Control of Plasmodium falciparum infection by human natural killer cells

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TECHNOLOGICAL
UNIVERSITY**

**CONTROL OF *PLASMODIUM FALCIPARUM*
INFECTION BY HUMAN NATURAL KILLER CELLS**

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2017

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School of Biological Sciences,

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Table of Contents

Acknowledgements.....	3
Table of Contents.....	5
List of Figures	10
List of Tables.....	13
Abbreviations	14
Abstract	15
Chapter 1. Introduction	17
1.1 Overview of Malaria.....	17
1.1.1 Global burden of malaria	17
1.1.2 Malaria vaccines.....	20
1.1.3 Human malaria parasite species	22
1.1.4 Clinical malaria	25
1.1.5 Host cell remodelling	26
1.2 Adaptive Immune Response to Malaria.....	34
1.2.1 Naturally acquired immunity to malaria.....	34
1.2.2 B cells.....	38
1.2.3 CD4 T cells.....	41
1.2.4 CD8 T cells.....	43
1.3 Host Innate Immunity Response to Malaria.....	46

1.3.1	Role of the innate immune system in malaria	46
1.3.2	Macrophage	49
1.3.3	Dendritic cells	53
1.3.4	Natural Killer Cells.....	56
1.4	Overview of Natural Killer Cells	61
1.4.1	NK cells overview	61
1.4.2	NK cell activation.....	62
1.4.3	Natural Cytotoxicity Receptor (NCR).....	64
1.4.4	Killer immunoglobulin-like Receptor (KIR)	67
1.4.5	NK cells differences between human and murine	68
1.4.6	NK cells and diseases.....	69
1.5	Humanized mice	75
1.6	Project Objectives	79
Chapter 2.	Materials and Methods.....	80
2.1	Generation of RBC-supplemented Immune Cell optimized Humanized (RICH) mice	80
2.2	Peripheral blood mononuclear cell (PBMC) purification.....	81
2.3	<i>P. falciparum</i> culture and infection.	82
2.4	In vitro NK cell experiments	83
2.5	Imaging.....	86

2.6	Microvesicle (MV) enrichment.....	87
2.7	mRNA transcriptional analysis	88
2.8	qPCR	89
2.9	KIR genotyping.....	90
Chapter 3. Results		93
3.1	RBC-supplemented Immune Cell optimized Humanized (RICH) mice support <i>P. falciparum</i> infection	93
3.2	Human NK cells control acute parasitemia in vivo.....	97
3.3	Human NK cells eliminate <i>P.falciparum</i> -iRBC in vitro.	99
3.4	Human NK cells specifically eliminate late stage <i>P.falciparum</i> - iRBC in vitro.	104
3.5	Over-expression of surface variant-antigen protects iRBC from NK cells.	107
3.6	Increased ICAM-4 expression on iRBC.....	111
3.7	NK cell activation and iRBC elimination required cell-to-cell contact.....	113
3.8	LFA-1 is involved in NK cell interaction and elimination of iRBC. 119	
3.9	Heterogenous Donor NK cell response to iRBC.	123
3.10	Involvement of pathogen recognition pathway in iRBC response. 129	

3.10.1	RIG-I/MDA signaling pathway	136
3.10.2	TLR signaling pathway	145
3.10.3	IFN signaling pathway	151
3.11	KIRs and NK cell response to iRBC	155
Chapter 4. Discussion		160
4.1	RICH mice support <i>P. falciparum</i> growth	160
4.2	NK cells are involved in parasitemia control in <i>P. falciparum</i> infected-RICH mice	161
4.3	In vitro control of parasitemia by NK cells	162
4.4	NK cells specifically eliminate late-stage iRBC	165
4.5	Variant surface antigen protects iRBC from NK cell elimination 166	
4.6	Increased ICAM-4 expression on iRBC	168
4.7	Contact-dependent lysis of iRBC by NK cells	170
4.8	LFA-1 is required for NK cell response to iRBC	171
4.9	Heterogenous donor response to iRBC	172
4.10	MYD88, TLR2 and TLR4 are required by NK cells to control parasitemia.	177
4.11	Involvement of TBK1/IKK ϵ in NK cell response to iRBC.	181

4.12 Under-representation of KIR2DS1 and KIR2DS2 in genotype of donors whose NK cells respond to iRBC	183
Chapter 5. Future Directions	185
5.1 NK cell-iRBC responsiveness / KIR and malaria severity association	185
5.2 Validation of NK cell receptors involved in iRBC recognition...	187
5.3 Verification of TLR2/4 involvement	189
5.4 Priorities in malaria research.....	190
Chapter 6. Conclusion	192
Supplementary Materials	196
Author's Publications	199
Posters Presentation	200
References.....	201

List of Figures

Fig. 1.1. Malaria Epidemiology 2015.....	19
Fig. 1.2 Life cycle of <i>P. falciparum</i>	28
Fig. 1.3 Impact of host immune cells on <i>P. c. chabaudi</i> AS parasitemia. ...	47
Fig. 1.4 Recognition of iRBC by cells of the innate immune system.	60
Fig. 2.1 Purity assessment of NK cells after negative selection.	84
Fig. 3.1 RICH mice supported robust human leukocyte and RBC reconstitution.	95
Fig. 3.2 RICH mice supported robust <i>P. falciparum</i> infection.....	96
Fig. 3.3 NK cells play critical role in acute control of parasitemia in vivo.	98
Fig. 3.4 iRBC activated NK cells, induced granulysin and increased IFN- γ secretion.....	102
Fig. 3.5 NK cells specifically eliminated late stage iRBC.	106
Fig. 3.6 Over-expression of surface variant antigens confer protection to NK cell elimination.	109
Fig. 3.7 Increase ICAM-4 expression on iRBC.	112
Fig. 3.8 NK cells used lytic granules to kill iRBC.....	115
Fig. 3.9 NK cell activation and iRBC elimination required cell-to-cell contact.....	117
Fig. 3.10 Involvement of LFA-1 in NK cell interaction and elimination of iRBC.....	121

Fig 3.11. Heterogenous response donor NK cell response to <i>P. falciparum</i> iRBC.....	128
Fig. 3.12 Gating strategy for FACS sorting of activated NK cells from iRBC co-culture.....	129
Fig. 3.13 Microarray data quality check.	131
Fig. 3.14 Transcriptomic changes correlated with protein changes observed on FACS.	133
Fig. 3.15 Involvement of pathogen recognition receptors in response to iRBC.....	135
Fig 3.16 RIG-I Like Receptor Signaling Pathway.	137
Fig. 3.17 Involvement of TBK1/IKK ϵ in sensing of foreign nucleic acids..	139
Fig. 3.18 Bx795 prevented NK cell control of parasitemia.....	141
Fig. 3.19. Uptake of MVs by NK cells through membrane fusion.....	144
Fig 3.20 TLR signaling pathway.	146
Fig. 3.21 MYD88-dependent control of parasitemia.	150
Fig. 3.22 Interferon signaling.....	153
Fig. 3.23 KIR genotyping by PCR-SSP.....	157
Fig. 3.24 KIR frequency in non-responder and responder donors.....	159
Fig. 4.1 <i>P. falciparum</i> development in RBC	166
Fig. 4.2 LFA-1 (CD11a/CD18) and DNAM-1 mediates NK cell-iRBC attachment.....	172
Fig. 4.3 Comparison of DEGs in children with mild and severe malaria against responder and non-responder.	175

Fig. 4.4 TLR2 and TLR4 are required for NK cell-iRBC response.	179
Fig. 4.5 Control of parasitemia by NK cells require TBK1/IKK ϵ pathway.	182
Fig. 5.1 NK92 cells can control iRBC parasitemia.....	188
Fig. 6.1 Proposed mechanism of NK cell response to iRBC.....	193

List of Tables

Table 2.1 Primers used for qPCR	90
Table 2.2 Primers used for KIR PCR-SSP	91
Table 2.3 Touchdown PCR cycling condition.....	92
Table 3.1 NK cells upregulated activation markers and effector molecules when exposed to iRBC.....	103
Table 3.2 Characteristics of parasites used for NK cell co-culture studies.	107
Table 3.3 NK cell response to different parasite strains/lines.....	110
Table 3.4 Donor profile of samples submitted for mRNA microarray.....	130
Table 3.5 qPCR results of selected genes involved in RIG-I like signalling pathway	138
Table 3.6 qPCR results of selected genes involved in TLR signalling pathway	147
Table 3.7 qPCR results of selected genes involved in IFN signalling pathway	153
Table 3.8 KIR genotype profile of responders and non-responders.	158
Table SI. List of DEGs between R _{iRBC} and NR _{iRBC}	196

Abbreviations

CMA	Concanamycin A
CMV	Cytomegalovirus
DNAM-1	DNAX Accessory Molecule-1
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cell
ICAM	Intercellular Adhesion Molecule
IDC	Intra-erythrocytic development cycle
iRBC	Infected red blood cells
KAHRP KO	Knob associated histidine-rich protein knock out
KIR	Killer immunoglobulin-like receptor
LFA-1	Lymphocyte function-associated antigen-1
MAHRP KO	Membrane associated histidine-rich protein knock out
MHC	Major histocompatibility complex
MV	Microvesicles
NCR	Natural cytotoxicity receptor
NK	Natural killer
PBMC	Peripheral Blood Mononuclear cells
PfEMPI	<i>Plasmodium falciparum</i> erythrocyte membrane protein 1
PfGPI	<i>P. falciparum</i> glycosylphosphatidylinositol
PRR	Pattern recognition receptor
RLR	RIG-I like receptor
TLR	Toll-like receptor

Abstract

Natural killer (NK) cells are key components of the innate immune system, and play critical roles in the control of acute infection by malaria parasites. It is known that the secretion of IFN- γ by NK cells is a key determinant for disease resistance. However, much less is known about its cytotoxicity on infected red blood cells (iRBC). Also, how NK cells identify and respond to iRBC remains to be characterized. Here we show that control of parasitemia by NK cells was contact-dependent and involved cytolytic granules. Video microscopy demonstrated osmotic lysis of iRBC by NK cells. Furthermore, NK cells preferentially lysed trophozoites and schizont-stage iRBC, and such lysis required complex interactions of multiple activation signals provided by LFA-1, TLR2 and TLR4, as well as signaling through MYD88-dependent and TBK/IKK ϵ -dependent pathways. Interestingly, NK cells from about 30% of malaria naïve donors do not eliminate iRBC, and upregulate the inhibitory NKG2A receptor instead of activation molecules, like CD69 and CD25. KIR genotyping further demonstrated increased frequencies of KIR2DS1 and KIR2DS2 in these donors. We further show that NK cell can control *P. knowlesi* parasitemia, demonstrating species-transcending immune control of this zoonotic parasite. In addition, over-expression of surface variant antigens by iRBC appeared to aid iRBC evasion from NK cell lysis. Together our study demonstrates the multifaceted interactions of NK

cells and *P. falciparum* iRBC, and identifies the interplay of activation and inhibitory signals in the control of parasitemia by NK cells.

