colour during degradation of hydrogen peroxide by HRP enzyme. 30 µl of 1M hydrochloric acid (HCl) was added to each well to stop the reaction and produced a very stable orange end solution whose absorbance was read at 490nm using the using the CYTATION|3 imaging reader and analysed using the Gen 5 3.02 software.

4.5.6. Competition phagocytosis assay

The assay procedure was similar to the opsonic phagocytosis described in section 4.5.3. Additionally, phagocytosis was compared between non competed and competed (preincubated with recombinant merozoite proteins) plasma samples in order to investigate the origin of parasite proteins on the ring culture cells that mediate the opsonic phagocytosis of rIEs and uEs in the ring culture.

4.6. Statistical analysis

All analysis was done using GraphPad PRISM (version 7.0). Kruskal-Wallis H test and Dunn's Multiple Comparison post hoc test were used to analyse the statistical significance of differences in opsonic phagocytosis between the different groups of the CHMI study.

4.7. Ethics

Ethical approval has been granted by the institutional research ethics committee, Kenya Medical Research Institute Scientific and Ethics Review Unit (KEMRI/SERU/ CGMR-C/029/3190) and by the sponsor of the study (University of Oxford) through the Oxford Tropical Research Ethics Committee (OxTREC, 2–16). Informed consent was obtained from participants. It was also approved for the samples to be used in Germany.

4.8. Results

4.8.1. Establishment of the opsonic phagocytosis assay (OPA) of ring-infected erythrocytes in our lab

To establish the OPA of rIEs assay in our laboratory, I tested hyperimmune plasma, naïve plasma, unopsonized and cytochalasin D treatment on using a set of different cultures conditions: uEs in fresh media, ring culture and uEs in used media. The proportion of THP-1 cells that phagocytosed uEs and rIEs were distinctly separated using a uniform flow cytometry gating strategy (Appendix 4.1) where the THP-1 stained positive for only the CellTrace dye and dual positive for both CellTrace and DHE dye respectively. The effector to target ratio (E:T) which was the monocyte to erythrocyte ratio, and the phagocytosis duration were optimized in order to yield an optimal condition for our assays that allowed a good difference of phagocytosis between opsonization of culture with hyperimmune plasma and naïve plasma (Appendix 4.2).

The validation of the successful establishment of the opsonic phagocytosis assay of rIEs in our lab was demonstrated by the percentage phagocytosis observed in the different culture conditions (Figure 4.4). In the culture of uEs in fresh media, the percentage of THP-1 cells that phagocytosed uEs was similar regardless of the different treatments, except for Cytochalasin D which was a negative control. In the ring culture (15% rIEs), the percentage of THP-1 cells that phagocytosed uEs and rIEs was higher especially after treatment with hyperimmune as compared to the naïve plasma and unopsonized conditions. However, the proportion of THP-1 cells that have phagocytosed uEs was higher compared to rIEs in all the treatment conditions. In the culture of uEs in used media (centrifuged at 4000 g for 15 minutes), the percentage of THP-1 cells that phagocytosed uEs was higher especially after treatment with hyperimmune as compared to the naïve plasma and unopsonized conditions.

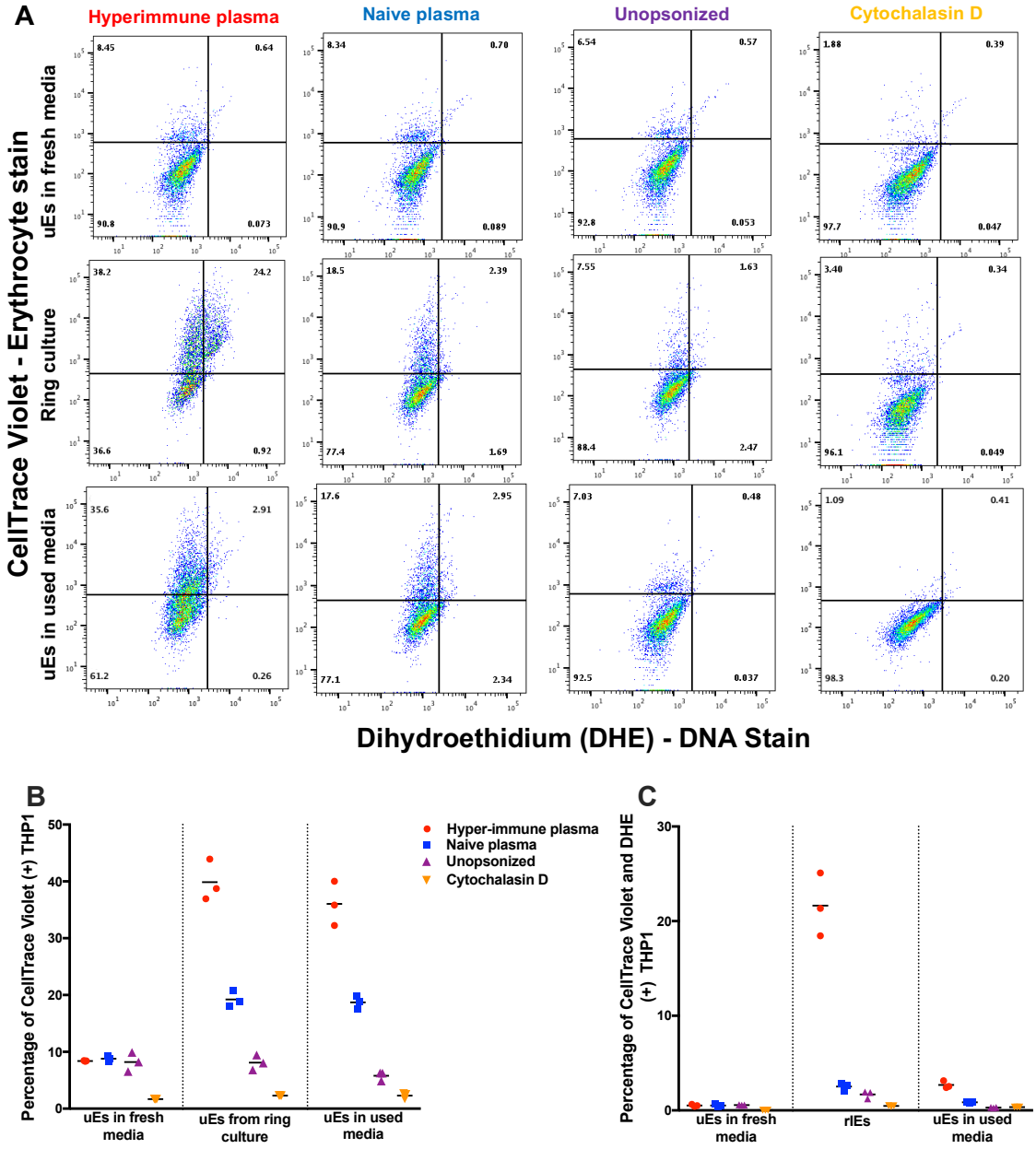

Figure 4. 4: Establishment of opsonic phagocytosis assay in our laboratory. A) Flow scatter plots showing the proportion of THP-1 cells that have phagocytosed uEs (stained positive for CellTrace Violet dye only - top left quadrant) and THP-1 cells that have phagocytosed rIEs (stained positive for both CellTrace Violet and DHE dyes - top right quadrant) after treatment of the different cultures (uEs in fresh media, ring culture and uEs in used media) with either hyperimmune plasma, naïve plasma, unopsonized or Cytochalasin D. In the culture of uEs in fresh media, the percentage of THP-1 cells that phagocytosed uEs was similar regardless of the different treatments, except for Cytochalasin D which was a negative control. In the ring culture (15% rIEs), the percentage of THP-1 cells that phagocytosed uEs and rIEs was higher especially after treatment with hyperimmune as compared to the naïve plasma and unopsonized conditions. However, the proportion of THP-1 cells that phagocytosed uEs was higher as compared to rIEs in all the treatment conditions. In the culture of uEs in used media (centrifuged at 4000 g for 15 minutes), the percentage of THP-1 cells that phagocytosed uEs was higher especially after treatment with hyperimmune as compared to the naïve plasma and unopsonized conditions. B) & C) graphical representation of the above flow scatterplots. B) The pattern of the percentage of THP-1 cells that phagocytosed uEs in ring culture or in used media was similar. C) The percentage of THP-1 cells that phagocytosed rIEs was higher after treatment with hyperimmune plasma.

4.8.2. Effect of opsonic phagocytosis of ring-infected erythrocytes on outcome of infection

Plasma samples from 189 individuals in the CHMI study were used to assess how the antibodies in the different test plasma would mediate *in vitro* phagocytosis of rIEs and how the opsonic phagocytosis of rIEs would correlate to the outcome of the study; symptoms, parasite density pattern and treatment. Individuals who were non-symptomatic had significantly higher ($P<0.0001$) phagocytosis of rIEs as compared to symptomatic individuals (Figure 4.5-A). Individuals whose parasite densities were >500 parasites/µl (treated category) had significantly lower opsonic phagocytosis of rIEs compared to individuals who had patent parasitemia although <500 parasites/µl (slow growth category), those who cleared the parasites by day 10 of the study (cleared category) and those who were parasite negative during the entire follow up period (highly immune category), $P<0.0001$, $P<0.0001$ and $P=0.0004$, respectively (Figure 4.5-B). Moreover, treated individuals who had high opsonic phagocytosis of rIEs were significantly fewer ($P<0.0001$) at any day beyond the tenth day post challenge compared to those with mid or low levels of opsonic phagocytosis of rIEs (Figure 4.5-C).

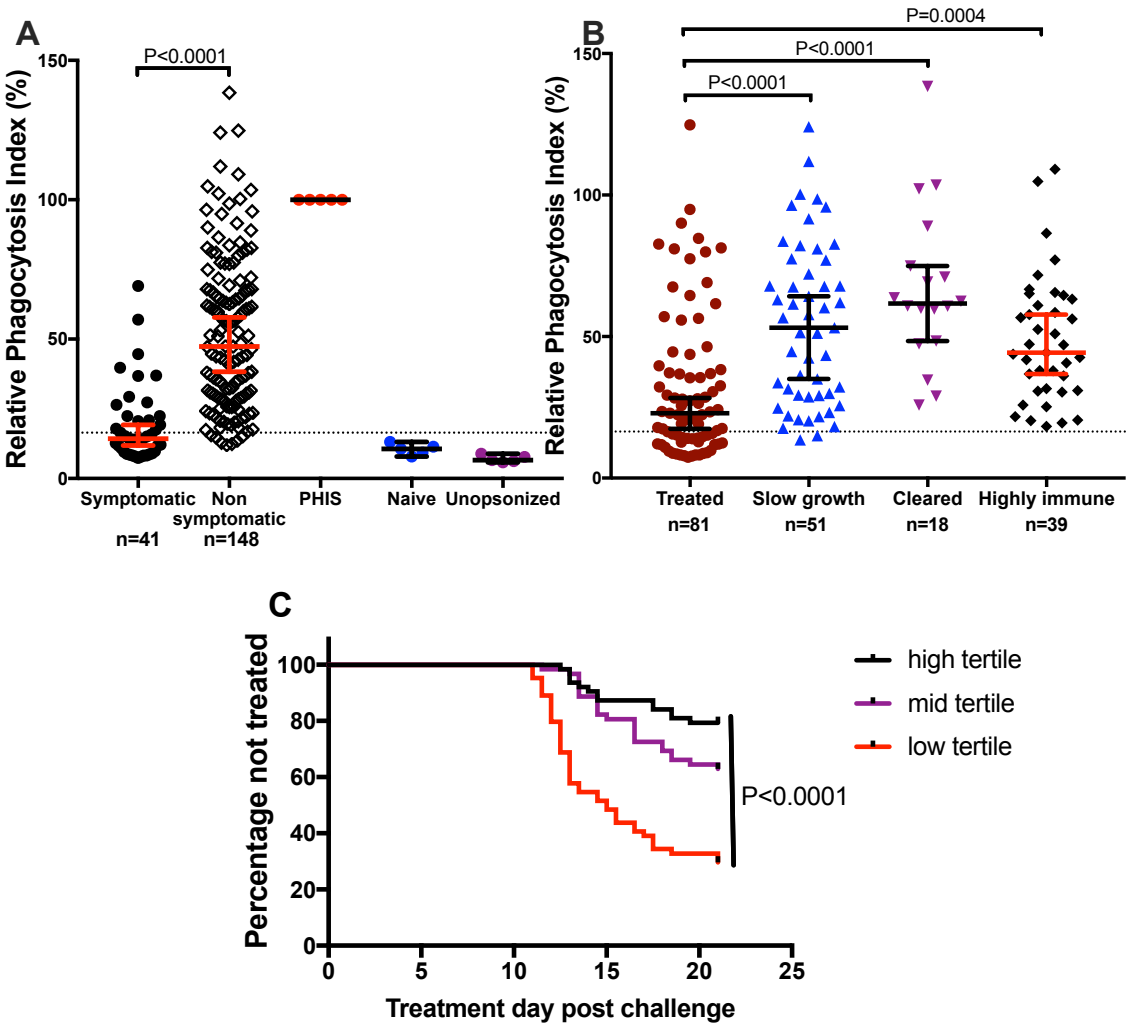

Figure 4. 5: Opsonic phagocytosis of ring-infected erythrocytes predicts outcome in a Controlled Human Malaria Infection (CHMI) study. A) Non-symptomatic individuals were shown to have significantly higher (P<0.0001, Mann Whitney test) phagocytosis of rIEs as compared to symptomatic individuals. B) Opsonic phagocytosis of rIEs was determined to be significantly lower in individuals who had >500 parasites/µl during the study as compared to those who had <500 parasites/µl (P<0.0001, Kruskal-Wallis test), specifically the slow growth category where the individuals had recurrent parasitemia although <500 parasites/µl, the cleared category where the individuals cleared the parasites by day 10 of the study and the high immune category where the individuals were parasite negative during the entire follow up period (P<0.0001, P<0.0001 and P=0.0004, Dunn's multiple comparison test) respectively. C) Treated individuals who had high opsonic phagocytosis of rIEs were significantly fewer (P<0.0001, Mantel-Cox test) at any day beyond the tenth day post challenge as compared to those with mid or low levels of opsonic phagocytosis of rIEs. The dotted horizontal line in graphs A and B is the seropositivity mark = {mean of the phagocytosis of rIEs using naïve plasma + 3(STDEV)} Statistics was calculated using GraphPad PRISM version 7.0.

Opsonic phagocytosis of rIEs showed a: 1) strong positive correlation of r=0.7796; P<0.0001 to IgG antibody response to merozoites, especially IgG1 and IgG3 (r=0.7774; P<0.0001 and r=0.6267; P<0.0001) respectively, but 2) weak correlation to IgG2 and IgG4 (Spearman r=0.451; P<0.0001 and Spearman r=0.1767; P=0.0150) respectively (Figure 4.6). A similar pattern was observed between opsonic phagocytosis of uEs to antibody responses to merozoites (Appendix 4.3).

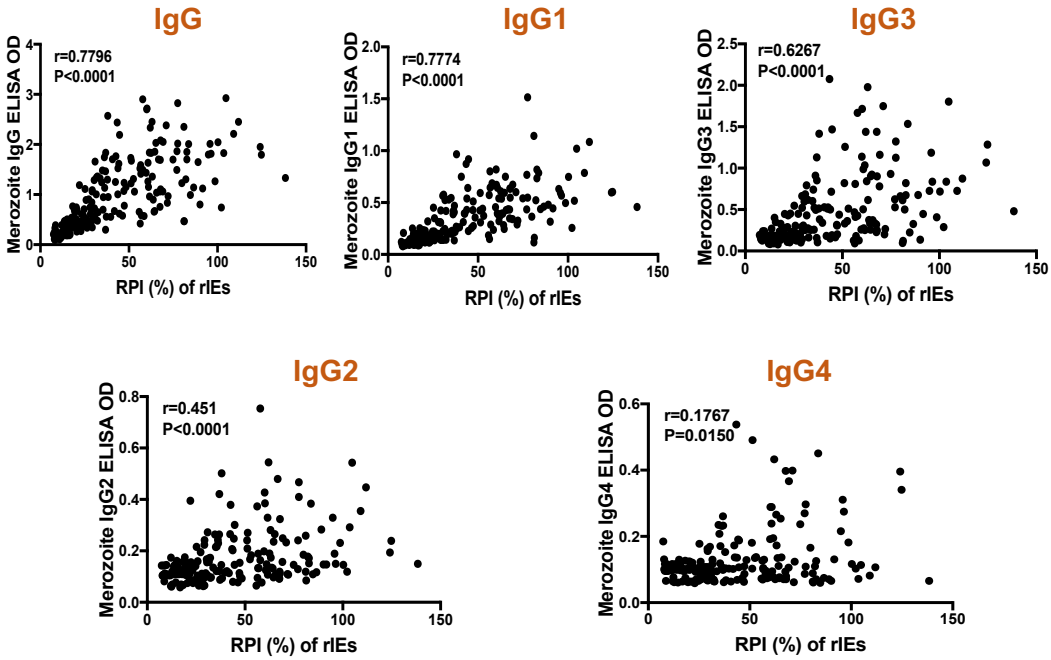

Figure 4. 6: Correlation of opsonic phagocytosis of ring-infected erythrocytes to antibody responses to merozoites. Opsonic phagocytosis of rIEs showed; a positive correlation (Spearman r=0.7796; P<0.0001) to IgG antibody responses to merozoites, especially IgG1 and IgG3 (Spearman r=0.7774; P<0.0001 and Spearman r=0.6267; P<0.0001) respectively, and a weak correlation to IgG2 and IgG4 (Spearman r=0.451; P<0.0001 and Spearman r=0.1767; P=0.0150) respectively. Statistics was calculated using GraphPad PRISM version 7.0.

Interestingly, individuals who were not treated had significantly (P<0.0001) high opsonic phagocytosis of uEs as compared to those who were treated (Figure 4.7-A) which is similar to that observed in regard to opsonic phagocytosis of rIEs. Generally, the opsonic phagocytosis of uEs was significantly higher (P<0.0001) compared to opsonic phagocytosis of rIEs between the treated and untreated groups (Figure 4.7-B). Moreover, I also observed a strong positive correlation (Spearman r=0.6418; P<0.0001) between the relative phagocytosis index of uEs to relative phagocytosis index of rIEs (Figure 4.7-C).

