Adaptation of Plasmodium falciparum in human RBC supplemented NSG mice

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**Adaptation of *Plasmodium falciparum* in human
RBC supplemented NSG mice**

Marvin Chew

SCHOOL OF BIOLOGICAL SCIENCES

2019

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supplemented NSG mice**

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SCHOOL OF BIOLOGICAL SCIENCES

A thesis submitted to the Nanyang
Technological University in partial fulfilment of
the requirement for the degree of Doctor of
Philosophy

2019

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Abbreviations

AS	Adapted Strain
CD	Cluster of differentiation
FACS	Fluorescence activated cell sorting
GOI	Gene of interest
IDC	Intra-erythrocytic development cycle
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
i.p.	Intraperitoneal
i.v	Intravenous
MAHRP	Membrane associated histidine-rich protein
MCSF	Macrophage Colony Stimulating Factor
MHC	Major histocompatibility complex
NK	Natural killer
PBMC	Peripheral Blood Mononuclear cells
<i>P. falciparum</i>	<i>Plasmodium falciparum</i>
PfEMP1	<i>P. falciparum</i> erythrocyte membrane protein 1
p.i.	Post-infection
RBC/iRBC	Red blood cell / infected red blood cell
RIFINs	Repetitive interspersed family protein
TNF	Tumor necrosis factor
TLR	Toll-like receptor
WT	Wildtype

Abstract

Human chimeric mouse models, also known as humanized mice, are powerful tools to study human obligate pathogens such as *Plasmodium falciparum*. In our chimeric mouse model, human RBC supplemented NSG mice, static *in vitro* cultured *P. falciparum* require adaptation to become competent to thrive in the mice. During the adaptation process, variant surface antigens (VSAs), known to play a major role in virulence and antigenic variation are upregulated. These VSAs belong to large multigenic families such as *P. falciparum* Erythrocyte Membrane Protein 1 (PfEMP1) and Repetitive Interspersed Family proteins (RIFINs) that have been shown to immune modulate effector function of host immune cells.

Adapted parasite upregulates VAR2CSA PfEMP1, a known immune modulator of macrophages. Using an *in vivo* conditional knockdown of *P. falciparum* membrane-associated histidine rich protein 1 (MAHRP1), surface expression of PfEMP1 was affected and diminished in adapted *P. falciparum*. This led to reduced competency of adapted parasites in the huRBC-NSG mice. *In vitro* phagocytosis assay also showed that adapted parasites are less recognized and phagocytosed by mouse and human macrophages compared to non-adapted parasites. MAHRP1 knockdown of adapted parasites also resulted in increased macrophage recognition and phagocytosis. We utilized the phagocytosis assay to screen known macrophage-polarizing compounds for increased phagocytosis of adapted parasites. Three compounds that polarize macrophages towards M1-like

and three towards M2-like macrophages were able to increase phagocytosis of adapted parasites after 24-hour treatment of M0 human primary macrophages.

Adapted parasites also upregulate A-type RIFINs that have been shown recently to ligate host immune inhibitory receptor, leucocyte immunoglobulin-like receptor B1 (LILRB1). Adapted parasites are less controlled by primary responder NK cell compared to non-adapted parasites. Moreover, anti-LILRB1 blocking antibody partially restores NK cell control of adapted parasites, indicating that LILRB1 is involved in the reduction of NK cell control of adapted parasites. Therefore, adapted *P. falciparum* infection in huRBC-NSG mice provides a powerful tool to elucidate host immune modulatory mechanisms of *P. falciparum* and provides new approaches for therapy and treatment.

Chapter 1. Introduction

1.1 Overview of Malaria

1.1.1 Global burden of Malaria

Malaria continues to be a devastating and important infectious disease of our time. After many years of steady decline, 2017 marks the first increase in the total number of malaria cases to 219 million across 87 countries, up from 217 million cases in 2016. The World Health Organization estimates malaria caused 435,000 deaths globally in 2017, mostly children under the age of five, which accounted for 61% of the total number of malaria deaths (World Health Organization 2018). It is because of the vulnerability of young children to malaria related severe disease and death that led to the development and approval of the world's first malaria vaccine, Mosquirix, otherwise known as RTS,S/AS01. Mosquirix by GlaxoSmithKline took 30 years and US\$1 billion dollars to develop. However, the vaccine does have its shortcomings which future malaria vaccines need to address. Mosquirix is intended only for children age of 5 to 17 months and requires four doses to achieve a 36.3% rate of protection (Theander and Lusingu 2015). Moreover, longer term studies are showing that protection wanes over time (Olotu, Fegan et al. 2016).

Currently, there are 6 species of *Plasmodium* that can infect humans. *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, *P. knowlesi* and more recently *P. cynomolgi*, where Malaysia has reported the very first zoonotic patient (Ta, Hisam et al. 2014). *P. falciparum* is responsible for the most virulent form of

malaria in humans, accounting for 99% of deaths caused by this disease (World Health Organization 2018). For the past few years, reports of partial drug resistant *P. falciparum* to the first-line antimalarial, artemisinin, have been reported in South East Asia. This partial drug resistance is characterized by delayed clearance of the parasite by artemisinin monotherapy or combinatorial therapy (Noedl, Se et al. 2008). Artemisinin-resistant *P. falciparum* has been associated with mutations in the *kelch13* propeller domain, although how the mutations confer artemisinin resistance to the parasite is unknown (Ariey, Witkowski et al. 2014). More recently and more worryingly, a single dominant lineage artemisinin resistant parasite with *kelch13* mutation C580Y has acquired piperazine resistance in Western Cambodia and has spread to Thailand and Vietnam, outcompeting other resistant strains (Imwong, Hien et al. 2017).

1.1.2 Malaria Lifecycle

Plasmodium has a complicated life cycle in the human and vector host. The pre-erythrocytic stage begins with a bite from an infected *Anopheles* mosquito. The parasite, in the form of sporozoites, is injected into the dermis and makes its way to the liver via the bloodstream and infects hepatocytes (Sinnis and Zavala 2012). After an incubation period of about 2 weeks, infected hepatocytes rupture and release merozoites, its next form. This asexual, erythrocytic stage of *Plasmodium* involves invading into red blood cells (RBCs). Once inside the RBC, the parasite matures through distinct stages, starting from ring stage to trophozoite stage, then to schizont stage and subsequently the

rupture of schizonts to release 16 to 32 daughter merozoites which invades new RBCs. This intra-erythrocytic development cycle (IDC) of *P. falciparum* typically takes about 48 hours. It is the rupture of schizonts and destruction of erythrocytes that is one of the causes of the clinical symptoms of malaria (Trampuz, Jereb et al. 2003). A portion of iRBCs commit to become gametocytes, leading to the transmission stage of the parasites back to the mosquito (Figure 1.1). The triggers for gametocytogenesis, either internally from the parasite through the AP2-G transcription factor (Josling and Llinas 2015) or externally from the host environment are unknown and currently an area of intense research (Dantzler, Ravel et al. 2015).

Figure 1.1: The lifecycle of *Plasmodium falciparum* and *Plasmodium vivax*. Lifecycle of *Plasmodium* depicted in human and mosquito host (Bousema and Drakeley 2011). Republished with permission from American Society for Microbiology Copyright © 2011

During its development within the RBC, *P. falciparum* extensively remodels the host RBC cell compartment and membrane to alter its rigidity and adhesion capabilities. It does so by exporting parasite proteins into the RBC cytosol and onto the RBC membrane surface, known as the parasite exportome. This parasite exportome plays an important role in determining the survivability and virulence of *P. falciparum* in its host (Maier, Rug et al. 2008). The parasite exportome makes up about 10% of the entire parasite proteome, attesting to the importance of remodeling the RBC (Spielmann and Gilberger 2015).

1.1.3 Variant Surface Antigens (VSAs)

Plasmodium falciparum exports parasite proteins and places them on the surface of the infected RBC. These parasite proteins are termed variant surface antigens (VSAs). VSAs allows the infected RBC to sequester to tissue and the microvasculature through interactions with host receptors, forming aggregates with other RBCs known as rosettes, as well as evade immune recognition (Chan, Fowkes et al. 2014). These VSAs are highly polymorphic and are part of multigenic families such as *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) (Leech, Barnwell et al. 1984), repetitive interspersed family (RIFIN) proteins (Kyes, Rowe et al. 1999), sub-telomeric variable open reading frame

(STEVAR) (Kaviratne, Khan et al. 2002) and surface-associated interspersed gene family (SURFIN) proteins (Winter, Kawai et al. 2005). These multigenic families are typically found in the sub-telomeric regions of the chromosomes, a region of great variability that undergoes constant changes through mitotic recombination (Claessens, Hamilton et al. 2014) during sexual-stage mating in the mosquito (Scherf, Lopez-Rubio et al. 2008). This provides the building blocks for new antigenic proteins to be formed, conferring the parasite a survival advantage through immune evasion or sequestration (Wahlgren, Goel et al. 2017). The *var* genes which codes for PfEMP1, comes after a region of telomere-associated repeat elements (TARE) and telomere repeats (Kraemer, Kyes et al. 2007). Interestingly, the other major multigenic families are encoded close to one another, highlighted by the fact that next to the *var* gene is a *rif* gene, that encodes for RIFINs (Fernandez, Hommel et al. 1999) or *stevor* gene, the codes for STEVARs (Kaviratne, Khan et al. 2002) (Fig. 1.2).

Figure 1.2: Schematic of the sub-telomeric regions of *P. falciparum* chromosomes.

Sub-telomeric regions showing the orientation and location of *var*, *rif* and *stevor*

genes in relation to one another (Scherf, Lopez-Rubio et al. 2008). Republished with permission from ANNUAL REVIEWS, INC, 2008.

1.1.4 *P. falciparum* Erythrocyte Membrane Protein 1 (PfEMP1)

PfEMP1 is the most extensively studied VSA and has been shown to be recognized as a target in naturally acquired immunity (Bull, Lowe et al. 1998). This high molecular weight protein of 200 kDa to 350 kDa is exported to the surface of the RBC and has been shown to be sensitive to mild trypsin treatment (Leech, Barnwell et al. 1984). PfEMP1 expression is mutually exclusive among its 60 members in the *var* gene family, with only one member being expressed on the iRBC surface at any given time. (Scherf, Hernandez-Rivas et al. 1998). This monoallelic expression of *var* genes adds weight to the notion that as a PfEMP1 on an infected RBC is being recognized by the host immune system, which leads to clearance, expression of other PfEMP1 on different iRBCs allows evasion of the immune system and clearance.

Export of PfEMP1 to the surface of the iRBC is complex due to its size and coordination required for it to pass through multiple membranes. PfEMP1 trafficks through the Maurer's cleft, a unique parasite structure located in the host red cell cytosol which functions as a sorting station for parasite exported proteins (Tilley, Sougrat et al. 2008). Disruptions to proteins associated with the Maurer's clefts have been shown to affect PfEMP1 trafficking to the surface (Cooke, Buckingham et al. 2006). One such Maurer's cleft protein is membrane-associated histidine-rich protein 1 (MAHRP1). Disruption of MAHRP1 prevents

PfEMP1 from reaching the surface of the iRBC and to the Maurer's cleft itself (Spycher, Rug et al. 2008). However, the disruption of MAHRP1 does not affect parasite survival in culture. Another Maurer's cleft protein that plays a role in PfEMP1 surface expression is skeleton binding protein 1 (SBP1). Disruption to SBP1 again prevents PfEMP1 from reaching the surface of the iRBC (Maier, Rug et al. 2007). Both MAHRP1 and SBP1 also affect the parasite's ability to bind to host receptors such as cluster of differentiation (CD) 36 in an *in vitro* binding assay.

PfEMP1 contains binding domains that are unique to the host receptor that they are capable of binding to. Extracellular domains of PfEMP1 consists of Duffy binding-like (DBL) domains, C2 domains and the cysteine-rich interdomain regions (CIDR) (Smith, Craig et al. 2000). DBL domains have 5 five sequence classes named α , β , γ , δ , ϵ and CIDR has 3 classes from α , β , γ . Binding to CD36 has been mapped to CIDR α 2-6 regions and other host receptors such as endothelial protein C receptor (EPCR) binding is through CIDR α 1. Intercellular Adhesion Molecule 1 (ICAM-1) binding has been linked to DBL $\alpha_{1.1/1.4}$ –CIDR $\gamma_{1.6}$ –DBL β_3 , also known as domain cassette 4 (DC4), (Avril, Bernabeu et al. 2016) whereas Platelet And Endothelial Cell Adhesion Molecule 1 (PECAM1) binding is through the DC5 cassette of DBL γ_{12} –DBL $\delta_{3/4}$ –DBL $\beta_{3/4}$ –DBL $\beta_{7/9}$ (Berger, Turner et al. 2013) (Fig. 1.3).

codes for a pseudogene, *var1*, that could bind to CSA. *UpsE* unique 5' sequences codes for a conserved PfEMP1 across most *P. falciparum* strains, *var2csa*. VAR2CSA binds to chondroitin sulfate A (CSA) that is found in abundance in the placenta and is involved in pregnancy-associated malaria (PAM) (Gamain, Trimmell et al. 2005).

1.1.5 Repetitive interspersed family (RIFINs)

RIFINs play a role as adhesins and sequestration, similar to PfEMP1. They are smaller at 28-45 kDa and about 150 copies can be found per genome in the sub-telomeric region. 70% of RIFINs belong to subgroup A termed A-RIFINs which has an insertion of 25 amino acids at the N terminus that is not found in the subgroup B (B-RIFINs) (Joannin, Abhiman et al. 2008). A-RIFINs contains the export sequence PEXEL, interacts with the Maurer's cleft that and is expressed on the surface of the iRBC whereas B-RIFINs are located internally within the parasite (Petter, Haeggström et al. 2007). Therefore, A-RIFINs have been implicated in immune evasion and rosetting. A-RIFINs have been shown to be involved in rosetting for type A and type O blood whereas PfEMP1 was shown to only be involved in type O blood rosetting (Goel, Palmkvist et al. 2015). Recently, A-RIFINs have been shown to interact directly with an immune inhibitory receptor, leucocyte immunoglobulin-like receptor B1 (LILRB1). By doing so, this interaction modulates immune cells such as B-cells and the NK cell line NKL and inhibits their effector functions (Saito, Hirayasu et al. 2017).

1.1.6 Sub-telomeric variable open reading frame (STEVOR)

The *stevor* multigene family is the third largest multigene family after PfEMP1 and RIFINs. It codes for a 30-40 kDa protein and each genome contains 30 to 40 copies. Like *var* and *rif* genes, they are located at the telomeric regions of the *P. falciparum* chromosomes. Multiple transcripts of *stevor* exist in a single parasite, similar to that of *rif* transcripts. STEVOR co-localizes with SBP1 at the Maurer's cleft. (Kaviratne, Khan et al. 2002) and is transported to the surface of the iRBC and is found to be sensitive to trypsin (Niang, Yam et al. 2009). STEVOR is closely related to RIFINs and resemble B-RIFINs in terms of number of copies in the genome and the absence of the 25 amino acid insertion sequence found in A-RIFINs. A-RIFINs and STEVORs binds to sialic acid on glycophorin A and C, respectively (Niang, Bei et al. 2014).

1.2 Naturally acquired immunity to malaria

Clinical symptoms of malaria are caused by the asexual stage of the infection as the parasites replicate in the blood. The disease pathogenesis caused by malaria is complex and not yet fully elucidated, but it is thought to be driven by sequestration and inflammation (Crompton, Moebius et al. 2014). Sequestration of parasites leads to obstruction in the vasculature causing local ischemia and inflammation in these organs, such as the brain. Systemic inflammation caused by the infection may also lead to increased sequestration by upregulating vascular adhesion receptors ICAM-1 and Vascular Cell Adhesion Protein 1 (VCAM-1). This brings about a multi-system disorder that is similar to sepsis (Mackintosh,

Beeson et al. 2004). It is unclear if these two processes are the main cause of pathogenesis and disease severity however they appear to mutually reinforce each other's effects on malaria disease pathogenesis (Crompton, Moebius et al. 2014). These effects are the etiologies of severe malaria such as cerebral malaria, severe anemia and severe metabolic acidosis.

With no prior exposure to *Plasmodium*, naïve individuals of any age can become ill on their first infection and develop febrile illness (Langhorne, Ndungu et al. 2008) and possibly severe disease. Therefore, young children in endemic areas are particularly susceptible to severe disease because only with repeated exposure do older children and adults develop protection against severe malaria (Gupta, Snow et al. 1999). Resistance to severe disease is typically acquired by the age of five. The endemic population still have repeated episodes of febrile malaria in adolescence before attaining clinical immunity by adulthood, although they still register low level parasitemia in their peripheral blood and typically do not exhibit sterile immunity. This contributes to the complexity of the disease where the immune system does not fully clear the parasite but rather confers protection from clinical symptoms (Doolan, Dobaño et al. 2009). Therefore, naturally acquired immunity (NAI) is defined as the cumulative outcome of exposure to multiple parasite infections that results in a diverse collection of strain-specific immune responses. Developing NAI requires antibody responses to VSAs as anti-VSA immunoglobulin (Ig) G levels are correlated with protection from malaria in clinical studies done in Ghana (Dodoo, Staalsoe et al. 2001), Kenya (Kinyanjui, Mwangi et al. 2004) and Tanzania (Magistrado,

Lusingu et al. 2007). Malaria naïve participants given a single *P. falciparum* infection generated antibodies that were reactive with 6 different *P. falciparum* strains that expressed different PfEMP1 on the surface (Elliott, Payne et al. 2007). Importantly, passive transfer of gamma globulins from hyperimmune serum taken from adults in West Africa and administered to East African children with severe malaria infection show a dramatic reduction in parasitemia levels to around 1% parasitemia by day 4, showing transferrable protection (McGregor, Carrington et al. 1963).

1.3 Innate immunity and malaria

The first symptoms of malaria, fever, headache, muscle pain, chills, vomiting and lethargy followed by malarial paroxysms are associated with high levels of circulating cytokines that originate from the innate immune system (Gazzinelli, Kalantari et al. 2014). The rupture of mature infected red blood cells coincide with the fever and headaches from systemic pro-inflammatory cytokine response. (Kwiatkowski, Bate et al. 2016). Both mouse and human studies have shown that the innate immune system responds quickly to blood-stage malaria infection. The initial innate immune response is crucial for the control of parasitemia in the host and also contributes to the initiation of the subsequent adaptative immune response (Stevenson and Riley 2004).

Several pathogen-associated molecular patterns (PAMPs) unique to *Plasmodium* have been identified and their subsequent trigger to pattern recognition receptors (PRRs) on innate immune cells.

Glycosylphosphatidylinositol anchors (GPI anchors) are glycolipids that anchor parasite surface proteins to the plasma membrane (Gowda 2007). GPI anchors are recognized by and triggers toll-like receptor (TLR) 2/TLR6 or TLR1/TLR2 heterodimers and to a lesser extent, TLR4 (Krishnegowda, Hajjar et al. 2005). This triggers mitogen-activated protein kinases and inhibitor of nuclear factor - κ b (NF- κ b) in macrophages that leads to TNF- α production. Hemozoin, another PAMP, is a by-product of parasite digestion of hemoglobin through a detoxification process of heme (Jani, Nagarkatti et al. 2008). When phagocytosed by macrophages, dendritic cells (DCs) or neutrophils in various organs (Frita, Rebelo et al. 2011), the quantity of hemozoin deposited within these phagocytes is an indication of parasite burden (Phu, Day et al. 1995). Hemozoin is able to induce pro-inflammatory cytokines TNF- α and interleukin (IL) -1 β from human monocytes and macrophages (Giribaldi, Prato et al. 2010) and intravenous injection of hemozoin in rats triggers TNF and IL-1 β production as well as thermal dysregulation, suggesting that hemozoin released from ruptured schizonts could be linked to the cyclical bouts of fever during a malarial infection (Sherry, Alava et al. 1995). Lastly, plasmodial DNA and RNA can activate innate immune responses. For *Plasmodium* DNA, the presence of immunostimulatory CpG motifs activates TLR9 through the phagocytosis of iRBCs and its degradation in the phagolysosome that leads to TNF- α production (Parroche, Lauw et al. 2007).