Chapter 1. Introduction

1.1 *Overview of Malaria*

1.1.1 Global burden of malaria

Malaria is the world's most important parasitic infectious disease, affecting 97 malaria endemic countries (Fig. 1.1A), and putting nearly half the world's population at risk of infection. Economically, international disbursement for malaria control and elimination amounted to US\$2.5 billion in 2015. WHO estimated there were 214 million cases of malaria and 438 000 deaths in 2015 (Fig. 1.1B), with 70% of deaths occurring in children younger than five. This represented a 48% reduction in malaria mortality compared to 2000, where mortality was at a high of 839000 (WHO 2015). It is worthwhile to note that despite apparent success in malaria mortality reduction, a greater problem of drug-resistant malaria parasites is slowly increasing, affecting 2 countries of the Greater Mekong Subregion (GMS) in 2010, to 5 countries in 2015. With the breaking down of geographical boundaries by air- and sea-travel, not only can there be rapid spread of drug-resistant parasites, but also expose non-malaria endemic metropolitan cities to malaria. For example, though Singapore is declared malaria-free by the World Health Organization (WHO) in 1982, there were still a total of 62 imported cases of malaria in 2014 (MOH 2015). The *Anopheles* mosquito, which is the vector for malaria transmission, is still found in clusters around Singapore (NEA 2008), and

if vector control and disease surveillance is insufficient, a resurgence of disease could occur. In a review of resurgences occurring in 61 countries, including Asian countries like China, South Korea, and Thailand, 91% of events were attributed to weakening malaria control program (Cohen, Smith et al. 2012). Also, according to the 2014 report on climate change by the Intergovernmental Panel on Climate Change (IPCC), increasing temperature, humidity and rainfall will likely exacerbate malaria transmission. More importantly, global warming will directly impact the distribution of mosquito-vectors, especially in high altitude areas, where malaria transmission has been restricted by low temperature, increasing the risk of malaria transmission in these places (Field and Barros 2014).

Malaria has plagued humanity since ancient times. Symptoms and treatments of malaria have been found recorded in one of the oldest Traditional Chinese Medicine text, the Huangdi Neijing, which is estimated to be composed around 475-221BC. In fact, it has infected human for so long, that it has become one of the strongest known selective pressure on the human genome, driving selection of hemoglobinopathies, such as sickle-cell anemia, glucose-6-phosphatase deficiency and thalassemia in areas where malaria is endemic (Kwiatkowski 2005).

Fig. 1.1. Malaria Epidemiology 2015. (A) Shown are the percentage of the population at risk of developing malaria. (B) Shown are total inpatient cases of malaria reported, with an overlay of malaria mortality. 88% of cases occurred in African region, followed by South East Asia (10%). Distribution of malaria deaths is similar. Made with Global Malaria Mapper (WHO 2015)

The importance of malaria research, has thus far given rise to a number Nobel laureate. Sir Ronald Ross was awarded the Nobel Prize for Physiology or Medicine in 1902 for the discovery that the malaria parasite is transmitted by the *Anopheles* mosquito. This was followed by Charles Louis Alphonse Laveran, whom was awarded the Nobel Prize for Physiology or Medicine in 1907 for his observations that the causative agent for malaria is a protozoan, which resides within red blood cells (RBC). And most recently, Youyou Tu won the 2015 Nobel Prize for

Physiology or Medicine for her discovery of artemisinin in the treatment of malaria.

1.1.2 Malaria vaccines

2015 also saw a significant milestone in malaria control being reached. In July 2015, RTS,S/AS01 (Mosquirix™), a malaria vaccine jointly developed by GlaxoSmithKline (GSK) and PATH Malaria Vaccine Initiative (MVI), is approved for use by the European Medicines Agency's (EMA) Committee for Medicinal Products for Human Use (CHMP) (EMA 2015), becoming not only the first licensed malaria vaccine, but also the first vaccine against a parasitic disease. On 23rd October 2015, World Health Organization's (WHO) Strategic Advisory Group of Experts on Immunization (SAGE) and the Malaria Policy Advisory Committee (MPAC) jointly endorsed the use of RTS,S in pilot projects in Africa to assess its feasibility as a form of complementary malaria control (WHO 2015).

RTS,S is a recombinant protein vaccine containing parts of the pre-erythrocytic stage circumsporozoite protein (CSP). Initial results from phase 3 clinical trials were encouraging, demonstrating a 50.4% vaccine efficacy after 3 doses in a 14 month follow up (Agnandji, Lell et al. 2011). However, RTS,S is far from being a perfect vaccine. In a recent 7 year follow-up of children whom received 3 doses of RTS,S, vaccine efficacy decreased over time, being close to 0% in the fourth year, and even counter-protective in the fifth year of follow-up. Overall vaccine efficacy

20

was estimated to be 28.3% (Olotu, Fegan et al. 2016). Such low vaccine efficacy was similarly observed previously, where a fourth booster dose was introduced into the vaccination regime. In a 4-year follow-up period, vaccine efficacy in children was 36% and 26% in infants after receiving all 4 shots. Without the fourth shot at an 18-months interval, vaccine efficacy dropped to 28% and 18% respectively (RTS 2015). One reason suggested for the low vaccine efficacy of RTS,S in Africa was the allelic mismatch between African *Plasmodium falciparum* (*P. falciparum*) strains and the strain which the RTS,S circumsporozoite protein (CSP) was modelled after. Vaccine efficacy against matched clinical malaria was 50.3%, while against mismatched clinical malaria the vaccine efficacy dropped to 33.4% (Neafsey, Juraska et al. 2015). In view of the 4-dose schedule, WHO released a position paper on the Mosquirix™ vaccine in 2016. In it, WHO recommended further evaluation of the vaccine in pilot implementation studies to determine if the efficacy observed in Phase 3 trials in children aged 5-17 months could be replicated in the context of routine health systems. Also, the vaccine was not recommended for use in infants (6-12 weeks) (WHO 2016). To fund such pilot studies, Gavi, the Vaccine Alliance, approved up to US\$27.5 million in funding support (Kelly 2016).

A close contender to Mosquirix™, is another pre-erythrocytic vaccine developed by Sanaria. It is a radiation-attenuated *Plasmodium falciparum* sporozoite (PfSPZ) vaccine which was first reported to have

over 90% protective efficacy (n=26) when administered by mosquito bites (Hoffman, Goh et al. 2002). However, a recent phase I clinical trial demonstrated only 50% protection (n=10) after 6 immunizations via mosquito bites (Hickey, Lumsden et al. 2016). To establish an injectable route of vaccine delivery, subcutaneous (SC), intradermal (ID), intramuscular (IM) or intravenous (IV) administration of PfSPZ were tested. A vaccine efficacy of 80% (n=15) was obtained by IV injection of 1.35×10^5 PfSPZ at 3 weeks after immunization (Seder, Chang et al. 2013). After 4 IV immunization with 2.7×10^5 PfSPZ, vaccine efficacy was 55% at 21 weeks after immunization (n=11) (Ishizuka, Lyke et al. 2016). In contrast, only 2 out of 80 volunteers were protected when PfSPZ was administered by SC or ID injections (Epstein, Tewari et al. 2011), and vaccine efficacy was 0% for IM immunization (Ishizuka, Lyke et al. 2016). Regarding heterologous challenge after PfSPZ vaccination administered by mosquito bites, Hoffman *et al* reported a heterologous protection efficacy of 67% (n=6) (Hoffman, Goh et al. 2002). In a 2015 study of 16 volunteers vaccinated by whole parasite immunization under chloroquine prophylaxis, heterologous protection was only 15% (n=13) (Schats, Bijker et al. 2015). Notwithstanding, studies are currently ongoing to further address this aspect (Ishizuka, Lyke et al. 2016).

1.1.3 Human malaria parasite species

Four species of parasites in the genus *Plasmodium* (*P.*) are traditionally responsible for human malaria – *P. ovale*, *P. malariae*, *P.*

vivax and *P. falciparum*. *P. ovale*, *P. vivax* and *P. falciparum* have a tertian (48 hrs) erythrocytic development cycle (EDC), while *P. malariae* has a quartan (72 hrs) EDC. *P. malariae* infections are on the decline, and cause disease mainly in Sub-Saharan Africa, where it accounts for only 2% of all malaria cases. Similarly, *P. ovale* infections are highly restricted to Sub-Saharan Africa, and account for about 8% of all cases (Carter and Mendis 2002). *P. vivax* invades reticulocytes, and is a major cause for clinical malaria, though mortality is rare. Of note is that both *P. ovale* and *P. vivax* can form hypnozoites (dormant liver stage), which are responsible for disease relapse after years of apparent cure. Unlike the other three species, *P. vivax* malaria is most prominent outside of Sub-Saharan Africa, comprising about 96% of cases in Asia, and 80-90% in the Middle East, and Central and South America (Carter and Mendis 2002). *P. falciparum* is the most virulent of the four species, and is mainly endemic in Sub-Saharan Africa, accounting for about 80% of cases (Carter and Mendis 2002, Snow and Omumbo 2006). South East Asia (SEA) contributes about 30% of global malaria cases annually, with India contributing 58% of these cases, and Indonesia contributing another 20% of cases in 2009 (Kumar, Chery et al. 2012). Historically, *P. vivax* was responsible for the majority of cases in India, with a *P. falciparum* to *P. vivax* ratio of 0.41 in 1985. Since then, this ratio has gradually increased, and in 2010 is at 1.01, representing an increase in *P. falciparum* cases (Das, Anvikar et al. 2012).