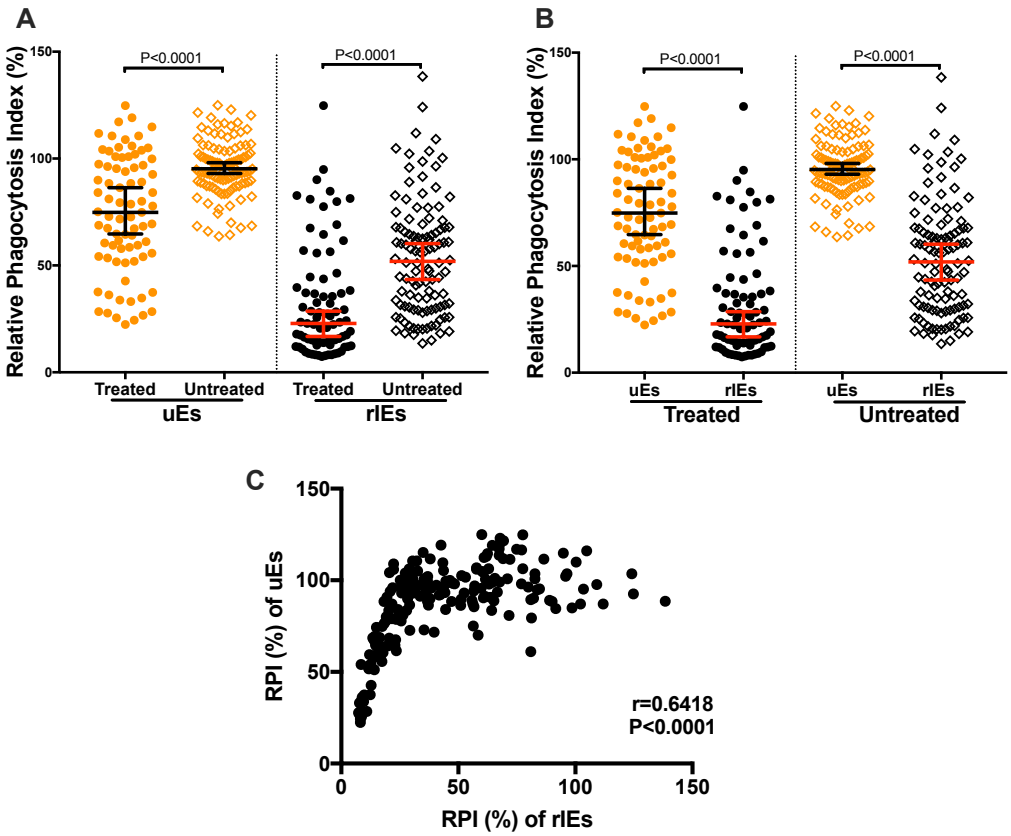

Figure 4. 7: Comparison of opsonic phagocytosis of uninfected erythrocytes and opsonic phagocytosis of ring-infected using samples from the Controlled Human Malaria Infection study. A) Individuals who were not treated were shown to have a significantly high (P<0.0001, Mann Whitney test) opsonic phagocytosis of uEs compared to those who were treated, and this was similar in regard to opsonic phagocytosis of rIEs. B) Opsonic phagocytosis of uEs was determined to be significantly higher (P<0.0001, Mann Whitney test) compared to opsonic phagocytosis of rIEs in both the treated and untreated categories. C) Relative phagocytosis index of uEs showed a strong positive correlation (Spearman r=0.6418; P<0.0001) to relative phagocytosis index of rIEs. Statistics was calculated using GraphPad PRISM version 7.0.

4.8.3. Origin of the antigens responsible for opsonic phagocytosis of the ring culture cells

Plasma samples of six individuals from the CHMI study were randomly selected based on their antibody responses to merozoites (18A0012 & 16K0036 - high; 17K0005 & 17K0028 - mid and 17K0074 & 17K0084 - low) for the competition opsonic phagocytosis assay. The selection of the merozoite recombinant antigens (CD4 tagged) for competition was guided by their availability in our lab and their potential as vaccine candidates (MSP1, MSP2, MSP3, EBA175 and AMA1). Pre-incubation of the individual plasma samples with the respective recombinant antigens showed a reduction in antibody response to the specific antigens, especially MSP1, EBA175 and

AMA1 (Figure 4.8). This validation was important before using the plasma samples for the competition opsonic phagocytosis assay.

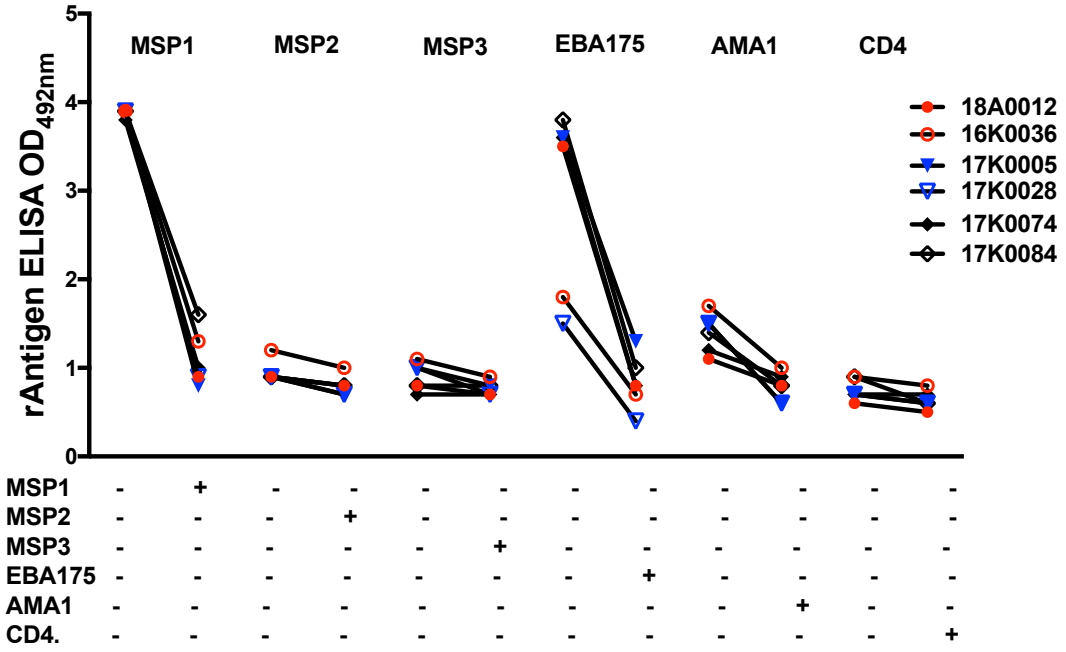

Figure 4. 8: Confirmation of depletion of merozoite specific antibodies. Pre-incubation of the individual plasma samples with the respective recombinant antigens showed a reduction in antibody response to the specific merozoite recombinant antigens, especially MSP1, EBA175 and AMA1. The CHMI study plasma samples used for the assay were selected based on their known antibody responses to merozoites (18A0012 & 16K0036 - high; 17K0005 & 17K0028 - mid and 17K0074 & 17K0084 - low). The selection of the merozoite recombinant antigens (CD4 tagged) for competition was guided by their availability in our lab and their potential as vaccine candidates.

The percentage phagocytosis of the ring culture using 1:100 dilution of the control plasma samples was higher after opsonization with hyperimmune plasma as compared to naïve plasma and unopsonized conditions (Figure 4.9-A). Additionally, opsonic phagocytosis in the presence of a pool of the recombinant antigens was similar to opsonic phagocytosis during the unopsonized condition (Figure 4.9-A). Relative phagocytosis index of uEs using individual plasma samples that were each pre-incubated with a pool of the merozoite recombinant antigens (competed) was significantly lower (P=0.0003) compared to using non-competed individual plasma samples (Figure 4.9-B). Relative phagocytosis index of rIEs using individual plasma samples that were each pre-incubated with a pool of the merozoite recombinant antigens (competed) was significantly lower (P=0.004) compared to using non-competed individual plasma samples (Figure 4.9-C). However, the significant difference observed in the relative phagocytosis index of the ring culture could not be

explained by pre-incubation of the individual plasma samples with a single merozoite recombinant antigen, except EBA175 which showed a similar finding as with pool of the antigens (Appendix 4.4 & Appendix 4.5).

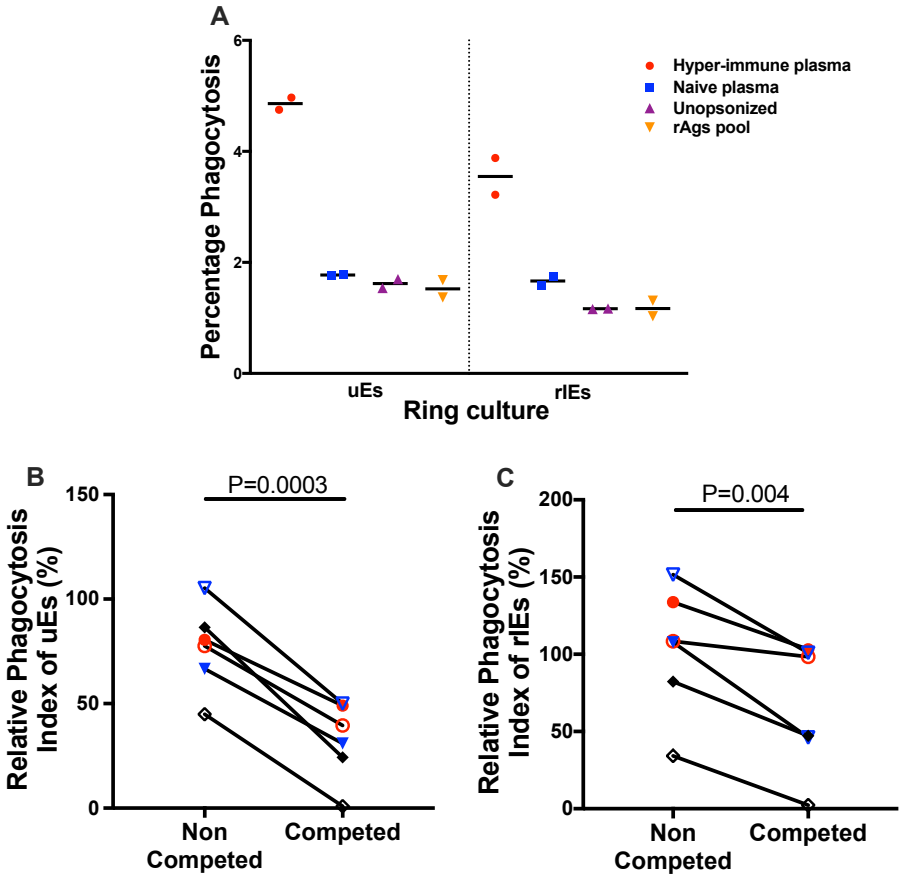

Figure 4. 9: Competition opsonic phagocytosis of the ring culture. A) The percentage phagocytosis of the ring culture using 1:100 dilution of the control plasma samples was higher after opsonization with hyperimmune plasma as compared to naïve plasma and unopsonized conditions. Relative phagocytosis index of (B) uEs and (C) rIEs using individual plasma samples that were each pre-incubated with a pool of the merozoite recombinant antigens (competed) was significantly lower (P=0.0003 and P=0.004, paired t-test) respectively, compared to using non-competed individual plasma samples. Statistics was calculated using GraphPad PRISM version 7.0.

In order to understand further which blood stage would be responsible for the observed opsonic phagocytosis of uEs cultured in used media, I compared phagocytosis of supernatant obtained from synchronized parasites of different blood stages of similar parasitemia. I found that the supernatant obtained from the parasite period between mature stage (35 hours) to the next cycle at about 10 hours post-invasion showed a higher percentage phagocytosis of uEs, especially when opsonized with hyperimmune plasma and malaria-immune IgG, as compared to supernatant obtained in the parasite period between ring stage (10 hours) to mature stage (34 hours) which was comparable to opsonic phagocytosis of uEs cultured in fresh media (Figure 4.10).

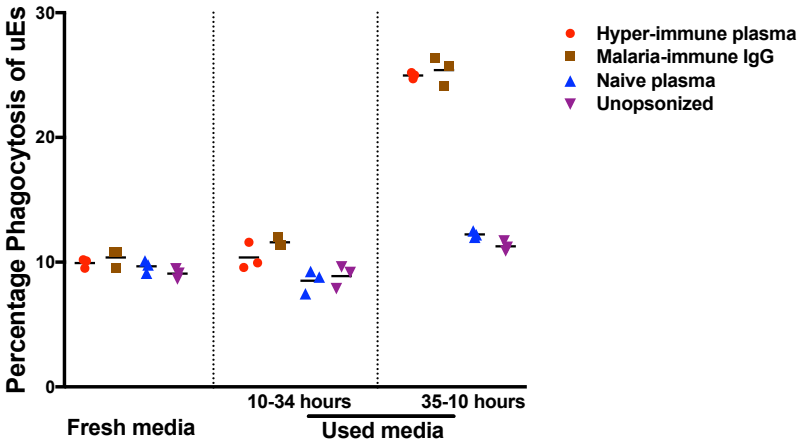

Figure 4. 10: Late blood stage parasite proteins mediate opsonic phagocytosis of uninfected erythrocytes. The supernatant obtained from the parasite period between mature stage (35 hours) to the next cycle at about 10 hours post-invasion showed a higher percentage phagocytosis of uEs, especially when opsonized with hyperimmune plasma and malaria-immune IgG, as compared to supernatant obtained in the parasite period between ring stage (10 hours) to mature stage (34 hours) which was comparable to opsonic phagocytosis of uEs cultured in fresh media.

4.9. Discussion

Establishment of opsonic phagocytosis assay (OPA) of rIEs in our laboratory

The successful establishment of the flow cytometry based OPA of rIEs was validated using various controls (Figure 4.4), and the observations confirmed previous findings which showed that phagocytosis of rIEs was elevated after incubation of the ring culture cells with hyperimmune sera (Chan et al., 2012). However, an older study showed no difference on phagocytosis of rIEs after incubation with malaria-immune serum (Celada et al., 1982). This could be explained by the limitation of the methods they used in quantifying for monocyte cells that have phagocytosed rIEs. The Giemsa-peroxidase stained smear is far less sensitive compared to the flow cytometry detection that was employed in my study. Flow cytometry differentiates between THP1 monocyte cells that have phagocytosed uEs from those that had phagocytosed young rIEs based on the different stains taken up by the uEs and rIEs in the ring culture.

Effect of OPA of rIEs on outcome of infection

The study participants employed were malaria-exposed volunteers who participated in a CHMI study (Kapulu et al., 2018). This study design is gaining prominence compared to cross sectional surveys of children living in malaria endemic areas for analyses aimed at identifying the correlates of protection.

Opsonic phagocytosis of rIEs in this study correlated with outcome of infection in the CHMI study. Findings from this study shows that the individuals whose plasma samples were capable of inducing high opsonic phagocytosis of rIEs were able to maintain low or no parasitemia, remain asymptomatic and require no treatment (Figure 4.5). Findings from this *in vitro* study could explain observations of previous *in vivo* studies which showed that IgG sensitized rIEs were rapidly cleared from peripheral circulation by the spleen during recovery from uncomplicated *P. falciparum* malaria in Thai adults (Lee et al., 1989), and thus confirming what was earlier hypothesised, circulating erythrocytes labelled with antibodies likely result in splenic clearance by interaction with Fc receptors (Douki et al., 2003). The findings from this study suggest that the correlation of the *in vitro* opsonic phagocytosis of rIEs to outcome of infection could highly likely contribute to parasite clearance and protection from clinical symptoms and treatment as manifested by the CHMI individuals. However, as other stages of the infection have been shown in other analyses to also correlate with protection, it would be important to try to calculate the contribution of the ring-stages to the total protection observed.

Understanding the OPA of uEs in ring culture and uEs incubated in used media

In this study, we did not employ 100% rIEs for the phagocytosis assay, but rather a ring culture of about 15% rIEs for technical reasons. Therefore, the phagocytosis of uEs in the ring culture was simultaneously analysed alongside rIEs, because the two populations were differentially detected by the flow cytometer based on their distinctive staining patterns. Phagocytosis of uEs in ring culture was shown to be elevated especially after opsonization with hyperimmune plasma (Figure 4.4) as shown in previous studies (Chan et al., 2012, Awah et al., 2011). This could explain the eventual consequence of earlier observations from other studies which reported that *P. falciparum* specific antibodies recognized the surface of uEs in ring culture (Layez et al., 2005, Douki et al., 2003), in a parasite dependent manner (Awah et al., 2009). The findings from my study further shows that the opsonic phagocytosis of uEs incubated in used media was also elevated after opsonization with hyperimmune plasma but this was not observed in case of uEs incubated in fresh media (Figure 4.4-B). This suggests that opsonic phagocytosis of uEs in ring culture and uEs incubated in used media is parasite specific and not a random phenomenon.

Opsonization of the ring culture cells using the CHMI plasma samples induced phagocytosis of uEs at a greater proportion than rIEs (Figure 4.7). This observation confirms findings from previous studies which implicated malarial anaemia to clearance of uEs (Awah et al., 2011, Awah et al., 2009), since the majority of erythrocytes lost during human malaria are actually uEs (Lamikanra et al., 2007), and specifically 10 fold greater than parasitized erythrocytes in case of severe malaria anaemia (Barnwell, 2006, Jakeman et al., 1999). Interestingly, findings from my study show that individuals who were not treated had higher level of phagocytosis of uEs (Figure 4.7). This is somehow consistent with earlier findings which showed that, in malaria endemic areas, the loss of uEs is coherent with the ability to clear parasite in children (Evans et al., 2006). Thus, an obvious question is the impact of the observed opsonic phagocytosis of uEs on anaemia in the CHMI study, and this can be answered by correlating the function, OPA of uEs, to haemoglobin levels of the individuals once the haematological data of the CHMI study are made available.

Origin of the antigens responsible for opsonic phagocytosis of ring culture cells

Opsonic phagocytosis of the ring culture cells showed a strong positive correlation to IgG antibody responses to merozoites, especially the cytophilic ones, IgG1 and IgG3, (Figure 4.6 and Appendix 4.3). Findings from this study also show that the parasite

proteins secreted in the media during the mature blood stages and merozoite invasion of the erythrocyte are responsible for opsonic phagocytosis of uEs incubated in used media (Figure 4.10). These findings support previous observations which showed that the ring surface protein 2 (RSP2) found on the surface of rIEs and uEs in ring culture was indeed of merozoite rhoptry origin (Douki et al., 2003), which was later confirmed to be similar to the rhoptry associated protein 2 (RAP2) (Sterkers et al., 2007).

Depletion of merozoite specific antibodies from the CHMI plasma samples showed a significant decrease in the phagocytosis of the ring culture cells (Figure 4.9). This observation is consistent with findings from a previous study which showed that competing off parasite specific antibodies by incubating a strongly positive immune serum with soluble fractions of sonicated *P. falciparum* merozoite preparation completely abolished surface immunofluorescence of young rIEs, and this inhibition was compensated by passaging the competed immune serum over an immunoadsorbent made from IgG fraction of serum of a healthy but *P. falciparum* hyperimmune Liberian donor (Perlmann et al., 1984). The findings from my study suggest that opsonic phagocytosis of ring culture cells and uEs incubated in used media is mediated by *Plasmodium* antigens on the surface of the ring culture cells and uEs incubated in used media which are highly likely of merozoite origin.