1.3.1 Macrophages

The monocytic lineage of innate immune cells such as monocytes, macrophages and dendritic cells, play crucial roles during malaria infection. Monocytes arise from hematopoietic stems cells (HSCs) from the bone marrow and are released into the peripheral blood circulation. During malaria infection, monocytes are recruited into tissues from circulation to replenish tissue resident macrophages (Lai, Sheng et al. 2018) in mouse malaria models. Macrophages control parasitemia during the initial infection by phagocytosis of infected RBCs. They are capable of both opsonic (Celada, Cruchaud et al. 1982) and non-opsonic phagocytosis (Malaguarnera and Musumeci 2002) of malaria infected RBCs. For non-opsonic phagocytosis, the Class B scavenger receptor, CD36, has been identified as the main receptor for macrophages to recognize iRBCs (Patel, Serghides et al. 2004) by recognizing PfEMP1 that is able to bind to CD36. PfEMP1 that do not bind to CD36, such as VAR2CSA, which binds to chondroitin sulphate A instead, have been shown to be less phagocytosed by macrophages (Serghides, Patel et al. 2006). Mutations that lead to CD36 deficiency in African populations have been associated with increased susceptibility to severe malaria (Aitman, Cooper et al. 2000). Opsonins such as complement factors and antibodies, bind to infected RBCs and signal to monocytes and macrophages for elimination of the bound target. Complement-mediated phagocytosis of ring-stage iRBCs has been shown to be mediated via complement receptor 1 (Silver, Higgins et al. 2010). Antibodies opsonized to iRBCs or merozoites lead to phagocytosis by macrophages through FcγRs.

Cytokine production by monocytes and macrophages in response to a malaria infection is important for disease outcome. High levels of IL-12 and IL-18 generated by monocytes and macrophages early in the infection prevented the progression to severe malaria (Torre 2009). IL-12 produced by macrophages in response to pathogens is considered as the key towards initiating cell-mediated immune responses against these pathogens by the proliferation and activation of T-helper (Th) 1 cells (Trinchieri 1995). IL-12 is regulated by the release of TNF- α that is produced by macrophages after being stimulated with GPI sequences (Schofield and Hackett 1993) or hemozoin (Pichyangkul, Saengkrai et al. 1994) and both are important co-stimulators for IFN- γ production by natural killer (NK) cells (Tripp, Wolf et al. 1993).

The spleen, in particular, plays a major immunological role in blood-borne infection such as *Plasmodium*. The spleen is divided into white pulp (WP) and red pulp (RP) that is separated by the marginal zone (MZ). The red pulp makes up to 80% of the volume of the spleen that consists of sinuses and cords that relates to its function. Splenic cords, also known as the cords of Billroth, are open spaces filled with tissue resident macrophages (Chadburn 2000). These spaces allow immune surveillance of blood-borne substances as blood flows from the arterioles to the cords and through the inter-endothelial slits of the venous sinuses. Abnormal or senescent RBCs that lose their deformability are unable to squeeze through the endothelial slits and are trapped in the cords for phagocytosis and clearance (Mohandas and Gallagher 2008). The process of squeezing erythrocytes through the endothelial slits is a way of preserving erythrocyte

viability through pitting where rigid material such as hemozoin, Howell Jolly bodies and intracellular malarial parasites are ejected from the erythrocyte (Schnitzer, Sodeman et al. 1972). Blood flowing through the red pulp is called open circulation and represents 10% of the blood that flows into the spleen via the splenic artery. The other 90% of circulation flows to the closed circulation. This fast pathway bypasses the red pulp and enters the marginal zone and into venous sinuses. There, it is joined by blood that flows from the closed circulation, where it drains to the trabeculae veins to the efferent splenic vein and leaves the spleen (Buffet, Safeukui et al. 2011).

The function of the spleen and its ability to clear iRBCs explains the need for the parasite to sequester to organs, thereby leaving the peripheral blood circulation to avoid splenic clearance. Sequestration of the less deformable mature stage parasite leads to their absence in the peripheral blood as they are more likely to be trapped and cleared by the spleen compared to ring stage parasites (Suwanarusk, Cooke et al. 2004). Splenectomized patients infected with *Plasmodium* offer insights into the importance of the spleen during an infection. One observation is that splenectomized patients show increased parasitemia during *P. falciparum* infection despite administration of antimalarial drugs. This shows that parasite clearance, even with anti-malarial therapy, involves splenic function (Demar, Legrand et al. 2004). Also, the appearance of trophozoite and schizonts in the peripheral blood of splenectomized patients indicates a lack of sequestration and change of VSA surface expression (Ho, Bannister et al. 1992). This observation was further supported by Cameroonian

RT-PCR analysis of a splenectomized Cameroonian patient, which showed a lack of expression of *var*, *rif-A* and *stevor* as well as the inability of the parasites to bind to CD36, ICAM-1, VCAM-1 and P-selectin. After 29 days of *in vitro* culture, the parasites showed restored expression of VSAs and binding ability to host receptors restored (Bachmann, Esser et al. 2009). In a parallel observation, *P. falciparum* infection in squirrel monkey showed that splenectomy affected cytoadherence as tissue sequestration was reduced in splenectomized monkeys (Hommel, David et al. 1983). Similarly, iRBCs taken from splenectomized monkeys failed to bind to CD36 compared to iRBCs taken from non-splenectomized monkeys (David, Hommel et al. 1983).

1.3.2 Natural Killer (NK) cells

NK cells develop in the bone marrow from the common lymphoid progenitor cell, the same progenitor cell as T and B lymphocytes, and are mainly found in the peripheral blood, the spleen and bone marrow (Moretta, Bottino et al. 2002). They constitute about 10% of peripheral blood mononuclear cells (PBMCs). They are called natural killer cells because unlike T cells, they do not need to be exposed to the antigen before they are able to engage and kill their target cells (Murphy and Weaver 2016). The main functions of NK cells during an infection are the production of cytokines and cytotoxicity against infected cells that are triggered by cytokine activation (Fehniger, Shah et al. 1999), antibody-dependent cell-mediated cytotoxicity (ADCC) (Arora, Hart et al. 2018)

and loss of inhibitory signal from MHC class I downregulation or mismatch, also known as the missing-self hypothesis (Kärre 2008).

NK cell response to *Plasmodium falciparum* infection in humans was first reported in the 1980s (Ojo-Amaize, Salimonu et al. 1981). NK cells are also able to mount cell-mediated cytotoxicity against iRBCs, causing lysis of the target iRBC (Orago and Facer 1991). There is evidence that NK cells are able to directly engage and kill iRBCs through cytotoxic activity. Distinct cytoplasmic granules of cytotoxic granzymes and pore-forming perforin in the NK cells are the main cytotoxic effectors against an infected RBC (Böttger, Multhoff et al. 2012). Cytotoxic granzymes have also been detected in the plasma of blood-stage infected patients, indicating that iRBCs are the targets of NK cells and CD8⁺ T cells. (Hermsen, Konijnenberg et al. 2003). Multiple groups have also reported the formation of stable conjugates between NK cells and iRBCs *in vitro*, supporting the notion of direct killing by NK cells (Artavanis-Tsakonas and Riley 2002, Korbel, Newman et al. 2005). These conjugates form within 30 minutes of exposure to iRBCs but the proportion of NK cells that forms conjugates varies significantly between donors, which suggests that the NK receptors involved in recognizing an infected RBC are polymorphic or have variable expression between donor NK cells (Artavanis-Tsakonas, Eleme et al. 2003, Korbel, Newman et al. 2005). This interaction also indicates the formation of the immune synapse which occurs before the migration of cytotoxic granules to the iRBC (Rak, Mace et al. 2011). Transwell experiments done previously in our group showed that NK cell control of parasitemia requires direct contact. Furthermore,

video microscopy of NK cells interacting and lysing iRBCs provides one of the clearest evidences yet that NK cells directly kill iRBCs and is one of the mechanisms by which they control parasitemia *in vitro* (Chen, Amaladoss et al. 2014).

LFA-1 has been identified as one of the NK cell receptors important for direct iRBCs interactions where blocking LFA-1 using anti-LFA-1 antibody showed reduced NK cell parasitemia control as well as a significant reduction in Nk cell contact time with the infected RBC (Chen, Amaladoss et al. 2014). However, the recognition receptor and parasite ligand responsible for direct lysis of an infected RBC has not been elucidated yet. PfEMP1 has thought to be an ideal candidate as the parasite ligand for NK cell receptor recognition because it is capable of binding to host receptors such as CD36, ICAM-1 and CSA and is recognized by macrophages for phagocytosis via the CD36 receptor (Patel, Serghides et al. 2004). However, PfEMP1 does not directly cause NK cell cytotoxic activity as measured by CD107a, a degranulation marker of NK cells (D'Ombrian, Voss et al. 2007).

1.3.3 NK cell heterogeneity

Donor NK cell response to iRBCs differ from donor to donor (Korbel, Newman et al. 2005). Specifically, donor NK cell release of IFN- γ during a 24-hour co-culture with iRBCs can be segregated into high responder for high IFN- γ release and low responder for low IFN- γ release. Moreover, CD69 upregulation correlates with IFN- γ release in CD56 dim NK cells, further illustrating that NK

cell response to iRBC in a co-culture is heterogenous. This well-documented heterogeneity in NK cell response points to the uniqueness of the NK cell as an innate lymphoid cell, particularly the genetic differences in the expression of activating and inhibitory receptors that sets the threshold of NK cell activation to iRBCs (Hoglund and Brodin 2010). Building on this observation, our lab showed that parasitemia control during NK cell co-culture with *P. falciparum* iRBCs is heterogenous from donor to donor. Responder donor NK cells are donor NK cells that were able to reduce parasitemia greater than 50% in a 96-hour co-culture. Otherwise, they are classified as non-responders. Differential expression of the Melanoma Differentiation-Associated protein 5(MDA5) pathway, an internal retinoic acid-inducible gene I (RIG-I) -like receptor that senses cytosolic RNA has been shown to contribute to the heterogeneous response of NK cells to iRBCs. MDA5 transcripts were significantly upregulated in responder NK cells. Subsequent experiments such as knockdown of MDA5 in responder cells led to loss of parasitemia reduction whereas non-responder given an MDA5 agonist led to increase parasitemia reduction in a dose-dependent manner. (Ye, Chew et al. 2018).

The production of pro-inflammatory cytokine interferon-gamma (IFN- γ) during malaria infection is an important response of NK cells. They are an early source of IFN- γ during an infection. The peak IFN- γ response of NK cells is 12 to 15 hours post infection compared to $\gamma\delta$ T cells and NKT cells whose IFN- γ production begins only at 24 to 48 hours. This indicates NK cells could be the early responding immune cells to initiate the cascade of innate immune responses

(Artavanis-Tsakonas and Riley 2002). The *P.chabaudi chabaudi* AS strain results in a non-lethal infection in the mouse model but in IFN- γ deficient mice, the infection becomes lethal (van der Heyde, Pepper et al. 1997). Furthermore, in the same mouse model when NK cells are depleted, peak parasitemia is higher as well reduced parasitemia clearing efficiency to resolve the infection (Mohan, Moulin et al. 1997).

1.4 Humanized mice

One of the main challenges of immunological studies of *P. falciparum* is that as an obligate human parasite, can only invade and replicate in human RBCs. Immunodeficient mice that allow the engraftment of human components such as human RBC, immune system or organ tissue began with the discovery of the mutant allele *Prkdc*^{scid} (protein kinase, DNA activated, catalytic polypeptide; severe combined immunodeficiency) (Bosma, Custer et al. 1983). With these genetically modified mice, it was shown for the first time the engraftment of human peripheral blood mononuclear cells, fetal hematopoietic tissues and human stem cells to make them “humanized”.

The first model of immunodeficient mice to sustain *P. falciparum* infection was established in 1995 (Tsuji, Ishihara et al. 1995). Human red blood cells were injected into *scid* mice intravenously and intraperitoneally to increase circulating levels of human RBC in the peripheral blood circulation. By doing so, they could sustain *P. falciparum* infection for two weeks in these mice. Current methods are similar to the technique employed by Tsuji et al. However,

it is the advancements made in generating more severely immunodeficient mice that have greatly improved this mouse model for *P. falciparum* studies. The NOD/SCID IL2 γ null (NSG) mice (Ishikawa, Yasukawa et al. 2005) with daily human RBC supplementation is capable of sustaining parasitemia levels for about 2 months (Jiménez-Díaz, Mulet et al. 2009). Sustaining *P. falciparum* infection with an *in vivo* system may yield important insights to the adaptation and expression of virulence factors. This is important as we know that laboratory-cultured *in vitro* parasites differ from parasites taken from patients (Peters, Fowler et al. 2007).

Moreover, NSG mice can be engrafted with components of the human immune system by irradiating newborn pups and injecting intracardially with CD34⁺CD133⁺ hematopoietic stem cells (HSCs). To reconstitute specific human immune cells, plasmids encoding human cytokines are delivered into the mice via hydrodynamic injections. Monocytes and macrophages can be reconstituted with MCSF-encoding plasmids. NK cells with IL-15 and FMS-like tyrosine kinase 3 ligand (Flt-3L) and dendritic cells with GM-CSF, IL-4, and Flt-3 (Chen, Khoury et al. 2009). Humanized mice for *P. falciparum* infections done previously in our lab have shown that NK cells, not monocytes, are important for the control of parasitemia in the humanized mice (Chen, Amaladoss et al. 2014).

1.5 Project Objectives

P. falciparum infection in immune compromised NSG mice supplemented with human RBC involves the adaptation of laboratory-cultured *in vitro* strains such as 3D7. Through adaptation, these parasites become competent in thriving in the mice without the need for treatment to deplete phagocytes in the mice. Understanding this process of adaptation by the parasites in human RBC-supplemented NSG mice allows us to investigate the interaction of host immune cells and pathogen. Therefore, this project aims to:

1. Understand and characterize the process of adaptation to elucidate the mechanism for adaptation in the human RBC-supplemented NSG mice.

2. *In vitro* and *in vivo* characterization of adapted *P. falciparum* in the context of host-pathogen interaction particularly to innate immune cell response.

Chapter 2. Materials and Methods

2.1 Adaptation of *P. falciparum* in huRBC-NSG mice

6-week-old female NSG mice were supplemented with 1ml of human RBC intraperitoneal injections daily comprising of 500µl whole RBCs, 250µl RPMI 1640 (Sigma-aldrich), 250µl human serum AB (Sigma-aldrich), 0.51mg hypoxanthine (Sigma-aldrich) filtered in 0.2µm filter units (Nalgene). After 10 days, peripheral blood human RBC reconstitution levels were checked by tail vein or submandibular bleed. 1µl of blood was stained PE human anti-CD235ab (Biolegend) staining for 15 mins at room temperature and washed 3 times in FACS buffer (0.2% BSA (Sigma-aldrich), 0.05% sodium azide (Sigma-aldrich) in 1xPBS). Mice with reconstitution levels above 20% were infected with *P. falciparum*. 1×10^7 mixed stage *P. falciparum* injected intravenously into the tail vein. Mice were periodically check for parasitemia via Giemsa Staining (10% Giemsa stain (Sigma-aldrich) in ultrapure water). Upon detection of parasitemia, mice were bled and adapted parasites were recovered and cultured in MCM until high parasitemia of above 5% parasitemia in culture before frozen down as stabulates in 50% freezing media (28% glycerol, 3% sorbitol, 0.65% NaCl in ultrapure water).

Clodronate treatment of huRBC-mice were done using Clodrosome® Liposomal Clodronate (Encapsula NanoSciences LLC). 25 µl per dose per mouse of 5mg/ml Clodrosome® was given via intraperitoneal injections at Day -6, Day-4 and Day -2 before infection with *P. falciparum* at Day 0.

In vivo imaging was captured on IVIS® Spectrum (PerkinElmer) with mice injected with RediJect D-Luciferin Bioluminescent Substrate (PerkinElmer) at 100 µl via intraperitoneal injection. Data was analyzed on LivingImage 3.2 (PerkinElmer).

Studies involving mice were approved by the institutional animal care and use committee (IACUC) of National University of Singapore (NUS), Agency of Science, Technology and Research (A*STAR) and Massachusetts Institute of Technology (MIT).

2.2 *P. falciparum* culture and infection

P. falciparum strains 3D7, 3D7attb, NF54attb cultured in 2.5% hematocrit human RBC in malaria culture media (MCM) comprising of 10.43g RPMI 1640 powder (Gibco), 25ml 1M HEPES (Gibco), 2g NaHCO₃ (Sigma-aldrich), 5g Albumax (Gibco), 0.05g hypoxanthine (Sigma-aldrich) and 25mg gentamicin (Gibco) in 1L milli-Q water. Cultures were incubated in Heracell 150 incubator (Thermo Scientific) at 37 in 5% CO₂, 3% O₂ and 92% N₂. Ring-stage iRBC were synchronized by using 5% D-sorbitol and schizont-stage iRBC were isolated by centrifugation on a 65% Percoll gradient at 2500 x g for 15 mins using brake-free settings.

3D7attb (Nkrumah, Muhle et al. 2006), NF54attb (Adjalley, Johnston et al. 2011) and NF54attb^{cas9+T7 Polymerase} (pCRISPR) (Sidik, Huet et al. 2016) were kindly shared with us by Prof Jacquin Niles and Prof David Fiddock. MAHRP KO is a kind gift from Prof Hans-Peter Beck (Spycher, Rug et al. 2008).

2.2.1 Adapted *P. falciparum* maintains adaptation after long term *in vitro* culture

Transfection of parasite utilized 50 µg of plasmid (either pMG56 or pSN054) per 300 µl packed red blood cells (RBCs), adjusted to 400 µl total volume in RPMI and placed in 0.2-cm electroporation cuvettes (Bio-rad). The electroporation of uninfected RBCs rather than directly on infected RBCs is termed pre-loading RBC technique (Deitsch, Driskill et al. 2001). Using Gene Pulser Xcell™ system (Bio-Rad), electroporation settings were eight square-wave pulses of 365 V for 1 ms, separated by 100 ms were used. Transfected RBCs were placed in 37°C water bath for 1 hour and washed twice with MCM. 500 µl of 1% parasitemia parasite culture was added to the transfected RBCs in normal MCM. After 4 days, they were placed in MCM with 2.5 mg Blasticidin S. Parasite transfected with the 10x Tet-R DOZI aptamer array were also maintained in 0.5 mM anhydrotetracycline (aTc) throughout culturing. No aTc conditions were done by washing the parasite culture twice and culturing in MCM without aTc for 24 hours before the assay was done.