Recently, it has been discovered that the zoonotic simian malaria parasite *P. knowlesi*, can cause disease in human (Singh, Kim Sung et al. 2004). In fact, it has been found to cause human malaria in most South East Asian countries, including Singapore (Ng, Ooi et al. 2008), and is a major cause in East Malaysia (Kantele and Jokiranta 2011, William, Menon et al. 2011). In East Malaysia, prevalence of *P. knowlesi* malaria increased from 703 cases in 2011 to 996 in 2013. This represented 62% all malaria notifications in 2013 (William, Jelip et al. 2014). In terms of virulence, it is comparable to *P. falciparum*, with 39% of patients developing severe malaria and has a case-fatality rate for severe malaria of 27% (William, Menon et al. 2011). Unlike the above four species, *P. knowlesi* has a quotidian (24 hrs) EDC.

Due to the species-specific tropism of *Plasmodium*, only humans and some non-human primates (NHP), such as chimpanzees and macaques, are susceptible to *Plasmodium* species that cause human malaria. Such host specific restriction is due to many factors such as distribution and habitat of the mosquito vector. Compatibility between host and parasite interactions, such as compatible red blood cell (RBC) receptors and parasite ligands are also determining factors in the propagation of infection. Notably, of the 430 *Anopheles* mosquito species, only 30-40 species transmit human malaria. The lifespan of adult female mosquitoes in nature is about 14-21 days, while the extrinsic incubation period for malaria parasites in the mosquitoes is about 10-21 days. This

takes up a considerable amount of the mosquitoes' lifespan, hence only a small proportion of mosquitoes survive long enough to transmit the infection. However, given the long period of untreated gametocytaemia in infected patients and availability of vectors, infectivity potential of the disease greatly increases (Macdonald 1957).

1.1.4 Clinical malaria

Most malarial-infections are clinically silent. It was estimated that in 2002, about 2.2 billion people were exposed to *P. falciparum* infection, but only 300-600 million (~10-20%) developed disease (Snow, Guerra et al. 2005), thus reflecting on the ability of the immune system in preventing disease. In highly endemic areas, young children and pregnant women are at highest risk of severe disease, while older children and adults often develop partial immunity from repeated exposure, and thus are lesser at risk of severe disease. Clinically, mild or uncomplicated malaria is often non-specific, and presents as febrile episodes accompanied by symptoms such as chills, malaise, fatigue, headache, cough, nausea, vomiting, abdominal pain, diarrhea, and arthralgia. The signs may include mild anemia and a palpable spleen, as well as mild jaundice. Laboratory tests may reveal a parasitemia of <0.1%, anemia, thrombocytopenia, elevated transaminases, blood urea nitrogen (BUN) and creatinine. In complicated or severe malaria, disease often spans a heterogeneous spectrum of disease syndromes, including cerebral malaria, severe malarial anemia, metabolic acidosis, pulmonary edema,

renal failure, hemoglobinuria (“blackwater fever”), shock-like syndromes and severe jaundice. In addition, pregnant women are susceptible to placental malaria, where it can cause fetal distress, premature labor, stillborn and low birth weight. Laboratory findings of severe malaria include hyperparasitemia (>5% parasitemia), acidosis, hypoglycaemia and coagulopathy, on top of those found in uncomplicated malaria (Breman 2014). However, with better malaria control, either by drug prophylaxis or vector control, parasitemia thresholds for attributing fever episodes to malaria was observed to be decreasing. This led to underestimation of *P. falciparum* episodes (Roucher, Rogier et al. 2012). As *P. falciparum* contributes to the most malaria-associated morbidity and mortality, I will be focusing on this species for the rest of my thesis.

1.1.5 Host cell remodelling

Infection in mammalian hosts begins with the asymptomatic exo-erythrocytic stage, initiated by the injection of sporozoites into the host’s blood during a blood meal taken by the female *Anopheles* mosquito (Fig. 1). These sporozoites migrate to the liver and infect hepatocytes. After an incubation period of about 2 weeks (for *P. falciparum*), infected hepatocytes rupture, releasing thousands of merozoites into the blood circulation, where they invade RBC and start the symptomatic intra-erythrocytic developmental cycle (IDC). The IDC is about 48 hrs for *P. falciparum*, during which the parasites develop through morphologically-distinct stages of ring, trophozoite and schizont. At the schizont stage,

about 16-30 merozoites form, which get released into the blood stream to invade other RBC and perpetuate the cycle (Miller, Good et al. 1994) (Fig. 1.2).

The IDC is responsible for the symptoms and pathologies of malaria, including the manifestations of severe disease. Naturally acquired antibodies against antigens expressed during the IDC are also correlated with parasitemia control (Chizzolini, Dupont et al. 1988). Hence, I will be focusing mainly on the IDC for my thesis.

Upon infection of RBC, the parasites make extensive modification to the host. The earliest morphological change is the formation of a parasitophorous vacuole in which the parasite resides, as well as numerous membranous structures in the RBC cytosol – Maurer's cleft (Mundwiler-Pachlatko and Beck 2013). This is followed by condensation of haemoglobin into foci of haemozoin,

Fig. 1.2 Life cycle of *P. falciparum*. Malaria infection in humans begins when a female *Anopheles* mosquito takes a blood meal and sporozoites get deposited mainly into the dermis. Sporozoites then invade the blood stream and migrate to the liver to infect hepatocytes (Amino, Thiberge et al. 2006). After about two weeks of incubation, infected hepatocytes rupture to release thousands of merozoites into the blood stream, where they invade erythrocytes and start the intra-erythrocytic developmental cycle (IDC). Here, throughout a 48 hrs period, the parasite matures through the stages of rings, trophozoites and schizonts. Schizonts ultimately rupture, releasing more merozoites to perpetuate the cycle. Some trophozoites differentiate into gametocytes, and is the form taken up by mosquitoes during their blood meal to start the sporogonic stage. The sporogonic stage lasts 2-3 weeks and ends with the formation of sporozoites which migrate to the mosquitoes' salivary glands.

and the formation of food vacuoles (Gruring, Heiber et al. 2011). The spectrin meshwork of the iRBC membrane and cytoskeleton are also remodeled resulting in increased rigidity and decreased deformability (Shi, Liu et al. 2013). Voltage dependent nutrient channels of the clag3 family are inserted into the iRBC membrane to facilitate nutrient uptake

28

and contribute to increase erythrocyte membrane permeability (Desai, Bezrukov et al. 2000, Nguitragool, Bokhari et al. 2011). Most noticeable of all, are the formation of knobs on the surface of the iRBC, which are composed mainly of knob-associated histidine rich proteins (KAHRP) (Rug, Prescott et al. 2006). KAHRP interacts with cytoskeletal proteins, like spectrin (Pei, An et al. 2005), to increase membrane rigidity and decrease deformability (Glenister, Coppel et al. 2002). These modifications seemed to path the way for the insertion of other parasite-derived proteins such as *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1), repetitive interspersed family protein (RIFIN) (Chen, Schlichtherle et al. 2000), and the subtelomeric variable open reading frame protein (STEVAR) (Niang, Yan Yam et al. 2009) on the iRBC surface. The reasons for the introduction of such neo-antigens on the iRBC surface, which expose the iRBC to increase immune recognition-stress, are not exactly known, but have been postulated to help iRBC avoid splenic clearance and evade the immune system.

One of the most antigenically diverse parasite protein unique to *P. falciparum* is PfEMP1. These variant antigens are 200-350 kDa, and are encoded by 59 *var* genes on multiple parasite chromosome in the 3D7 genome (Su, Heatwole et al. 1995, Gardner, Hall et al. 2002). These genes had been found to have high duplication, shuffling and mutation rates, thus adding to their antigenic heterogeneity (Flick and Chen 2004). *Var* genes are transcribed as early as in the ring-stage, and peak at the late

rings stage (Kyes, Rowe et al. 1999). In a process similar to allelic exclusion, only one variant of PfEMP1 is expressed at any one time in a population of parasites through epigenetic regulation (Scherf, Hernandez-Rivas et al. 1998) (Pasternak and Dzikowski 2009). Switching of PfEMP1 expression occurred at about 2.4% per generation *in vitro* (Roberts, Craig et al. 1992), and this phenomenon of clonal antigenic variation has been implicated in immune evasion.

PfEMP1 had been shown to bind various host receptors, such as inter-cellular adhesion molecule 1 (ICAM-1), chondroitin sulphate A (CSA), and CD36 (Moxon, Grau et al. 2011). This leads to the phenomena of 1) rosetting, where an iRBC surrounds itself with normal RBC; 2) cytoadherence, where iRBC is observed to line the walls of vasculature; and 3) sequestration, where late stages of iRBC do not circulate in the peripheral blood, but cytoadhere to endothelium of the post-capillary venules (Berendt, Ferguson et al. 1990). Such promiscuity of binding is in part due to the different adhesion domains present on the extracellular portion of PfEMP1 (Miller, Baruch et al. 2002). The general organization of PfEMP1 is highly conserved. The Duffy Binding Like domains (DBL), and cysteine-rich inter-domain regions (CIDR) are responsible for the adhesion phenotype of PfEMP1 (Kraemer and Smith 2006). Different variant of PfEMP1 have different compositions of these adhesion domains, hence some variants have better binding of CSA, while others prefer CD36. The resultant sequestration of iRBC in the microvasculature of the

brain, lung, liver, kidney and placenta has been implicated in the pathology of severe malaria.