Summary

I successfully established the flow cytometry based opsonic phagocytosis assay of rIEs in our laboratory. I show for the first time that opsonic phagocytosis of rIEs *in vitro* was correlated with the outcome of infection (parasite densities, symptoms and need for treatment) in a CHMI study. Interestingly, I show that phagocytosis of uEs was higher in untreated individuals as compared to the treated category. It is thereby important to investigate the impact of OPA of uEs on malarial anaemia in this CHMI study, and this will be addressed once the haematological data of the study are available. Additionally, I also show that merozoite specific antibodies mediated the observed opsonic phagocytosis of the ring culture cells and uEs incubated in used media. In Chapter 5, I investigate the specific parasite antigens on the surface of ring culture cells and uEs incubated in used media.

Chapter 5

The identification of parasite proteins on ring culture cells and uninfected erythrocytes incubated in used media

5.1. Introduction

The human erythrocyte gets modified after *P. falciparum* invasion (Przyborski and Lanzer, 2005). The parasite increases the permeability of the host's plasma membrane by inducing new permeability pathways (NPPs) needed for growth and propagation of the parasite (Krugliak and Ginsburg, 2006, Staines et al., 2006), and remodels the host cell by trafficking proteins to the erythrocyte compartment which play a role in the pathogenesis of the disease and immune acquisition (Knuepfer et al., 2005). The trafficking of the parasite proteins is made possible by the maturation of membranous structures of parasite origin within in the host erythrocyte cytoplasm, Maurer's clefts, which begins from the young trophozoite stage (Mundwiler-Pachlatko and Beck, 2013, Gruring et al., 2011), and thus expressed on the surface of mIEs during the intraerythrocytic development of *P. falciparum* (Kriek et al., 2003). The majority of these surface antigens are highly polymorphic and encoded by multigene families (Chan et al., 2014), and are thought to mediate vascular adhesion, sequestration and rosette formation (Chen et al., 2004).

The initial dogma, the presence of antigenic determinants on the surface of late intracellular asexual blood stages of *P. falciparum* (Hommel et al., 1982), was challenged by findings from early studies which gave the first evidence of the possibility of parasite proteins on the surface of rIEs (Perlmann et al., 1984). The findings from the study showed that some of the antigens previously shown to be released during schizont rupture and merozoite invasion are deposited on the erythrocyte surface during invasion probably because of their affinity of receptors structures on the erythrocyte membrane. Following this preliminary evidence, other studies confirmed the hypothesis using immunofluorescence assays, (Douki et al., 2003, Thompson et al., 2001, Sam-Yellowe and Perkins, 1991, Sam-Yellowe et al., 1988, Klotz et al., 1989) suggesting that antigens found on the surface of rIEs are transferred during merozoite invasion into the erythrocyte. Moreover, the parasite antigens have also been observed on the surface on uEs in culture or used culture media using radio labelling and immunofluorescence techniques (Sterkers et al., 2007, Camus and Hadley, 1985). It is therefore important to explore current technology to

identify new and/or previously described parasite antigens on the surface of rIEs and uEs in culture and used media.

5.2. Rationale

In this chapter, I investigated whether there are parasite proteins on the surface of ring culture cells and uninfected erythrocytes incubated in used media. To test this, I employed a surface trypsinization method, followed by mass spectrometry analysis of the supernatant containing peptides. Due to technical challenges, I decided to focus on the ring culture and not to sort out the rIEs from the ring culture because only small volumes (2 µl cells/ml which is equivalent to 10 Million cells/ml) of the ring culture can be reliably sorted using the fluorescent cells sorting (FACS) at low flow rates (90 minutes/ml). This was technically impractical because large volumes of the rIEs were required in the surface trypsinization protocol. Moreover, I considered it reasonable to work on the ring culture based on my hypothesis: the immunogenicity of the rIEs and uEs in the ring culture are comparable, because the rIE is not known to transport any parasite proteins to its surface and only comes in contact with parasite proteins during merozoite invasion and rupture of mIEs similarly to the uEs in the ring culture. I was also interested to find out whether the shaved parasite proteins identified on the surface of ring culture cells would be similar to those obtained from shaving uEs incubated in used media.

Surface trypsinization

Surface trypsinization of infected erythrocytes has been previously used to quantitatively remove *P. falciparum* Erythrocyte Membrane Protein 1 (PfEMP1) with limited cleavage of major human erythrocyte proteins (Fernandez et al., 1999), and shave off a novel polymorphic antigen, SURFIN, encoded by the surface-associated interspersed genes (*surf* genes) (Winter et al., 2005).

Mass spectrometry assay

The assay was conducted at the Deutsches KrebsForschungszentrum (DKFZ)-genomics & proteomics core facility at Heidelberg, where the shaved surface proteins were identified using a mass spectrometry-based proteomics approach which employed nano-flow (high performance liquid chromatography) HPLC, a Q-Exactive HF-X mass spectrometer and a combination of specialized workflows that ensured a very sensitive and in-depth analysis of the proteins.

5.3. Overall objective

To investigate parasite proteins on the surface of the ring culture cells and uEs incubated in used media.

5.4 Specific objectives

- To investigate whether the surface trypsinization method efficiently removes surface proteins on the different cultures.
- To investigate the presence of known and/or novel parasite proteins on the ring culture and uEs incubated in used media.
- To investigate the difference between identified parasite proteins on the surface of the ring culture and uEs incubated in used media.

5.5. Laboratory methods

5.5.1. *P. falciparum* culture and synchronization

The *P. falciparum* 3D7 strain was used for surface trypsinization and mass spectrometry analysis because its genome is well annotated and available at the Plasmodium database (Plasmodb). The parasites were cultured as explained in chapter 3 (section 3.5.1). Parasite synchronization and stage specific separation was done using D-sorbitol lysis method and label free magnetic-activated cell sorting (MACS) method respectively. Cultures were maintained at high parasitemia and synchronized every 46 hours with 5% D-sorbitol as described in chapter 3 (section 3.5.1). Once a parasitemia of at least 20% of mIEs was achieved, the mIEs was further separated by MACS in order to get rid of the uEs. The mIEs contain larger paramagnetic hemozoin crystals (non-toxic and polymerized form of haem, obtained from the catabolism of host haemoglobin by the parasite) compared to rIEs and uEs, and thus mIEs can be purified from culture by use of commercial magnets. The eluate containing mIEs (40-45 hours) was incubated with two times volume of fresh uEs and cultured as explained in chapter 3 (section 3.5.1) for 12 hours to allow invasion by the released merozoites from the ruptured mIEs. The resultant ring culture (containing rIEs of 0-12 hours) was purified by MACS in order to get rid of any possible mIEs that did not rupture, followed by a slight D-sorbitol treatment to further get rid of any mIEs still present in the ring culture flow through after the MACS as shown in Appendix 5.1.

Fresh uEs were incubated in media that was previously used for 24 hours. The used media was obtained from a mixed culture (where merozoite invasion was evident & contained at least 10% rIEs). The used media was centrifuged at 4000 rpm for 15 minutes (allows erythrocytes and merozoites to precipitate/pellet) and used to incubate fresh uEs for 24 hours. Fresh uEs were also incubated in fresh/unused media to serve as a control.

5.5.2. Surface trypsinization of the ring culture

Surface trypsinization for each experimental condition (ring culture containing approximately 20% rIEs of ≤ 12 hours, uEs in used media and uEs in fresh media) was done in four independent replicates. A mock-shaved condition of the exact trypsin-shaved ring culture was also included to control for the experiment. The cultures were washed gently with ice cold 1X PBS. The washed cells, 300 µl, were resuspended to make a final volume of 1 ml and digested with 1 µg/ml of porcine-modified trypsin at 37°C for 30 minutes. The modified trypsin specifically cleaves at the carboxylic side of lysine and arginine residues which is essential for protein identification, and most importantly modifies the lysine residues by reductive methylation to ensure that it is stable and resistant to autolytic cleavage. The trypsinization reaction was terminated by addition of 10 mg/ml of soybean trypsin inhibitor, and the cells were centrifuged at 725 g for 30 seconds in order to pellet the erythrocytes. The resultant supernatant was centrifuged again at 16,000 g for 3 minutes at 4°C and quantified using the CYTATION|3 imaging reader and analysed using the Gen 5 3.02 software.

5.5.3. Mass spectrometry assay

The samples (10 µg/sample) were applied to 1 dimensional - sodium dodecyl sulfate-polyacrylamide gel (1D-SDS-PAGE) in order to fractionate, followed by excision of the gel pieces whose cysteine residues were reduced (disulphide bond breakage) and carbamidomethylated by addition of dithiothreitol (DTT) and iodoacetamide respectively. Carbamidomethylation is a deliberate post-translational modification (alkylation) introduced to cysteine residues to allow identification and characterization of proteins in peptide mass fingerprinting. The processed gel pieces were digested overnight with trypsin. The resulting peptides were loaded on a cartridge trap column packed with Acclaim PepMap300 C18, 5 µm, 300 Å wide pore and separated in a 120

min gradient from 3% to 40% ACN on a nanoEase MZ Peptide analytical column (300 Å, 1.7 µm, 75 µm x 200 mm, Waters). Eluting peptides were analysed by an online coupled Q-Exactive-HF-X mass spectrometer. A tandem mass spectrometer generally generates numerous ions from the test sample, separates the ions based on their mass-to-size ration (m/z), and finally records the relative abundance of each unique ion type (MSMS).

5.6. Statistical analysis

Data analysis was carried out by MaxQuant (version 1.6.3.3). Match between runs option was enabled to allow transfer of peptide identification across raw files based on accurate retention time and m/z. Quantification was done using a label free quantification approach based on the MaxLFQ algorithm which employed delayed normalization and extraction of maximal ratio information from peptide signals in order to attain the highest possible accuracy of quantification (Cox et al., 2014).

5.7. Results

5.7.1. Quantification of sample protein concentration before mass spectrometry

As a requirement of the mass spectrometry facility, the protein concentration of the test samples was measured using a nanodrop, and readings recorded as shown in Table 5.1. There was no distinct difference in the measurement of the protein concentration of samples among the different experimental conditions. However, the supernatant obtained after trypsinization of uEs incubated in fresh media appeared to have a low concentration compared to the other experimental conditions (uEs incubated in used media, trypsin-shaved and mock-shaved ring culture).

Table 5. 1: Quantification of protein concentration of the trypsin shaved samples

Sample Number	Sample name	Protein Concentration [µg/µl]	Sample Volume [µl]	Buffer composition	Comment
1	uEs in used medium	0.655	700	1X PBS	Shaved with tripsin
2	uEs in used medium	0.607	700	1X PBS	Shaved with tripsin
3	uEs in used medium	0.514	700	1X PBS	Shaved with tripsin
4	uEs in used medium	0.417	700	1X PBS	Shaved with tripsin
5	uEs in fresh medium	0.494	700	1X PBS	Shaved with tripsin
6	uEs in fresh medium	0.445	700	1X PBS	Shaved with tripsin
7	uEs in fresh medium	0.592	700	1X PBS	Shaved with tripsin
8	uEs in fresh medium	0.365	700	1X PBS	Shaved with tripsin
9	Ring culture shaved	0.761	700	1X PBS	Shaved with tripsin
10	Ring culture shaved	2.098	700	1X PBS	Shaved with tripsin
11	Ring culture shaved	1.012	700	1X PBS	Shaved with tripsin
12	Ring culture shaved	0.743	700	1X PBS	Shaved with tripsin
13	Ring culture mock-shaved	0.583	700	1X PBS	Final supernatant tripsinized
14	Ring culture mock-shaved	2.138	700	1X PBS	Final supernatant tripsinized
15	Ring culture mock-shaved	1.249	700	1X PBS	Final supernatant tripsinized
16	Ring culture mock-shaved	0.747	700	1X PBS	Final supernatant tripsinized

5.7.2. Identification of peptides and proteins by mass spectrometry

A total of 2882 peptides and 478 proteins were identified by MSMS based on a false detectable rate (FDR) cut off of 0.01 on peptide level and 0.01 on protein level (Figure 5.1-A and Figure 5.1-B respectively). A minimum number of quantified peptides was required for protein quantification and thus a total of 472 proteins could be quantified. Proteins that had 2 identified razor or unique peptides (based on Occam's razor principle) were referred to as identified protein2peptides (Figure 5.1-B). The number of identified peptides, proteins, protein2peptides, and quantified proteins appeared to be lower in the mock-shaved ring culture category compared to the other experimental groups (uEs in used media, uEs in fresh media and trypsin-shaved ring culture).

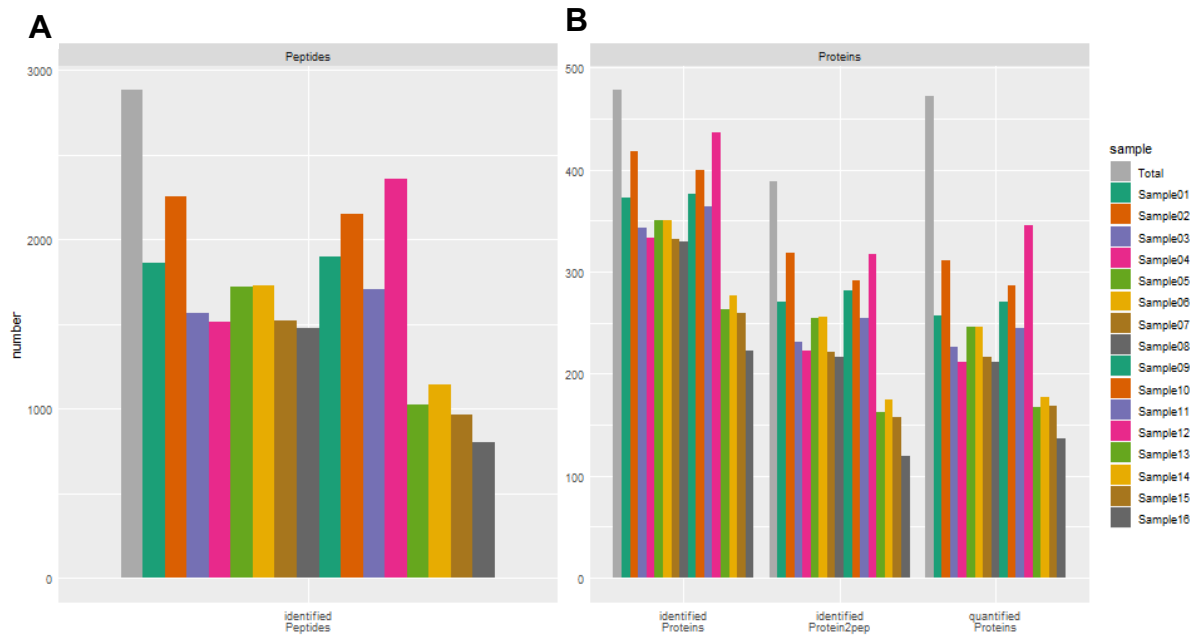

Figure 5. 1: Identified peptides and proteins by MSMS. A total of (A) 2882 peptides and (B) 478 proteins were identified by MSMS based on a false detectable rate (FDR) cut off of 0.001 on both peptide and protein level. However, only 472 protein were quantified since they had the minimum number of quantified peptides required for protein quantification. Protein2peptides were proteins that had 2 identified razor or unique (based on Occam's razor principle). The samples were supernatant obtained after shaving the different cultures: samples 01-04 from uEs in used media; samples 05-08 from uEs in fresh media; samples 09-12 from trypsin-shaved ring culture and samples 13-16 from mock-shaved ring culture. Samples obtained from the mock-shaved ring culture category showed the least number of detectable proteins and peptides.

The identified peptides were searched using MaxQuant-andromeda peptide search engine, which matched the MSMS spectra to peptide sequences in the Human and *Plasmodium* genome databases, based on a probabilistic scoring of peptides spectrum matches. The identified proteins included 444 human proteins and 34 *Plasmodium* proteins. The human proteins were categorised based on the gene ontology of biological processes: 19 - for actin cytoskeleton organization, 49 - for activation of the immune system, 159 - for biological regulation processes, 154 - for metabolic activities and 63 - for other cellular biological processes. The *Plasmodium* proteins were divided between proteins that were differentially identified (12 proteins) and those that were comparably identified (22 proteins) across the different experimental conditions. The comparably identified *Plasmodium* proteins resembled proteins from other *Plasmodium* species: 2 - *P. inui San Antonio*, 2 - *P. yoelli yoelli*, 1 - *P. berghei*, 1- *P. knowlesi*, 3 - *P. cymologi*, 3 - *P. vivax* and 11 - *P. falciparum*. Ten of the differentially identified *Plasmodium* proteins resembled *P. falciparum* and only one resembled *P. cymologi* (Figure 5.2).

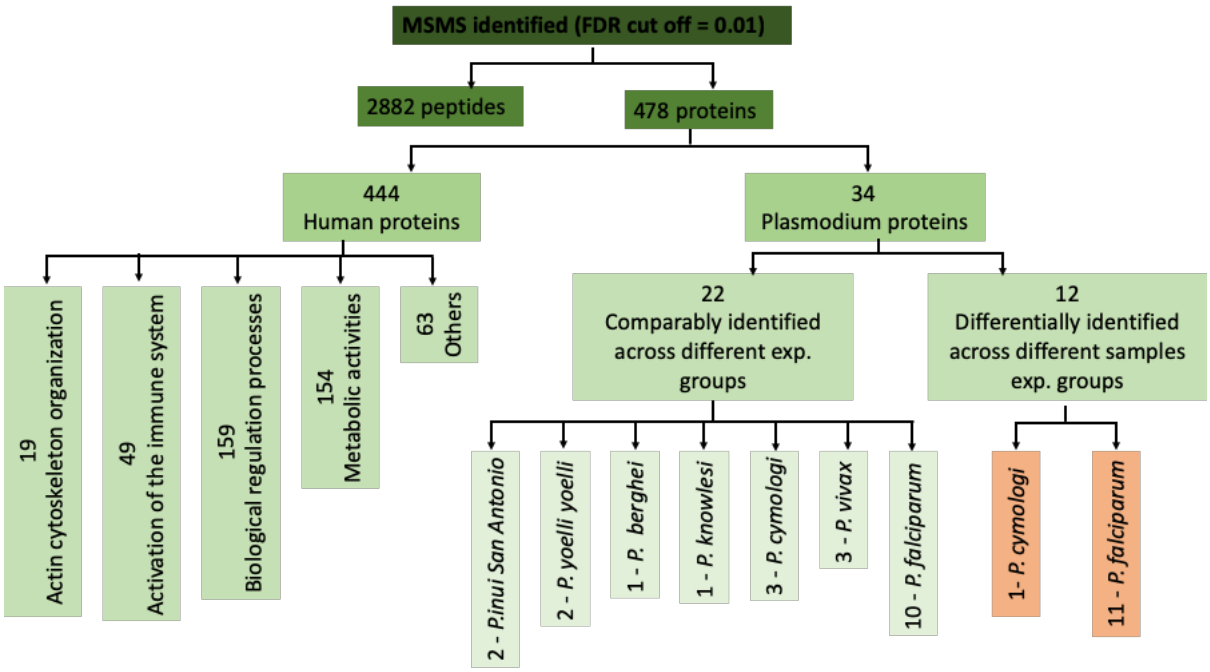

Figure 5. 2: Categorization of the identified proteins. Among the 478 identified peptides, there were 444 human proteins and 34 *Plasmodium* proteins. The human proteins were categorised based on the gene ontology of biological processes; 19 - for actin cytoskeleton organization, 49 - for activation of the immune system, 159 - for biological regulation processes, 154 - for metabolic activities and 63 - for other cellular biological processes. The *Plasmodium* proteins were divided between proteins that were differentially identified (12) and those that were comparably identified (22) across the different experimental conditions. The comparably identified *Plasmodium* proteins resembled proteins from various *Plasmodium* species; 2 - *P. inui San Antonio*, 2 - *P. yoelli yoelli*, 1 - *P. berghei*, 1- *P. knowlesi*, 3 - *P. cymologi*, 3 - *P. vivax* and 11 - *P. falciparum*. Ten of the differentially identified *Plasmodium* proteins resembled *P. falciparum* and only one resembled *P. cymologi*.