2.2.2 Single parasite cloning

Single parasite cloning of a parasite culture was done by determining the parasitemia of the culture in percentage. Count the neat culture in hemocytometer to determine total RBC and iRBC per ml. Add 1×10^6 cells into 10ml of warm MCM (Solution A). Determine the volume of solution A to add to the master mix using the formula:

Vol of solution A = Number of wells x (parasite per well)/parasitemia (in decimal) x cell/L

We have set the parasite per well as 0.5. Once the volume of solution A is determined. Master mix calculation is as follows:

MCM to add = (96x100 µl) – (Vol of solution A) – (200 µl packed RBCs)

Where 200 µl is the amount of packed RBCs needed in a 96 well plate at 2% hematocrit and total well volume is set at 100 µl. MCM and solution A and the packed RBCs are mixed well and aliquoted into a 96 well plate. Wells with parasites are determined by Giemsa staining and subsequently expanded.

2.3 Whole genome sequencing of *P. falciparum*

P. falciparum genomic DNA was isolated from high parasitemia cultures using NucleoSpin® Blood Columns with Collection Tubes (Macherey-Nagel) using manufacturer's protocol. Whole genome next generation sequencing of adapted and non-adapted *P. falciparum* were done at Singapore Centre for Environmental Life Sciences Engineering Sequencing Core using Illumina Miseq Run V3; 2x300bp and Illumina DNA Library Preparation. FASTQ files of sequencing reads were aligned to the *P. falciparum* 3D7 reference genome available, at PlasmoDB, using bowtie2 v2.3.2. SAM file generated from the alignment is converted to BAM files using samtools v1.3. SNP variant calling on the BAM files was done using freebayes v1.0.1 and SNP filtering based on

QUAL using vcfilter. Kinship coefficient analysis on SNPs were kindly helped by Neslihan Kaya from Genome Institute of Singapore.

2.4 *P. falciparum* microarray and analysis

Highly synchronized parasite cultures cultured in 25ml MCM and 2.5% hematocrit were centrifuged and washed in 1X PBS and snapped frozen in liquid nitrogen capturing six time points (TP1 to TP6). TP1 is collected at 8 hours and the final collection, TP 6, is at 48 hours. Blood pellets were stored in -80°C. Parasite RNA was isolated by thawing blood pellet samples at 65°C for 5 min and adding TRIzol reagent (Invitrogen) at a ratio of 10:1 to blood sample. A 1:5 ratio of chloroform (Fisher Scientific) to TRIzol mixture was added and incubated on ice for 5 min. Centrifugation at 3,900 x g for 10 min at 4°C followed by the recovery of the top aqueous phase that contains the RNA was collected and added with equal volume of isopropanol (Fisher Scientific) and kept at -20°C for overnight RNA precipitation. After centrifugation at 11,000 x g for 1 hour at 4°C, the RNA pellet was washed with cold 70% ethanol (Merck), air dried and subsequently resuspended in RNAase-free water. The integrity of the RNA was determined on RNA 6000 Nano chips (Agilent) and read on the 2100 Bioanalyzer Instrument (Agilent).

Amplification of cDNA was carried out using Switching Mechanism At 5' end of RNA Template (SMART) polymerase chain reaction (PCR) to ensure the synthesis of full-length cDNA products from the mRNA template. This is first done with first strand synthesis by reverse transcription involved 500ng of

total RNA with SMART primer mix (25 μ M SMART-oligo-dT, 25 μ M SMART-N9 and 50 μ M TS-oligo) to a total volume of 8 μ l and incubated at 65°C for 5 min and chilled on ice for 10 min. Subsequently, 4 μ l of 5X First Strand reverse transcription buffer, 2 μ l of 0.1M DTT, 2 μ l of 3 mM dNTPs (dATP, dCTP, dGTP, dTTP), 40 units of RNaseOUT™ Recombinant Ribonuclease Inhibitor (Invitrogen) were added to make a total volume of 19 μ l. The mixture was incubated at room temperature for 2 min followed by the addition of 200 units of SuperScript® II Reverse Transcriptase at 42°C for 50 min.

2.5 μ l of the cDNA product mixed with 0.45mM dATP, 0.225mM dTTP, dGTP, dCTP and 0.225mM amino-allyl-dUTP (Biotium, USA), 6 μ M Primer IIa, 10 μ l of 10X Thermopol® Buffer and 10 units of Taq DNA polymerase (New England Biolabs) to a total of 100 μ l volume reaction. PCR cycling conditions were at 1 cycle at 95°C for 5 min. 60°C for 1 min and 68°C for 10 mins, 19 cycles of 95°C for 30 sec, 60°C for 30 sec and 68°C for 5 mins, 1 cycle at 72°C for 5 min and hold at 4°C. Amplified PCR products were visualized on 1% agarose gel with products ranging from 100-1500 bp. PCR products were purified with MinElute PCR purification kit (Qiagen) according to manufacturer's protocol and eluted in 16 μ l of elution buffer.

The reference pool was created by adding equal amounts of RNA from each timepoint from the parental strain and undergoing the same method for cDNA generation and PCR amplification as stated earlier.

4 µg of SMART-amplified DNA samples were labelled with Cy3 or Cy5 (GE Healthcare) in 0.1M NaHCO₃ pH 9 for 4 hours at room temperature in the dark. Timecourse samples were labeled with Cy5 and reference pools were labeled with Cy3. Labeled samples were purified using the MinElute PCR purification kit (Qiagen) and eluted in 15 µl of elution buffer.

Cy5-labeled samples were mixed with Cy3-labeled reference pool and boiled at 100°C for 3 min and cooled at room temperature for 5 min. 95 µl of elution buffer and 125 µl of 2X Hi-RPM Hybridization Buffer (Agilent) were added to the combine labeled samples and hybridized on post-processed microarray chips for 20 hr at 65°C using the Agilent hybridization system (Agilent). The chips were then washed in 0.6X saline-sodium citrate (SSC)/0.03% (sodium dodecyl sulfate) SDS for 5 min and in 0.06X SSC for another 5 min. The chips were spun dry at 600 rpm for 6 min and then scanned using PowerScannerTM (Teacan).

The microarray scanned images were analyzed using GenePix Pro 6.0 program (Axon Instruments) and the data was local regression LOESS normalized and filtered for signal intensity over the background noise using R package LIMMA. The expression profile for each gene was represented by an average expression of all the probes represented on the chip.

Differentially expressed genes were determined using Significance Analysis of Microarray (SAM) (Tusher, Tibshirani et al. 2001).

2.5 Cloning of pSN054 plasmid

Plasmid pSN054 (kindly shared and in collaboration with Prof Jacquin Niles) was constructed in 3 segments, namely left homology region (LHR), right homology region (RHR) and single guide RNA region (sgRNA). LHR consist of the 500bp homology region and the 300bp recoded region. This constitute to the last 800bp at the 3' end of the gene of interest with the stop codon removed. The recoded region contains the sgRNA cut site and was recoded to prevent continuous recognition by the sgRNA:cas9 complex. Recoding was done using codon juggling software (Gene Design) with organism set to *Saccharomyces cerevisiae*. RHR consist of the 500bp region in the 3' UTR of the gene of interest immediately downstream of the stop codon. The sgRNA region consist of the 20bp variable sgRNA with 40bp overhangs on both side to the pSN plasmid, creating a 100bp insert into the pSN054 plasmid.

P. falciparum genomic DNA was isolated from high parasitemia cultures using NucleoSpin® Blood Columns with Collection Tubes (Macherey-Nagel) using manufacturer's protocol. Forward and reverse primers for RHR consisted of 25bp overhangs to the plasmid at the I-SceI cut site. 10ng of genomic DNA in a 50 µl PCR reaction using LongAmp® Taq DNA Polymerase (New England Biolabs) according to manufacturer's protocol. Thermocycling conditions were 95 for 1 min, 30 cycles of 95°C for 5sec, 50°C for 20 sec, 45°C for 20 sec and 60°C for 4 min and 30 sec. Final extension at 60°C for 10 mins.

PCR product was loaded and ran in a 1% agarose gel at 120V for 40 mins. PCR band was confirmed according to size and gel extracted using NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel). To insert RHR region into pSN054, 4 µg of original pSN054 was incubated overnight at 37°C with restriction enzyme I-SceI (New England Biolabs) and heat inactivated at 65°C for 20 mins and cleaned up using NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel). 50ng of I-SceI cut vector pSN054 and 30ng of PCR RHR insert were Gibson assembled using NEBuilder® HiFi DNA Assembly Master Mix (New England Biolabs) according to manufacturer's protocol and incubated for 60 minutes at 50°C. 4µl of the reaction mix was then added to 50 µl of BigEasy®-TSA™ Electrocompetent Cells (Lucigen Corporation) and pulsed in Gene Pulser Xcell™ system (Bio-Rad) in 1.0mm cuvettes at 10µF, 600 Ohms and 1800V. Cells were allowed to recover for 1hr shaking in Recovery Medium (Lucigen Corporation) at 37°C. Cells were then plated in Terrific Broth (Life Technologies) agar plates with chloramphenicol selection antibiotic (35µg/ml; Sigma-aldrich) overnight at 37°C. Colonies were picked and expanded in 5ml of Terrific Broth with chloramphenicol and L-(+)-arabinose (10µg/ml; Sigma-aldrich) and incubated overnight at 37°C. Plasmids were extracted using QIAprep Spin Miniprep Kit (Qiagen) and sent for sequencing using sequencing primer "SeqP3 PSN054 RHR Upstream" = 5'-tagcataacccttggggcctctaa-3'. Plasmid with the correct insertion proceeded for LHR insertion.

LHR forward primer consisted of 25bp overhang region to the pSN plasmid and reverse primer to the 5' of the recoded region of the gene of interest.

PCR of LHR region with overhangs was done using LongAmp® Taq DNA Polymerase and gel extracted as previously mentioned. 4 µg of RHR was incubated overnight at 37°C with restriction enzymes FseI and AsisI (New England Biolabs) and heat inactivated at 65°C for 20 mins and cleaned up using NucleoSpin® Gel and PCR Clean-up. 50ng of cut RHR pSN054 vector and 30ng of PCR LHR insert and 30ng of gBlock (Integrated DNA Technologies) recoded region were Gibson assembled in one pot using NEBuilder® HiFi DNA Assembly Master Mix (New England Biolabs) according to manufacturer's protocol and incubated for 60 minutes at 50°C. Again, 4µl of the reaction mix was then added to 50 µl of BigEasy®-TSA™ Electrocompetent Cells and pulsed in Gene Pulser Xcell™ system (Bio-Rad) in 1.0mm cuvettes at 10µF, 600 Ohms and 1800V. Cells were allowed to recover for 1hr shaking in Recovery Medium at 37°C. Cells were then plated in Terrific Broth agar plates with chloramphenicol selection antibiotic overnight at 37°C. Colonies were picked and expanded in 5ml of Terrific Broth with chloramphenicol and L-(+)-arabinose and incubated overnight at 37°C. Plasmids were extracted using QIAprep Spin Miniprep Kit (Qiagen) and sent for sequencing using sequencing primer "Valseq HA Tag PRev" (Appendix) to confirm presence of LHR and recoded region. Plasmid with the correct insertion proceeded for sgRNA insertion.

The insert for sgRNA is made from two 60bp primers with a 20bp overlap that is the sgRNA target sequence. These primers are made into double stranded DNA using Klenow Fragment (3'→5' exo-) (New England Biolabs) with CutSmart® Buffer and dNTPs (New England Biolabs) using the following

protocol. Primers and dNTPs were primed for 5mins at 98°C followed by the addition of Klenow fragment and further incubated for 1 hour at 37°C and then 75°C for 20 mins. The reaction was cleaned up using NucleoSpin® Gel and PCR Clean-up.

sgRNA Klenow Reaction	
Cutsmart Buffer	4
dNTPs	1.32
Primer 1 (100uM)	0.7
Primer 2 (100uM)	0.7
Klenow Polymerase	2
dH ₂ O	33.78
Total	42ul

4µg LHR and RHR integrated plasmid was incubated with AflIII restriction enzyme (New England Biolabs) overnight at 37°C and heat inactivated at 65°C for 20 mins. 50ng of cut LHR and RHR pSN054 vector and 30ng of sgRNA product were Gibson assembled NEBuilder® HiFi DNA Assembly Master Mix according to manufacturer's protocol and incubated for 60 minutes at 50°C. Again, 4µl of the reaction mix was then added to 50 µl of BigEasy®-TSA™ Electrocompetent Cells and pulsed at 10µF, 600 Ohms and 1800V. Cells were allowed to recover for 1hr shaking in Recovery Medium at 37°C. Cells were then plated in Terrific Broth agar plates with chloramphenicol selection antibiotic overnight at 37°C. Colonies were picked and expanded in 5ml of Terrific Broth with chloramphenicol and L-(+)-arabinose and incubated

overnight at 37°C. Plasmids were extracted using QIAprep Spin Miniprep Kit and sent for sequencing using sequencing primer “SeqP5 PSN054 sgRNA upstream” = 5'-tgcataatcaggtacgtcataaggata-3' to confirm presence of sgRNA insert. This is the final construct for the pSN054 plasmid and was expanded in 100ml of Terrific Broth with chloramphenicol and L-(+)-arabinose at 37°C overnight and plasmids extracted using Plasmid Plus Midi Kit (Qiagen).

2.6 Peripheral blood mononuclear cell (PBMC) purification.

Whole blood donated by healthy non-malarial immune adult volunteers at the National University Hospital of Singapore Blood Donation Center. Informed consent was obtained from all donors in accordance with approved protocol and guidelines. Project approval was obtained from the Institutional Review Board of National University of Singapore (NUS-IRB 10-285). Whole venous blood collected in Citrate-Phosphate Dextrose-Adenine-1 (CPDA-1, JMS) and PBMC were isolated from whole blood over Ficoll-Paque PLUS (GE Healthcare) density gradient. Pelleted RBC were washed twice in RPMI 1640 (Sigma-Aldrich) and stored 1:1 in MCM. Remaining RBCs within the PBMC fraction were lysed with ACK lysis buffer (Life Technologies) and purified PBMC were washed twice with RPMI 1640. PBMCs were counted and cryopreserved at a concentration of 1×10^8 cells/ml in 1:1 RPMI and PBMC freezing medium of 85% fetal bovine serum (Gibco) and 15% dimethyl sulfoxide (Sigma-Aldrich) in liquid nitrogen.

2.7 Monocyte-derived Macrophage and phagocytosis assay

Human donor monocytes were isolated using EasySep™ Human Monocyte Isolation Kit (StemCell Technologies) according to manufacturer's protocol. Monocytes were seeded into 96 well plate at 100,000 cells per plate and cultivated in the presence or absence of human recombinant Macrophage Colony Stimulating Factor (MCSF), (20ng/ml; Peprotech) in RPMI 10% FBS. Non-adherent cells were washed off during the media change every other day. Infected RBCs were first sorbitol-treated with 5% sorbitol (Sigma-aldrich) w/v in Ultrapure water for 15mins. 24hrs later, late trophozoites were enriched using a Percoll (Sigma-aldrich) gradient of 65% and 35% layered over each other (Miao and Cui 2011). 35% Percoll layer removed haemozoin pellets and debris and 65% Percoll enriched late trophozoites and schizonts iRBCs. Infected RBCs were then stained with DAPI (4',6-Diamidino-2-Phenylindole, Dilactate (Life Technologies)) according to manufacturer directions at 1:1000 ratio for 15 mins and washed off in MCM for 3 times. Infected RBCs were then seeded at 5 iRBCs (target) to 1 macrophage (effector) (therefore 500,000 iRBCs per well) and co-cultured in MCM for 90 mins. After the co-culture, the macrophages were washed in PBS to remove excess iRBCs and lifted off the well using cell detaching solution Accutase (StemCell Technologies) at room temperature for 15 minutes incubation. Macrophages were stained with anti-human CD14⁺ PE (Clone 63D3; Biolegend) marker and quantified using Attune NxT (Life Technologies). Phagocytosis index is calculated as the percentage of

macrophages that is now DAPI-positive after ingesting DAPI-stained parasites, counting for 5,000 events in the macrophage gate. Data was analyzed using FlowJo (Becton, Dickinson and Company)

Compounds for polarizing macrophages were dissolved according to manufacturer's protocol and seeded into each well of M0 macrophages at a concentration of 1 μ M for 24 hours. After which, compounds were washed off with 1xPBS for 3 times and iRBCs added as mentioned previously for phagocytosis assay.

2.8 Primary NK cell parasitemia reduction co-culture

Primary NK cells were isolated from donor PBMCs using EasySep™ Human NK Cell Enrichment Kit (Stemcell Technologies) according to manufacturer's protocol. iRBCs for the co-culture were synchronized to late trophozoite by sorbitol treatment for 15mins, performed 24 hours before the co-culture. We seeded 0.5% parasitemia per well at 2% hematocrit and NK cells (effector) to iRBCs (target) ratio was set at 1:10 (5×10^4 : 5×10^5) in 200 μ l MCM per well in a 96-well round bottom plate. Infected RBCs with no NK cell to track parasitemia increase in the absence of NK cells were seeded in triplicate wells. MCM media was changed every other day. Quantification of parasitemia was done by flow cytometry using 2 μ l of iRBC pellet stained for 15 mins at room temperature with human anti-CD45 Pe-Cy7 (Clone 2D1; Biolegend), human anti-CD56 APC (Clone MEM-188; Biolegend) for NK cell surface staining and with Hoechst 33342 (1:1000 dilution; Life Technologies) for iRBC staining and

analyzed using Attune NxT (Life Technologies) and data analyzed in FlowJo (Becton, Dickinson and Company). NK Cell Activation and Expansion System (NKAES) NK cells were kindly given by Prof Dario Campana and cultured in RPMI+10%FBS in recombinant IL-2 (100U/ml; R&D Systems, Inc). Anti-LILRB1 blocking antibody was seeded at 500ng/ml per well (R&D Systems, Inc).

2.9 Immunofluorescence assay (IFA)

Smears of late stage parasite cultures were made of glass slides and methanol fixed on ice for 15 mins and air dried. Smears were blocked in 3% bovine serum albumin (BSA; Sigma-aldrich) in 1xPBS for 1 hour at room temperature and washed three times with 1xPBS. Primary antibody incubation was done overnight at 4°C. Primary antibodies of Rat Anti-HA (Roche) at 1:100 and Mouse Anti-PfEMP1 ATS at 1:500. After 3 washes with 1xPBS, secondary antibodies of Goat anti-Rat IgG (H+L) Alexa Fluor 488 (1:500; Invitrogen) and Goat anti-Mouse IgG (H+L) Alexa Fluor 647 (1:500; Invitrogen) with Hoechst 33342 (1:1000) were incubated for 1 hour at room temperature and washed 3 times. The slides were then mounted in VECTASHIELD® Antifade mounting media (Vector Laboratories) and imaged on LSM710 confocal microscope (Carl Zeiss) and data analyzed on ZEN 2 (Carl Zeiss).