RIFINs are polypeptides of 30-45kDa, and encoded by genes of the *rif* family, which have 149 copies in the 3D7 genome (Fernandez, Hommel et al. 1999, Gardner, Hall et al. 2002). Transcription of *rif* occurs during the late rings to early trophozoite stages (Kyes, Rowe et al. 1999). Like PfEMP1, RIFINs are also clonally variant (Fernandez, Hommel et al. 1999, Kyes, Rowe et al. 1999), and might undergo allelic exclusion too (Goel, Palmkvist et al. 2015). Through binding assays, it was recently established that a *rif* gene (PFIT_bin05750) preferentially bound blood group A RBC over group O RBC, and may be involved in microvasculature sequestration *in vivo* (Goel, Palmkvist et al. 2015).

Like RIFINs, STEVORs are also small polypeptides of 30-40kDa (Cheng, Cloonan et al. 1998). Although there are 28 copies of the gene in the 3D7 parasite genome (Gardner, Hall et al. 2002), only a subset of *stevor* genes are transcribed in the mid-trophozoite stage (Kaviratne, Khan et al. 2002), and they might undergo transcriptional antigenic switching (Lavazec, Sanyal et al. 2007). STEVOR are found to be present in the Maurer's cleft and exported onto iRBC surface during late-trophozoite and schizont stage (Blythe, Yam et al. 2008, Niang, Yan Yam et al. 2009). Like PfEMP1 and RIFIN, clonal variation at the iRBC surface also occurs for STEVOR, leading to different antigenic properties (Niang, Yan Yam et al. 2009). Though STEVOR is not required for parasite

survival *in vitro* and is detected in <30% of culture-adapted parasites, >90% of iRBC from fresh field isolates are found to have STEVOR expression, thus indicating a possible role in immune evasion (Blythe, Yam et al. 2008). Synthetic STEVOR peptides had been shown to bind 30-40 kDa proteins present on RBC membrane, suggesting a role in rosette formation (Garcia, Puentes et al. 2005). This was later confirmed by Niang *et al*, showing that STEVOR mediated PfEMP-1-independent rosetting, and bound glycophorin C on RBC surface (Niang, Bei et al. 2014). Over-expression of STEVOR was also linked to increased iRBC rigidity and reduced deformability, which might promote microvasculature sequestration (Sanyal, Egee et al. 2012). In addition to the expression STEVOR on iRBC surface, STEVOR was also found to be on the surface of merozoites, and STEVOR antibodies could block merozoite invasion (Niang, Bei et al. 2014).

Related to but distinct from the RIFINs and STEVORs are the PfMC-2TM proteins. They are a family of proteins encoded by 13 *Pfmc-2tm* genes. They are mainly expressed from late rings to late trophozoite stages. Like RIFINs and STEVORs, PfMC-2TM also possess two transmembrane domains. However, the extracellular loop of PfMC-2TMs are only about three residues long, compared to those of RIFINs and STEVORs which are 170 and 60 residues respectively (Sam-Yellowe, Florens et al. 2004). Most genes of *Pfmc-2tm* are transcribed at the same time, albeit at varying levels (Lavazec, Sanyal et al. 2007).

Taken together, infection of RBC by *P. falciparum* result in drastic changes in the RBC, with presentation of various classes of parasitic variant antigens on the RBC surface which have roles in iRBC survival and immune evasion.

1.2 ***Adaptive Immune Response to Malaria***

1.2.1 Naturally acquired immunity to malaria

Early observations on rhesus monkeys infected with primate *plasmodia* demonstrated a protective effect of sera from chronically infected *rhesus* monkey (Coggeshall and Kumm 1937). This was later demonstrated in human children, where passive transfer of pooled gamma-globulins from malaria immune adults alleviated malaria symptoms and parasitemia (Cohen, Mc et al. 1961). When pooled immune African globulins were passively transferred to malaria-infected Thai patients, symptoms of fever and parastiemia decline as fast as drug treatment. Interestingly, this also demonstrated a lack of geographical-restriction for protective antibodies (Bouharoun-Tayoun, Attanath et al. 1990). It was then shown that exposure to placental malaria during the first pregnancy can induce antibodies that may protect against placental malaria in subsequent pregnancy (Fried, Nosten et al. 1998). Infants below 6 months old were also seemingly protected from malaria, suggesting a role in protective maternal antibodies (Snow, Nahlen et al. 1998). It is thus evident that humoral immunity constitutes an important aspect towards malaria immunity.

Naturally acquired immunity (NAI) to malaria is first suggested by Robert Koch in 1900, and is most often observed in endemic areas of high malaria transmission (Doolan, Dobano et al. 2009). However, it soon became clear, that in such cases of NAI, the protection conferred, was

strain-specific (Bull, Lowe et al. 1998), as evident by studies using homologous and/or heterologous strains of *P. vivax* in treatment of neurosyphilis (Collins, Jeffery et al. 2004). In a recent study involving whole parasite immunization under chloroquine prophylaxis, it was shown that only 15% of volunteers remained protected from a heterologous challenge one year after the initial immunization (Schats, Bijker et al. 2015). Overall, NAI to malaria is observed to be species and stage specific, requiring at least 15 years of uninterrupted exposure to malaria (Druilhe and Khusmith 1987, Doolan, Dobano et al. 2009). Indeed, it was observed that reactive antibodies to malarial antigens were fast to develop, but have no correlation with clinical immunity (Druilhe and Khusmith 1987, Bouharoun-Tayoun, Attanath et al. 1990, Bull, Lowe et al. 1999). With reduced malaria transmission due to efforts in vector control, there had been a decline in malaria antibody seropositivity leading to decreasing NAI (Diop, Richard et al. 2014). This also led to a shift of malaria cases towards later childhood and into adulthood (Trape, Tall et al. 2011, Griffin, Ferguson et al. 2014).

As mentioned previously, titers of reactive antibodies against IDC antigens correlated with parasitemia control (Chizzolini, Dupont et al. 1988). Reactive antibodies have been shown to react to antigens on surface of iRBC causing a phenomenon of agglutination. Of the different assays used to gauge the “protectiveness” of reactive antibodies, titres of agglutinating reactive antibodies have the highest association with

clinical protection (Marsh, Otoo et al. 1989). The repertoire of reactive antibodies in each infected individual is highly diverse, and mostly are reactive only to the individual's homologous parasite (Marsh and Howard 1986). This suggest a large of degree antigenic diversity, and probably reflect the parasites' immune evasion capability (Marsh and Howard 1986). However, certain strains of parasite were easily agglutinated by plasma of different individuals, suggesting that though the antigenic targets were diverse, there might be some dominant epitopes involved (Bull, Lowe et al. 1999).

Hyperimmune sera obtained from malaria-immune patients were able to recognize normal iRBC, but not RBC infected with mutant strains that cannot express PfEMP1 (Piper, Roberts et al. 1999, Chan, Howell et al. 2012). Patients with reactive antibodies to PfEMP1 were also noted to have reduced risk of clinical malaria (Chan, Howell et al. 2012). Apart from PfEMP1, reactive antibodies against RIFIN were also detected, albeit at lower prevalence (Abdel-Latif, Khattab et al. 2002). In longitudinal studies, it was observed that high anti-RIFIN titers correlated with increased parasite clearance time, though they had no effect on reinfection rates (Abdel-Latif, Dietz et al. 2003). In longitudinal follow-ups of 9-month old infants, no protective effect was observed for STEVOR antibodies, though there was a strong positive correlation between presence of STEVOR antibodies and parasitemia (Schreiber, Khattab et al. 2008).

With the observed protective effect of anti-PfEMP1 antibodies, attempts were made to identify protective conserved epitopes. It was shown that serum antibodies from Ghanaian children recognized a conserved peptide of the Duffy-binding like domain α (DBL α) of PfEMP1, although there was no correlation between clinical protection and antibodies to this peptide (Dodoo, Staalsoe et al. 2001). Similar results were obtained when 5 other DBL domains were tested. Despite the presence of reactive antibodies to these recombinant peptides, there was no observed protective association (Mackintosh, Christodoulou et al. 2008). The cysteine-rich interdomain region 1 (CIDR1) is another domain present on PfEMP1, and is known to be the CD36 binding domain (Baruch, Ma et al. 1997). Using a 179-amino acid fragment of CIDR1, Baruch *et al* demonstrated that vaccination of Aotus monkey with the CIDR1 fragment cloned from Malayan Camp (MC) *P. falciparum*, induced immunity to homologous parasite challenge, but not to heterologous strain challenge by Vietnam Oak Knoll (FVO) *P. falciparum* (Baruch, Gamain et al. 2002). However, repeating the vaccination with FVO-CIDR1 failed to protect Aotus monkey even from homologous challenge from FVO *P. falciparum* challenge (Makobongo, Keegan et al. 2006).

The mode of action of such protective antibodies are myriad. Antibodies were noted to prevent iRBC adhesion to other RBC in a phenomenon known as rosetting. In a study comparing anti-rosetting ability of plasma collected from children with cerebral malaria or mild

malaria, it was found that plasma from children with mild malaria possessed anti-rosetting activity, suggesting that such anti-rosetting antibodies had a protective effect against cerebral disease (Carlson, Helmby et al. 1990). Serum from pregnant women inhibited iRBC binding to CSA (Fried, Nosten et al. 1998), and improved birth outcomes (Duffy and Fried 2003). Naturally acquired antibodies were also noted to inhibit merozoite invasion. Reactive antibodies to merozoite surface proteins (MSP) had been shown to block merozoite invasion of RBC in a complement-dependent manner (Boyle, Reiling et al. 2015). These antibodies were also involved in opsonic phagocytosis of merozoites or iRBC by monocytes (Celada, Cruchaud et al. 1982, Bouharoun-Tayoun, Attanath et al. 1990, Bouharoun-Tayoun, Oeuvray et al. 1995).

1.2.2 B cells

The humoral system mentioned above, is just one part of the adaptive immune system, typically attributed to B cells. As mentioned, continuous high density exposure to malaria is often required for generating long-lasting immunity. This hinted at the possibility that malaria infection can impair B cell response, leading to defective or suboptimal B cell memory. Indeed, it was observed that antibody response to antigens, especially merozoites antigen, were very short-lived; and antibody response to malaria antigens was more short-lived in children less than three years-old than in older children (Kinyanjui, Conway et al. 2007, Akpogheneta, Duah et al. 2008).