The *Plasmodium* proteins were categorised based on two major categories: 1) the differentially identified proteins which referred to proteins that had a higher LFQ intensities/upregulated in the positive control experimental conditions (uEs in used media and trypsin-shaved ring culture) compared to the negative control experimental conditions (uEs in fresh media and mock-shaved ring culture), and 2) the comparably identified proteins which referred to proteins that had a similar LFQ intensities/no change in the positive control conditions (uEs in used media and trypsin-shaved ring culture) as well as the mock-shaved ring culture negative control condition (Table 5.3). The differentially identified proteins and the comparably identified proteins were predicted to be mainly involved in the entry of the parasite into the host cell and metabolic biological processes respectively. Most of the differentially identified proteins were predicted to have signal peptide and/or transmembrane helix, unlike the comparably identified proteins where majority were predicted to have neither signal peptide nor a transmembrane helix (Table 5.2).

The data quality of the samples had to be validated using two approaches before proceeding with the statistical analysis: 1) the distribution of the quantitative data had to be close to the normal distribution and centred around the same value over the different samples (Appendix 5.2), and 2) the samples had to have a good general correlation of the LFQ intensities (Appendix 5.3). Moreover, the identified proteins had to pass a selection criterion in order to merit for statistical analysis: 1) they had to have a minimum number of 2 unique peptides in order to get a LFQ value, and 2) the LFQ values needed not to be 0 in three out of four independent replicates for at least one experimental condition. Therefore, out of the 34 identified *Plasmodium* proteins, there were 8 differentially identified proteins and 3 comparably identified proteins that proceeded for further statistical analysis (Table 5.4).

Table 5. 2: Table showing the 34 identified *Plasmodium* proteins

PlasmoDB identifier	Product description	Molecular weight (kDa)	Protein length	Signal peptide	Trans-membrane Helix prediction	Predicted function
PF3D7_1324900	Lactate dehydrogenase	34.107	316	yes	yes	carbohydrate metabolic process
PF3D7_0929400	High molecular weight rhoptry protein 2	162.663	1378	yes	no	entry into host cell
PF3D7_0905400	High molecular weight rhoptry protein 3	104.854	897	yes	no	entry into host cell, host cell surface receptor binding
PF3D7_0731500	Erythrocyte binding antigen 175	174.586	1502	yes	yes	entry into host cell; host cell surface receptor binding
PF3D7_1301600	Erythrocyte binding antigen 140	140.594	1210	yes	yes	host cell surface receptor binding
ENO	Enolase	50	446			entry into host cell
PF3D7_1410400	Rhoptry-associated protein 1	90.052	782	yes	no	protein binding
PF3D7_0501600	Rhoptry-associated protein 2	46.738	398	yes	no	entry into host cell; protein binding
PF3D7_0302500	Cytoadherence linked asexual protein	167.240	1417	yes	no	cytoadherence to microvasculature, mediated by symbiont protein
PF3D7_1027300	Peroxiredoxin	43.929	393	no	no	entry into host cell & antioxidant activity
PCYB_112350	Elongation factor 1	48.011	434	no	no	translational elongation
PF3D7_0818900	Heat shock protein 70;70kDa	73.914	677	no	no	response to stress; ATPase activity

PF3D7_0917900	Heat shock protein 70;78kDa	72.386	652	yes	no	response to drug
PF3D7_0626800	Pyruvate kinase	55.660	511	no	no	carbohydrate metabolic process
PF3D7_0216400	Vacuolar protein sorting associated protein 45	86.255	722	no	no	vesicle docking involved in exocytosis
PF3D7_1311800	M1-family alanyl aminopeptidase	126.061	1085	no	yes	Proteolysis; response to drug
PF3D7_1029600	Adenosine deaminase	42.465	367	no	no	purine ribonucleoside monophosphate biosynthetic process; deaminase activity
PF3D7_1012400	Hypoxanthine-guanine phosphoribosyl Transferase	26.362	231	no	no	purine ribonucleoside salvage; hypoxanthine phosphoribosyltransferase activity
PF3D7_1451100	Elongation factor 2	93.521	832	no	no	translational elongation
PF3D7_1406500	WD repeat-containing protein 65	181.532	1527	no	no	cytoskeletal protein binding
PF14_0425	Fructose-bisphosphate aldolase	40.104	369	no	no	carbohydrate metabolic process; cytoskeleton organisation
PF3D7_1462800	Glyceraldehyde 3-phosphate dehydrogenase	36.635	337	no	no	carbohydrate metabolic process
PKH_093790	Myosin heavy chain subunit	199.670	1718	no	no	Myosin complex
PY07151	Uncharacterized protein	81.098	690	no	no	no data available
PVX_089505	Suppressor of Ras1 3-9	30.220	262	no	no	protein domain specific binding
PVX_123890	Uncharacterized protein	137.424	1265	no	no	chromatin binding; DNA binding

PVX_081792	Uncharacterized protein	576.792	5031	no	no	microtubule-based movement; ATPase activity
C922_03767	CTP synthase	95.801	850	no	no	pyrimidine nucleotide biosynthetic process
C922_03524	Uncharacterized protein	398.898	3500	no	no	Protein binding
PCYB_147060	Actin	39.931	356	no	no	no data available
PCYB_142080	ATP synthase subunit gamma	31.331	274	no	no	ATP synthesis coupled proton transport
PCYB_053170	Uncharacterized protein	660.712	5758	no	no	no data available
PY02078	Uncharacterized protein	79.631	674	no	yes	no data available
PBANKA_134200	SNARE protein	44.948	416	no	yes	no data available

KEY:

orange differentially identified across the experimental groups

green comparably identified across the experimental groups

Table 5. 3: Table showing number of razor + unique peptides and Label free quantification values (LFQ) of the 34 identified *Plasmodium* proteins

PlasmoDB identifier	Product description	Razor + unique peptides				Label Free Quantification (LFQ) Log2 values			
		uEs_used	uEs_fresh	Ring culture _shaved	Ring culture _mock shaved	uEs_used	uEs_fresh	Ring culture _shaved	Ring culture _mock shaved
PF3D7_1324900	Lactate dehydrogenase	0,3,3,3	0,0,0,0	3,3,3,3	0,1,1,0	0,22.9, 23.9,24.3	0,0,0,0	23.8,23.5 24.1,25.3	0,0,0,0
PF3D7_0929400	High molecular weight rhoptry protein 2	1,3,0,0	0,0,0,0	4,3,2,8	1,0,0,1	0,0,0,0	0,0,0,0	25,23.7, 23.8,24.2	0,0,0,0
PF3D7_0905400	High molecular weight rhoptry protein 3	0,2,0,1	0,0,0,0	4,3,2,5	2,1,1,0	0,21.7,0,0	0,0,0,0	24.2,22.4, 22.7,23.4	23.9,0,0,0
PF3D7_0731500	Erythrocyte binding antigen 175	9,15,10,9	0,0,0,0	4,9,7,10	3,5,4,5	26.8,26.3, 26.3, 25.9	0,0,0,0	22.5,25, 23.8,25.3	23.1,23.6, 24,24.9
PF3D7_1301600	Erythrocyte binding antigen 140	8,11,7,7	0,0,0,0	3,9,6,12	1,1,0,1	26.1,26.3 26.1,26.4	0,0,0,0	24.4,25.6 25.4,26.1	0,0,0,0
ENO	Enolase	0,0,0,0	0,0,0,0	3,5,3,5	0,0,0,0	0,0,0,0	0,0,0,0	23,22.8, 22.6,24.8	0,0,0,0
PF3D7_1410400	Rhoptry-associated protein 1	0,1,0,0	0,0,0,0	0,1,0,2	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,21.5	0,0,0,0
PF3D7_0501600	Rhoptry-associated protein 2	1,1,1,1	0,0,0,0	0,1,1,2	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,22.7	0,0,0,0
PF3D7_0302500	Cytoadherence linked asexual protein	0,0,0,0	0,0,0,0	3,1,1,1	1,0,0,0	0,0,0,0	0,0,0,0	24.5,0,0,0	0,0,0,0
PF3D7_1027300	Peroxiredoxin	0,0,0,0	0,0,0,0	0,2,2,4	0,0,0,0	0,0,0,0	0,0,0,0	0,20.9,0, 23	0,0,0,0
PCYB_112350	Elongation factor 1	1,2,1,1,	0,0,0,0	1,2,2,4	0,0,1,1	0,23.8,0,0	0,0,0,0	0,24, 24.7,25.1	0,0,0,0

PF3D7_0818900	Heat shock protein 70;70kDa	0,0,0,0	0,0,0,0	3,0,2,3	0,1,0,0	0,0,0,0	0,0,0,0	23.6,0, 23.7,24	0,0,0,0
PF3D7_0917900	Heat shock protein 70;78kDa	3,3,2,2	3,2,3,3	5,6,6,9	3,4,4,2	27.8,27.7, 27.1,27	27.8,27, 27.7,27.3	28.3,28.1, 27.7,27	28.7,28.6, 28.4,27.4
PF3D7_0626800	Pyruvate kinase	0,0,0,0	0,0,0,0	0,0,0,2	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,21.6	0,0,0,0
PF3D7_0216400	Vacuolar protein sorting-associated protein 45	1,1,1,1	1,1,1,1	1,1,1,1	1,1,1,1	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,26.8
PF3D7_1311800	M1-family alanyl aminopeptidase	0,0,0,0	0,0,0,0	0,0,0,1	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,20.8	0,0,0,0
PF3D7_1029600	Adenosine deaminase	0,0,0,0	0,0,0,0	0,1,1,1	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,21	0,0,0,0
PF3D7_1012400	Hypoxanthine-guanine phosphoribosyl Transferase	0,0,0,0	0,0,0,0	0,0,0,2	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,21.7	0,0,0,0
PF3D7_1451100	Elongation factor 2	1,1,2,1	0,0,0,0	0,0,0,2	0,1,1,0	0,0,0,0	0,0,0,0	0,0,0,22.1	0,0,0,0
PF3D7_1406500	WD repeat-containing protein 65	1,1,1,1	1,1,1,1	1,1,1,1	1,1,1,1	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,26.8
PF14_0425	Fructose-bisphosphate aldolase	0,0,0,0	0,0,0,0	0,0,0,1	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,22.5	0,0,0,0
PF3D7_1462800	Glyceraldehyde 3-phosphate dehydrogenase	1,2,2,1	1,0,1,1	1,1,2,4	0,1,0,0	0,0,0,0	0,0,0,0	0,0,27.6,0	0,0,0,0
PKH_093790	Myosin heavy chain subunit	1,1,1,1	1,1,1,1	1,1,1,1	1,1,1,1	25.6,25, 25.8,26	26,0, 26.3,0	25.4,25.1, 26,25.5	26.1,26.3, 26.2,25.6
PY07151	Uncharacterized protein	1,1,1,1	1,1,1,1	1,1,1,1	1,1,1,1	28.6,28.4, 0,28.6	0,28.7, 29,0	29,0, 0,28.7	0,0, 0,28.8

PVX_089505	Suppressor of Ras1 3-9	0,0,0,0	0,0,0,0	0,0,0,1	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,21.5	0,0,0,0
PVX_123890	Uncharacterized protein	1,0,1,0	1,1,1,1	1,0,1,0	0,1,1,1	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,23
PVX_081792	Uncharacterized protein	0,0,0,0	0,0,1,1	0,1,0,0	0,0,1,1	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0
C922_03767	CTP synthase	1,1,1,1	1,1,1,1	1,1,1,1	1,1,1,1	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,24.3
C922_03524	Uncharacterized protein	1,0,0,1	0,0,0,0	1,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0
PCYB_147060	Actin	0,1,0,1	0,0,0,0	0,1,0,0,	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,22.3	0,0,0,0
PCYB_142080	ATP synthase subunit gamma	1,0,0,1	0,0,0,0	1,1,0,0	0,1,1,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,23.0,0
PCYB_053170	Uncharacterized protein	0,0,1,1	0,0,0,0	0,0,0,0	0,0,0,1	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,22.5
PY02078	Uncharacterized protein	0,0,1,1	1,1,1,1	0,0,1,1	0,1,1,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,24.3,0
PBANKA_134200	SNARE protein	0,0,0,0	0,0,0,0	1,1,0,0	0,1,1,1	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,27.9

KEY:

orange differentially identified across the experimental groups green comparably identified across the experimental groups

Table 5. 4: Table showing list of *Plasmodium* proteins that passed the selection criterion for further statistical analysis

PlasmoDB identifier	Product description	Razor + unique peptides				Label Free Quantification (LFQ) Log2 values			
		uEs_used	uEs_fresh	Ring culture _shaved	Ring culture _mock shaved	uEs_used	uEs_fresh	Ring culture _shaved	Ring culture _mock shaved
PF3D7_1324900	Lactate dehydrogenase	0,3,3,3	0,0,0,0	3,3,3,3	0,1,1,0	0,22.9, 23.9,24.3	0,0,0,0	23.8,23.5 24.1,25.3	0,0,0,0
PF3D7_0929400	High molecular weight rhoptry protein 2	1,3,0,0	0,0,0,0	4,3,2,8	1,0,0,1	0,0,0,0	0,0,0,0	25,23.7, 23.8,24.2	0,0,0,0
PF3D7_0905400	High molecular weight rhoptry protein 3	0,2,0,1	0,0,0,0	4,3,2,5	2,1,1,0	0,21.7,0,0	0,0,0,0	24.2,22.4, 22.7,23.4	23.9,0,0,0
PF3D7_0731500	Erythrocyte binding antigen 175	9,15,10,9	0,0,0,0	4,9,7,10	3,5,4,5	26.8,26.3, 26.3, 25.9	0,0,0,0	22.5,25, 23.8,25.3	23.1,23.6, 24,24.9
PF3D7_1301600	Erythrocyte binding antigen 140	8,11,7,7	0,0,0,0	3,9,6,12	1,1,0,1	26.1,26.3 26.1,26.4	0,0,0,0	24.4,25.6 25.4,26.1	0,0,0,0
ENO	Enolase	0,0,0,0	0,0,0,0	3,5,3,5	0,0,0,0	0,0,0,0	0,0,0,0	23,22.8, 22.6,24.8	0,0,0,0
PCYB_112350	Elongation factor 1	1,2,1,1,	0,0,0,0	1,2,2,4	0,0,1,1	0,23.8, 0,0	0,0,0,0	0,24, 24.7,25.1	0,0,0,0
PF3D7_0818900	Heat shock protein 70;70kDa	0,0,0,0	0,0,0,0	3,0,2,3	0,1,0,0	0,0,0,0	0,0,0,0	23.6,0, 23.7,24	0,0,0,0
PF3D7_0917900	Heat shock protein 70;78kDa	3,3,2,2	3,2,3,3	5,6,6,9	3,4,4,2	27.8,27.7, 27.1,27	27.8,27, 27.7,27.3	28.3,28.1, 27.7,27	28.7,28.6, 28.4,27.4
PKH_093790	Myosin heavy chain subunit	1,1,1,1	1,1,1,1	1,1,1,1	1,1,1,1	25.6,25, 25.8,26	26,0, 26.3,0	25.4,25.1, 26,25.5	26.1,26.3, 26.2,25.6
PY07151	Uncharacterized protein	1,1,1,1	1,1,1,1	1,1,1,1	1,1,1,1	28.6,28.4, 0,28.6	0,28.7, 29,0	29,0, 0,28.7	0,0, 0,28.8

NOTE:

To pass the selection criterion, the proteins had to have:

- a minimum of 2 unique peptides in order to get a LFQ value.
- LFQ values (not 0) in three out of four independent replicates for at least one experimental condition.

KEY:

orange differentially identified across the experimental groups

green comparably identified across the experimental groups

The 11 selected *Plasmodium* proteins were displayed using a principle component analysis (PCA) and a heatmap (Figure 5.3 and Figure 5.4 respectively). The PCA reduced the dimensionality of the multivariable data by finding the best projection to represent the LFQ intensity variance in between the samples in two dimensions giving an overview about the similarity of the samples and the general discrimination complexity. The samples obtained from uEs in used media (used_01-04) show some similarity to samples obtained from trypsin-shaved ring culture (shaved_09-12) because they tend to cluster together, and samples obtained from uEs in fresh media (fresh_05-08) show some similarity to samples obtained from mock-shaved ring culture (mock_13-16) which also tend to cluster together (Figure 5.3). However when we considered all the selected human and *Plasmodium* proteins together the PCA analysis showed that the shaved samples; uEs in used media (used_01-04), uEs in fresh media (fresh_05-08) and trypsin-shaved ring culture (shaved_09-12) showed some similarity by clustering together and away from the mock-shaved ring culture (mock_13-16) as shown in Appendix 5.4.

The heatmaps was generated using Log-2 LFQ values that had been Z-score normalized and hierarchical clustering was performed on row and columns using the Euclidean distance and ward.D2 (the default parameters on the Z-scored LFQ values were used to calculate the Euclidean distance and the branch length which represents the average distance to the next sample). It allowed global visualization of the data in order to identify clusters of proteins and/or samples with consistent behaviour and gave an overall view of relative protein LFQ intensities across the samples. Generation of heatmap using only the selected *Plasmodium* proteins, the samples clustered well based on their experimental conditions and the 3 comparably identified Plasmodium proteins had similar protein LFQ intensities across the samples in the different experimental conditions whereas the 8 differentially identified *Plasmodium* proteins were relatively upregulated in the trypsin-shaved ring culture and uEs in used media and absent/down regulated in uEs in fresh media and mock-shaved ring culture. (Figure 5.4). Moreover, generation of heatmap using all the selected human and *Plasmodium* proteins, the samples clustered well based on their experimental conditions as observed previously with the PCA analysis where the mock-shaved ring culture was clearly discriminated from the other three groups (uEs in used media, uEs in fresh media and trypsin-shaved ring culture) which clustered fairly well within their respective groups (Appendix 5.5).