2.10 Western Blot

Late trophozoite parasites cultured with and without aTc were isolated using a 65% Percoll gradient and centrifugation at 2500 x g for 15 mins using brake-free settings. Recovered parasites were washed 3 times with RPMI and added to 200µl of 7.5mM Tris-HCL pH8.0 and placed in -80°C overnight. Frozen samples were centrifuged at 20,000 x g. The pellet was washed 3 times in 1xPBS and placed in 2% SDS and centrifuged for 900 x g for 5 mins. 11 µl of the aqueous layer was added with 5 µl of 4x Laemmli Sample Buffer (Bio-rad) and 4 µl of β-mercaptoethanol (Sigma-aldrich) and loaded on a 10% Mini-PROTEAN® TGX™ Precast Protein Gel and ran for 80V for 15 mins and raised to 110V for 45 mins. Samples were transferred to 0.2 µm Polyvinylidene difluoride (PVDF) membrane using Trans-Blot Turbo Transfer System (Bio-rad) at mixed molecular weight setting of 1.3A for 7 mins. PVDF blot is then washed in 0.1% PBS-Tween (PBS-T) and blocked in 5% skim milk in PBS-T for 1 hour at room temperature. Primary antibody probing using Rat anti-HA tag (Roche) and Mouse anti-Actin (Invitrogen) at 1:3000 in 2% Bovine Serum Albumin (BSA) in PBS-T overnight at 4°C on a rocker. The blot was washed 3 times in PBS-T for 10 mins each time and probed with secondary antibodies Goat Anti-Rat HRP (Biolegend) and Goat Anti-mouse HRP (Biolegend) at 1:10,000 in 2% BSA PBS-T for 1 hour at room temperature and again washed for 3 times. The blot was imaged on ChemiDoc MP (Bio-rad) in Clarity Max Western ECL Substrate (Bio-rad). Western blot analysis was done using Image Lab v6.0 (Bio-rad).

2.11 qPCR

200 ng of RNA is reverse transcribed to cDNA by SuperScript II Reverse Transcriptase (ThermoFisher) using the same method as the microarray workflow. Real-time PCR of 20 µl total volume with 1 µl of the cDNA added to 10 µl of iQ Sybr Green Master Mix (Bio-Rad) with 0.5 µl of forward and reverse primers (500nM) and 8 µl of ddH₂O. Thermocycling started at 95°C for 2 mins and 40 cycles of 95°C for 10 sec, 55°C for 15 sec and 72°C for 30 sec on the CFX1000 Real-Time PCR Detection System (Bio-Rad). *P. falciparum* reference gene used was PF3D7_0717700 seryl-tRNA synthetase using forward primer 5'-AAGTAGCAGGTCATCGTGGTT-3' and reverse primer 5'-TTCGGCACATTCTTCCATAA-3' (Salanti, Staalsoe et al. 2003). VAR2CSA qPCR primers used were forward primer 5'-CACGACATTAACAATACATGCAGA-3' and reverse primer 5'-CATTGCATTACAGACATTGG-3' (Rottmann, Lavstsen et al. 2006). qPCR results were analyzed using REST2009 analysis program (Qiagen).

Chapter 3. Characterization of adapted *P. falciparum* in huRBC-NSG mice

3.1 Adaptation of *P. falciparum* in huRBC-NSG mice

To support *P. falciparum* infection in NSG mice supplemented with human RBCs (huRBC-NSG), two requirements need to be fulfilled. Either the NSG mice are treated with clodronate to deplete existing phagocytes in the mice (Arnold, Tyagi et al. 2011) or using competent *P. falciparum* that have been adapted in the huRBC-NSG mice (Angulo-Barturen, Jiménez-Díaz et al. 2008). Adaptation of *P. falciparum* strains for thriving in the huRBC-NSG mice and humanized mice with human reconstituted immune system is a more attractive method because using clodronate or other immune modulating therapy would add another layer of complexity. Therefore, we adapted our existing laboratory-cultured strains such as 3D7, 3D7attb, NF54attb, pCRISPR, W2mef and T994 for competent growth in the huRBC-NSG mice.

3.1.1 Generating huRBC-NSG mice and *P. falciparum* infection

To produce huRBC-NSG mice suitable for *P. falciparum* infection, 1ml of 50% hematocrit human RBCs were injected intraperitoneally into NSG mice for 10 to 14 days. Mice with more than 20% human RBC reconstitution, as determined by flow cytometry, are used for parasite infection (Fig. 3.1A). As shown in Fig 3.1B, human RBC reconstitution in NSG mice progressively

increased in the mouse peripheral blood from 0% on day 0, to 14.2% on day 7 and 34.7% on day 10.

Figure 3.1: NSG mice supplementation with human RBCs. (A) Schematic of events for human RBC-supplementation of NSG mice. (B) Representative FACS plot of human CD235ab to determine levels of human RBC in huRBC-NSG mice peripheral blood.

HuRBC-NSG mice with human RBC reconstitution of more than 20% were infected with 1×10^7 iRBCs non-adapted parasites on Day 0 (Fig. 3.2A). Peripheral blood of infected mice was analyzed every 5 days by blood smears for presence of parasites (Fig. 3.2B). For parasite strain T994, we do not detect any parasite in the peripheral blood at Day 7, consistent with the clearance of the

iRBC by the mice. At Day 19, we detected very low number of parasites and at Day 23, the number of parasites increases. As shown in Figure 3.2A, positive peripheral blood smear for T994 parasites was observed on Day 19; for W2mef on Day 23; and for 3D7 on Day 36. The apparent clearance of iRBC from the peripheral blood, as seen in all three parasite strain in Figure 3.2A and the reappearance of parasites in the peripheral blood, is consistent with existing literature (Angulo-Barturen, Jiménez-Díaz et al. 2008). The reappearance and increase in parasitemia in the huRBC-NSG mice indicate that the parasite has been adapted and are now competent for growth in the huRBC-NSG mice.

B)

Figure 3.2: Parasitemia of non-adapted *P. falciparum* after infection in the huRBC-NSG mice. (A) In the initial 2 weeks, no parasitemia can be seen in the peripheral blood. After Day 17 for T994, Day 21 for W2mef and Day 23 for 3D7, parasites can be seen in the blood film Giemsa smear, which stains DNA as dark purple and cytoplasm as pale blue. (B) Representative thick Giemsa stain blood film of peripheral huRBC-NSG mouse blood infected with T994, showing no parasites at Day 7, the appearance of a single parasite at Day 19 and increasing parasitemia at Day 23.

For huRBC-NSG mouse-adapted *P. falciparum*, we added the “AS” designation to the strain name to indicate that it has been adapted. Non-adapted parasites were given the designation “WT” for wildtype.

3.1.2 Adapted *P. falciparum* maintains adaptation after long term *in vitro* culture

We wanted to determine if adapted parasites would revert to become non-adapted parasites again after being cultured *in vitro* for multiple generations. Therefore, adapted parasites were bled from the huRBC-NSG mice and *in vitro* cultured for expansion for 10 generations and infected into new huRBC-NSG mice. Adapted parasites were able to initiate infections immediately and sustain parasitemia in huRBC-NSG mice for 37 days before we terminated the experiment (Fig. 3.3B). This demonstrates that adapted parasites were able to maintain adaptation even though they were cultured *in vitro* for multiple generations. The different stages of iRBC, from rings to schizonts, were seen in the peripheral blood of mice during the course of infection (Fig. 3.3A), similar to what Angulo-Barturen *et al* had described.

Figure 3.3: Parasitemia of adapted *P. falciparum* after infection in the huRBC-NSG mice. (A) Giemsa stain of huRBC-NSG mouse peripheral blood smear showing the presence of rings (white arrow), trophozoites (blue arrow) and schizonts (red arrow) in

the peripheral blood circulation. (B) Parasitemia of two T994 adapted strains and two W2mef adapted strains infections were tracked in huRBC-NSG mice for up to 37 days.

3.1.3 Adaptation of *P. falciparum* to huRBC-NSG mice not due to genetic heterogeneity of parasite cultures

We wanted to know if adaptation is due to the selection of a unique parasite from a genetically heterogenous culture. *In vitro* cultured *P. falciparum* have been in continuous culture cumulatively for many years. This could lead to genetic heterogeneity within a culture (Yeda, Ingasia et al. 2016).

3D7 B7, a single parasite clone which we attained from our *P. falciparum* 3D7 culture using limiting dilutions represents a genetically homogenous culture. Adaptation of 3D7 B7 took 26 ± 5.1 days, similar to our earlier adaptation of parasites. 3D7 B7 AS parasites were then bled from the mice and expanded in *in vitro* culture for more than 10 generations and re-infected into new huRBC-NSG to confirm that it is indeed adapted (Fig. 3.4). Therefore, adapted parasites were able to be derived from a genetically homogenous non-adapted culture.

Figure 3.4: Parasitemia of adapted 3D7 B7 AS in huRBC-NSG mice. Parasitemia of 3D7 B7 AS infection in huRBC-NSG mice after adaptation and *in vitro*.

3.2 Adapted parasites are genetically identical to parental non-adapted parasites

Since adaptation did not come from the selection of genetically unique parasites within a heterogenous culture, we wanted to compare if non-adapted and adapted parasites were genetically different from one another due to adaptation. We hypothesized that the genetic difference could be gain of function SNPs and would be conserved in all the huRBC-NSG adapted strain. Therefore, we conducted whole genome sequencing of three pairs of *P. falciparum* strain, before and after adaptation in the huRBC-NSG mice. They were 3D7 B7 WT and 3D7 B7 AS, 3D7attb WT and 3D7attb AS and NF54attb WT and NF4attb AS. 3D7 is a clone of NF54 derived by limiting dilutions (Rosario 1981). Hence, all our sequencing reads can be aligned to the available *P. falciparum* 3D7 reference genome.

Illumina MiSeq paired end 2x300bp reads generated 44-50 million reads, an output of 15Gb that provides about 218 times coverage for the entire 22.9 Mb genome for the six parasite strains. The standard workflow for SNP variant calling analysis was firstly to map the raw reads FASTQ files to the *P. falciparum* 3D7 reference genome using bowtie 2.3.2. We subsequently sort the read aligned SAM files to BAM files using samtools 1.3 ready for variant calling. SNP variant calling was done using freebayes1.0.1 and filtering of the called SNPs based on

quality threshold with vcfFilter. The threshold for quality and read depth with GATK was set at Quality > 500, Read Depth >75.

SNPs called in the parental non-adapted parasite strain (e.g. 3D7attb WT), were also found in their corresponding adapted parasite (e.g. 3D7attb AS). This shows that the adapted parasites are related to their corresponding non-adapted parasites. However, analysis of all the unique SNPs found in the adapted parasite were not conserved among the 3 adapted parasite strains. This indicated that there was no conserved genetic change that was responsible for adaptation in all three adapted parasite strains.

Using a kinship coefficient analysis termed Kinship based INference for Genome-wide association studies (KING) (Manichaikul, Mychaleckyj et al. 2010), SNPs called from each parasite strain were analyzed to determine their identity by descent (IBD) and determine their relatedness to one another (Henden, Lee et al. 2018). Non-adapted and adapted parasite pairs did not meaningfully diverge, therefore they had high kinship coefficient values to one another. (Fig 3.5A). Kinship coefficient values of above 0.354 corresponds to duplicate or monozygotic twin (Yang, Xu et al. 2011). All our kinship coefficient values were greater than 0.48, which shows that they are genetically identical to one another. Therefore, adaptation did not genetically diverge non-adapted and adapted parasites and adaptation is not due to genetic changes in the adapted parasite.

Figure 3.5: Kinship coefficient analysis of SNPs in adapted and non-adapted parasites. (A) Kinship coefficient values of the different *P. falciparum* strains compared to a specific strain. (B) Heat map of kinship coefficient values compared across the different strains.

3.3 Gene expression differences between non-adapted and adapted parasites enrich for variant surface antigens

Since genetic changes are not the cause of adaptation in these parasites, we compared the gene expression of adapted parasites to non-adapted parasites using microarray analysis. 3D7 B7 WT and AS, 3D7attb WT and AS and pCRISPR WT and AS were highly synchronized and RNA harvested at 8 hour intervals across the intraerythrocytic development cycle (IDC) to generate 6 timepoints per parasite strain. Differentially expressed genes (DEGs) were determined using Significance Analysis of Microarray (SAM) (Tusher, Tibshirani et al. 2001). Using a delta of 0.065 selects differentially expressed genes with $FDR < 0.25$ (Fig. 3.6A), SAM revealed 267 significantly upregulated genes (Fig. 3.6B) and 321 significantly downregulated genes (Fig. 3.6C).

A)

Figure 3.6: Differentially expressed genes in adapted *P. falciparum* compared to non-adapted. (A) SAM analysis of observed and expected score for each *P. falciparum* gene to determine DEGs that are upregulated (in red) and downregulated (in green) set at delta 0.065. (B) Heatmap of six timepoints across 48-hour IDC gene expression of upregulated DEGs in adapted parasites (right of white line) compared to non-adapted parasites (left of white line). (C) Heatmap of six timepoints across 48-hour IDC gene expression for downregulated DEGs in adapted parasites (right of white line) compared to non-adapted parasites (left of white line)

Gene Ontology (Ashburner, Ball et al. 2000, Consortium 2018) biological processes analysis (Mi, Muruganujan et al. 2018) of the adapted parasite DEGs indicates adaptation of *P. falciparum* in hu-RBC NSG mice involves processes such as cytoadherence to microvasculature, modulation of symbiont host erythrocyte aggregation, modulation by symbiont of host cellular process and antigenic variation (Fig. 3.7). These processes are mediated primarily by parasite variant surface antigens (VSAs).

	Plasmodium falciparum (REF)	upload_1 (▼ Hierarchy NEW! ?)				
GO biological process complete	#	#	expected	Fold Enrichment	+/-	raw P value
cytoadherence to microvasculature mediated by symbiont protein	57	16	5.36	2.98	+	3.67E-04
↳ adhesion of symbiont to host	83	21	7.81	2.69	+	2.42E-04
↳ symbiotic process	294	55	27.67	1.99	+	7.07E-06
↳ interspecies interaction between organisms	301	55	28.32	1.94	+	1.38E-05
↳ multi-organism process	306	55	28.79	1.91	+	1.72E-05
modulation by symbiont of host erythrocyte aggregation	80	21	7.53	2.79	+	1.04E-04
↳ regulation of erythrocyte aggregation	80	21	7.53	2.79	+	1.04E-04
↳ regulation of homotypic cell-cell adhesion	80	21	7.53	2.79	+	1.04E-04
↳ regulation of cell-cell adhesion	80	21	7.53	2.79	+	1.04E-04
↳ regulation of cell adhesion	80	21	7.53	2.79	+	1.04E-04
↳ modification by symbiont of host morphology or physiology	81	21	7.62	2.76	+	1.21E-04
↳ modification of morphology or physiology of other organism involved in symbiotic interaction	81	21	7.62	2.76	+	1.21E-04
↳ modification of morphology or physiology of other organism	82	21	7.72	2.72	+	2.27E-04
antigenic variation	180	36	16.94	2.13	+	9.57E-05
↳ passive evasion of immune response of other organism involved in symbiotic interaction	180	36	16.94	2.13	+	9.57E-05
↳ evasion or tolerance of immune response of other organism involved in symbiotic interaction	182	37	17.13	2.16	+	6.13E-05
↳ evasion or tolerance of defense response of other organism involved in symbiotic interaction	182	37	17.13	2.16	+	6.13E-05
↳ evasion or tolerance of defenses of other organism involved in symbiotic interaction	182	37	17.13	2.16	+	6.13E-05
↳ avoidance of defenses of other organism involved in symbiotic interaction	182	37	17.13	2.16	+	6.13E-05
↳ response to defenses of other organism involved in symbiotic interaction	182	37	17.13	2.16	+	6.13E-05
↳ response to other organism	182	37	17.13	2.16	+	6.13E-05
↳ response to external biotic stimulus	182	37	17.13	2.16	+	6.13E-05
↳ response to external stimulus	188	37	17.69	2.09	+	8.81E-05
↳ response to stimulus	404	63	38.02	1.66	+	2.04E-04
↳ response to biotic stimulus	182	37	17.13	2.16	+	6.13E-05
↳ response to immune response of other organism involved in symbiotic interaction	182	37	17.13	2.16	+	6.13E-05

Figure 3.7: Gene ontology analysis of differentially expressed genes between adapted and non-adapted parasites. Gene ontology biological processes that were significantly enriched by differentially expressed genes of the adapted parasites compared to the non-adapted parasites from our *P. falciparum* microarray analysis. Fisher's Exact with False Discovery Rate (FDR) multiple test correction. FDR < 0.05.

Since VSAs play a major role in these biological processes, we analyzed the gene expression of multigenic variant surface antigen families and found significant changes in gene expression between the non-adapted and adapted for two of the main VSA family, PfEMP1 and RIFINs. Of the 71 gene IDs that are classified under the PfEMP1 family, including pseudogenes, only *var2csa* (PF3D7_1200600) had a paired T-test significance after FDR correction set at <0.10 (adjusted p-value=0.069) (Fig. 3.8). Our timecourse of PfEMP1 expression also agrees with the current peak mRNA expression of PfEMP1 in late rings at

18 hpi (Bozdech, Zhu et al. 2003). Highest expressing PfEMP1 for non-adapted parasites in the three non-adapted strain switch expression from group B or C PfEMP1 to *var2csa* expression in their respective adapted strains.

Figure 3.8: Microarray heatmap of PfEMP1 family gene expression between non-adapted (WT) and adapted (AS) parasites. Six timepoints (TP1 to TP6) at 8-hour

intervals representing the entire IDC of *P. falciparum*. PF3D7_1200600 (*var2csa*, indicated by black arrow) is the highest expressing PfEMP1 and only PfEMP1 that is statistically significant within the PfEMP1 family comparing non-adapted and adapted. (Paired T-test, Benjamini-Hochberg's FDR correction. Adjusted p-value<0.10).

Table 3.1: Highest expressing PfEMP1 in non-adapted and adapted parasites.

Strain	Non-adapted (WT)	Group	log2FC	Adapted (AS)	log2FC	Fold Difference
3D7 B7	PF3D7_1240900	C	1.03	PF3D7_1200600	2.33	2.3x
3D7attb	PF3D7_0412400	B	0.75	PF3D7_1200600	1.95	2.6x
pCRISPR	PF3D7_0420700	C	1.20	PF3D7_1200600	2.33	1.94x

The 115 member RIFIN family showed 61 differentially expressed genes that were upregulated or downregulated within the RIFIN family (paired T-test, Benjamini-Hochberg's FDR correction. Adjusted p-value<0.20) (Fig. 3.9). Interestingly, the majority of the upregulated gene members are A-type RIFINs, with the top 10 expressing RIFINs found in the adapted parasite being A-type RIFINs (Table 3.2). A-type RIFINs are surface expressing RIFINs compared to their counterpart, B-type RIFINs which are not (Petter, Haeggström et al. 2007). Therefore, A-type RIFINs could play a role in binding or immune evasion. A-type RIFINs PF3D7_0223100 and PF3D7_1254800, have been identified as immune modulating RIFINs (Saito, Hirayasu et al. 2017), are found in the top 10 expressed RIFINs in the adapted parasite (Table 3.2). The 3 most downregulated RIFINs are B-type RIFINs that are non-surfacing expressing RIFINs (Petter, Haeggström et al. 2007). Unlike PfEMP1, RIFIN expression is varied among the

different members (Llinas, Bozdech et al. 2006). Therefore, in the RIFIN family, adaptation led to an upregulation of the A-type surface expressing RIFINs and a downregulation of the B-type, non-surface expressing RIFINs.

Figure 3.9: Microarray heatmap of RIFIN family members. Differentially expressed genes of the 115 member RIFIN family that are significantly upregulated or downregulated in the adapted parasite (AS) compared with the non-adapted parasite (WT). Paired T-test, $FDR \leq 0.20$

Table 3.2: Table of highest and lowest expressing differentially expressed RIFINs.