In a lethal *P. yoelii* model, it was shown that B-cell activating factor (BAFF) expression levels on dendritic cells (DC) was reduced, suggesting a reduced capacity for memory B cell (MBC) support and differentiation (Liu, Stacey et al. 2012). The cysteine-rich interdomain region 1 α (CIDR1 α) of PfEMP1 had been shown to interact with B cells partially through the B-cell receptor, leading to persistent activation and possibly exhaustion of B cells *in vivo*, especially in chronic malaria (Simone, Bejarano et al. 2011).

Apart from reduced survival of MBCs, *Plasmodium* infection also seemed to alter MBC population proportion. In chronically infected patients, there was an expansion of atypical hyporesponsive MBC (CD10⁻CD19⁺CD20⁺CD21⁻CD27⁻), which comprised about 17% of total mature circulating B cells (Weiss, Crompton et al. 2009, Illingworth, Butler et al. 2013, Portugal, Tipton et al. 2015). Such atypical MBCs expressed higher levels of inhibitory receptors, and expanded less in response to B cell receptor cross-linking or CD4 T cell stimulation compared to naïve B cells (Moir, Ho et al. 2008). In response to malaria infection *in vivo* or CpG stimulation *in vitro*, atypical MBCs did not actively secrete antibodies (Portugal, Tipton et al. 2015), thus suggesting that *P. falciparum* infection can disrupt the acquisition of an efficient humoral response. However, in contrast to Portugal *et al*, Muellenbeck *et al* utilizing single cell sorting and antibody cloning demonstrated that *P. falciparum* infection not only stimulated the development of both classical and atypical MBC, but also

caused both MBC subtypes to secrete polyreactive anti-*Pf* IgG that bound iRBC, and inhibited *in vitro* merozoite invasion (Muellenbeck, Ueberheide et al. 2013). As such the contribution of atypical MBC to malaria-specific B cell memory remained to be solved.

In Madagascar, following a 30-year period of complete malaria control, people aged over 40, whom had prior exposure to malaria, were observed to be more resistant to clinical malaria, suggesting that some aspect of malaria-immunity might be long-lived (Deloron and Chougnet 1992). This was verified by Wipasa *et al*, who showed the maintenance of long lasting antigen-specific memory B cells even in the absence of reinfection (Wipasa, Suphavitai et al. 2010). Using elispot against merozoite surface proteins, Ndungu *et al* showed that exposure of Swedish residents to malaria during travel can induce long-lasting MBC for up to 16 years without re-exposure, even as the corresponding serum antibody is absent (Ndungu, Lundblom et al. 2013). In a *P. chabaudi* C57BL/6 mice infection model, it was shown that after *P. chabaudi* infection, merozoite surface protein 1 (MSP-1) specific MBC expanded rapidly, and persisted with a half-life of 221 days, long after parasitemia was cleared on day 15. Upon re-challenge, these MSP-1-specific MBC expanded and undergo somatic hypermutation and class-switching to produce high-affinity IgM and IgG (Krishnamurthy, Thouvenel et al. 2016). It remained to be seen if these long-lasting MBCs could contribute to protective immunity against malaria challenge.

1.2.3 CD4 T cells

It's long been known that CD4⁺ T cells are crucial in malaria immunity. CD4⁺ T cell clones stimulated with *P. chabaudi* antigens were able to confer protection to athymic nude mice against *P. chabaudi* infection (Brake, Long et al. 1988). Similar results were obtained when adoptive transfer of CD4⁺ T cells from *P. chabaudi* infected BALB/c mice into athymic *nu/nu* mice protected the mice from lethal challenge, and cleared parasitemia to sub-patent levels (Meding and Langhorne 1991). In humans, it was shown that very low dose of *P. falciparum* iRBC can induce protective immunity against the homologous strain, even though there were almost no detectable anti-malarial antibodies. This was characterized by interferon gamma (IFN- γ) secretion and expansion of both CD4⁺ and CD8⁺ T cells (Pombo, Lawrence et al. 2002).

CD4⁺ T cells are typically divided into IFN- γ producing T_H1 cells involved in cell-mediated immunity against intracellular pathogens, and IL-4-producing T_H2 cells which work with B cells in humoral immunity (Mosmann and Coffman 1989). In a *P. chabaudi* infection model in either resistant C57B/6 mice or susceptible A/J mice, it was observed that a predominant T_H1 response occurred during the acute phase of infection, while a T_H2 response predominated after 3 weeks (Langhorne, Gillard et al. 1989, Stevenson and Tam 1993, Sponaas, Cadman et al. 2006). This switch in T_H1/T_H2 response had been attributed to the presence of different dendritic cell (DC) subtypes. It was observed that during the

acute phase of infection, CD11c⁺CD8⁺ DC predominated and drove mainly a T_H1 response. Towards the later stage, there was increased apoptosis of CD11c⁺CD8⁺ DC coupled with increased proliferation of CD11c⁺CD8⁻ DC, which skewed CD4 response to T_H2 (Sponaas, Cadman et al. 2006). Of late, evidence is increasing about the plasticity of T_H1/T_H2 cells (Murphy and Stockinger 2010), and it was suggested that CD4 subsets interconvert depending on cytokine patterns and parasite burden, thus evolving at different stage of malaria infection (Perez-Mazliah and Langhorne 2014). The observation of T_H1 CD4 T cells (IFN- γ ⁺T-bet⁺) co-expressing IL-10, which is typically a T_{reg} cytokine in malaria-infected children seemed to lend support to this hypothesis (Jagannathan, Eccles-James et al. 2014).

Recently much attention had been focused on a subset of CD4⁺ T cells, known as Follicular Helper CD4 T cells (T_{FH}), characterized by their expression of CXCR5. Apart from IFN- γ and IL-4, these T_{FH} also secreted IL-21, and were required for the maintenance of germinal center and B cell differentiation to plasma and memory B cells (Crotty 2011, Locci, Havenar-Daughton et al. 2013). These cells had been observed in malaria-immune Malian children, and the CXCR3⁻ T_H2 polarized T_{FH} cells indeed provided better B cell help. In acute malaria, there was proliferation and preferential activation of the less functional T_H1 polarized CXCR3⁺ T_{FH} cells (Obeng-Adjei, Portugal et al. 2015). However, in severe malaria in the *P. chabaudi* model, it was observed that there is overall suppression of T_{FH} differentiation. Neutralization of inflammatory cytokines, i.e. tumor

necrosis factor alpha (TNF- α) and IFN- γ , restored their differentiation (Ryg-Cornejo, Ioannidis et al. 2016). These studies suggested that malaria can impair germinal centre responses, and affect effective B cell response.

1.2.4 CD8 T cells

CD8⁺ T cells, which are responsible for cell-mediated immunity, have been shown to be critical for the protection against malaria sporozoite during the pre-erythrocytic phase of infection in the liver (Schofield, Villaquiran et al. 1987, Weiss, Sedegah et al. 1988). However, their role during the erythrocytic cycle is less clear. Using the *P. yoelii* 17X parasite, which cause a self-limiting disease, T cell depletion led to severe disease and increased mortality in CBA/CaJ mice. Via adoptive transfer of T cell subsets, Jayawardena et al showed that the subset of T cell involved in protection lies within the Ly1 (CD4⁺) subset (Jayawardena, Murphy et al. 1982). This was later refuted by Mogil et al, which showed that when Ly2 (CD8⁺) T cell subset were adoptively transferred into T cell depleted mice, protection was restored (Mogil, Patton et al. 1987). In support of Jayawardena's finding, Vinetz et al showed that protection lay within the CD4⁺ T cell population, and that adoptive transfer of CD8⁺ T cells from immune BALB/c, C57BL/10 or CBA/CAj mice into their nude or antibody depleted counterpart conferred no protective effect on *P. yoelii* 17X infection (Vinetz, Kumar et al. 1990). Similar results were obtained with *P. chabaudi* infection in NIH mice by Taylor-Robinson et al. They showed that CD8⁺ T cell depletion had no effect on parasitemia and survival,

whereas both T_H1 and T_H2 cells were required to control parasitemia albeit through different mechanisms (Taylor-Robinson, Phillips et al. 1993). This conundrum was reexamined in 2010 by Imai et al. In agreement with observations of Jayawardena and Vinetz et al, CD8⁺ T cells from naïve mice that survived one round of lethal *P. yoelii* infection did not confer protection. However, when these I7XNL-recovered mice were boosted twice, adoptive transfer of their CD8⁺ T cells conferred protection in naïve mice in a IFN- γ - and macrophage-dependent manner (Imai, Shen et al. 2010). This suggested that CD8⁺ T cells were not activated during primary infection, and required a prime-boost regime to generate functionally active antigen-specific CD8⁺ T cells. Similarly, when CD8⁺ T cells were adoptively transferred 21 days post-*P. chabaudi* infection, protection could be transferred to naïve mice (Horne-Debets, Faleiro et al. 2013). The authors further showed that PD-1, a cell surface receptor on T cells, was involved in T cell exhaustion in chronic *P. chabaudi* infection (Horne-Debets, Faleiro et al. 2013).

Since iRBC do not express major histocompatibility complex (MHC), it is interesting how CD8⁺ T cells recognize and interact with iRBC. To this end, Lundie et al generated transgenic *P. berghei* constitutively expressing MHC I- or II-restricted epitopes from hemagglutinin (HA) of the influenza virus PR8. They showed that these transgenic antigens were efficiently presented to CD8⁺ T cells via CD8 α DCs, leading to CD8 proliferation (Lundie, de Koning-Ward et al. 2008).

In a *P. yoelii* model, it was shown that *P. yoelii* infected erythroblasts interacted with murine CD8⁺ T cells in a Fas-FasL dependent manner leading to externalization of phosphatidylserine, which enhanced their phagocytosis by macrophage (Imai, Ishida et al. 2015). All in all, it seemed that during the IDC stage of malaria-infection, CD8⁺ T-cells do not directly participate in iRBC elimination, but execute their effects through macrophages and DCs.

1.3 ***Host Innate Immunity Response to Malaria***

1.3.1 Role of the innate immune system in malaria

As alluded to before, B cell deficient mice developed a chronically relapsing parasitemia (von der Weid, Honarvar et al. 1996) (Fig. 1.3).