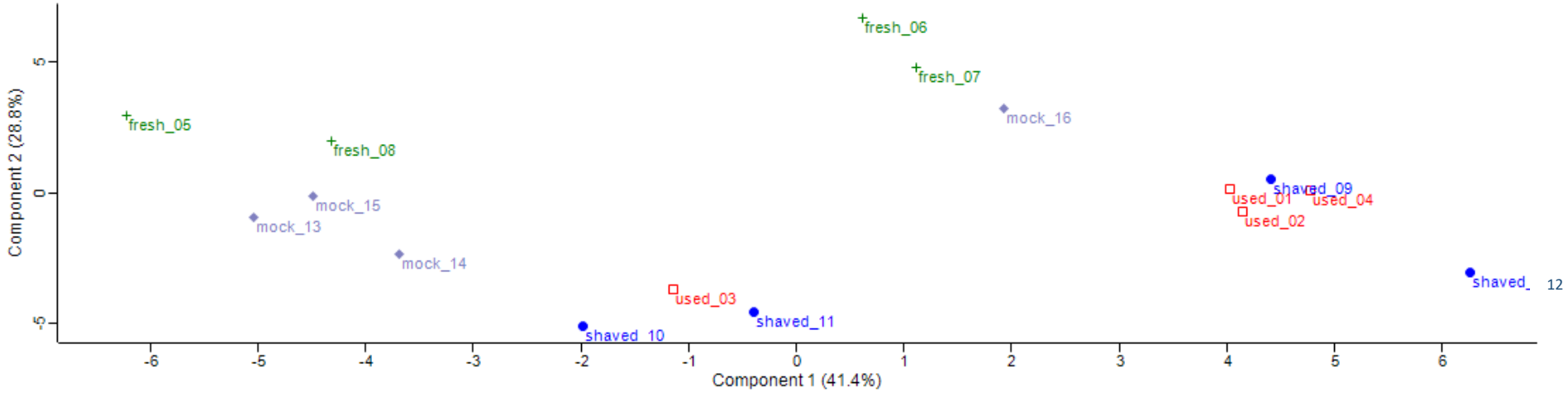

Figure 5. 3: Principle component analysis using only the selected *Plasmodium* proteins.

The samples obtained from uEs in used media (used_01-04) show some similarity to samples obtained from trypsin-shaved ring culture (shaved_09-12) because they tend to cluster together, and samples obtained from uEs in fresh media (fresh_05-08) show some similarity to samples obtained from mock-shaved ring culture (mock_13-16) which also tend to cluster together. The proteins used for the principal component analysis were those that passed the selection criterion for statistical analysis.

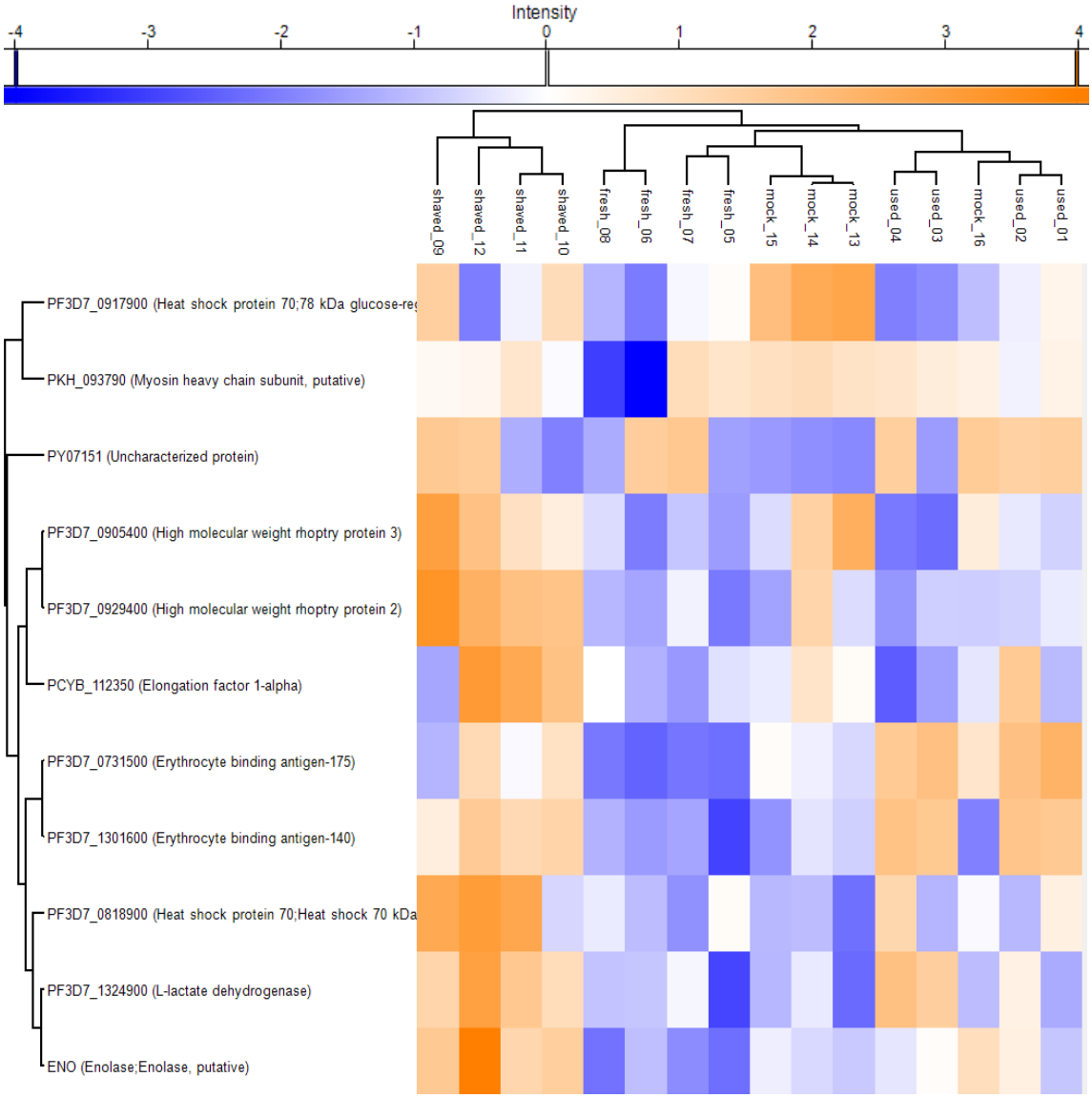

Figure 5. 4: Heatmap generated using only the selected *Plasmodium* proteins. The heatmap was generated using Log-2 LFQ values that had been Z-score normalized and hierarchical clustering was performed on row and columns using the Euclidean distance and ward.D2. Quantitative values have been colour coded according to the colour key shown on the top. Protein are labelled by their gene-name and accession number. Sample names are shown on the top of the plot. The samples clustered well based on their experimental conditions, and the 3 comparably identified Plasmodium proteins had similar protein LFQ intensities across the samples in the different experimental conditions whereas the 8 differentially identified *Plasmodium* proteins were relatively upregulated in the trypsin-shaved ring culture and uEs in used media and down regulated in uEs in fresh media and mock-shaved ring culture. The samples were supernatant obtained after shaving the different cultures: used_01-04 from uEs in used media; fresh_05-08 from uEs in fresh media; shaved_09-12 from trypsin-shaved ring culture and mock_13-16 from mock-shaved ring culture. The proteins used to generate the heatmap were those that passed the selection criterion for statistical analysis.

5.7.3. Identification of significantly upregulated *Plasmodium* proteins between the different experimental conditions

The mean LFQ values of the selected human and *Plasmodium* proteins were compared between all the possible combinations of the experimental conditions using student's t-test in order to determine how each identified protein differs between any two possible experimental groups. The obtained p-values were used to generate volcano plots so as to determine the significantly upregulated protein(s) between any two experimental conditions. There were no significantly upregulated *Plasmodium* proteins identified in uEs in fresh media culture when compared to uEs in used media or trypsin-shaved ring culture, and there were also no significantly upregulated *Plasmodium* proteins identified in mock-shaved ring culture when compared to trypsin-shaved ring culture. Thus, these findings validated the assay because they passed well as controls. There were significantly upregulated *Plasmodium* proteins identified in trypsin-shaved ring culture as compared to mock-shaved ring culture and uEs in fresh media, and there were also significantly upregulated *Plasmodium* proteins identified in uEs in used media as compared to uEs in fresh media. Moreover, there were two rhoptry associated proteins (PF3D7_0929400 - High molecular weight rhoptry protein 2 and PF3D7_0905400 - High molecular weight rhoptry protein 3) that were significantly upregulated in trypsin-shaved ring culture compared to uEs in used media (Figure 5.5). Similar findings we observed when generating volcano plots using only selected *Plasmodium* proteins (Appendix 5.6).

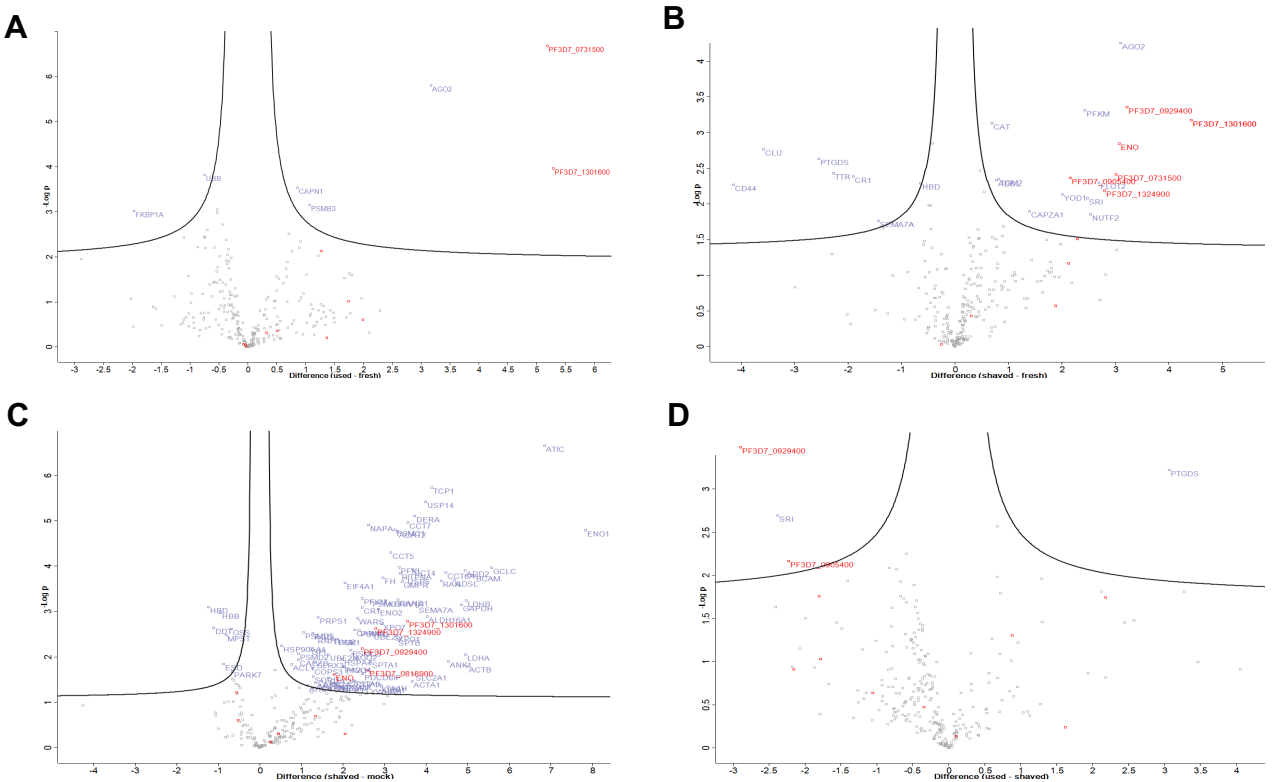

Figure 5. 5: Volcano plots generated using all the selected human and *Plasmodium* proteins. The volcano plots were generate using the -Log p values derived from the p-values obtained during Student's *t*-test analysis where the mean LFQ values of each of the selected human and *Plasmodium* proteins were compared between all the possible combinations of the experimental conditions. There were significantly upregulated Plasmodium proteins identified in (A) uEs in used media (B) trypsin-shaved ring culture when compared to uEs in fresh media culture. Additionally, (C) there were also significantly upregulated Plasmodium proteins identified in trypsin-shaved ring culture when compared to mock-shaved ring culture. (D) There were two rhoptry associated proteins that were significantly upregulated in trypsin-shaved ring culture compared to uEs in used media. The significantly upregulated proteins denoted in blue and red were from human and *Plasmodium* respectively, whereas the proteins in grey were the human proteins that were not significantly upregulated between any given two experimental conditions. The gene names of the various accession numbers of the *Plasmodium* hits are as follows; PF3D7_0929400 - Lactate dehydrogenase, PF3D7_0929400 - High molecular weight rhoptry protein 2, PF3D7_0905400 - High molecular weight rhoptry protein 3, PF3D7_0731500 - Erythrocyte binding antigen 175, PF3D7_1301600 - Erythrocyte binding antigen 140, ENO - Enolase, PCYB_112350 - Elongation factor 1, PF3D7_0818900 - Heat shock protein 70;70kDa, PF3D7_0917900 - Heat shock protein 70;78kDa, PKH_093790 - Myosin heavy chain subunit and PY07151 - Uncharacterized protein. The proteins used to generate the volcano plots were those that passed the selection criterion for statistical analysis.Key: red - significantly upregulated *Plasmodium* proteins; blue - significantly upregulated human proteins and grey - proteins with no change between any given two experimental conditions.

5.8. Discussion

Evaluation of the success of the surface proteomics approach

This being the first surface proteomics study conducted on ring culture cells, it was important to assess the successful establishment of the assay in our laboratory. Findings from the following control strategies validated the assay: 1) the mock-shaved ring culture category had the least number of identified proteins and peptides (Figure 5.1 and Appendix 5.5), 2) the samples clustered well depending on whether they were obtained from experimental conditions that contained parasite material or not (Figure 5.3 and Appendix 5.4), 3) there were no significantly upregulated *Plasmodium* proteins identified in uEs in fresh media culture when compared to uEs in used media or trypsin-shaved ring culture (Figure 5.5), and 4) there were also no significantly upregulated *Plasmodium* proteins identified in mock-shaved ring culture when compared to trypsin-shaved ring culture (Figure 5.5).

Differentially identified *Plasmodium* proteins

The differentially identified parasite proteins in the study were of utmost interest because they were relatively upregulated in the uEs in used media and/or trypsin-shaved ring culture but absent/down regulated in uEs in fresh media and mock-shaved ring culture. Moreover, most of them have already been identified in previous different studies as possible surface parasite proteins discharged on the early intracellular asexual blood stage from merozoite organelles during invasion, and some have been proposed to bind to uEs in culture and in used media.

The components of the high molecular weight rhoptry complex, RhopH2 and RhopH3, were upregulated in trypsin-shaved ring culture, whereas the first member of the complex, cytoadherence linked asexual protein (CLAG3.1) also known as RhopH1, was interestingly identified only in the trypsin shaved ring culture but didn't merit the criteria for further statistical analysis (Figure 5.3). The RhopH3 was first suggested to be inserted into the membrane of the host erythrocyte during invasion where immunofluorescence findings showed that the rhoptry protein was discharged from the merozoite and spread around the surface of the erythrocyte upon contact with the merozoite (Sam-Yellowe et al., 1988). Moreover, the entire high molecular weight rhoptry complex was found to bind to newly infected erythrocyte membranes ghost (Sam-Yellowe and Perkins, 1991) and remained intact for at least 18 hours post-invasion (Lustigman et al., 1988). Recent findings have clarified that RhopH3 performs

a major role in invasion (Ito et al., 2017), and the entire RhopH complex contributes to the new permeability pathway (NPP) which serves as a nutrient channel important for parasite growth and proliferation (Counihan et al., 2017, Ito et al., 2017).

The members of the low molecular weight rhoptry complex, rhoptry-associated proteins 1 and 2 (RAP1 and RAP2 respectively), were upregulated in trypsin-shaved ring culture and identified in both trypsin-shaved ring culture and uEs in used media (Figure 5.3). However, they didn't meet the criteria for further statistical analysis. The RAP2, was first identified as ring-surface protein 2 (RSP2) by immunoprecipitation assay of surface-iodinated rIEs extracts (Pouvelle et al., 2000). Other immunofluorescence studies confirmed that RSP2 is indeed a rhoptry-derived protein and is discharged on the membrane of a newly invaded erythrocyte during contact with merozoites (Douki et al., 2003), and RAP1 and RAP2/RSP2 were described to positively stain the membrane of uEs in culture (Sterkers et al., 2007). Recently, RAP2 has been shown to be a ligand of a receptor on the erythrocyte membrane, basigin, whose interaction inhibition completely abolished parasite invasion (Zhang et al., 2018). This confirms why RAP2 was considered refractory to deletion (Sterkers et al., 2007).

The merozoite micronemal proteins, erythrocyte binding antigens 140 and 175 (EBA140 and EBA 175 respectively), were upregulated in both trypsin-shaved ring culture and uEs in used media (Figure 5.3). The EBA175 protein was first suggested to bind to erythrocytes after incubation with supernatant fluids of a cultured human malaria parasite containing a 175KDa merozoite binding antigen which was shown to be depleted after the supernatants were absorbed on normal human erythrocytes (Camus and Hadley, 1985). On the other hand, EBA140 protein was first described to bind to erythrocyte surface by incubating erythrocytes with radiolabelled supernatants from merozoites (Thompson et al., 2001). The parasite ligands, EBA175 and EBA140 bind to specific erythrocyte sialoglycoprotein receptors, glycophorin A (Sim, 1995), and glycophorin C (Thompson et al., 2001) respectively, during merozoite invasion. However, EBA140 has been suggested not to be essential for normal growth and merozoite invasion in *P. falciparum* infection (Thompson et al., 2001). Recently, the interaction of EBA175 and glycophorin A has been shown to be the major invasion and evasion pathway for *P. falciparum* merozoites (Jaskiewicz et al., 2019).

Additionally, another merozoite protein, peroxiredoxin, was upregulated in the trypsin-shaved ring culture and interestingly identified only in the trypsin-shaved ring culture (Figure 5.3). However. it didn't meet the criteria for further statistical analysis. Peroxiredoxin was previously known as merozoite capping protein 1 (MCP1) which was first described to be located at the attachment site between the erythrocyte and the merozoite apical region (Klotz et al., 1989). The immunofluorescence findings from the study showed that MCP1 moved in the plane of the membrane around merozoites during invasion and persisted at the pole in newly invaded erythrocyte and was speculated to be among the components that form the tight junction complex. However, other contradictory findings recently showed that MCP1 was localized to the nucleus and thus it was renamed peroxiredoxin (PfnPrx) and suggested its putative role in the nuclear DNA protection against oxidative stress (Richard et al., 2011). Despite this controversy, an obvious question would be whether peroxiredoxin/MCP1 serves any role in parasite growth because it is polymorphic and has been recently shown to be refractory to deletion (Sanderson and Rayner, 2017).