Rank	Gene ID	Type	Expression Average (log2FC)
1	PF3D7_0617700	A	0.646278
2	PF3D7_1000500	A	0.583521
3	PF3D7_0223400	A	0.464824
4	PF3D7_0732900	A	0.449698
5	PF3D7_1300500	A	0.422573
6	PF3D7_1254800*	A	0.416045
7	PF3D7_0732200	A	0.407138
8	PF3D7_0223100*	A	0.402106
9	PF3D7_0324400	A	0.396897
10	PF3D7_0401400	A	0.372172
53	PF3D7_1373000	A	-0.42614
54	PF3D7_1373100	A	-0.43368
55	PF3D7_0401600	Unknown	-0.43819
56	PF3D7_0402700	Pseudogene	-0.4383
57	PF3D7_0600300	A	-0.45029
58	PF3D7_0632100	A	-0.47539
59	PF3D7_0300200	A	-0.51679
60	PF3D7_0532900	B	-0.58125
61	PF3D7_1372600	B	-0.58683
62	PF3D7_1040600	B	-0.59675

3.4 Adapted parasites maintain adaptation after plasmid transfection

To determine the importance of these DEGs in adapted parasites for thriving in the huRBC-NSG mice, we developed a method for *in vivo* conditional knockdown of *P. falciparum* genes. Firstly, we wanted to know if transfection and genetic modification of adapted parasites impacted the adaptation status of the parasite? Transfecting parasites is a harsh process where after positive drug selection, only very few parasites integrate or carry the plasmids that would confer resistance to the drug. We utilized a conditional knockdown system called TetR-DOZI aptamer system. This system uses the tetracycline repressor protein (TetR) fused with the *P. falciparum* development of zygote inhibited (DOZI) protein, an RNA helicase that is involved in regulating eIF4E-dependent translation, to control translation. (Ganesan, Falla et al. 2016). Ten tandem repeats of RNA aptamers (10x aptamer arrays) with specific conformation which allows TetR-DOZI to bind to them, are inserted into the 3' UTR of the gene (Fig. 3.10A). At the mRNA level, TetR-DOZI protein will bind to the 10x aptamer array and prevent translation of the tagged gene (Fig. 3.10B). However, the addition of anhydrotetracycline (aTc) induces a conformational change in TetR to prevent TetR-DOZI from binding to the 10xaptamer array and allows translation of the gene (Fig. 3.10C).

Figure 3.10: RNA schematic overview of how TetR-DOZI translational control occurs in *P. falciparum*-tagged genes. (A) Gene of interest (GOI) is tagged at the 3' end with the 10x RNA aptamer array that has a specific RNA conformation for binding by TetR protein. The arrow upstream of the GOI indicates translation and the end of the

mRNA is after the 10x aptamer array. (B) In the absence of aTc, TetR-DOZI fusion protein (shown here as TetR) will bind to the 10x aptamer, preventing translation. (C) In the presence of aTc (blue circle), aTc binding to TetR leads to a conformational change, preventing TetR-DOZI from binding to the secondary RNA aptamer structure, allowing translation to proceed.

We transfected adapted parasites 3D7attB AS with the plasmid pMG56. Plasmid pMG56 contains attP sites that integrate into the attB site in the 3D7attB parasites within the *cg6* gene (Nkrumah, Muhle et al. 2006) for fast integration of the plasmid into the parasite genome. This process is achieved using the Bxb1 mycobacteriophage integrase-mediated recombination that would integrate the attP containing plasmid into the parasite attB site, creating stable integration into the parasite genome, generating an attL and attR site in the process (Fig. 3.11).

Figure 3.11: Schematic of plasmid pMG56 containing attP site integration into attB site. AttP containing pMG56 integrates into attB containing *P. falciparum* (such as 3D7attB) parasites at the dispensable cg6 gene locus. Integration of attP site into the attB site produces attL and attR sites flanking the integrated plasmid. (Figure adapted from Adjalley, Sophie H., et al. "Quantitative assessment of *Plasmodium falciparum* sexual development reveals potent transmission-blocking activity by methylene blue." *Proceedings of the National Academy of Sciences* 108.47 (2011): E1214-E1223.)

The pMG56 plasmid contains the firefly luciferase (FLuc) gene under the translational control of the 10x TetR-DOZI aptamer array. Plasmid pMG56 demonstrate a 70 fold higher induction of FLuc signal with aTc (+aTc) compared to no aTc (Ganesan, Falla et al. 2016). After transfection, parasites were placed under blasticidin (BSD) drug selection. Transfectants that integrated the plasmid are able to survive the BSD drug selection and are labelled as 3D7attB AS pMG56 parasites.

3D7attB AS pMG56 parasites were seeded into 96 well plates to determine if Rediject Bioluminescent substrates, which are *in vivo* mouse luciferase substrates that are designed to enter the cell without cell lysis, are compatible with our transfected parasite line (Figure 3.12). At 5×10^6 parasites detection levels, D-luciferin which detects the presence of Firefly luciferase, had a total flux that was 67x higher in the +aTc wells (541,500 p/s) compared to the no aTc wells (7962 p/s). The +aTc wells are also 692.25x higher in total flux compared to uninfected RBCs alone (781 p/s). When compared to the constitutively expressed Renilla Luciferase (RLuc), that is present in the pMG56

plasmid but not under the TetR-DOZI aptamer control, the total flux of iRBCs containing wells (1.16×10^7 p/s) versus uninfected RBCs (1.66×10^6 p/s) were only 5.96x higher for detection at 5×10^6 parasites, which represents a low signal to noise ratio and unsuitable for utilizing RLuc subsequently for our *in vivo* mouse assay (Fig 3.12B).

Figure 3.12: Adapted parasites transfected with pMG56 scanned with IVIS. Transfected 3D7attbAS pMG56 adapted parasites of decreasing concentration were analyzed in IVIS to determine difference in luminescence between +aTc and no aTc in

the presence of A) Rediject D-Luciferin substrate (Firefly) or B) Rediject Coelenterazine h substrate (Renilla) in all wells.

3D7attb AS pMG56 parasites were infected into 2 groups of huRBC-NSG mice. Group 1 was given aTc (+aTc) and Group 2 was not (no aTc). Our control group were huRBC-NSG mice not infected with any parasite. These transfected adapted parasites were able to initiate infections immediately and sustain parasitemia. Comparing Group 1 (+aTc) and Group 2 (no aTc), max flux of around 63,900 p/s occurred at the snout and majority of the rest of the signal at the foot pads and tail (Fig. 3.13A). Total flux of Group 1 is 4.7×10^6 p/s and Group 2 is 408,333 p/s (p-value=0.0312, Unpaired T-test; Fig. 3.13B). Control Group 3 had a total flux of 431,000 p/s which is comparable to Group 2 (no aTc) and therefore not significantly different (p=0.8788, Unpaired T-test, Fig. 3.13B). All mice had parasitemia above 5% except for the control mice, which were not infected. Therefore, adapted parasite 3D7attb AS was able to maintain adaptation after transfection of pMG56 DNA plasmid and subsequent drug selection, generating the parasite line 3D7attb AS pMG56. Also, we have established an *in vivo* inducible knockdown of the FLuc gene using aTc. This shows that the Tet-R DOZI 10x aptamer array system can be used for *in vivo* conditional knockdown of tagged *P. falciparum* genes in the huRBC-NSG mice.

Figure 3.13: Transfected pMG56 adapted parasites (3D7attb AS pMG56) in huRBC-NSG mice. (A) IVIS image of 3D7attb AS pMG56 showing regions with less fur having a stronger FLuc signal in the +aTc mice (Group 1). No aTc (Group 2) and RBC only (Group 3) had similar low luminescence signal. (B) The +aTc (Group 1) mice

had significantly higher FLuc signal compared to no aTc in mice > 5% parasitemia. Data shown as mean $\pm$ SD, n=3; * p<0.05, ns=not significant.

3.5 PfEMP1 surface expression affects adapted parasite growth in huRBC-NSG mice

Since *in vivo* translational control of *P. falciparum* genes using TetR-DOZI 10x aptamer array has been validated in our huRBC-NSG *P. falciparum* infection model, we selected three *P. falciparum* gene targets that would perturb VAR2CSA and PfEMP1 surface expression on iRBCs and evaluate how this would affect adapted parasite growth in the huRBC-NSG mice. These 3 genes are *Plasmodium* translation-enhancing factor (PTEF; PF3D7_0202400), skeleton-binding protein 1 (SBP1; PF3D7_0501300) and membrane-associated histidine rich protein 1 (MAHRP1; PF3D7_1370300). PTEF is the VAR2CSA translation enhancing factor and in PTEF knock out parasites, VAR2CSA protein expression is abolished (Chan, Frasch et al. 2017). SBP1 (Maier, Rug et al. 2007) and MAHRP1 (Spycher, Rug et al. 2008) are required for the transport of PfEMP1 to the surface of the iRBC and knock out of these genes prevented PfEMP1 from reaching the surface, causing them to accumulate within the parasite plasma membrane.

To quickly and efficiently tag these genes with the 10x aptamer array for *in vivo* expression control, we utilized CRISPR-cas9 to execute homology directed repair (HDR) using DNA donor templates. *P. falciparum* strain NF54attb transfected to express the cas9 protein and T7 RNA polymerase (pCRISPR) was transfected with pSN054 plasmids that contains specific homology regions to each of the three genes of interest. The plasmid consists of

a 500bp left homology region (LHR), a 300bp recoded region, 2xHA tag, 10x aptamer array, BSD resistance cassette, TetR-DOZI fusion protein, guide RNA (gRNA) under the T7 promotor and a 500bp right homology region (RHR) (Fig. 3.14).

The 3' end of the endogenous gene is selected for sgRNA recognition based on a CRISPR on-target score (Doench, Fusi et al. 2016) that maximizes on-target activity and an off-target score (Hsu, Scott et al. 2013) that minimizes off-target effects. The last 300bp of the gene of interest is recoded (termed Recoded Region) by codon juggling, which keeps the translated protein product the same but changes the codons used for coding the amino acids. This to prevent continued recognition and cutting by the sgRNA:cas9 complex of the target sequence once the genetic modifications are inserted.

Figure 3.14: Schematic of plasmid pSN054. (A) Plasmid pSN054 is flanked by left and right homology arms to integrate the plasmid into the gene of interest, introducing a 2xHA tag as well as the 10x aptamer array into the 3' end of the gene.

3.5.1 Plasmid construction of pSN054_PTEF, MAHRP1 and SBP1

During pSN054 plasmid construction, only pSN054_PTEF and pSN054_MAHRP1 were successfully obtained. SBP1 contains a region of high repeats (genomic position 976bp – 1047bp) that after multiple attempts were constantly truncated after transformation into competent *E. coli* (Fig. 3.15). This truncation effect is common in *E.coli* transformation for tandem and inverse repeats (Bzymek and Lovett 2001).

Figure 3.15: Sequencing alignment of pSN054_SBP1 showing truncations. SBP1 gene contains multiple repeats that leads to truncations in that region in plasmid pSN054_SBP1 (dotted black box).

Plasmid pSN054_PTEF and pSN054_MAHRP1 were transfected in non-adapted and adapted pCRISPR parasites. Transfectants that survived BSD drug selection were designated pCRISPR WT_PTEF, pCRISPR AS_PTEF, pCRISPR WT_MAHRP and pCRISPR AS_MAHRP. Since pCRISPR WT_PTEF came up

first during the drug selection, the parasites were harvested for genomic DNA and using diagnostic PCR to check for integration with primers upstream of the LHR and downstream of the RHR.

For pCRISPR WT_PTEF, diagnostic PCR with primers upstream of the LHR and downstream of the RHR in the 3' UTR would yield PCR products of 1.5kbp for the unedited locus and an 8.5kbp band for the edited locus. The diagnostic PCR showed both bands (Fig. 3.16A), suggesting a heterogenous population of unedited and edited parasites. Both bands were sent for sequencing and the 8.5kb band indeed contain the gene of interest, PTEF, tagged with the 2xHA tag and the 10x aptamer (Fig. 3.16B) whereas the 1.5kb band was the unedited PTEF gene, without the recoded region including the stop codon, that would have been removed in the edited locus (Fig. 3.16C).

alignment of 1.5kbp PCR product shows perfect alignment to the unedited locus and absence of any modification.

Therefore, we obtained single parasite clones using limiting dilutions for pCRISPR WT_PTEF and pCRISPR AS_PTEF. 2 clones of the non-adapted parasites pCRISPR WT_PTEF_G6, pCRISPR WT_PTEF_F11 and 3 clones of the adapted parasite pCRISPR AS_PTEF_C2, pCRISPR AS_PTEF_C8 and pCRISPR AS_PTEF_E3 parasite cultures were made. New diagnostic PCR primers were made with the forward primer found on the native locus (LHR region) and the reverse primer on the edited HA tag region that would yield a 1kb PCR product band. Absence of this band indicates initial parasite clone contains the unedited PTEF locus. For all the parasite clonal cultures, the 1kb band was present (Fig. 3.17A) and sequenced to show that they indeed carry the genetic modifications, shown here by the non-native recoded region (Fig. 3.17B).

Figure 3.17: Transfectants from single clone origin show edited PTEF locus. (A) Diagnostic PCR single 1 kb PCR products, indicating presence of edited PTEF locus. (B) Sequencing alignment of the 1 kb PCR product shows edited locus containing the

non-native recoded region and that all single clone origin parasite cultures carry the edited PTEF locus.

We probed pCRISPR WT_PTEF_G6 and pCRISPR AS_PTEF_C2 for protein expression of PTEF by detecting the HA tag. Although there was correct integration at the PTEF locus (Fig. 3.17B), we were unable to detect the HA tag in IFA and in Western blot. It was found that PTEF could not be detected in certain parasite lines by Western blot because the gene's Kozak sequence. Since it has a pyrimidine (U) in the +4 position, the translation initiation of the gene is poor and most of the scanning ribosomes would not initiate translation in the putative start codon (Kozak, Evans et al. 1991). This suggest that translation of PTEF could be under the regulation of another mechanism as codon optimization of PTEF which reduces the presence of GU wobble codons led to enhanced translation (Chan, Ch'ng et al. 2017).

3.5.2 MAHRP1 knockdown in no aTc condition affects surface expression of PfEMP1

For MAHRP1, we obtained clone pCRISPR AS_MAHRP_C10 by limiting dilutions and confirmed integration of the 2xHA tag and 10x aptamer array into the *mahrp1* gene by diagnostic PCR of a 750bp product and sequencing (Fig. 3.18A). To confirm inducible gene expression of *mahrp1* with aTc, we cultured pCRISPR AS_MAHRP_C10 in the presence (+aTc) and absence (no aTc) of aTc.

Anti-HA tag western blot analysis of pCRISPR AS_MAHRP1_C10 parasite lysate cultured in +aTc or no aTc show an 86.7% knockdown of MAHRP1 (Fig. 3.18B).

Figure 3.18: Western blot of Anti-HA tag in pCRISPR AS_MAHRP1_C10. (A) Aligned sequence of pCRISPR AS_MAHRP1_C10 genomic DNA diagnostic PCR showing the integration of the recoded region and the HA tag. (B) In the presence of aTc, HA tag shows a robust signal compared to no aTc were knocked down of 86.7% was quantified. Representative data from of three western blots.

Anti-HA tag IFA showed that the HA-tagged protein localized to the erythrocyte membrane, in agreement with previous published results (Spycher, Rug et al. 2008). The absence of aTc showed a detectable but much lower anti-HA signal by IFA (Fig. 3.19), agreeing with our western blot analysis. PfEMP1 localization appears to be affected by the knockdown of MAHRP1. In the presence of aTc where MAHRP1 is expressed, PfEMP1 forms the typical punctate pattern and localizes to the surface of the erythrocyte membrane. However, in the absence of aTc, MAHRP1 expression is greatly reduced and we observe the accumulation of PfEMP1 within the parasite plasma membrane or parasitophorous vacuole (Fig. 3.19).

Figure 3.19: pCRISPR AS_MAHRP1_C10. Smears of iRBCs cultured in aTc (top row) and no aTc (bottom row) probed with antibodies recognizing PfEMP1 (red), HA (green) and the DNA stain DAPI (blue). Scale bar = 5µm

3.5.3 MAHRP1 conditional knockdown adapted parasites affects growth in huRBC-NSG mice

We wanted to determine if this change of PfEMP1 surface expression, by conditional knockdown of MAHRP1, affects growth of adapted parasites in the huRBC-NSG mice. We infected pCRISPR AS_MAHRP1_C10 in huRBC-NSG mice with (+aTc) and without (no aTc). In +aTc mice, parasitemia increased in 2 out of the 3 mice to around 3% parasitemia at Day 14. One mouse had increasing parasitemia till Day 6, peaking at 1.5% parasitemia, before decreasing to 0.6% at the end of experiment at Day 14 (Fig. 3.20A). However, for mice that had no aTc, parasitemia increased at first and peaked at Day 6 to 0.3% in two mice and 0.9% in one mouse but steadily declined to around 0.2% after 14 days for all 3 mice (Fig. 3.20A). This indicates that surface expression of PfEMP1 affects adapted parasite growth in the huRBC-NSG mice.

Figure 3.20: Parasitemia (%) of huRBC-NSG mice infected with pCRISPR AS_MAHRP1_C10 in +aTc or no aTc conditions. (A) In +aTc mice (black lines),

parasitemia rose in 2 out of 3 mice compared to no aTc (blue lines) that had stagnating parasitemia in 3 mice. Each line represents one mouse. (B) Mean parasitemia values in the +aTc mice (black lines) show steadily increasing parasitemia but not for no aTc mice (blue lines). Data shown as mean $\pm$ SD, n=3.

3.6 Discussion

3.6.1 Adaptation of *P. falciparum* in huRBC-NSG is not a result of genetic changes

P. falciparum adapted in the huRBC-NSG mice demonstrates a unique phenotype compared to non-adapted parasites for their ability to thrive in non-phagocyte depleted huRBC-NSG mice (Fig. 3.2, 3.3). We were able to adapt multiple *P. falciparum* laboratory cultured strains in the huRBC-NSG mice. Whole genome sequencing and SNPs analysis reveals that this adapted phenotype is not driven by a particular genotype enriched from a genetically heterogeneous population (Fig. 3.4) or by mutation (Fig. 3.5) as adapted parasites remain genetically identical to their parental non-adapted strain.

3.6.2 Adaptation of *P. falciparum* in huRBC-NSG due to change in adapted parasite gene expression

Laboratory cultured blood stage *P. falciparum* parasites originated from infected patients before they were adapted to continuous *in vitro* culture (Trager and Jensen 1976). However, not all parasites isolated from patients are able to adapt and survive in continuous culture (Jensen and Trager 1978, Nsoby, Kiggundu et al. 2008). Effects of this adaptation from *ex vivo* to *in vitro* continuous culture include gene expression changes, particularly of PfEMP1 expression. Overall expression of PfEMP1 is downregulated compared to *ex vivo* parasites taken from patients (Peters, Fowler et al. 2007). There is also a switch of the type of PfEMP1 expressed in long term *in vitro* culture, switching towards

CD36-binding PfEMP1 the longer the parasite is cultured (Zhang, Zhang et al. 2011).