Despite the obvious conclusion for the importance of B cell in long term parasitemia control, it is also evident that the innate immunity can control parasitemia, thus preventing an ever-increasing parasitemia. In a study in Papua New Guinea, where multiple strains of *Plasmodium* often infect the same host, it was revealed that peak parasitemia of different species follows a sequential pattern. Parasitemia of one species would increase to a threshold where it then plateaus before declining. This decline then sparked the increase in parasitemia of another species, which then continued the pattern. Such “density-dependent” regulation of parasitemia suggested that the innate immunity system had a detection threshold, crossing which activated it and kept parasitemia in check, until the adaptive immune system catches up (Bruce and Day 2003).

The host innate immune system is the first line of defense against *Plasmodium* infection, and plays critical roles in limiting the acute infection. In fact, the outcome of early host–parasite interaction is a strong determinant for later immunopathology and adaptive immune responses (Urban, Ing et al. 2005). Critical to this, is the role of cytokines, which not only determine the activation, proliferation and functions of circulating immune cells, but may also contribute to malaria pathogenesis

in the host (Miller, Good et al. 1994). In non-lethal models of *P. yoelii* and *P. chabaudi* malaria, parasitemia peaked by the second

Fig. 1.3 Impact of host immune cells on *P. c. chabaudi* AS parasitemia. Time course of *P. chabaudi* parasitemia in wild-type mice (A); IFN- γ deficient mice (B); B cell depleted mice (C); NK cell depleted mice (D); CD8-depleted mice (E); and C4-depleted mice (F). Figure created from data in (Süss, Eichmann et al. 1988, Taylor-Robinson and Phillips 1994, Favre, Ryffel et al. 1997, Mohan, Moulin et al. 1997, van der Heyde, Pepper et al. 1997)

week, and subsequently got cleared. In these models, it was shown that

T_H1 cytokines, especially IFN- γ , are required in the control of acute

parasitemia (van der Heyde, Pepper et al. 1997). In IFN- $\gamma^{-/-}$ mice, *P.*

chabaudi infection led to higher mortality, but had no effect on

immunoglobulin production and reinfection susceptibility (Favre, Ryffel

et al. 1997). When C57BL/6 mice infected with the lethal *P. yoelii* 17XL parasite were treated with a neutralizing anti-IFN- γ antibody, all mice died earlier despite having the same level of parasitemia as control mice (Kobayashi, Ishida et al. 2000). Similarly, in *P. chabaudi* models, IFN- γ neutralization led to death of all mice (Stevenson, Tam et al. 1995). In humans, IFN- γ was significantly increased in patients during the acute phase of malaria (Torre, Speranza et al. 2002). Also, when malaria-naïve volunteers were experimentally infected with *P. falciparum*, it was observed that soluble granzymes and IFN- γ were significantly increased (Hermesen, Konijnenberg et al. 2003).

Another important cytokine is TNF- α . It is initially named ‘cachectin’ for its role in cachexia. TNF- α is an endogenous pyrogen produced by mononuclear cells, and mediates a range of inflammatory processes in the host, including endotoxic shock (Beutler and Cerami 1987, Kwiatkowski, Cannon et al. 1989). High plasma TNF- α levels were observed in children with acute malaria, and its release from mononuclear cells was associated with rupture of iRBC (Kwiatkowski, Cannon et al. 1989). In cases of severe malaria, pro-inflammatory cytokines, like TNF- α , IL-6 and IL-12(p70) were significantly elevated over uncomplicated or healthy controls (Shaffer, Grau et al. 1991, Lyke, Burges et al. 2004).

TNF- α has been associated with both positive and negative outcomes in malaria. In cerebral malaria, low doses of TNF- α were

observed to be protective in murine models, while high doses of TNF- α could induce overexpression of ICAM-1 on brain endothelial cells resulting in iRBC sequestration and vessel occlusion (Gimenez, Barraud de Lagerie et al. 2003). Anti-TNF- α therapy though inhibited fever in cases of cerebral malaria, had no effect on cerebral malaria mortality (Kwiatkowski, Molyneux et al. 1993), and might even increase possibility of residual neurological sequelae in survivors (van Hensbroek, Palmer et al. 1996). In severe malaria anemia, TNF- α directly suppressed erythropoiesis (Perkins, Were et al. 2011), and promoted dyserythropoiesis – the presence of abnormal erythroid precursors (Clark and Chaudhri 1988). Hence, unlike the importance of IFN- γ , TNF- α seemed to be more associated with malaria symptoms and contribute to disease pathology instead of protection.

1.3.2 Macrophage

In response to iRBC, macrophages had been shown to secrete soluble mediators like IL-1, TNF- α (Pichyangkul, Saengkrai et al. 1994), reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Prada, Malinowski et al. 1996). TNF- α and nitric oxide (NO) had been shown to be released by macrophages in response to the glycosylphosphatidylinositol (GPI) anchor of *P. falciparum* (*Pf*GPI) (Schofield and Hackett 1993, Tachado, Gerold et al. 1996). The corresponding receptor utilized by macrophage to interact with *Pf*GPI had been identified to be Toll-like receptor 2 (TLR2), and to a lesser

extent TLR 4 (Krishnegowda, Hajjar et al. 2005). Triggers for cytokine secretion include the phagocytosis of iRBC. Parasite microparticles were also potent activators of murine macrophage via MYD88 and TLR-4 dependent pathways (Couper, Barnes et al. 2010).

Phagocytosis is another key function of macrophages, and serves to directly reduce parasitemia. Via opsonic phagocytosis, macrophages had been observed to phagocytose iRBC (Lunel and Druilhe 1989) (Bouharoun-Tayoun, Attanath et al. 1990) and merozoites (Zhou, Ludlow et al. 2012). Opsonins involved included immune complexes (Mibei, Orago et al. 2005), IgG (Waitumbi, Opollo et al. 2000), and components of the complement pathway, such as C1q (Srichaikul, Puwasatien et al. 1975) and C3d (Facer, Bray et al. 1979). Indeed, in experimental human malaria infection, increased complement activation in both classical and alternative pathways was observed (Roestenberg, McCall et al. 2007). Defects in complement regulatory proteins, such as CRI and CD55, were also observed in children with severe malaria (Owuor, Odhiambo et al. 2008).

Non-opsonic phagocytosis of iRBC also occurred, and involved the scavenger receptor, CD36 (McGilvray, Serghides et al. 2000), which recognized the trypsin-sensitive parasite protein, *Pf*EMP1 (Patel, Serghides et al. 2004). However, the efficiency of non-opsonic phagocytosis was less than opsonic phagocytosis (McGilvray, Serghides et al. 2000). This was in part due to the variant of *Pf*EMP1 expressed by the

50

parasite. For example, parasites involved in placental-malaria, preferentially expressed PfEMP1 that binds chondroitin sulphate A (CSA) instead of CD36, thereby allowing them to escape early clearance by macrophages (Serghides, Patel et al. 2006). Interestingly, non-opsonic phagocytosis via CD36 did not induce TNF- α secretion by macrophages (McGilvray, Serghides et al. 2000, Erdman, Cosio et al. 2009).

Another much less characterized mechanism utilized by monocytes to control parasitemia is antibody-dependent cellular inhibition (ADCI). It was first described by Bouharoun-Tayoun *et al*, and involved the phenomenon where certain protective antibodies only exert their effect in the presence of monocytes. (Bouharoun-Tayoun, Attanath et al. 1990). Furthermore, this resulted in the secretion of soluble mediators by the monocytes which caused growth arrest of the parasite at the trophozoite stage (Bouharoun-Tayoun, Oeuvray et al. 1995). Antibodies against merozoite-surface protein 1 (MSP1), a protein on the surface of merozoite, had been shown to contribute to ADCI (Galamo, Jafarshad et al. 2009).

In malaria-infected patients, macrophages and monocytes are laden with hemozoin, and the proportion of hemozoin-laden macrophages appeared to correlate with disease severity (Metzger, Mordmuller et al. 1995, Awandare, Ouma et al. 2007). It was observed that hemozoin uptake by macrophage increased the transcription of cytokines, such as IL-1 β , TNF- α and IL-1RA, and chemokines, such as

51

MCP-1 and MIP-1 α (Giribaldi, Prato et al. 2010). Murine macrophages were also observed to produce NO in response to hemozoin in the presence of IFN- γ (Jaramillo, Gowda et al. 2003). However, hemozoin had also been shown to impair macrophage activation, function (Prada, Malinowski et al. 1996), and responsiveness to IFN- γ (Schwarzer, Alessio et al. 1998). This resulted in impairment of MHC upregulation, which affected antigen presentation and led to immunodepression (Schwarzer, Alessio et al. 1998). Increased frequencies of hemozoin-laden macrophage in malaria-infected children had been linked to decreased serum RANTES (Were, Davenport et al. 2009) and MIF levels (Awandare, Ouma et al. 2007). In contrast to murine macrophages, human macrophages did not express inducible nitric oxide synthase (iNOS) nor NO when ingesting hemozoin (Skorokhod, Schwarzer et al. 2007). It was also suggested that hemozoin itself had no immunomodulatory effect, instead it was contaminants, like fibrinogen, that served to activate macrophages/monocytes (Barrera, Skorokhod et al. 2011). In view of the varied experimental protocols utilized, such as type of hemozoin (naturally purified hemozoin vs synthetic hemozoin), presence of contaminants in hemozoin, concentration of hemozoin, incubation time, and culture conditions, there is no consensus on the 'true' biological effect of hemozoin, and whether it is immune activating or suppressing remains to be elucidated (Boura, Frita et al. 2013).

Macrophages have also been implicated in the pathogenesis of severe malaria. Regardless of the controversies surrounding the biological effect of hemozoin on macrophage, it is generally agreed that in the pathogenesis of severe malaria anemia (SMA), hemozoin-laden macrophage had an altered cytokine secretion profile, characterized by increased TNF- α , NO, IL-10 and IL6, which resulted in inhibition of erythropoiesis (Awandare, Kempaiah et al. 2011, Perkins, Were et al. 2011).