Finally, one of the highly expressed enzymes associated with multiple subcellular compartments, *P. falciparum* enolase, was upregulated in the trypsin-shaved ring culture and interestingly identified only in the trypsin-shaved ring culture (Figure 5.3). Enolase was previously detected in every stage of the *P. falciparum* life cycle (Bhowmick et al., 2009), and interestingly shown to be located on the cell membrane of the three main invasive stages of the *P. falciparum* parasite: merozoite (Pal-Bhowmick et al., 2007), sporozoite (Bhowmick et al., 2009) and ookinetes (Ghosh et al., 2011). Moreover, *P. falciparum* enolase could be a potential protective antigen because findings from other studies showed that it: was a correlate of natural protective immunity, played a role in the growth of *P. falciparum in vitro* cultures, and conferred partial protection against malaria in mice during vaccination with recombinant *P. falciparum* enolase (Pal-Bhowmick et al., 2007). The moonlighting functions of *P. falciparum* enolase have been recently implicated to be mediated by tryptophan residues present in a pentapeptide insert of the enzyme (Dutta et al., 2017). The findings from my study, identification of enolase as one of the parasite proteins on ring culture cells, is consistent with previous hypothesis which stated that its localization on the merozoite may indicate a possible role in red blood cell invasion (Bhowmick et al., 2009). I therefore speculate that it could be transferred on the

surface of newly infected erythrocytes during invasion, but this could be confirmed by immunofluorescence assay.

Summary

I conducted the first surface proteomics study on ring culture cells and in this one study I identified all the parasite proteins on rIEs and uEs that were previously identified in isolation. My data conclusively shows that the merozoite is the main source of the surface parasite proteins on ring culture cells and uEs in used media. I speculate highly that this proteins could be responsible for the opsonic phagocytosis of ring culture cells observed in the previous chapter, because antibody depletion of one of the differentially identified merozoite protein, EBA175, showed a reduction of the opsonic phagocytosis of ring culture cells similar to competition with the pool of several merozoite recombinant proteins (Appendix 4.4 and Appendix 4.5). Most of these differentially identified proteins are already predicted to be involved in invasion and/or shown to be correlates of protective immunity. However, there are two interesting parasite proteins identified only in the trypsin-shaved ring culture: 1) enolase, whose presence on the surface of newly invaded erythrocytes have not been previously shown, and 2) peroxiredoxin, whose possible role in parasite clearance and protective immunity has not been explored. It is thereby noteworthy for future studies to be conducted as this could inform vaccine development.

Chapter 6

Conclusion and recommendations

6.1. Summary of findings

The blood stage is responsible for the clinical symptoms of *P. falciparum* malaria, possibly due to the exponential parasite growth every 48 hours. However, individuals in malaria endemic areas, maintain low levels of circulating ring-infected erythrocytes (rIEs) during natural asymptomatic infections. The low parasite densities were thought to result from the host's immune defence against the mature asexual blood stages, and the potential contribution of rIEs to parasite clearance had not been explored. The experiments and analyses presented in this thesis were aimed at addressing this knowledge gap by investigating the significance of the clearance of rIEs in protective immunity.

I found out that a proportion of rIEs are comparably retained between lab and field isolates in an artificial spleen, suggesting that this may be a mechanism of parasite clearance that contributes to the low parasite densities observed in malaria endemic areas. Although antibodies had no direct killing effect on rIEs, they bound on the erythrocyte and mediated *in vitro* opsonic phagocytosis. This activity predicted clinical outcome in a malaria challenge study and provides the first evidence that it may be important for protective immunity. Additionally, I discovered parasite proteins of biological relevance on the surface of the ring culture cells which could highly likely be responsible for the proposed role of rIEs in protective immunity. However, the possibility that some of these antigens could be involved in anaemia during malaria infection cannot be ruled out due to the clearance of the tagged uEs by the immune system.

6.2. Recommendations

This study contributes to the understanding of the role of rIEs in protective immunity by correlating an *in vitro* antibody mediated function of rIEs to outcome of *P. falciparum* infection in a CHMI study. However, it will be important to determine the proportion contribution of the rIEs clearance in the overall protective immunity observed in the CHMI study. This can be done by mathematical modelling where all the antibody mediated functions that are being investigated in the CHMI study could be analysed in a model and by use of mathematical concepts help predict the individual contribution

of each function to the total protective immunity. Additionally, in regard to the observed opsonic phagocytosis of uEs in the ring culture, it will be important to investigate its impact on anaemia in the CHMI study. This can be done by correlating the observed clearance of uEs to haemoglobin levels of the individuals during the study once the haematological data are available. This will be essential to inform vaccine development, especially because this study shows evidence of shared targets between the ring culture cells and the merozoite which is one of the most studied invasive form of the parasite. Measures to compensate the possible loss of circulating uEs during malaria infection can be considered during the implementation of potential vaccine candidates that are known to be shared immune targets between the merozoites and the uEs during malaria infection. Furthermore, it would be of prime importance to investigate whether circulating rIEs and uEs during malaria infection are indeed phagocytosed *in vivo*. This can be done by conducting opsonic phagocytosis using peripheral blood samples freshly obtained from individuals in malaria endemic areas. This would strengthen the evidence of the physiological relevance of rIEs in protective immunity demonstrated in this study.

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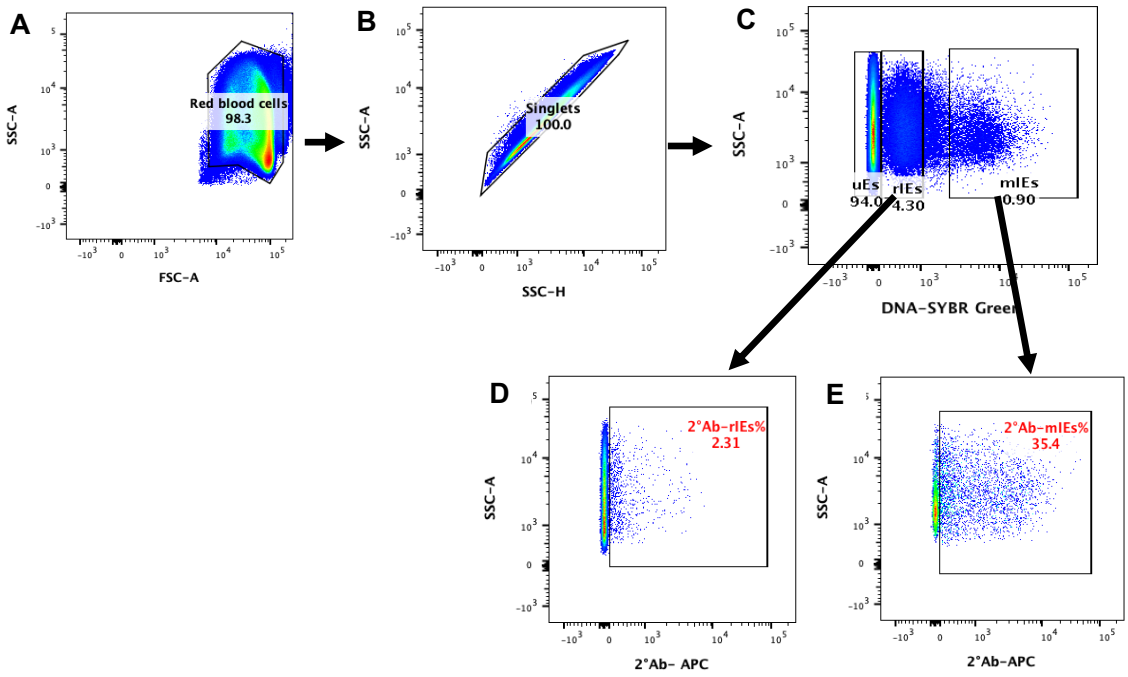
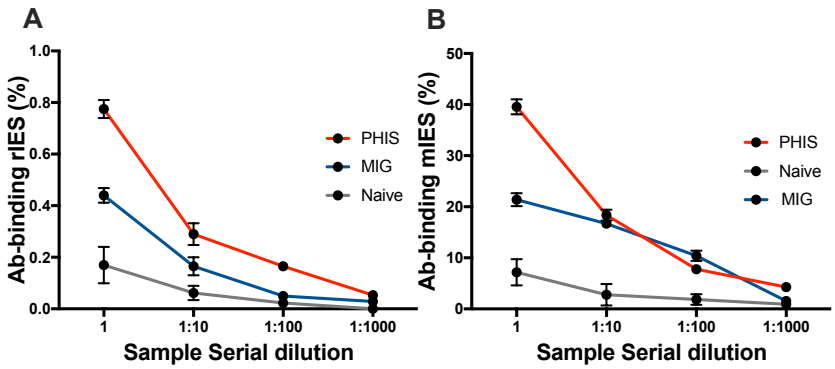
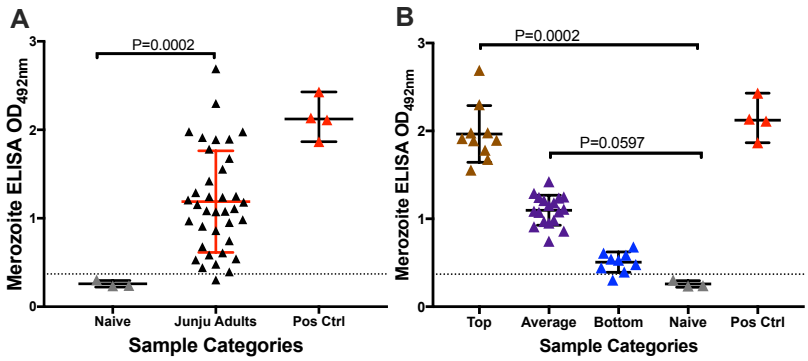


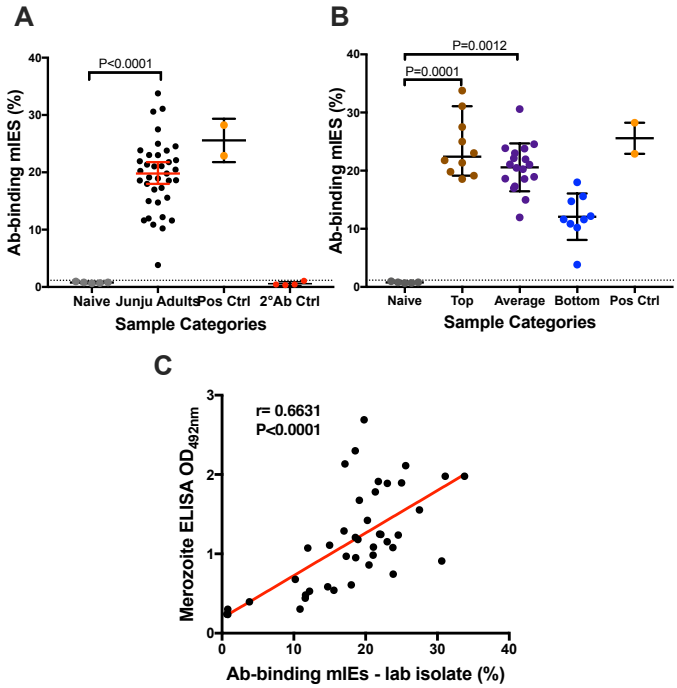
Appendix 3. 1: Flow cytometry gating strategy for antibody binding to ring-infected erythrocytes and mature-infected erythrocytes. (A) The photomultiplier tube PMT voltages of the forward scatter and side scatter were adjusted to ensure that the entire population of interest (red blood cells) was visible within the scatter plot. (B) Only single cells that passed the laser beam in the flow cell were gated. (C) The PMT voltage for the SYBR Green channel was also adjusted using relevant controls to identify the uEs and IEs populations, and further distinguish between the single invaded rIEs and mIEs. The PMT voltage for the APC channel was also adjusted using relevant controls to ensure that the antibody binding to IEs was correctly detected. The percentage of rIEs and mIEs that were detected by antibody in malaria-immune plasma were gated (D) and (E) respectively.



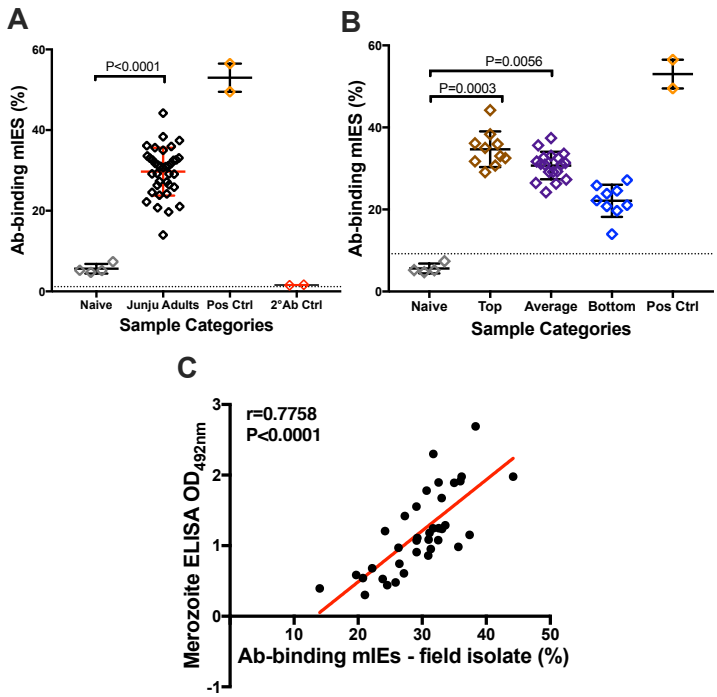
Appendix 3. 2: Antibody binding to FCR3-lab isolate infected erythrocytes is dose dependant. The percentage of antibody binding to (A) rIEs and (B) mIEs, decreased with increase in the dilution of test samples; Pool of hyper immune plasma (PHIS) obtained from Kenyan individuals, Malaria-immune immunoglobulins obtained from Malawian individuals (MIG) and malaria-naïve plasma obtained from German individuals.



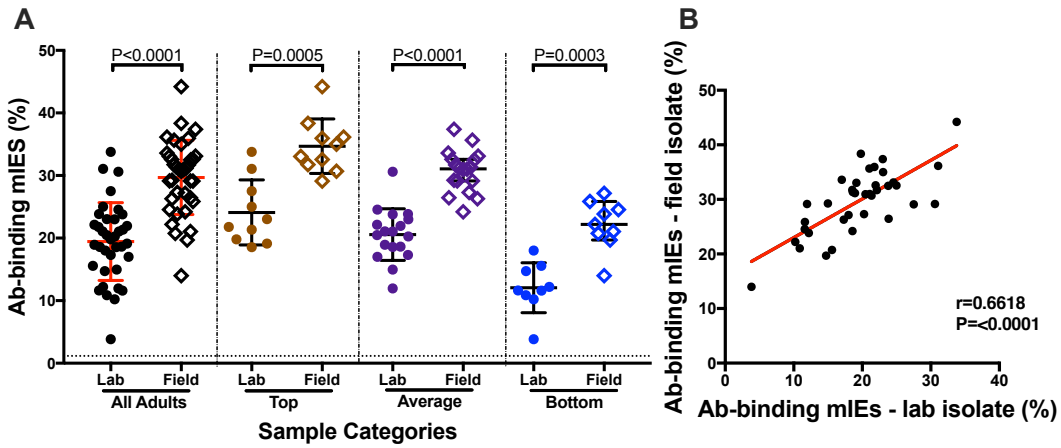
Appendix 3. 3: Antibody response to NF54 merozoites. A) Detection of antibody response to NF54 merozoites using all Junju adult's plasma was determined to be significantly higher (P=0.0002, Mann Whitney test) compared to malaria-naïve plasma. B) The Junju adults were categorized based on their merozoite ELISA OD values; top, average and bottom with (>1.5, 0.7-1,5 and <0.7) OD ranges respectively, and the antibody responses to the merozoites of the different categories of the Junju adults was determined to be significantly higher compared to malaria-naïve plasma (P<0.0001, Kruskal-Wallis test), specifically; the top and average responders (P=0.0002 and P=0.0597 respectively, Dunn's multiple comparison test). The positive control was a pool of hyper immune plasma samples of Junju adults from Kilifi Kenya. The dotted horizontal line is the seropositivity mark = {mean of the antibody binding to rIEs using naïve plasma + 3(STDEV)}. Statistics was calculated using GraphPad PRISM version 7.0.



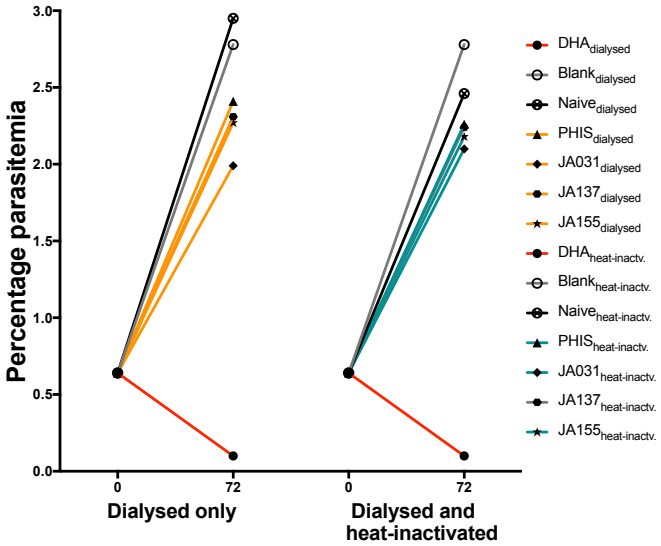
Appendix 3. 4: Antibody binding to FCR3-lab isolate mature-infected erythrocytes. A) Detection of antibody binding using all Junju adult's plasma was determined to be significantly higher (P<0.0001, Mann Whitney test) compared to malaria-naïve plasma, and detection of secondary antibody binding in absence of plasma was negligible indicating a high sound to noise ratio. B) Detection of antibody binding focussing on the different categories of the Junju adults based on the antibody response to merozoites was determined to be significantly higher compared to malaria-naïve plasma (P<0.0001, Kruskal-Wallis test), specifically; the top and average responders (P=0.0001 and P=0.0012 respectively, Dunn's multiple comparison test). C) Antibody binding to mIEs showed a strong positive correlation (spearman r=0.6631; P<0.0001), with antibody response to NF54 merozoites. The positive control was a pool of hyper immune plasma samples of Junju adults from Kilifi Kenya. The dotted horizontal line in graphs A and B is the seropositivity mark = {mean of the antibody binding to rIEs using naïve plasma + 3(STDEV)}. Statistics was calculated using GraphPad PRISM version 7.0.