Our microarray analysis revealed that adapted parasites showed significant changes to the gene expression of VSAs such as PfEMP1 and RIFIN compared to long-term cultured non-adapted parasites. For PfEMP1, non-adapted parasites highest expressing PfEMP1 are group B and C PfEMP1, which are CD36-binding PfEMP1 (Howell, Levin et al. 2008). After adaptation, VAR2CSA was the highest expressing PfEMP1 with almost two-fold increase in expression compared to non-adapted parasites, agreeing with Fowler et al.'s observation.

Surface expressing A-type RIFINs are upregulated in adapted parasites compared to non-adapted parasites (Table 3.1) with RIFIN expression in adapted up to 2.6 times higher compared to non-adapted parasites (Fig. 3.9). RIFIN expression, similar to PfEMP1 expression, are downregulated in *in vitro* culture compared to *in vivo* infection in patients (Daily, Le Roch et al. 2005). Therefore, static *in vitro* cultures downregulate RIFIN expression but adaptation and infection in the huRBC-NSG mice upregulates RIFIN expression, that is also observed in *in vivo* human infection.

Therefore, VSA genes expression are overexpressed in *in vivo* human infection compared to *in vitro* static culture (Daily, Le Roch et al. 2005). We observe this as well in our adapted parasites for PfEMP1 and RIFIN VSA expression compared to *in vitro* non-adapted parasites. This suggest that VSAs

have an important role during in human infection and are upregulated by the parasite. This could be due to encountering the host immune system or spleen and therefore upregulate VSAs for immune evasion or sequestration to avoid being cleared by the spleen. These elements are not present in *in vitro* cultures, hence these VSAs are downregulated. However, in the infection of the huRBC-NSG mice, the parasites encounter the host immune system, as well as the spleen, which triggers the parasites to upregulate certain VSAs.

3.6.3 Adapted parasites can be genetically modified without affecting competency in huRBC-NSG mice

Adapted parasites remained adapted to huRBC-NSG mice even after transfection and drug selection, as shown by 3D7attb AS pMG56 infection in huRBC-NSG mice. Luminescence signal is strongest at the tail, footpads and snout, areas that have less fur (Fig. 3.13). This indicates that the adapted parasite burden is mainly found in the peripheral blood circulation and not sequestered to any particular organ, similar to liquid and blood cancer IVIS luminescence patterns found in peripheral blood circulation (Habets, de Bock et al. 2019).

In vivo gene silencing of the firefly luciferase gene under the control of the TetR-DOZI 10x aptamer array in 3D7attb AS pMG56 in huRBC-NSG mice (Fig. 3.13) validate our conditional knockdown approach to silence *P. falciparum* genes that are essential for adapted parasites to thrive in the huRBC-NSG mice. TetR and anhydrotetracycline have proven to be an effective conditional knockdown system in mice previously as well (Gandotra, Schnappinger et al.

2007). Since VSAs have been implicated to be important for *P. falciparum* adaptation to huRBC-NSG mice, we utilized the Tet-R-DOZI 10x aptamer array conditional knockdown to determine *P. falciparum* genes essentiality for *in vivo* adaptation.

3.6.4 Surface PfEMP1 expression needed for adapted *P. falciparum* to thrive in huRBC-NSG mice

The knockdown of MAHRP1 using the TetR-DOZI 10x aptamer array in no aTc condition (Fig. 3.18) led to PfEMP1 remaining within the parasite plasma membrane and not reach the surface of the iRBC (Fig. 3.19). This confirms the need for MAHRP1 in the export of PfEMP1 to the surface of the iRBC (Spycher, Rug et al. 2008). MAHRP1 knockdown in adapted parasite infection in huRBC-NSG mice with no aTc led to reduced parasitemia compared to +aTc mice (Fig. 3.20). Therefore, surface expression of PfEMP1 in adapted parasites is needed for thriving in the huRBC-NSG mice. Adapted parasites highly upregulated VAR2CSA PfEMP1 and loss of VAR2CSA PfEMP1 surface expression diminishes parasite growth. PfEMP1 plays an important role for antigenic variation (Biggs, Gooze et al. 1991) and immune evasion (Urban, Ferguson et al. 1999) during infection in humans and this could be the case for our adapted parasite huRBC-NSG mice infection.

VAR2CSA is a unique PfEMP1 because it is phylogenetically classified on its own based on its upstream promotor (*upsE*) and having its own distinct extracellular domains. VAR2CSA is also one of the three PfEMP1 that are

conserved across the different *P. falciparum* strains (Rowe, Kyes et al. 2002). VAR2CSA expressing parasites are found sequestered in the placenta of malaria infected pregnant women (Tuikue Ndam, Salanti et al. 2005) and binds to chondroitin sulfate A (CSA) found in the placenta (Clausen, Christoffersen et al. 2012). VAR2CSA expressing parasites have also been found in men and children (Beeson, Ndungu et al. 2007) and therefore not just in pregnant women. The function of VAR2CSA goes beyond just adhesion to CSA. VAR2CSA expressing iRBCs have been shown to modulate monocyte and macrophage cytokine and chemokine responses (Sampaio, Eriksson et al. 2018) and less phagocytosed by both mouse and human macrophages (Serghides, Patel et al. 2006). Moreover, *var2csa* has been implicated as the main PfEMP1 the parasite would switch to when *var* specific heterochromatin are perturb, owing to the fact it has the lowest threshold for activation among the *var* genes (Ukaegbu, Zhang et al. 2015).

Therefore, *var2csa* expression of the adapted parasites in our huRBC-NSG mouse model suggest that *var2csa* expression aids adapted parasites to avoid clearance by the mouse immune system and spleen. Surface expression of VAR2CSA PfEMP1 is required by the adapted parasite to avoid being cleared. We next sought to understand the interaction of macrophages and the adapted parasites and how that relates to clearance in the next chapter.

Chapter 4. Macrophage phagocytosis of adapted *P. falciparum*

4.1 Adapted parasites do not accumulate in the spleen and lungs in huRBC-NSG mice

Since PfEMP1 surface expression is important in adapted parasite growth in the huRBC NSG mice, we wanted to know if there is a difference in organ sequestration between non-adapted and adapted parasites. We infected via tail vein injection pMG56 adapted and non-adapted parasites at 1.5×10^7 iRBCs in 200 μ l. One-hour post infection, we harvested mouse organs such as lung, heart, spleen, kidney, liver and brain and utilized IVIS to detect for accumulation of parasite FLuc signal. In the non-adapted parasite infection, higher FLuc signal could be seen in the spleen and the lungs compared to the adapted parasite infection (Fig. 4.1A). Non-adapted parasite total flux seen at the spleen is at 8946 ± 2937 p/s compared to the adapted at 2707 ± 1166 p/s (p-value=0.0267, unpaired T-test, Fig 4.1B). Non-adapted total flux in the lungs are at $53,177 \pm 34371$ p/s compared to adapted at 9705 ± 8964 p/s (p-value=0.1014, unpaired T-test; Fig. 4.1B). Therefore, adapted parasites avoid sequestration and clearance in the spleen and potentially in the lung, which allows the adapted parasite to thrive in the huRBC-NSG mice.

A)

B)

Figure 4.1: Ex vivo IVIS of huRBC-NSG mice organs. (A) Ex vivo IVIS image huRBC-NSG mice infected with pMG56 non-adapted and adapted parasites showing sequestration/trapping in the lung and spleen but not in other organs for the non-adapted parasites. (B) Total flux showing parasite burden in the lung and spleen for non-adapted parasites.

parasites whereas adapted parasites have significantly less parasite burden in the spleen and difference in the lungs. Data shown as mean $\pm$ SD, n=3; * p<0.05.

4.2 Need for parasite adaptation abolished in clodronate-treated huRBC-NSG mice

Adapted parasites avoid sequestration in the spleen unlike non-adapted parasites. Since the spleen is involved in the clearance of iRBCs (Chotivanich, Udomsangpetch et al. 2002), depletion of macrophages and other phagocytes in the spleen using clodronate treatment would allowed non-adapted parasites to grow more efficiently in huRBC-NSG mice, as shown previously (Arnold, Tyagi et al. 2011).

In our clodronate-treated mice, we saw depletion in the red pulp macrophages in spleen defined as F4/80^{hi} CD11b^{int} in the splenic CD45⁺ population (Lai, Sheng et al. 2018) (Fig. 4.2 A), from 19.87% $\pm$ 2.7% of the CD45⁺ splenocyte population to 6.05% $\pm$ 2.93% (Fig. 4.2B). After 2 rounds of treatment of clodronate, huRBC-NSG mice were infected with non-adapted 3D7attb WT and clodronate was administered every other day for the duration of the experiment. In line with previous studies, 3D7attb WT did not require adaptation with parasitemia in the peripheral blood rising to 4.06% $\pm$ 2.27% (Fig. 4.2C). However, in the non-clodronate treated control group mice, 3D7attb WT did not increase in parasitemia and was not detectable in the peripheral blood after Day 1. Therefore, existing phagocytes such as macrophages in the spleen

play a role in the control of non-adapted parasites in the huRBC-NSG mice and control their growth in the mice.

Figure 4.2: Clodronate-treated huRBC-NSG mice. (A) Representative FACS plot of CD45⁺ splenocytes for mouse F4/80 and mouse CD11b with polygon gate indicating tissue resident red pulp macrophages (F4/80^{hi} CD11b^{int}) population being depleted by clodronate treatment. (B) Red pulp macrophage population depletion quantified by flow cytometry. Data shown as mean $\pm$ SD, n=3; ** p<0.01. (C) Parasitemia values of 3D7attb

WT (non-adapted) in clodronate-treated mice and control mice treated with PBS-liposome. Data shown as mean $\pm$ SD, n=3.

4.3 *In vitro* non-opsonic macrophage phagocytosis assay using flow cytometry

Since clodronate-treated mice are depleted of tissue resident macrophages that allows non-adapted iRBCs to thrive in the huRBC-NSG mice, we wanted to determine if there is a difference in macrophage recognition and non-opsonic phagocytosis of non-adapted and adapted iRBCs. The current method of determining macrophage phagocytosis of iRBCs relies on Giemsa staining of macrophages followed by counting each macrophage individually using light microscopy to determine if they have taken up a parasite (McGilvray, Serghides et al. 2000, Mimche, Thompson et al. 2012). This method of detection is tedious, difficult to quantify (Fig. 4.3A) and low throughput. The use of various fluorescent dye to stain iRBCs before co-culture with macrophages and detect uptake of the dye as a measure of phagocytosis have been used but faces challenges such as high auto-fluorescence of macrophages, especially in the shorter wavelength channels such as FITC (Blue laser) and DAPI (UV laser) (Fig. 4.3B).

A)

B)

Figure 4.3: Primary monocyte-derived human macrophages co-cultured with infected RBCs. (A) Giemsa stain of human macrophages and infected RBCs co-culture showing the phagocytosis of iRBCs (black arrows) but the presence of possible parasite internalized structures (red arrows) that makes quantification challenging. (B) FACS

panel FSC and SSC showing the larger macrophage gate and smaller iRBCs gate (Top panel). Mean fluorescence intensity (MFI) of the iRBC population (red) compared to the macrophage population (blue) shows the MFI differences in FITC (left bottom panel) and DAPI (right bottom panel) in unstained conditions. Infected RBCs have lower MFI in FITC and DAPI compared to macrophages.

We sought to develop a robust and high-throughput method using flow cytometry and semi-permeable dyes to reliably quantitate phagocytosis of iRBC. We initially tested with Hoechst 33342 and DHE (Chan, Rénia et al. 2012) and discovered that Hoechst 33342 would inadvertently stain macrophage DNA during the co-culture (Fig. 4.4B). Instead, we utilized DAPI, a DNA dye that has been used to stain iRBC for IFA and measure parasitemia using flow cytometry but not used in the context for iRBC staining and co-culture with macrophages for macrophage phagocytosis flow cytometry assay.

DAPI staining of iRBCs was similar to Hoescht stained iRBCs (Fig. 4.4A) and does not lead to unintentional staining of macrophages during the co-culture unlike Hoechst (Fig. 4.4B).

Figure 4.4: DAPI staining compared to Hoechst 33342 staining. (A) Infected RBCs stained with Hoechst or DAPI gave similar parasitemia readings. (B) Primary MDM flow cytometry plot when co-cultured with iRBC stained with DAPI did not lead to unintentional staining unlike Hoechst stained iRBC co-culture led to the entire macrophage population becoming Hoechst positive.

To address the issue of autofluorescence of macrophages in the DAPI channel, we utilized the linear relationship of the FITC and DAPI channel where

the autofluorescence seen in one channel is related in a linear fashion to the other channel (Paradis 2017). Therefore, by gating on macrophages (upper panel, Fig. 4.5), comparing FITC and DAPI channel, we see a linear relationship form along a 45-degree axis (bottom left panel, Fig. 4.5). However, if the macrophage phagocytoses an iRBC stained with DAPI, the linear relationship changes and increases in DAPI intensity compared to FITC (bottom right panel, Fig. 4.5). We can also see that DAPI does not stain macrophages during the co-culture with DAPI stained uninfected RBC (bottom center panel, Fig. 4.5).

Figure 4.5: Primary human monocyte-derived macrophage DAPI and FITC channel properties. Macrophage gated based on FSC and SSC (top panel) and subsequently visualized on the DAPI against FITC channel show a linear 45-degree relationship (bottom left panel). DAPI-stained RBCs do not affect this relationship (middle bottom panel) but DAPI- stained iRBCs does with macrophages now entering into the polygon gate, indicating that the autofluorescence linear relationship is lost and now higher in DAPI signal.

To attribute the uptake of DAPI signal to phagocytosis, we added cytochalasin D, an actin polymerization inhibitor, to the co-culture to inhibit human macrophage phagocytosis of iRBCs (Ribes, Ebert et al. 2010). Phagocytosis percentage dropped to $5.9\% \pm 1.139\%$ from $42.24 \pm 7.24\%$ with the addition of cytochalasin D to the co-culture (Fig. 4.6).

Figure 4.6: Effect of Cytochalasin D on human monocyte-derived macrophage and iRBC co-culture and DAPI-based flow cytometry assay. (A) FACS panel show no

Cytochalasin D (top left) and with Cytochalasin D (top right) percentage phagocytosis in polygon gate. (B) Difference in percentage phagocytosis with and without Cytochalasin D. Data shown as mean $\pm$ SD of duplicate wells and are representative of 2 independent experiments; *** $p < 0.001$.

4.4 Adapted parasites are less phagocytosed by mouse and human macrophages compared to non-adapted parasites

Next, we co-cultured non-adapted 3D7attb WT and adapted 3D7attb AS parasites in mouse RAW macrophage cells and human monocyte-derived macrophages (MDM) to determine non-opsonic phagocytosis of iRBC. Using the flow cytometry assay developed here, we quantified the percentage phagocytosis (phagocytic index) of macrophages for non-adapted and adapted iRBCs. RAW mouse macrophage cell line had a percentage phagocytosis of $19.33 \pm 2.71\%$ for non-adapted parasites whereas adapted parasites showed $12.59 \pm 4.42\%$ ($p=0.0006$; paired T-test; Fig. 4.7).

Figure 4.7: Phagocytosis assay of RAW mouse macrophage cell line comparing non-adapted and adapted parasites. FACS panel indicating gating for RAW mouse macrophages (top panel) and the FITC and DAPI linear relationship of macrophages from co-culture with uninfected RBCs (middle left panel), non-adapted iRBCs (middle

center panel) and adapted iRBCs (middle right panel) and DAPI positive macrophages in the polygon gate. Difference in percentage phagocytosis by RAW macrophages between non-adapted and adapted parasites. Data shown as mean $\pm$ SD of triplicate wells and are representative of 3 independent experiments; ** $p < 0.01$.

For *P. falciparum* iRBC phagocytosis assay with primary monocyte-derived macrophages (MDM), human monocytes are cultured for 7 days without the addition of any growth factors such as macrophage colony-stimulating factor (Serghides, Patel et al. 2006). These MDMs showed percentage phagocytosis of $30.28\% \pm 4.99\%$ for non-adapted parasites and $17.6\% \pm 3.03\%$ for adapted parasites ($p = 0.0046$, paired T-test; Fig. 4.8). When compared to our 10x aptamer-tagged MAHRP1 adapted parasite pCRISPR AS MARHP1_C10 with aTc (+aTc Adapted) showed similar phagocytosis percentage to the adapted strain at $19.51\% \pm 4.9\%$ with no statistical significance between them ($p = 0.3827$, unpaired T-test; Fig. 4.8). When cultured in no aTc, adapted pCRISPR AS MARHP1_C10 (no aTc Adapted) percentage phagocytosis increase to $23.21\% \pm 4.12\%$ which is statistically significant compared to +aTc Adapted ($p = 0.0494$, paired T-test; Fig. 4.8).

Figure 4.8: Phagocytosis assay of human monocyte-derived macrophages comparing non-adapted and adapted parasites. FACS panel indicating FSC and SSC

profile of macrophages without MCSF and gating strategy (top panel). FITC and DAPI linear relationship of macrophages from co-culture with uninfected RBCs (middle left panel), non-adapted iRBCs (middle center panel) and adapted iRBCs (middle right panel) and DAPI positive macrophages in the polygon gate. Difference in percentage phagocytosis by macrophages between non-adapted, adapted, pCRISPR AS_MAHBP1_C10 +aTc (+aTc adapted) and no aTc (no aTc adapted) parasites. Data shown as mean $\pm$ SD of triplicate wells and are representative of 3 independent experiments; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns=not significant.

MDMs can also be cultured with growth factor MCSF for 7 days, which is known to increase their phagocytic capabilities (Bober, Grace et al. 1995). These macrophages are considered M0 or resting macrophages similar to tissue-resident macrophages (Dong, Zhao et al. 2013). The percentage phagocytosis of MCSF-cultured MDMs for non-adapted parasites is at $46.6 \pm 6.3\%$ and adapted parasites at $38.4 \pm 4.39\%$. Therefore MCSF-cultured MDMs have increased phagocytosis of iRBCs, although adapted parasites were significantly less phagocytosed compared to non-adapted parasites ($p = 0.0018$, paired T-test; Fig. 4.9). For +aTc adapted parasites, phagocytosis percentage is at $38.62 \pm 5.56\%$ which is not significantly different compared to adapted parasites ($p = 0.9262$, unpaired T-test, Fig. 4.9) and statistically significant when compared to no aTc adapted whose percentage phagocytosis is $45.56 \pm 5.09\%$ ($p = 0.0083$; paired T-test; Fig. 4.9D).

A) Monocyte-derived Macrophages

B)

C)

D)

Figure 4.9: Phagocytosis assay of human monocyte-derived macrophages with MCSF comparing non-adapted and adapted parasites. (A) FACS panel indicating FSC and SSC profile of macrophages cultured with MCSF and gating strategy. (B) Phase contrast live IFA showing DAPI stained parasite (blue) internalized by an MDM on the left and the one on the right has not internalized any parasite. (C) FITC and DAPI linear relationship of macrophages from co-culture with uninfected RBCs (middle left panel), non-adapted iRBCs (middle center panel) and adapted iRBCs (middle right panel) and DAPI positive macrophages in the polygon gate. (D) Difference in percentage phagocytosis by macrophages between non-adapted, adapted, pCRISPR AS_MAHPR1_C10 +aTc (+aTc adapted) and no aTc (no aTc adapted) parasites. Data shown as mean $\pm$ SD of triplicate wells and are representative of 3 independent experiments; * $p < 0.05$, ** $p < 0.01$, ns=not significant.