1.3.3 Dendritic cells

Like macrophages, dendritic cells (DCs) are derived from monocytes, and are key antigen presentation cells (APCs), serving a central role in bridging innate and adaptive responses. They can be further divided into conventional DCs (CD11c⁺BDCA1⁺ or CD11c⁺BDCA3⁺), which are highly efficient APCs; and plasmacytoid (CD11c⁺BDCA2⁺BDCA4⁺) DCs, which secrete type I interferon (Dzionek, Fuchs et al. 2000, Vu Manh, Bertho et al. 2015). In response to iRBC, DCs had been shown to directly phagocytose iRBC (Urban, Ferguson et al. 1999), resulting in secretion of IL-12 and IFN- γ (Ing, Segura et al. 2006), as well as TH1/TH2 polarization of CD4⁺ T cells, and priming of CD8⁺ T cells mentioned above.

Early observations on the effect of iRBC on DC showed that DC maturation was impaired when exposed to *P. falciparum* iRBC, and were less efficient in promoting T-cell proliferation (Urban, Ferguson et al. 1999). Elliott *et al* then demonstrated that such inhibition of DC

maturation was dose-dependent, and further determined that the inhibition was *Pf*EMP1-independent (Elliott, Spurck et al. 2007). *In vivo* mouse model using *P. yoelii*, showed suppression of CD8⁺ T cell activation by DCs isolated from *P. yoelii* infected spleen (Ocana-Morgner, Mota et al. 2003). Millington *et al* went on to show that hemozoin was the component of iRBC that inhibited DC function both *in vitro* and *in vivo* (Millington, Di Lorenzo et al. 2006). In adults with acute *P. falciparum* malaria, it was observed that IL-10 was elevated, leading to reduction in circulating DCs and accumulation of immature DC (Pinzon-Charry, Woodberry et al. 2013).

Interestingly, Perry *et al* reported contradictory findings. They showed that DCs from *P. yoelii* infected mice not only showed more upregulation of activation markers, but also stimulated naïve T cells to secrete IL-2 and IFN- γ (Perry, Rush et al. 2004). *P. chabaudi* infection in C57BL/6 mice also showed expansion of DCs leading to T cell activation (Guermonprez, Helft et al. 2013). Pouniotis *et al* pulsed murine DCs with either *P. yoelii* or *P. chabaudi* iRBC and demonstrated that although DC maturation was impaired, they were able to induce both T_H1 and T_H2 responses, and conferred long-term protection in naïve mice against the corresponding *Plasmodium* species *in vivo* (Pouniotis, Proudfoot et al. 2004). *In vitro* experiments demonstrated that human plasmacytoid DCs were activated by *P. falciparum* iRBC and secreted IFN- α in response. The authors further demonstrated that murine bone marrow DC responded to

P. falciparum schizont extracts via a TLR9- and MYD88-dependent pathway (Pichyangkul, Yongvanitchit et al. 2004). In children with acute malaria, total number of peripheral blood DCs was the same as controls, but there was notable increase in proportion of conventional DCs (Urban, Cordery et al. 2006). To solve the conundrum of whether DCs are activated or inhibited by malaria parasites, Wykes and Good suggested that DC functions were only inhibited by virulent or lethal parasites (Wykes and Good 2008).

Like macrophages, interaction of TLR2/4 on DCs and *Pf*GPI triggered release of inflammatory cytokines (Kumar, Gowda et al. 2012). Utilizing transgenic mice with various TLR knockouts, Coban *et al* demonstrated that hemozoin purified from *P. falciparum* activated murine DCs via a TLR9- and MYD88-dependent pathway, and proposed that hemozoin was a novel non-DNA ligand for TLR9 (Coban, Ishii et al. 2005). Franklin *et al* showed that MYD88^{-/-} murine DCs had impaired secretion of cytokines in response to *P. chabaudi* iRBC (Franklin, Rodrigues et al. 2007). However, it was later discovered that *P. falciparum* hemozoin was contaminated with malarial DNA, and that nuclease treatment of hemozoin abolished its TLR9 stimulatory effects (Parroche, Lauw et al. 2007). Indeed, by treating hemozoin with DNase and trypsin, Wu *et al* demonstrated that purified hemozoin was unable to activate DCs (Wu, Gowda et al. 2010). Instead, a protein-DNA complex was the

activating ligand for DCs in a TLR9-dependent pathway (Wu, Gowda et al. 2010).

1.3.4 Natural Killer Cells

In a *P. yoelii* 17X NL irradiated-sporozoite challenge BALB/c mouse model, it was shown that when NK cells were depleted by anti-asialo-GM1 (ASGM1) anti-serum, protective immunity was partially abrogated (Doolan and Hoffman 1999). Similar results were also obtained with *P. chabaudi* infection, where higher peak parasitemia was observed when NK cells were depleted in disease-resistant B6 mice (Fig. 1.3) (Mohan, Moulin et al. 1997). In another *P. chabaudi* model, using susceptible A/J mice, exogenous recombinant IL-12 administration led to amelioration of infection dependent on IFN- γ and TNF- α secretion, suggesting that a T_H1 response was critical for disease resistance (Stevenson, Tam et al. 1995). Interestingly, the depletion of NK cells in this model reversed the protection conferred by IL-12, suggesting that NK cell activity was central to immunity (Mohan, Moulin et al. 1997). However, care should be taken when interpreting these data, as NK1.1 and ASGM1 have been noted to also be expressed on some T cells, and consequently might be depleted as well (Slifka, Pagarigan et al. 2000). To circumvent this issue, a model utilizing SCID mice, demonstrated that NK cell-depletion led to faster disease progression with higher *P. yoelii* parasitemia, lower IFN- γ levels, and the mice succumbed to disease earlier than controls (Choudhury, Sheikh et al. 2000). Similarly, using transgenic IL-12p40 knockout mice,

56

Su *et al* showed that impairment of IFN- γ secretion by NK cells prolonged the course of *P. chabaudi* infection (Su and Stevenson 2002). These experiments thus provided evidence supporting the importance of NK cells in the control of acute disease, possibly through the secretion of IFN- γ .

In human, the ability to mount a robust IFN- γ response was also associated with lower reinfection rate in children that had a mild malaria episode (Luty, Lell et al. 1999). NK cells are major secretors of IFN- γ , and are the earliest source of IFN- γ , with secretions detected as early as 6 hrs when PBMC were co-cultured with iRBC (Artavanis-Tsakonas and Riley 2002). *In vitro* experiments showed that when PBMC were co-cultured with iRBC, NK cells formed conjugates with iRBC, which was necessary for IFN- γ secretion (Artavanis-Tsakonas, Eleme et al. 2003). Artavanis-Tsakonas *et al* also showed that neutralization of IL-12 reduced the percentage of IFN- γ^+ NK cells within PBMC, suggesting a requirement of IL-12 for NK cell response to iRBC (Artavanis-Tsakonas and Riley 2002). Secretion of IFN- γ by purified NK cells in response to iRBC was also noted to require the presence of monocytes as well as the MYD88 adaptor protein (Baratin, Roetynck et al. 2005). Hence, human NK cells seem to respond to iRBC through the secretion of IFN- γ .

Apart from NK cells' secretory role, it was observed that in *P. chabaudi* resistant mice, NK cell number and cytotoxicity activity greatly increased during infection, as compared to susceptible mice (Eugui and

57

Allison 1980). In human, it was observed that malaria-infected children had elevated NK cell counts and that their control of parasitemia was positively correlated with NK cell lytic activity (Ojo-Amaize, Salimonu et al. 1981). Orago *et al* then demonstrated NK cell lysis of *P. falciparum* schizont-staged iRBC via chromium-release assay (Orago and Facer 1991). Mavoungou *et al* subsequently showed that NK cell control of parasitemia required the presence of immune serum from malaria semi-immune patients (Mavoungou, Luty et al. 2003). NK cell cytolytic ability also seemed to have a protective effect against human malaria. In patients presenting with acute malaria, NK cell cytotoxicity was reduced, and cannot be boosted by IFN- γ stimulation (Ojo-Amaize, Salimonu et al. 1981, Orago and Facer 1991). This observation hinted at the possibility that either the NK cells had already been maximally stimulated in the patient, or due to genetic and/or other reasons the NK cells have lower protective potential. Similarly, in a study of Gabonese pregnant women, it was observed that women with NK cells that have reduced cytotoxicity against iRBC have higher serum cortisol levels and more likely to have positive blood smear for malaria parasites (Bouyou-Akotet, Issifou et al. 2004). It is thus apparent, that apart from NK cells' secretory role, NK cell cytotoxicity also played a role in iRBC response.

In vitro experiments showed heterogeneity in donor NK cells' ability to secrete IFN- γ in response to iRBC. The percentage of non- to low-responders ranged from 30-60% (Artavanis-Tsakonas and Riley

2002, Artavanis-Tsakonas, Eleme et al. 2003). In a study of 23 individuals, out of the 8 individuals with the inhibitory KIR3DL2*002 allele, it was noted that 5 of them (62.5%) belonged to the high IFN- γ responder group (Artavanis-Tsakonas, Eleme et al. 2003, Korbel, Newman et al. 2005). Whether such heterogeneity of NK cell response has any impact on malaria disease outcomes remain to be studied.

In the search for receptors that NK cells used to recognize iRBC, Mavoungou *et al* generated NKp30- and NKp46-Ig fusion peptides, and showed that these NCRs bound to DBLs of PfEMP1 (Mavoungou, Held et al. 2007). The authors further showed that these NKp30- and NKp46-Ig fusion peptides could reduce the lysis of iRBC by IL-2-activated human NK cells (Mavoungou, Held et al. 2007). However, the role of PfEMP1 in NK cell activation remained controversial, as DC-J parasites, which did not express PfEMP1, could still up-regulate CD69 on NK cell surface (Baratin, Roetyneck et al. 2007). Similarly, D'Ombra *et al* using another PfEMP1⁻ parasite line, showed that the absence of PfEMP1 had no effect on NK cell degranulation (D'Ombra, Voss et al. 2007). Apart from the NCRs, the role of TLR2 in NK cell recognition of iRBC was also investigated. Baratin *et al* co-cultured NK cells and iRBC in the presence of a TLR2 blocking antibody, and demonstrated no reduction in IFN- γ secretion, suggesting dispensability of TLR2 (Baratin, Roetyneck et al. 2005). However, it should be noted that in all these cases, PBMC was used for the iRBC-stimulation assay, and it is possible that NK cell was

being activated by other immune cell types. As such the role of NCRs and TLRs on NK cell activation remains to be elucidated.