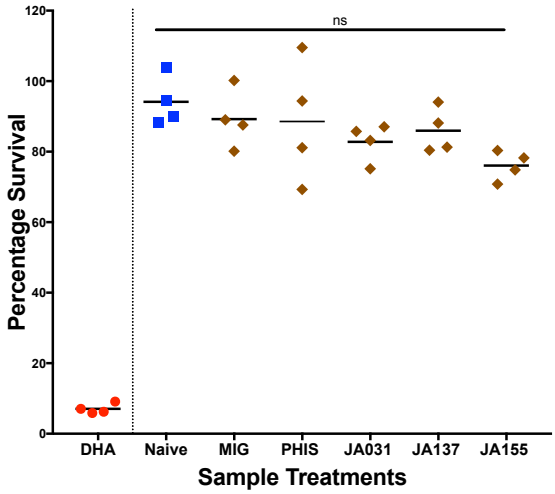
Appendix 3. 5: Antibody binding to field isolate mature-infected erythrocytes obtained from Kilifi. A) Detection of antibody binding using all Junju adult's plasma was determined to be significantly higher (P<0.0001, Mann Whitney test) compared to malaria-naïve plasma, and detection of secondary antibody binding in absence of plasma was negligible indicating a high sound to noise ratio. B) Detection of antibody binding focussing on the different categories of the Junju adults based on the antibody response to merozoites was determined to be significantly higher compared to malaria-naïve plasma (P<0.0001, Kruskal-Wallis test), specifically; the top and average responders (P=0.0001 and P=0.0012 respectively, Dunn's multiple comparison test). C) Antibody binding to mIEs showed a strong positive correlation (spearman r=0.7758; P<0.0001), with antibody response to NF54 merozoites. Positive control was a pool of hyper immune plasma samples of Junju adults from in Kilifi Kenya. The dotted horizontal line in graphs A and B is the seropositivity mark = {mean of the antibody binding to rIEs using naïve plasma + 3(STDEV)}. Statistics was calculated using GraphPad PRISM version 7.0.



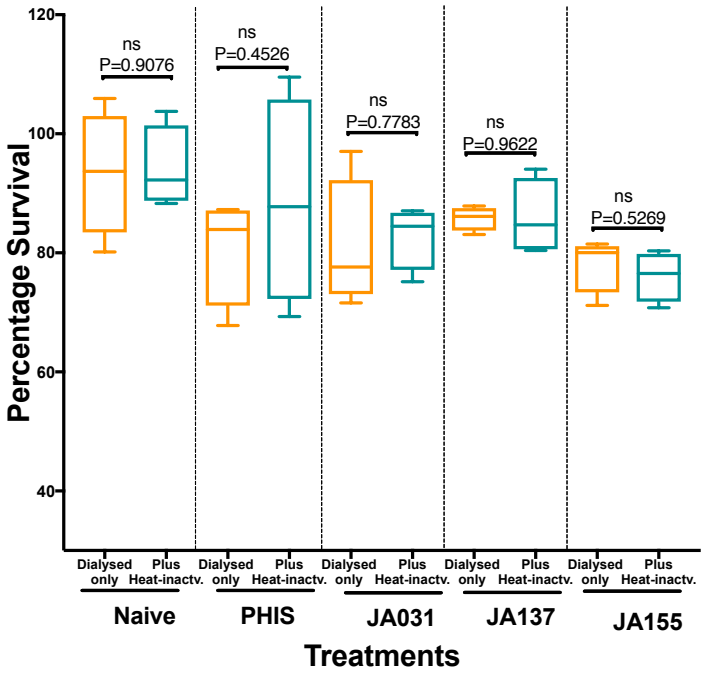
Appendix 3. 6: Comparison of antibody binding between laboratory and field isolate mature-infected erythrocytes. A) The percentage of antibody binding to field rIEs was determined to significantly higher focussing on all Junju adults, top, average and bottom responders (P<0.0001, P=0.0005, P<0.0001 and P=0.0003 respectively, Mann Whitney test) as compared to antibody binding to lab rIEs. B) Antibody binding to field mIEs showed a strong positive correlation (spearman r=0.6618; P<0.0001), with antibody binding to lab mIEs. Statistics was calculated using GraphPad PRISM version 7.0.



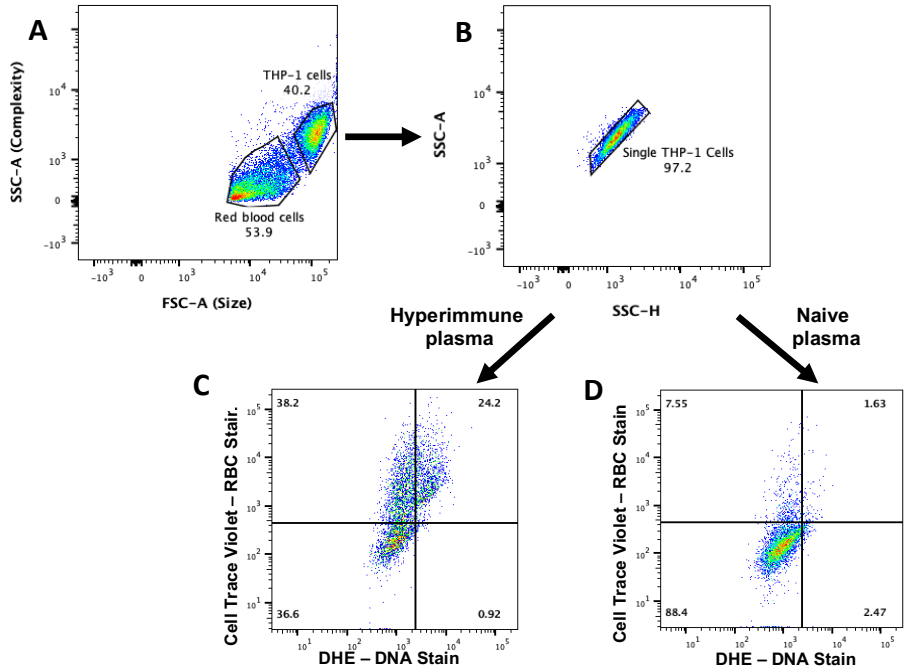
Appendix 3. 7: Effect of dialysed only and dialysed plus heat-inactivated plasma on parasitemia. Exposure of rIEs to dialysed only or dialysed plus heat-inactivated plasma enhanced parasite growth is a similar manner in contrary to exposure to DHA.



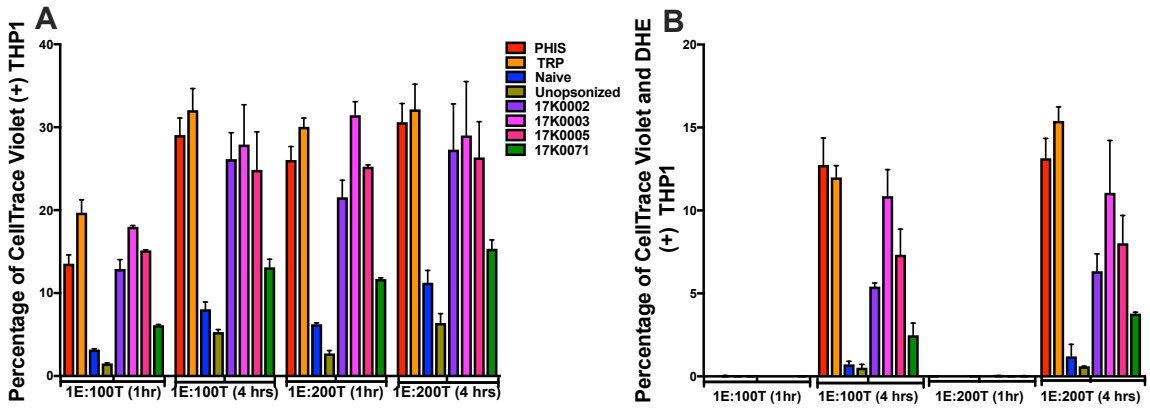
Appendix 3. 8: Effect of dialysed and heat-inactivated immune plasma on ring survival. Percentage survival of rIEs when treated with dihydroartemisinin (DHA, positive control), naïve plasma (negative control), PHIS (pool of hyper immune plasma), MIG (purified malaria immunoglobulins) and randomly selected plasma of malaria-immune adults from Kilifi (JA031, JA137, JA155). The percentage survival rate was comparable when parasites were exposed to naïve plasma, pool of hyper immune serum, purified malaria immunoglobulins and plasma from randomly selected malaria-immune adults from Kilifi, Kenya, (P = 0.2691, one-way ANOVA). Statistics was calculated using GraphPad PRISM version 7.0.



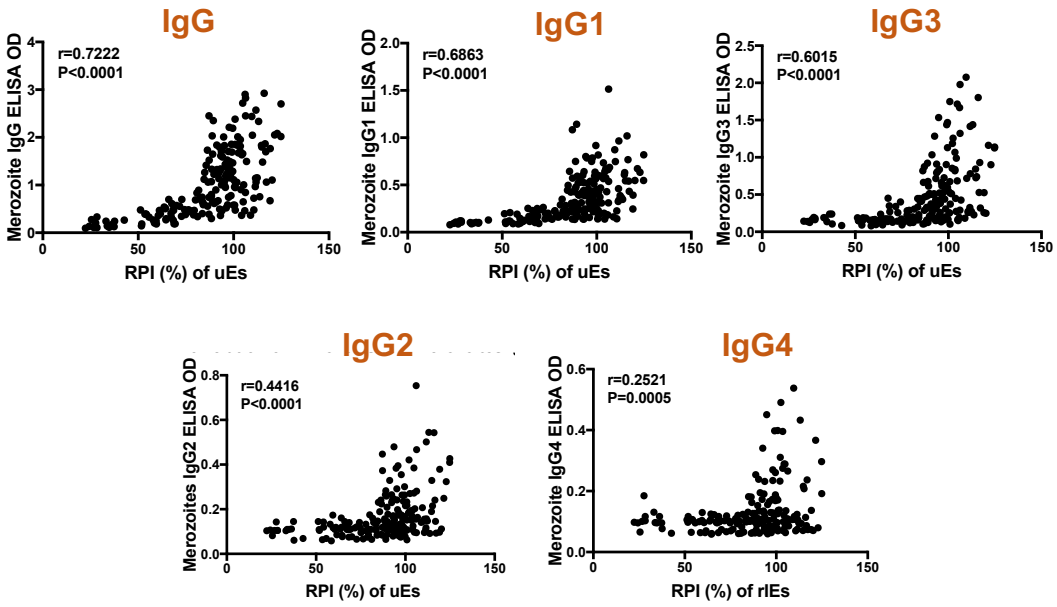
Appendix 3. 9: Comparison of the effect of dialysed only or dialysed plus heat-inactivated immune plasma on ring survival. The parasites survival rates were similar, determined by unpaired t-test, when exposed to the dialysed only or dialysed and heat-inactivated state of the each of the test plasma.



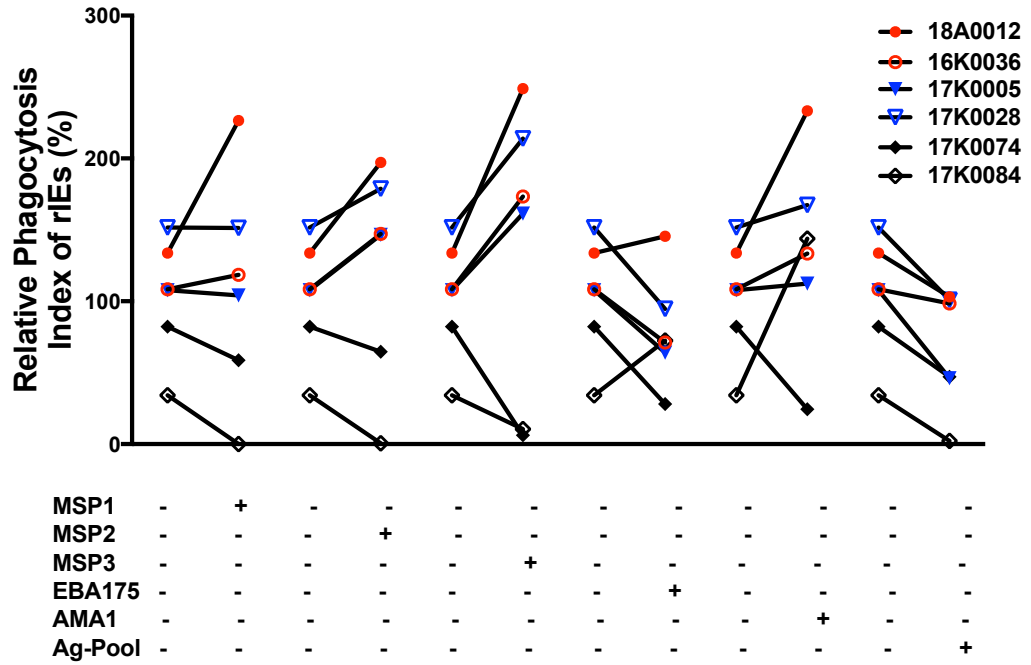
Appendix 4. 1: Flow cytometry gating strategy for opsonic phagocytosis of ring-infected erythrocytes. A) The photomultiplier tube PMT voltages of the forward scatter and side scatter were adjusted to ensure that the population of THP-1 cells and red blood cells were well discriminated based on their difference in size and complexity. B) Only the single cells of our population of interest (THP-1 cells) that passed the laser beam in the flow cell were gated. The PMT voltage of CellTrace Violet and DHE dyes were adjusted using relevant controls in order to identify and correctly discriminate the true population of THP-1 cells that phagocytosed uEs (top right quadrant) from those that phagocytosed rIEs (top left quadrant) after opsonization with (C) hyperimmune and (D) naïve plasma.



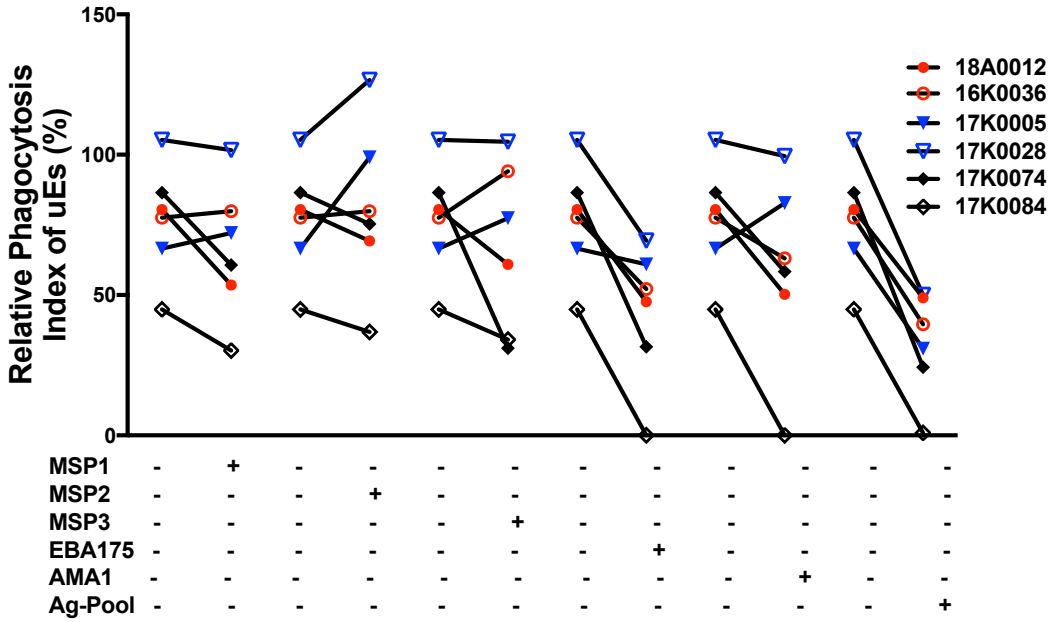
Appendix 4. 2: Optimization of effector to target ratio and phagocytosis duration. A) The effect of different effector to target ratios (1E:100T & 1E:200T) and phagocytosis durations (one & four hours) on the percentage of THP-1 cells that have taken up uEs which are positive for CellTrace dye only, and B) the percentage of THP-1 cell that have taken up rIEs which are positive for both CellTrace Violet and DHE dyes was determined. The plasma samples used during the optimization were: two pools of hyperimmune plasma from Kenya (PHIS and TRP), naïve plasma from German donors, and four randomly selected individuals obtained from the CHMI study (17K0002, 17K0003, 17K0005 AND 17K0071). The effector to target ratio: 1E:200T, and four hours of phagocytosis was considered optimum for a good difference of phagocytosis of rIEs between opsonization of culture with hyperimmune and naïve plasma. The percentage parasitemia of the ring culture used was 8%.



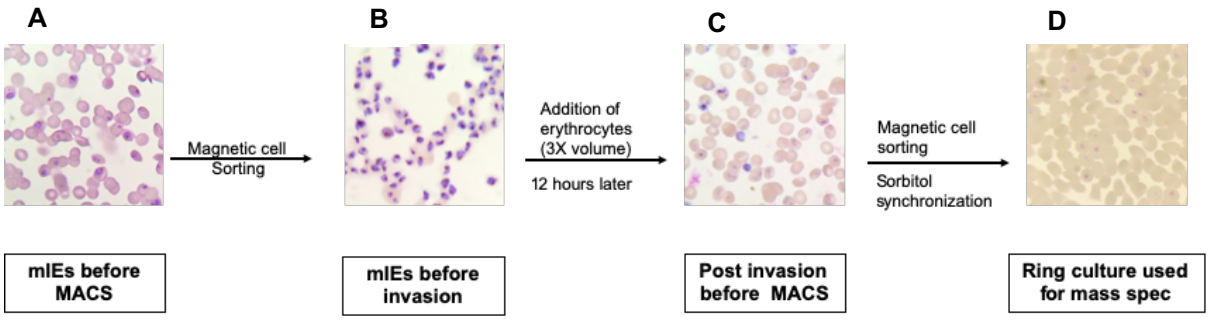
Appendix 4. 3: Correlation of opsonic phagocytosis of uninfected erythrocytes to antibody responses to merozoites. Opsonic phagocytosis of uEs showed; a positive correlation (spearman r=0.7222; P<0.0001) to IgG antibody responses to merozoites, especially IgG1 and IgG3 (spearman r=0.6863; P<0.0001 and spearman r=0.6015; P<0.0001) respectively, but a weak correlation to IgG2 (spearman r=0.4416; P<0.0001) and IgG4 (spearman r=0.2521; P=0.0005). The data was statistically analysed by GraphPad PRISM version 7.0.