4.5 Screening macrophage polarizing compounds that increases phagocytosis of adapted parasites

As a way of developing new immunotherapies that targets host immune cells to enhance their effectiveness against *P. falciparum*, we screened macrophage polarizing compounds for their ability to increase non-opsonic phagocytosis of adapted parasites using our *in vitro* phagocytosis assay. Initial test on FDA approved and non-approved compounds ability to polarize M0 macrophages to become M1-like or M2-like, that were based on macrophage morphology and RNA-seq analysis (Data done at Prof Jianzhu Chen lab at MIT (unpublished data)). From that test, a list of 16 compounds which polarizes

macrophages to M1-like or M2-like were used to determine if polarization of M0 macrophages increases phagocytosis of adapted parasites (Table 4.10).

Table 4.1: List of macrophage polarizing compounds tested

Name	Polarization	FDA Approval
C1	M2	Yes
C2	M2	Yes
C4	M2	Yes
C5	M1	No
C6	M1	No
C7	M2	No
C9	M1	No
C10	M1	Yes
C11	M2	No
C12	M2	No
C15	M1	Yes
C16	M1	No
C17	M2	No
C19	M1	No
C20	M1	Yes
C22	M1	No

Type of compound	Total
M1—FDA approved	3
M1—non-FDA approved	6
M2—FDA approved	3
M2—non-FDA approved	4

After 7 days of culture in MCSF, M0 macrophages are treated with the compounds for 24 hours. The macrophages were then washed with PBS and co-cultured with the adapted iRBCs for 90 minutes (Fig. 4.10). M1-like macrophages are round in appearance whereas M2-like macrophages are elongated (Fig. 4.10).

Figure 4.10: Overview of polarization of human macrophage with compounds.

Flowchart on the left indicates workflow for polarization of M0 macrophages with

polarizing compounds. Phase contrast images on M0 (top right) macrophages before exposure to polarizing compounds and after polarization to M1-like (middle right) and M2-like (bottom right).

Of the 16 compounds screened, 6 compounds were found to increase phagocytosis of adapted parasites (Fig. 4.11). Compounds C7, C11, C12, C17, C20 and C22 were found to significantly increase macrophage phagocytosis of adapted iRBCs (Table 4.2).

Figure 4.11: MCSF-cultured MDMs percentage phagocytosis of adapted parasites after exposure to polarizing compounds. Polarizing compounds affects MDMs phagocytosis of adapted parasites. Data shown as mean $\pm$ SD of triplicate wells and are representative of 2 independent experiments; * $p < 0.05$, ** $p < 0.01$

Table 4.2: Summary of six macrophage polarizing compounds that increases phagocytosis of adapted parasites.

Name	Polarization	% Phagocytosis	P-value
No Drug	M0	41.43 $\pm$ 8.540	-
C7	M2	54.30 $\pm$ 1.102	0.0244
C11	M2	49.20 $\pm$ 7.733	0.0037
C12	M2	50.70 $\pm$ 6.511	0.0059
C17	M1	53.77 $\pm$ 7.753	0.0287
C20	M1	53.96 $\pm$ 6.255	0.0238
C22	M1	56.64 $\pm$ 9.125	0.0159

Compounds C7 and C22 were further validated for their ability to increase macrophage phagocytosis with non-adapted iRBCs. Without any polarizing compounds, phagocytosis of non-adapted iRBC was 48.15% $\pm$ 8.40%. Compound C7 increase the phagocytic index to 58.87% $\pm$ 3.76% (p-value=0.0477, paired T-test; Fig. 4.12) and compound C22 to 57.58% $\pm$ 3.86% for (p-value=0.0389, paired T-test; Fig. 4.12) compared to no compound treatment.

Figure 4.12: MCSF-cultured MDMs percentage phagocytosis of non-adapted parasites after exposure to polarizing compounds C7 and C22. Polarizing compounds affects MDMs phagocytosis of non-adapted parasites. Data shown as mean $\pm$ SD of triplicate wells and are representative of 2 independent experiments; * $p < 0.05$.

4.6 Discussion

4.6.1 Non-adapted parasites accumulate to lung and spleen of huRBC-NSG mice

PfEMP1 mediates sequestration to tissue and organs through binding of host receptors such as CD36. In the huRBC-NSG mice, non-adapted parasites were found to accumulate in the lung and spleen unlike adapted parasites (Fig. 4.1). One reason could be due to the expression of VAR2CSA, a non-CD36 binding PfEMP1, by the adapted parasites (Fig 3.8). In mouse malaria *Plasmodium berghei*, CD36 mediates binding of iRBC to the lungs in C57BL/6 mice as CD36^{-/-} mice showed markedly reduced lung parasite burden compared to wildtype mice (Franke-Fayard, Janse et al. 2005). In a similar experiment, *P. berghei* knockout of orthologs of SBP1 and MAHRP1 prevented binding of *P. berghei* iRBCs to CD36 and reduced parasite burden in the lungs compared to wildtype parasite (De Niz, Ullrich et al. 2016). Therefore, CD36-binding PfEMP1 expressed in non-adapted parasites led to accumulation in the lungs and spleen unlike the adapted parasites expressing VAR2CSA that is non-CD36-binding.

4.6.2 Macrophages in huRBC-NSG mice prevents non-adapted *P. falciparum* infection

Clodronate treatment in mice depletes macrophages in the spleen and liver (Mönkkönen, van Rooijen et al. 1991). We show that red pulp macrophages are depleted in clodronate-treated NSG mice (Fig. 4.2A, B). Non-adapted *P.*

falciparum requires clodronate-treated huRBC-NSG mice to thrive (Arnold, Tyagi et al. 2011, Duffier, Lorthiois et al. 2016). This is consistent with remaining macrophages in the NSG mice being capable of clearing non-adapted parasites upon infection (Fig 4C). Macrophages in the NSG mice are still functional as they have been shown to clear human RBC during supplementation via phagocytosis and clodronate treatment helped improved human RBC reconstitution (Hu, Van Rooijen et al. 2011). This effect was also seen in NOD/SCID mice infection with *P. falciparum* where *P. falciparum* infection was shown to be high pro-inflammatory and monocytes and macrophages are found to be critical in controlling *P. falciparum* and human RBC reconstitution in the mice (Arnold, Tyagi et al. 2010).

4.6.3 Adapted parasites are less phagocytosed by mouse and human macrophages

Since remaining macrophages in the huRBC-NSG mice prevents non-adapted parasites from increasing in parasitemia but not adapted parasites, we hypothesized that adapted parasites are able to evade macrophage recognition and phagocytosis, preventing them from being cleared in the huRBC-NSG mice. In our macrophage phagocytosis assay, adapted parasites are less recognized and phagocytosed by mouse (Fig. 3.25) and human macrophages (Fig. 3.26 & 3.27). For non-opsonic phagocytosis, CD36 is the main receptor for recognition and phagocytosis of parasitized iRBC by macrophages (Patel, Serghides et al. 2004). When compared to iRBCs expressing CD36-binding PfEMP1, VAR2CSA

expressing iRBCs have been shown to be less phagocytosed by mouse and human primary macrophages previously (Serghides, Patel et al. 2006) through reduced recognition via CD36. Therefore, by expressing VAR2CSA PfEMP1, adapted parasites reduce CD36 receptor recognition and phagocytosis and evades clearance in the huRBC-NSG mice. Moreover, VAR2CSA expression have also been shown to modulate macrophage immune response. VAR2CSA expressing iRBCs reduces NF- κ B activation and cytokine responses during co-cultures (Sampaio, Eriksson et al. 2018). Therefore, VAR2CSA expression in adapted parasite is important for immune evasion and clearance by remaining mouse macrophages.

To determine the essentiality of VAR2CSA surface expression in adapted parasites, we mentioned previously that adapted parasite pCRISPR AS_MAHBP1_C10 in no aTc condition has been shown to knockdown MAHBP1 and prevents PfEMP1 expression on the surface of the iRBC (Fig. 3.19). In our developed phagocytosis assay, MAHBP1 knockdown and no PfEMP1 surface expression in the no aTc condition led to a significant increase in human macrophage phagocytosis compared to +aTc condition. This supports the hypothesis that surface expressing VAR2CSA PfEMP1 on the adapted iRBC reduces phagocytosis by macrophages and survival in the huRBC-NSG mice without the need for clodronate treatment. This result explains why infection of adapted pCRISPR AS_MAHBP1_C10 in huRBC-NSG mice with no aTC was unable to increase in parasitemia compared to mice with aTc.

Therefore, in the absence of surface expressing PfEMP1, macrophages recognize and phagocytose iRBC through a different pathway. CD47, a marker of self on the RBCs, engages the inhibitory receptor signal regulator protein alpha (SIRP α) on macrophages that prevents phagocytosis. Murine RBCs that lack CD47 are cleared by splenic red pulp macrophages (Oldenborg, Zheleznyak et al. 2000). Senescent RBCs have been shown to have 30% lower mean expression of CD47 and is a way for macrophages to identify and remove old RBCs from circulation. To show that phagocytes play a role in this, clodronate treatment in mice decreased the overall expression of CD47 in circulating RBCs as well as increased the accumulation of senescent RBCs in the mice. (Khandelwal, Van Rooijen et al. 2007). *P. falciparum* infected RBCs have been shown to have significantly lower levels of CD47 compared to uninfected RBCs and CD47 expression decreases proportionately as the parasites matures within the iRBC. Therefore, surface expression of PfEMP1 could be a method by which the parasite uses to mitigate the loss of CD47 expression and evade macrophage phagocytosis and clearance. In the case of the VAR2CSA-expressing adapted parasites, upon the loss of surface expression of VAR2CSA, reduced CD47 expression of the iRBC which leads to recognition and phagocytosis by macrophages.

Therefore, macrophages play a key role in the control of *P. falciparum* iRBCs in huRBC-NSG mice. This is shown with clodronate treatment, parasitemia is able to increase (Arnold, Tyagi et al. 2011) compared to no clodronate treatment in non-adapted parasites (Fig. 3.4C). Clodronate-treated

NSG mice shows a 70% reduction in the percentage of red pulp macrophages from mouse splenocytes (Fig. 3.4B) and induces considerable changes to the spleen in terms of size and internal architecture (Hu, Van Rooijen et al. 2011). The spleen plays a crucial role in the clearance of iRBCs in human infection (Israeli, Shapiro et al. 1987) and PfEMP1 expression is reduced in splenectomized patient (Bachmann, Esser et al. 2009). These factors show the connection between the spleen, iRBC clearance and PfEMP1. Therefore, a proposed model for huRBC-NSG *P. falciparum* adaptation could be the process of overcoming splenic clearance. Clodronate-treatment drastically reduces the spleen's ability to clear non-adapted parasites, hence they are able to grow and expand in the mouse without adaptation. However, without clodronate treatment and splenic function is not impaired, parasites adapt to express VAR2CSA, reducing macrophage phagocytosis and clearance by the spleen. The significant reduction of iRBC sequestration in the spleen by adapted parasites compared to non-adapted parasites supports this notion (Fig. 3.14).

4.6.4 Macrophage polarizing compounds increases non-opsonic phagocytosis of adapted parasites in human macrophages

Polarizing macrophages as a therapy comes from the concept of repolarization of tumor associated macrophages at the site of the tumor microenvironment from tumor promoting (M2) to tumor killing (M1) (Sica, Larghi et al. 2008). This is a broad generalization of the concept, although we know now that polarized macrophages at the tumor microenvironment exist as a

spectrum on the M1-M2 continuum and to determine their state would require transcriptional profiling (Qian and Pollard 2010). One example of immunomodulatory drug targeting of macrophages for increase non-opsonic phagocytosis of *P. falciparum* iRBCs is rosiglitazone. Rosiglitazone increases CD36 expression on human and mouse macrophages and in a dose-dependent manner, increases phagocytosis of iRBCs (Serghides, Patel et al. 2009). This study builds on the earlier work that macrophage recognition of iRBCs is through the parasites CD36 PfEMP1 VSA. However, in our case where parasites shifted away from expressing CD36 PfEMP1 to VAR2CSA, could enhancement of non-opsonic phagocytosis be possible? We observed that MDMs cultured in MCSF increased non-opsonic phagocytosis of both non-adapted and adapted iRBCs (Fig. 4.9) compared to MDMs cultured in no MCSF (Fig. 4.8). This agrees with the transcriptional analysis of MCSF cultured MDMs compared to those cultured without MCSF, which shows upregulation of phagocytic receptors and capacity (Dong, Zhao et al. 2013).

In our screen of sixteen compounds that polarizes M0 MDMs to either M1-like or M2-like and using non-opsonic phagocytosis as the parameter for increased anti-parasitic function, three compounds that polarized MDMs to M1-like and three compounds to M2-like had significant increases in phagocytosis of adapted parasites. One potential candidate, compound C22, is a selective ceramide kinase inhibitor that polarizes macrophages towards the M1-like state. Ceramide kinase (CERK) produces ceramide 1-phosphate (C1P) (Bajjalieh, Martin et al. 1989) that is a bioactive sphingolipid and play key roles in cellular

processes in macrophages (Gómez-Muñoz, Kong et al. 2004). Along with glucosylceramide synthase (GCS) and sphingomyelin synthases (SMS-1 and -2), CERK regulates ceramide metabolism in cells such as macrophages. Interestingly, along the different activation states of macrophages, ceramide metabolism switches towards upregulation of CERK and downregulation of GCS or vice versa. In mouse monocytes, ceramide metabolism favors GCS and downregulates CERK. Culturing monocytes in MCSF tilts the metabolism to upregulate CERK and downregulate GCS. However, activating macrophages with LPS or IFN- γ , towards the classically activated M1 macrophage, shifts ceramide metabolism towards upregulating GCS (Rovina, Graf et al. 2010) and downregulating CERK (Rovina, Jaritz et al. 2010). By inhibiting CERK, compound C22 shifts ceramide metabolism towards the M1-like, which are classically activated macrophages.

Both M1-like and M2-like polarizing compound enhanced macrophage phagocytosis of adapted parasites (Table 4.2). As we only measured the macrophage phagocytic capability, in the context of host immunity, M1 and M2 macrophages play a much wider role in concert with other immune effector cells and cytokine signaling during the infection (Stevenson and Riley 2004). Pro-inflammatory M1 macrophages, induced early on by IFN- γ , produces TNF α and reactive oxygen intermediates that helps control parasitemia (Steevels and Meyaard 2011). IFN- γ levels correlates to anti-inflammatory cytokine IL-10 (Day, Hien et al. 1999), which induces anti-inflammatory M2 macrophages and limits inflammation and oxidative damage (Lokken, Mooney et al. 2014).

However, there is a heterogeneity in the innate immune response of *P. falciparum* infection in patients. Patients with a more pro-inflammatory response are able to better control parasitemia but increases the likelihood of severe clinical symptoms. Conversely, patients that developed a more anti-inflammatory response has higher parasitemia but have less severe clinical symptoms (Walther, Woodruff et al. 2006). Therefore, macrophage polarizing compounds identified here that increases non-opsonic phagocytosis could aid in both scenarios. In a pro-inflammatory response that leads towards severe clinical symptoms, polarization towards an anti-inflammatory response by M2-like polarizing compounds while still maintaining parasitemia control could be clinically beneficial. In patients with anti-inflammatory response with high parasitemia, we could potentially polarize towards a more pro-inflammatory response to induce parasitemia control with the M1-like polarizing compounds. To determine if this application is a possibility, our next steps are to further validate our six polarizing compounds for efficacy in the huRBC-NSG mice as well as human immune system reconstituted humanized mice.

Chapter 5. Parasitemia control of adapted parasites by primary NK cells

5.1 Adapted parasites demonstrate reduced parasitemia control by responder primary NK cells compared to non-adapted parasites

Based on our microarray data, VSAs involved in immune evasion and host-pathogen interaction were enriched in our adapted parasites compared to non-adapted parasites. In particular, expression of two RIFIN VSA family member proteins, PF3D7_0223100 and PF3D7_1254800 were upregulated. (Table 3.1) These had been found to interact with immune inhibitory receptor leukocyte immunoglobulin-like receptor-B1 (LILRB1) (Saito, Hirayasu et al. 2017). With that, we tested the ability of primary NK cells to control parasitemia of non-adapted and adapted parasites by measuring percentage parasitemia reduction of each unique donor NK cells. This assay has been previously described (Ye, Chew et al. 2018) where parasites are co-cultured with and without NK cells at a starting parasitemia of 0.5%. After 96 hours, 2 full cycles of the parasite asexual lifecycle, we measured the parasitemia of the co-culture using DNA stain Hoechst 33342 for staining iRBCs and a CD45 negative gate to remove CD45⁺ NK cells from the RBC gate (Fig. 5.1).

Figure 5.1: Gating strategy of primary NK cells and iRBCs co-culture. FACS gating strategy to determine parasitemia reduction of NK cells and iRBC co-culture comparing parasitemia of non-adapted and adapted parasite growth with and without NK cell co-culture.

In Figure 5.1, NK cells were shown to be able to control non-adapted parasites, reducing parasitemia from 3.7% to 0.43% after 96 hours. To calculate the parasitemia reduction by NK cells, the parasitemia value from the no NK cell (iRBC only) culture is subtracted from the parasitemia value from the iRBC and NK cell co-culture. This difference is then divided by the no NK cell (iRBC only) parasitemia to determine the percentage parasitemia reduction by NK cells. This is summarized by the formula below.

$$\% \text{Parasitemia reduction} = \frac{\text{Parasitemia}_{\text{no NK}} - \text{Parasitemia}_{\text{NK}}}{\text{Parasitemia}_{\text{no NK}}} \times 100$$

However, there are heterogenous responses of primary donor NK cells to iRBCs. Donor NK cells can be classified as responder and non-responder NK cells addressed previously by our group. The percentage parasitemia reduction of responder NK cells to non-adapted 3D7 is 71% $\pm$ 12% whereas non-responder NK cell is at 25% $\pm$ 7.7% (Ye, Chew et al. 2018).

We evaluated six responder donor NK cells responses to non-adapted 3D7attB parasites and the percentage parasitemia reduction is 73.63% $\pm$ 16.95%. The same six donor NK cell parasitemia reduction for the adapted 3D7attB AS is at 31.42% $\pm$ 15.04% (p-value=0.0041, paired T-test; Fig. 5.2).

Figure 5.2: Parasitemia reduction of non-adapted and adapted parasites by responder NK cells. Co-culture of non-adapted and adapted parasites with six responder donor NK cells show a reduction of percentage parasitemia reduction of adapted parasites. Data shown as mean $\pm$ SD and represents 6 unique responder NK cells from 2 independent experiments; ** $p < 0.01$.

5.2 3D7 parasites selected for PF3D7_1254800 RIFIN expression show reduced parasitemia control by responder NK cells but no reduction in macrophage phagocytosis

Responder NK cells had reduced capacity to control huRBC-NSG mice adapted parasites compared to non-adapted parasite. We wanted to know if non-adapted parasites expressing the immune modulating RIFIN PF3D7_1254800 would also demonstrate reduced parasitemia control by responder NK cells.

Certain RIFINs such as PF3D7_1254800 have been identified to have immune modulating properties on certain immune cells. Immune modulating refers to the protein's ability to modulate the response of the immune cell towards its target cell. Saito *et al* (Saito, Hirayasu et al. 2017) showed that iRBC transduced to express immune modulating RIFIN PF3D7_1254800 inhibited IgM production in a co-culture with primary B cells compared to a non-immune modulating RIFIN, PF3D7_1000500. Also, K562 target cells transduced to express RIFIN PF3D7_1254800 led to reduced lysis by NK cell line NKL compared to another non-immune modulating RIFIN PF3D7_1254200.