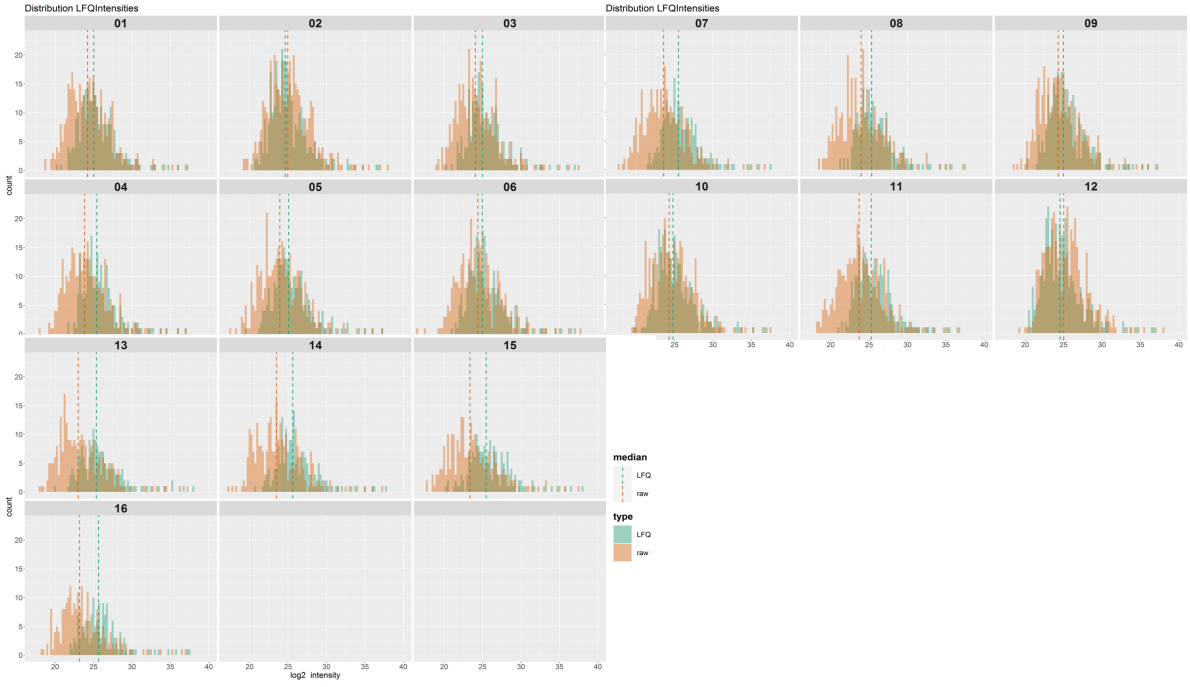
Appendix 4. 4: Merozoite specific antibodies mediate opsonic phagocytosis of ring-infected erythrocytes. Relative phagocytosis index of rIEs using individual plasma samples that were each pre-incubated with individual merozoite recombinant antigens and a pool of the recombinant antigens. The CHMI study plasma samples used for the assay were selected based on their known antibody responses to merozoites (18A0012 & 16K0036 - high; 17K0005 & 17K0028 - mid and 17K0074 & 17K0084 - low). The selection of the merozoite recombinant antigens for competition was guided by their availability in our lab and their potential as vaccine candidates.



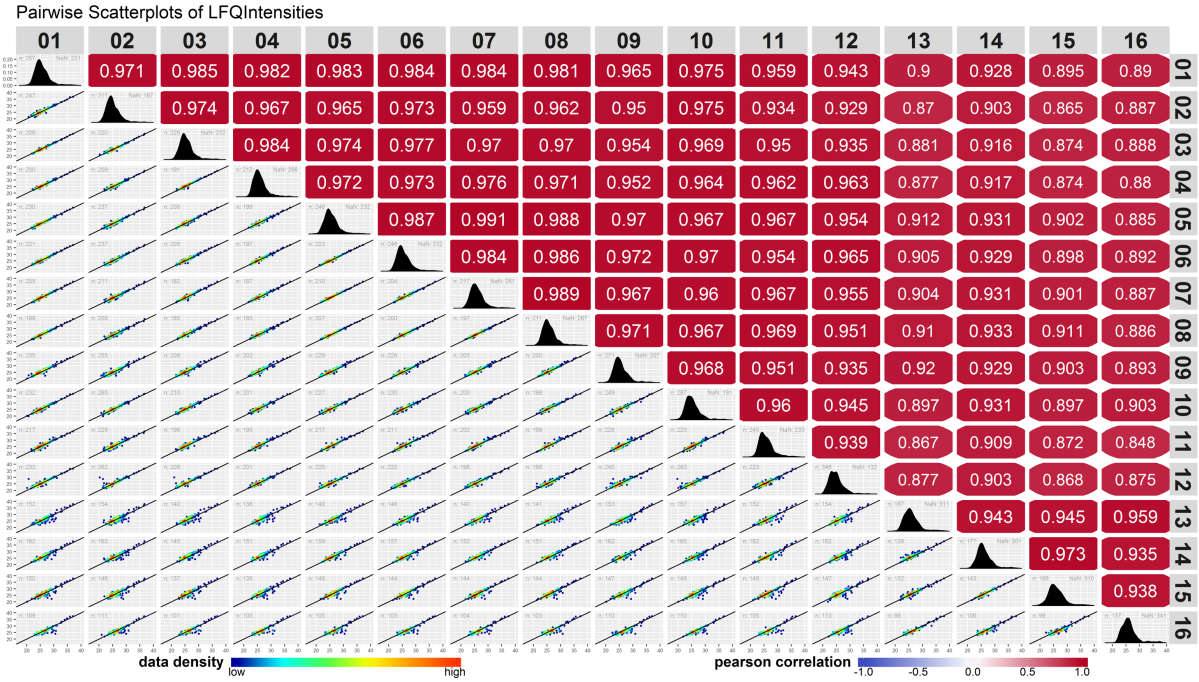
Appendix 4. 5: Merozoite specific antibodies mediate opsonic phagocytosis of uninfected erythrocytes. Relative phagocytosis index of uEs using individual plasma samples that were each pre-incubated with individual merozoite recombinant antigens and a pool of the recombinant antigens. The CHMI study plasma samples used for the assay were selected based on their known antibody responses to merozoites (18A0012 & 16K0036 - high; 17K0005 & 17K0028 - mid and 17K0074 & 17K0084 - low). The selection of the merozoite recombinant antigens for competition was guided by their availability in our lab and their potential as vaccine candidates.



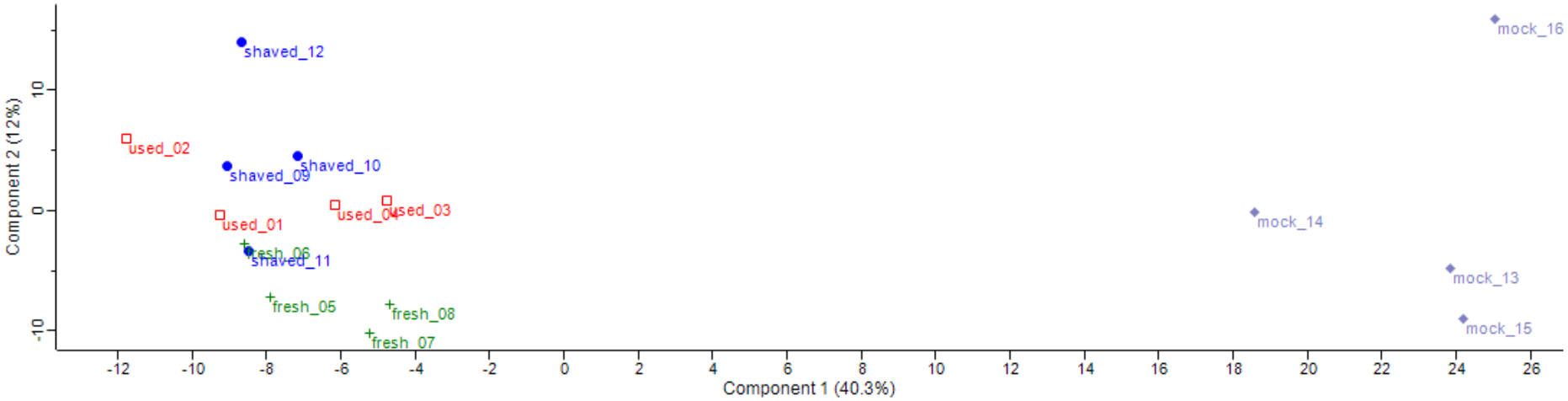
Appendix 5. 1: Experimental flow of how to obtain ring culture for mass spectrometry study. Culture of about 20% mIEs was obtained after previous D-sorbitol synchronization 46 hours apart (A). The mIEs are separated from uEs by magnetic cell sorting to obtain almost 90% pure mIEs (B) and maintained in culture until the mIEs reach about 40 hours post-invasion before adding fresh uEs to allow invasion by the released merozoites after schizont rupture. About 12 hours post invasion (C), magnetic cell sorting followed by sorbitol synchronization was conducted to get rid of mIEs that did not rupture, yielding a ring culture (0-10 hours post-invasion) which was used for mass spectrometry assays (D).



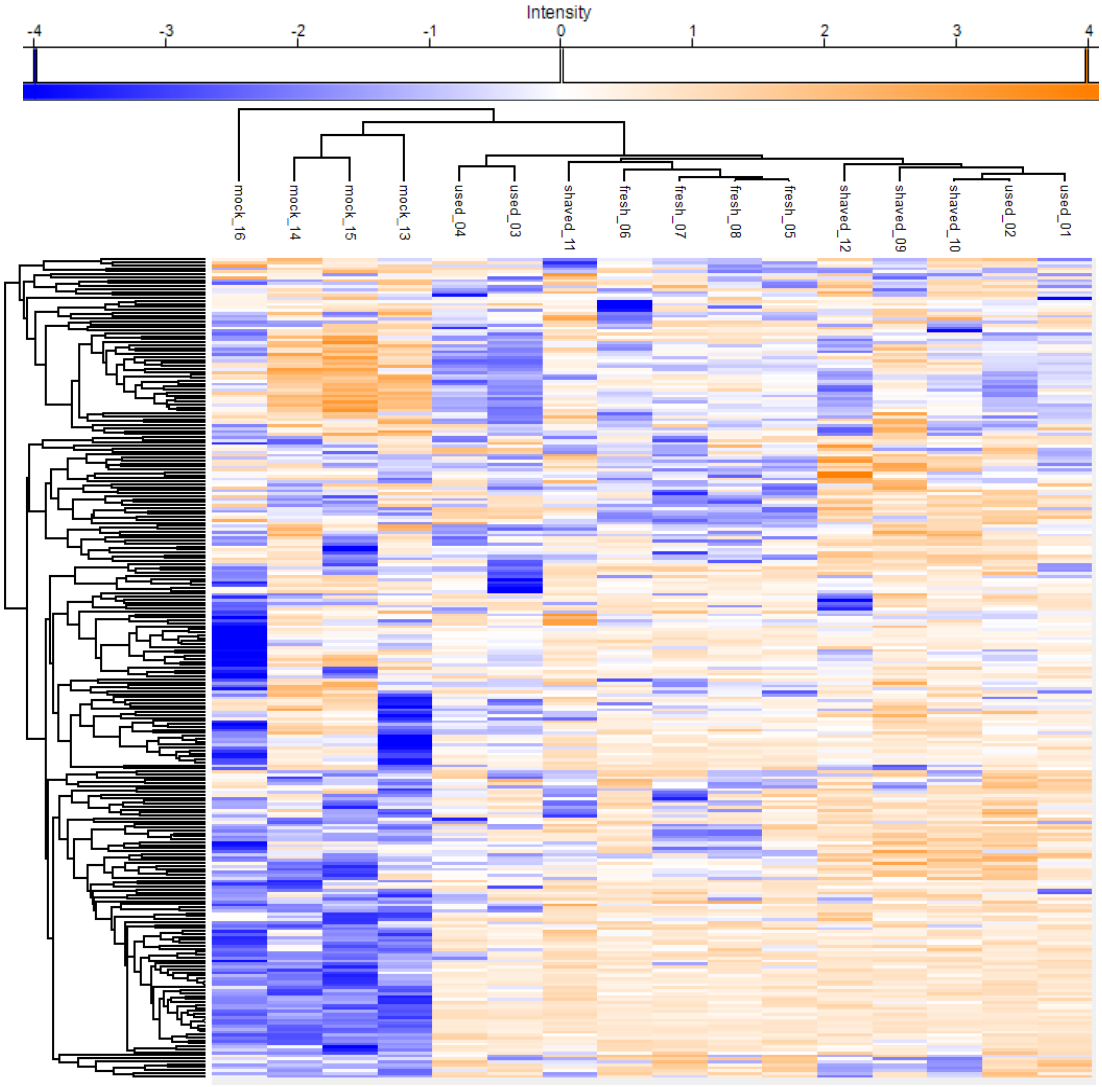
Appendix 5. 2: Histograms showing data quality of samples. The graphs show the log2 transformed LFQ intensities on x-axis and the count per bin on y-axis. The width of each bin was set to 0.25. The red-dotted line marks the median of the data. The data was close to normal distribution and centred around the same value over the different samples, which was a prerequisite for further statistical testing of the data. The samples were supernatant obtained after shaving the different cultures: samples 01-04 from uEs in used media-samples; samples 05-08 from uEs in fresh media; samples 09-12 from trypsin-shaved ring culture and samples 13-16 from mock-shaved ring culture.



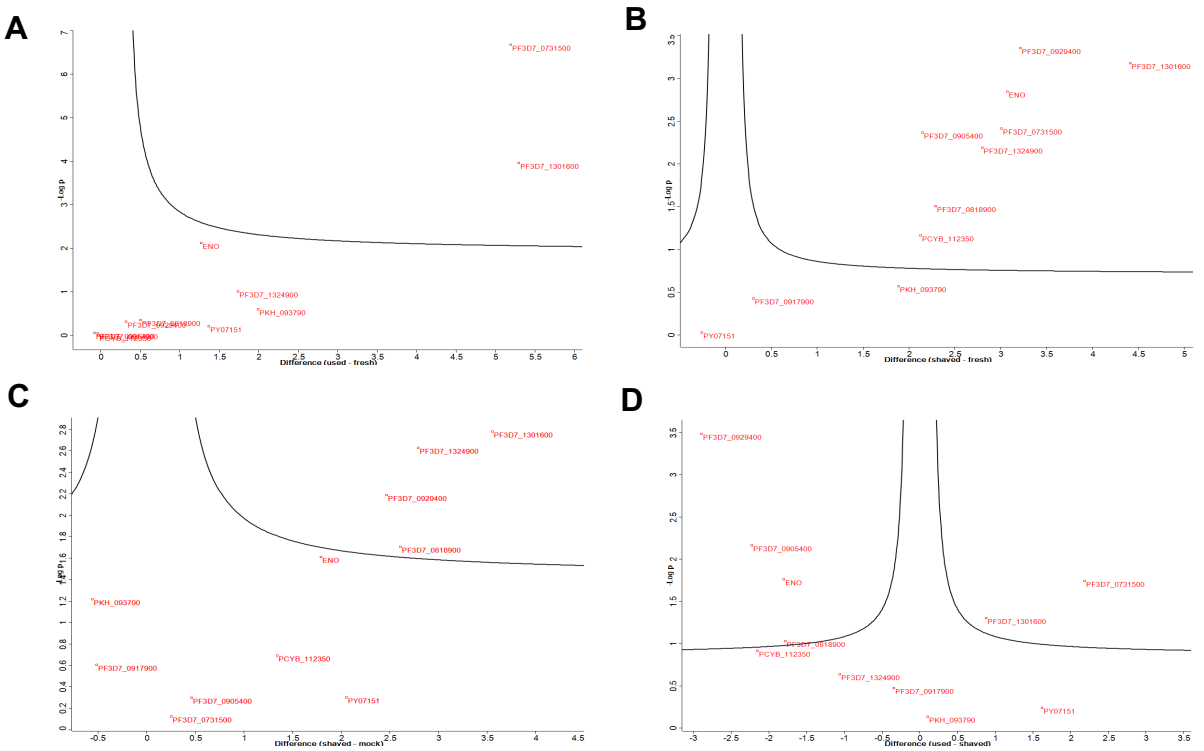
Appendix 5. 3: Multi-scatterplot showing data quality of samples. Pairwise scatter plots of log2 transformed LFQ intensities of each sample combination on the lower left half of the figure, density plot of each sample on the diagonal, and Pearson correlation for all sample combinations on the upper-right half. The samples show strong positive general correlation with each other, which was a prerequisite for further statistical testing of the data. The samples were supernatant obtained after shaving the different cultures: samples 01-04 from uEs in used media-samples; samples 05-08 from uEs in fresh media; samples 09-12 from trypsin-shaved ring culture and samples 13-16 from mock-shaved ring culture.



Appendix 5. 4: Principle component analysis using all the selected human and *Plasmodium* proteins. The shaved samples; uEs in used media (used_01-04), uEs in fresh media (fresh_05-08) and trypsin-shaved ring culture (shaved_09-12) showed some similarity by clustering together and away from the mock-shaved ring culture (mock_13-16). The proteins used for the principal component analysis were those that passed the selection criterion for statistical analysis.



Appendix 5. 5: Heatmap generated using all the selected human and *Plasmodium* proteins. The heatmap was generated using Log-2 LFQ values that had been Z-score normalized and hierarchical clustering was performed on row and columns using the Euclidean distance and ward.D2. Quantitative values have been colour coded according to the colour key shown on the top. Protein are labelled by their gene-name and accession number. Sample names are shown on the top of the plot. The samples clustered well based on their experimental conditions as observed previously with the PCA analysis where the mock-shaved ring culture was clearly discriminated from the other three groups (uEs in used media, uEs in fresh media and trypsin-shaved ring culture) which clustered fairly well within their respective groups. The samples were supernatant obtained after shaving the different cultures: used_01-04 from uEs in used media-samples; fresh_05-08 from uEs in fresh media; shaved_09-12 from trypsin-shaved ring culture and mock_13-16 from mock-shaved ring culture. The proteins used to generate the heatmap were those that passed the selection criterion for statistical analysis.



Appendix 5. 6: Volcano plots generated using only selected *Plasmodium* proteins. The volcano plots were generate using the -Log p values derived from the p-values obtained during Student's *t*-test analysis where the mean LFQ values of each of the selected human and *Plasmodium* proteins were compared between all the possible combinations of the experimental conditions. There were significantly upregulated Plasmodium proteins identified in (A) uEs in used media (B) trypsin-shaved ring culture when compared to uEs in fresh media culture. Additionally, (C) there were also significantly upregulated Plasmodium proteins identified in trypsin-shaved ring culture when compared to mock-shaved ring culture. (D) There were two rhoptry associated proteins that were significantly upregulated in trypsin-shaved ring culture compared to uEs in used media. The significantly upregulated proteins denoted in blue and red were from human and *Plasmodium* respectively. The gene names of the various accession numbers of the *Plasmodium* hits are as follows; PF3D7_0929400 - Lactate dehydrogenase, PF3D7_0929400 - High molecular weight rhoptry protein 2, PF3D7_0905400 - High molecular weight rhoptry protein 3, PF3D7_0731500 - Erythrocyte binding antigen 175, PF3D7_1301600 - Erythrocyte binding antigen 140, ENO - Enolase, PCYB_112350 - Elongation factor 1, PF3D7_0818900 - Heat shock protein 70;70kDa, PF3D7_0917900 - Heat shock protein 70;78kDa, PKH_093790 - Myosin heavy chain subunit and PY07151 - Uncharacterized protein. The proteins used to generate the volcano plots were those that passed the selection criterion for statistical analysis.

Key: red - significantly upregulated *Plasmodium* proteins; blue - significantly upregulated human proteins and grey - proteins with no change between any given two experimental conditions.