Selection of 3D7 parasites expressing immune modulating RIFIN PF3D7_1254800 (SLI-RIFIN) or non-immune modulating PfEMP1 PF3D7_0421300 (SLI-PfEMP1) were generated and validated by Radoslaw Igor Omelianczyk in Prof Peter Presier's lab (manuscript under review). Selection for expression of the gene of interest utilizes a drug resistance cassette tagged to the 3' end of the gene separated by a skip peptide (Birnbaum, Flemming et al. 2017). Since the expression of the drug resistance cassette is under the endogenous promotor of the gene of interest, when the gene of interest is expressed, the drug resistance cassette be expressed as well. Therefore, parasites that are able to survive the drug selection expresses the gene of interest (Figure S2).

Three responder donor primary NK cells were co-cultured with non-adapted, adapted, SLI-RIFIN and SLI-PfEMP1 parasites for 96 hours and parasitemia values quantified using flow cytometry. Non-adapted parasite

percentage parasitemia reduction was $61.89\% \pm 7.0\%$ whereas adapted parasite parasitemia reduction was $38.49\% \pm 15.58\%$ that is significant compared to non-adapted parasite (p-value=0.0258, paired T-test; Fig. 5.3). SLI-RIFIN parasitemia reduction was $39.13\% \pm 16.18\%$, similar to the adapted parasite and significant when compared to non-adapted parasitemia reduction (p-value=0.0341, unpaired T-test; Fig. 5.3) and SLI-PfEMP1 parasitemia reduction (p-value=0.0465, paired T-test; Fig. 5.3). SLI-PfEMP1 parasitemia reduction was $54.52\% \pm 19.79\%$ and is not significant compared to non-adapted parasitemia reduction (p-value=0.4551, unpaired T-test; Fig. 5.3). Therefore, selection of immune modulating RIFIN Pf3D7_1254800 (SLI-RIFIN) showed significant reduction in NK cell parasitemia control. However, selection of non-immune modulating PfEMP1 PF3D7_0421300, did not lead to a significant reduction in parasitemia control by NK cells.

Figure 5.3: Responder NK cells parasitemia reduction of VSA-expressing parasites.

Non-adapted and adapted parasitemia reduction were compared along with RIFIN PF3D7_1254800 (SLI-RIFIN) expressing and PfEMP1 PF3D7_0421300 (SLI-PfEMP1) by responder NK cells in a 96-hour co-culture. Data shown as mean $\pm$ SD of 3 unique responder NK cells from 2 independent experiments; * $p < 0.05$.

To determine if SLI selected VSAs affects macrophage phagocytosis of iRBC, primary monocyte-derived macrophage without MCSF phagocytosis assay were co-cultured with SLI-PfEMP1 and SLI-RIFIN and compared to non-adapted and adapted 3D7. No significant difference between phagocytosis were seen between of non-adapted 3D7attb ($29.0\% \pm 5.7\%$) and SLI-PfEMP1 (25.9%

$\pm 3.2\%$; $p=0.3827$, unpaired T-test $p=0.2805$) and SLI-RIFIN ($26.5\% \pm 5.0\%$; $p=0.4385$) summarized in Figure 5.4.

Figure 5.4: Human primary monocyte-derived macrophage phagocytosis assay of SLI-PfEMP1 and SLI-RIFIN parasites. Phagocytosis of non-adapted 3D7attb were compared along with SLI selected expression of RIFIN PF3D7_1254800 (SLI-RIFIN) and PfEMP1 PF3D7_0421300 (SLI-PfEMP1). Data shown as mean $\pm$ SD of 2 unique donors from 2 independent experiments; * $p<0.05$.

5.3 LILRB1 inhibitory receptor affects NK cell parasitemia reduction of adapted parasites

Immune modulating RIFIN PF3D7_1254800 binds to and activates LILRB1 inhibitory immune receptor (Saito, Hirayasu et al. 2017). We wanted to determine if LILRB1 is involved in the reduction of parasitemia control of adapted parasites by NK cells. In order to have sufficient primary NK cells for our assay, we obtained expanded primary donor NK cells termed NK Cell Activation and Expansion System (NKAES) NK cells from our collaborator. These NK cells are expanded and activated, expressing the activation marker CD69 and considered more potent than unstimulated and IL-2 stimulated NK cells (Fujisaki, Kakuda et al. 2009).

Two donor expanded NKAES-NK cells, D280317 and D140417 were used for co-culture with non-adapted and adapted iRBCs. NKAES-NK cells were able to control non-adapted 3D7attb WT parasites at $64.25\% \pm 14.61\%$ parasitemia reduction, similar to the responder donor NK cells shown previously. Adapted 3D7attb AS parasite parasitemia reduction is at $44.75\% \pm 12\%$, which is significantly different from the non-adapted parasite ($p=0.0491$, paired T-test, Fig. 5.4).

Anti-LILRB1 blocking antibody was added to the co-culture to block binding and activation of LILRB1 on NK cells. Anti-LILRB1 treatment had no significant effect on parasitemia control of non-adapted parasites ($65.59\% \pm 11.8\%$) when compared to no treatment ($64.25\% \pm 14.61\%$). NK cell parasitemia

reduction with anti-LILRB1 treatment in adapted parasites ($54.78\% \pm 8.18\%$) was not significant when compared to no treatment ($44.75\% \pm 12.0\%$; $p=0.2515$, paired T-test, Fig. 5.4), although mean parasitemia control increased by 10.03%. Therefore, anti-LILRB1 treatment restores NK cell parasitemia reduction of adapted parasite to levels comparable to non-adapted parasitemia reduction ($p=0.1091$, paired T-test, Fig. 5.4).

To attribute this increase in parasitemia reduction to the specificity of anti-LILRB1 blocking antibodies, non-specific antibody mouse IgG1 isotype control was added to the co-culture. Parasitemia reduction between non-specific antibody treated non-adapted ($63.69\% \pm 10.17\%$) and adapted ($42.82\% \pm 13.32$) remained significant ($p=0.0459$, paired T-test; Fig 5.4), showing that anti-LILRB1 antibody specificity led to the increase in NK cell parasitemia reduction of adapted parasites.

Figure 5.5: Percentage parasitemia reduction between non-adapted and adapted parasites by NKAES-NK cells. Adapted parasites show a difference in parasitemia reduction by NKAES-NK cells compared to non-adapted parasites. The addition of anti-LILRB1 antibody added in the co-culture increases parasitemia reduction of adapted parasites whereas addition of a non-specific mouse isotype control does not. Data shown as mean $\pm$ SD of duplicate wells and are representative of 2 independent experiments; * $p < 0.05$.

To ascertain the LILRB1 expression of the two donor NKAES-NK cells, both donors were stained with the same anti-LILRB1 blocking antibody used for

the NK cell co-culture. Donor D280317 had a higher percentage of NK cell expressing LILRB1 at 62.4% compare to donor D140417 at 52.5%, showing a heterogeneity in the expression of LILRB1 on NK cells. Staining of a different immune inhibitory receptor, leukocyte-associated Ig-like receptor 1 (LAIR1), both donors showed similar expression at 98.1% for D280317 and 99.4% for D140417 (Fig. 5.5).

Figure 5.6: Flow cytometry staining showing expression of immune inhibitory receptors LILRB1 and LAIR1 on NKAES-NK cells. LILRB1 expression on two donors of NKAES-NK cells by staining with anti-LILRB1 antibody showing difference in LILRB1 expression between donors (middle row) and similar expression of LAIR1 (bottom row).

5.4 Discussion

Work done previously in our lab showed that primary NK cell control of iRBCs can be classified as responder and non-responder through an *in vitro* co-culture assay where greater than 50% reduction in parasitemia growth classifies the NK cell donor as a responder (Ye, Chew et al. 2018). From 6 responder primary NK cell co-culture, adapted parasites showed reduced parasitemia control compared to non-adapted parasites (Fig. 5.2). Therefore, adapted parasites are able to evade NK cell killing compared to non-adapted parasites.

Microarray analysis of adapted parasites revealed DEGs involved in antigenic variation and immune evasion (Fig. 3.8) as well as an upregulation of members of RIFINs (Table 3.2). RIFINs have recently been shown to interact with human inhibitory immune receptors such as LILRB1. The effect of these interactions has led to the modulation of immune cells such as B-cell responses to iRBCs (Saito, Hirayasu et al. 2017). LILRB1 is part of the leukocyte receptor complex (LRC) that codes for both activating and inhibitory receptors (Wilson, Torkar et al. 2000). LILRB1 contain protein motifs for immunoreceptor tyrosine-based inhibitory motifs (ITIMs) that upon ligation of the receptor, becomes phosphorylated and inhibit NK cell lysis of target cells (Riteau, Menier et al. 2001). Inhibition of immune cell response via ligation of LILRB1 has been exploited by viruses such as human cytomegalovirus (Yang and Bjorkman 2008) and dengue virus (Chan, Ong et al. 2014) that has led to reduced effector functions.

In our adapted parasites, the top ten highest expressing RIFINs are surface expressing A-type RIFINs, which could play a role in host receptor interaction, unlike B-type RIFINs (Petter, Haeggström et al. 2007). Among the top ten expressing are RIFIN member PF3D7_1254800 and PF3D7_0223100, which have been shown to interact with LILRB1 and modulate immune cell responses (Saito, Hirayasu et al. 2017). Other VSAs such as PfEMP1 are not involved in NK cell recognition and killing of iRBCs when comparing with 3D7 and 3D7 PfEMP1 knockdown parasite (D'Ombrian, Voss et al. 2007). Moreover, in a co-culture with responder NK cells and other nine parasites strains AP2KO6, A4, DD2, HB2, KAHRP KO, T994, T996, W2mef and MAHRP KO, only AP2KO6 was able to reduce parasitemia control of responder NK cells significantly (Ye 2017). AP2KO6 is a 3D7 parasite line with a truncated ApiAP2 transcription factor that led to significant upregulation of variant surface antigens RIFINs and STEVORs (Martins, Macpherson et al. 2017).

Immune modulating RIFIN PF3D7_1254800 (SLI-RIFIN) was upregulated using selection-linked integration and found to reduce parasitemia control of NK cells significantly compared to non-adapted 3D7 and non-immune modulating PfEMP1 PF3D7_0421300 (SLI-PfEMP1) (Fig. 5.3). Therefore, SLI-RIFIN, similar to adapted parasites, were able to significantly reduce responder NK cell parasitemia control. PF3D7_1254800 immune modulating effects of NK cells were determined by transfecting NK cell target cell K562 with PF3D7_1254800, from amino acid residue 166 to 275, which showed significant reduction in NK cell line NKL cytotoxicity compared to non-transfected K562

(Saito, Hirayasu et al. 2017). Here, we show that SLI-RIFIN shows PF3D7_1254800 ability to immune modulate primary responder NK cells by upregulation of PF3D7_1254800 in 3D7 parasites.

Since the immune modulating RIFINs upregulated in the adapted parasites ligates LILRB1, we utilized anti-LILRB1 blocking antibody to determine the involvement of LILRB1 engagement on reduction of NK cell control by adapted parasites. Anti-LILRB1 blocking antibody during the NK cell co-culture was able to rescue activated and expanded primary NK cell control of adapted parasite parasitemia reduction to $54.78\% \pm 8.18\%$ from $44.75\% \pm 12.0\%$ (Fig. 5.4). Although this difference is not statistically significant, it did abolish the non-adapted and adapted percentage parasitemia reduction statistical significance. These results are based on two donor NK cells only, thus more donor samples are required to establish the effect LILRB1 has on NK cell control of parasitemia via anti-LILRB1 antibody blocking. Also, since there are differences in the expression of LILRB1 between the two NKAES-NK cell donors (Fig. 5.5), anti-LILRB1 blocking antibody rescue on NK cell parasitemia control could be varied from donor to donor. Previous study has shown NK cell LILRB1 staining of 23% to 77% from six different donors, suggesting that LILRB1 is expressed in a subset of CD56⁺ NK cells and not all NK cells (Colonna, Navarro et al. 1997). This is unlike LAIR1 staining in NK cells which has been shown to stain the whole CD56⁺ NK cell population (Meyaard, Adema et al. 1997). RIFINs can also interact with LAIR1, however, we do not know if they are able to immune modulate NK cells (Saito, Hirayasu et al. 2017). There

could also be other RIFIN members or VSAs that affect NK cell control of adapted parasites, hence anti-LILRB1 blocking could not restore the full effect of NKAES-NK cell control of adapted parasite. With more donor NK cell samples in our future work, including SLI-RIFIN to determine if anti-LILRB1 blocking treatment is effective would help us determine the extent of LILRB1 interaction with RIFINs is involved in the reduction of NK cell control of parasitemia.

Chapter 6. Future Directions and Conclusions

We have characterized the process of adaptation of *in vitro* cultured *P. falciparum* in huRBC-NSG mice that reveals the parasite's ability to evade clearance by the residual host immune cells of the huRBC-NSG mice through changes in VSA expression. Using MAHRP1 conditional knockdown as a method for affecting PfEMP1 surface expression on the iRBC, we have shown that the knockdown of MAHRP1 and the absence of PfEMP1 on the surface of the iRBC led to adapted parasites reduced competency in the huRBC-NSG mice. *In vitro* characterization of adapted parasites with mouse and human macrophages shows reduced recognition and phagocytosis compared to non-adapted parasites. Primary human NK cell co-culture with adapted parasites also shows reduced parasitemia control compared to non-adapted parasites.

Hence, adapted *P. falciparum* infection in huRBC-NSG mice provided us two insights to *P. falciparum* immune evasion of host immune cells and highlights the shortcomings of *in vitro* culture in the study of VSAs in virulence and antigenic variation. Firstly, surface expression of VAR2CSA in adapted parasites plays a role in the reduction of macrophage phagocytosis, as revealed in the reduced competency in huRBC-NSG mice. Secondly, we show that inhibitory immune receptor LILRB1 is involved in the reduced NK cell control of adapted parasites.

Therefore, *P. falciparum* infection in the huRBC-NSG mice mirrors closer the natural human infection compared to static *in vitro* cultures, allowing

new areas of host-pathogen studies as well as new therapies to be discovered and evaluated. One example is the paradigm shifting immunotherapy that targets host cells and receptors rather than the pathogen, in the case of immune cell checkpoint blockade used in cancer immunotherapy, to unleash the immune system against cancer cells (Ishida, Agata et al. 1992, Leach, Krummel et al. 1996). We show that *in vitro* enhancement of primary human macrophage phagocytosis of adapted parasites using macrophage polarizing compounds. Although further validation is required in the human immune system reconstituted humanized mice, macrophage polarization using FDA approved compounds and non-approved compounds provides a potential new form of anti-malarial therapy. Since this therapy does not target the parasite, development of resistance would not be a significant concern.

In the context of NK cell control of *P. falciparum*, inhibitory receptor blocking, such as LILRB1 blockade could enhance NK cell control. Since huRBC-NSG mice do not have NK cells, we plan to use the human immune system reconstituted humanized mice, reconstituting human NK cells, as a next step to evaluate the therapeutic efficacy of anti-LILRB1 blocking in adapted *P. falciparum* infection in NSG mice. Other A-type RIFINs were upregulated in our adapted parasites and their effect towards NK cells or macrophages have not yet been elucidated. There could potentially be more parasite interactions towards host receptors that we would not have uncovered as these RIFINs are not upregulated in static culture. Evaluating these RIFINs, in the context of innate immunity, is one of our next area of focus using the SLI method of single

activation and selection of individual RIFINs. Moreover, further refinements to our genetic approaches can be made to determine the importance of VAR2CSA for modulating NK cell control. Since adapted parasites enriches for VAR2CSA, could VAR2CSA or other PfEMP1 have a role in modulating NK cell control as well or co-expression of immune modulating RIFIN is required? Although attempts have been made to use SLI-selection and RNA aptamer-based knockdowns to directly manipulate *var2csa*, these have not been successful.

Adapted *P. falciparum* in the huRBC-NSG mice is a powerful tool to study immune modulation of host immune cells by the parasites. This thesis presented two examples of how adapted parasites, which have upregulated immune modulating VSAs, evade recognition and clearance by macrophages and NK cell. From this study, potential new therapies that target host immune cells to enhance effector function could benefit vulnerable groups infected with *P. falciparum*, such as children under the age of 5 who are most susceptible to mortality by malaria infection (World Health Organization 2018). Early IFN- γ production in children correlates to immunity and protection (D'Ombrian, Robinson et al. 2008) for which NK cells are a major source of IFN- γ in the early stages of malaria infection (Artavanis-Tsakonas and Riley 2002). Enhancing NK cell control via anti-LILRB1 therapy could be a way to control parasitemia in the early stages. Moreover, macrophage polarizing compound could potentially augment the immune response towards a pro-inflammatory or anti-inflammatory response, whichever best improves clinical outcomes for the young patient.

Another vulnerable group are primigravidas with placental malaria (Duffy and Desowitz 2001) that affects the development of the fetus (Leach, Krummel et al. 1996). In placental malaria, VAR2CSA expressing parasites bind to CSA found mainly in the placenta (Fried and Duffy 1996). The placenta is an immune privileged organ with mainly innate immune cells present such as NK cells, macrophages and dendritic cells (Bulmer, Denise et al. 1988). Reduction of parasite burden has been shown to provide better outcomes for primigravidas and the child (Greenwood, Armstrong et al. 1992) which these therapies may provide.

Supplementary Materials

Figure S1: qPCR analysis of var2csa expression of adapted compared to non-adapted parasites. (A) Bar graph of adapted parasites var2csa expression at timepoint 2 compared to non-adapted parasites. (B) Analysis of qPCR var2csa expression with an expression ratio of 8.846 compared to non-adapted parasites at timepoint 2 of the microarray IDC.

Figure S2: Transcriptomic analysis of SLI-PfEMP1 and SLI-RIFIN parasite lines.

(A) Microarray analysis heatmap of SLI-PfEMP1 *var* gene family at 18 hours, 24 hours and 30 hours timepoint in the IDC showing repression of *var* gene members in SLI-PfEMP1 (right of white line) compared to parental 3D7 line (left of white line) before SLI selection. The black arrow indicates Pf3D7_0421300, the PfEMP1 selected for expression. (B) Expression of RIFIN Pf3D7_1254800 by qRT-PCT (in red box) in SLI-PfEMP1 (left), which shows no expression, and SLI-RIFIN (right), which shows expression of the selected RIFIN. Data shown in this figure is reproduced with

permission from Mr Radoslaw Igor Omelianczyk 's PhD Thesis entitled "Role of STEVOR and its interacting partners in parasite virulence", (Omelianczyk 2019).

Author's Publication

1. Ye W, **Chew M**, Hou J, Lai F, Leopold SJ, Loo HL, Ghose A, Dutta AK, Chen Q, Ooi EE, White NJ. Microvesicles from malaria-infected red blood cells activate natural killer cells via MDA5 pathway. PLoS pathogens. 2018 Oct 4;14(10):e1007298. (Co-first author)

Poster Presentations

1. 11th Singaporean Society for Immunology (SgSI) Annual Symposium. Singapore. May 2019. Poster Presentation: "Plasmodium falciparum Evasion of NK cells through LILRB1 Inhibitory Receptor Interaction"

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