In summary, NK cells play critical roles during the acute phase of malaria infection, with secretion of IFN- γ and increased cytotoxic capability being associated with better outcomes. However, as opposed to macrophages and dendritic cells, where the mechanisms of activation by iRBCs have been identified, how NK cells recognize and interact with iRBCs is less well characterized (Fig. 1.4).

Fig. 1.4 Recognition of iRBC by cells of the innate immune system. iRBC is recognized by macrophage and dendritic cells (DC) through the interaction between the scavenger receptor, CD36, and the parasite variant surface antigen, PfEMP1. Additionally, PfGPI is recognized by TLR2 and TLR4, triggering the release of inflammatory cytokines, like IL-12 and TNF- α , from macrophages and DCs. DCs are also activated by PfDNA through TLR9. Natural killer (NK) cells interact with iRBC resulting in IFN- γ production, which induces DC maturation, activates macrophages, and activates the adaptive immune response. Unlike macrophages and DCs, the mechanisms of NK cell-iRBC interaction are less defined. Though NKp30 has been suggested to bind PfEMP1, other studies have disputed its involvement.

1.4 ***Overview of Natural Killer Cells***

1.4.1 NK cells overview

Natural killer (NK) cells are part of the innate immune system, and are found in both lymphoid and nonlymphoid tissues. Morphologically, NK cells appear as large granular cells, with an abundance of secretory granules containing perforin and granzymes. In human, they constitute about 10-18% of peripheral blood mononuclear cells (PBMC). In other compartments, they constitute 20-30% of total lymphocytes in the liver, 10-20% in lungs, 4-10% in the spleen, 0.5-1% in bone marrow, ~0.2% in lymph nodes, and almost non-detectable in thymus (Westermann and Pabst 1992, Carrega and Ferlazzo 2012). Similar proportions are observed in mice (Gregoire, Chasson et al. 2007).

They were first named in 1975 from observations of their cytolytic activity against tumor cells without prior stimulation (Kiessling, Klein et al. 1975) and without MHC restriction (Lanier, Phillips et al. 1986). Phenotypically, mature human NK cells are defined by the presence of CD56 (Lanier, Testi et al. 1989) and absence of CD3 (Lanier, Phillips et al. 1986). More recently, under a suggested common innate lymphoid cell (ILC) nomenclature proposed by Spits *et al*, NK cells belonged to group 1 ILCs, characterized by their IFN- γ secretion (Spits, Artis et al. 2013). In mice, due to the absence of CD56, they are defined by either NK1.1 or DX5 (Walzer, Bléry et al. 2007). Because of these variations, it is suggested that NKp46, which is expressed by both human and murine NK cells, be

61

used as a unifying NK cell marker across species (Walzer, Bléry et al. 2007). However, whether it is a better NK cell marker is still open to debate, as NKp46 expression does not appear to be exclusively restricted to NK cells (Hudspeth, Silva-Santos et al. 2013).

Apart from their cytotoxic ability, they also play an important role in the activation of the adaptive immune response via the secretion of a myriad of cytokines including interferon (IFN)- γ , tumor necrosis factor (TNF)- α , interleukin (IL)-13, and the chemokines CCL3 (macrophage inflammatory protein 1 α), CCL4 (macrophage inflammatory protein 1 β), and CCL5 (RANTES). Another important function of NK cells are their roles in contact-dependent costimulation of T and B cells, serving as an important link between innate and adaptive immune system (Zimmer 2010).

1.4.2 NK cell activation

NK cell activation depends on the dynamic interplay of activating and inhibitory signals. The “missing-self” hypothesis proposes that MHC class I molecules present an inhibitory signal to NK cells preventing their activation. Upon down-regulation of MHC class I, NK cells become activated (Ljunggren and Karre 1990). The discovery of p58 molecules (Moretta, Vitale et al. 1993), which are later classified as killer immunoglobulin-like receptor (KIR) or CD158 (Wagtmann, Biassoni et al. 1995), substantiated the hypothesis. The C-type lectin-like CD94-NKG2 heterodimer is another group of NK receptors which recognize HLA-E,

which present peptides derived from other HLA molecules, thus allowing for the global monitoring of HLA expression (Lanier 1998, Ravetch and Lanier 2000, Lanier 2005).

Inhibitory signals can be overcome by activation of NK cell activating receptors (Lanier 2005). Cytotoxicity and cytokine secretion in NK cells is a tightly controlled and regulated function. It requires simultaneous engagement of sequential signals to coordinate the process of granule polarization and degranulation, each of which requires the engagement of different NK activating receptors with their corresponding ligands (Bryceson, March et al. 2005). Apart from the surveillance of 'self-molecules', it is emerging that NK cells also recognize 'non-self' pathogen-derived antigens (Colucci, Di Santo et al. 2002). The natural cytotoxicity receptor (NCR) NKp44 has been shown to be involved in dengue virus interaction (Hershkovitz, Rosental et al. 2009), as well as bind hemagglutinin of influenza virus (Arnon, Lev et al. 2001). The homodimer NKG2D receptor binds UL16-binding proteins (ULBP) of human cytomegalovirus (Cosman, Mullberg et al. 2001). NK cells also express toll-like receptors (TLRs), which are ubiquitous receptors for numerous pathogen-associated products, and their ligation leads to NK cell activation (Lauzon, Mian et al. 2007). When exposed to lipophosphoglycan (LPG) from the protozoan parasite *Leishmania major*, TLR2 on NK cells are up-regulated, accompanied with increase IFN- γ and TNF- α secretion (Becker, Salaiza et al. 2003).

1.4.3 Natural Cytotoxicity Receptor (NCR)

NCR belongs to the immunoglobulin superfamily (Ig-sf). However, apart from their observed function in NK cell activation and killing of tumor cells, they do not share much similarities in terms of sequence or structure. Nonetheless, they bear certain common features: 1) they are type I transmembrane proteins. 2) they contain a positively charged amino acid residue in their transmembrane domain allowing interaction with immunoreceptor tyrosin-based activation motif (ITAM)-containing adaptor molecules. NKp30 and NKp46 utilize the CD3 ζ and Fc ϵ R γ adaptors, while NKp44 utilizes DAP12 adaptors (Moretta, Biassoni et al. 2000, Kruse, Matta et al. 2014).

The first NCR identified is NCR1 or NKp46 (CD335). The cross-linking of NKp46 leads to Ca²⁺ mobilization, cytotoxicity and cytokine release (Sivori, Vitale et al. 1997). Sivori *et al* further showed via antibody blocking assays that NKp46 is involved in NK cell cytotoxicity towards allogenic and xenogenic HLA-class I-negative cells (Sivori, Pende et al. 1999). Antibody masking of NKp46 revealed three groups of target tumor cells. In the first group, where NKp46 blocking led to >70% inhibition of lysis by NK cells, were tumors of murine origin. In the second group, with 30-60% inhibition of lysis, were tumor cell-lines from human lung, liver, breast, melanomas and Epstein-Barr virus-transformed cells. In the third group, where anti-NKp46 had no effect on NK cell tumor lysis, were other human tumor cell lines, such as the ovarian tumor cell-line, IGROV

(Pessino, Sivori et al. 1998). Heparan sulfate proteoglycans (HSPG), which had been found to be upregulated on surface of certain tumor cells (Blackhall, Merry et al. 2001), were found to be binding targets of NKp46-immunoglobulin fusion protein (NKp46-Ig) (Bloushtain, Qimron et al. 2004), as well as NKp44-Ig and NKp30-Ig (Hecht, Rosental et al. 2009). Apart from tumor cells, it is also shown that NKp46-Ig binds to haemagglutinin (HA) of influenza virus (Mandelboim, Lieberman et al. 2001).

The second NCR identified is NKp44 (CD336). Unlike NKp46 which is constitutively expressed on NK cells, NKp44 appears to be expressed on NK cells in response to IL-2 stimulation (Vitale, Bottino et al. 1998). Unlike the other 2 NCRs, the intracellular domain of NKp44 contains an immunoreceptor tyrosine-based inhibitory motif (ITIM), although it was found to be non-functional (Campbell, Yusa et al. 2004). Using NKp44-Ig, it was demonstrated that one of its cellular ligands was proliferating cell nuclear antigen (PCNA) (Rosental, Brusilovsky et al. 2011). The next cellular ligand of NKp44 identified via a yeast two-hybrid screen was NKp44L, which appeared to be an isoform of the mixed-lineage leukemia-5 protein (Baychelier, Sennepin et al. 2013). Interestingly, PCNA expression is restricted to within the nucleus, while NKp44L is a cytosolic protein. As such, their contribution to actual NK cell activation is unknown (Kruse, Matta et al. 2014). As for pathogen recognition, NKp44-Ig was shown to bind West Nile Virus (WNV) and

65

Dengue Virus (DV) envelope protein (Hershkovitz, Rosental et al. 2009). NKp44-Ig also interacted with cell components of MTB and *Mycobacterium bovis* (BCG), although anti-NKp44 blocking of NK cells failed to abolish NK cell activation by the respective bacteria (Esin, Batoni et al. 2008, Esin, Counoupas et al. 2013).

The third NCR identified is NKp30 (CD337). Its expression profile on NK cells mirrors that of NKp46. Its crosslinking also led to NK cell cytotoxic activity. The authors further went on to show that NKp30 is responsible for NK cell activity against tumor cells that don't engage NKp46 or NKp44 (Pende, Parolini et al. 1999). As mentioned, NKp30-Ig had been shown to bind HSPG (Bloushtain, Qimron et al. 2004). However, such NKp30-HSPG interaction had no bearing on NK cell killing of tumor cells, thus questioning the validity of HSPG as ligands for activating NKp30 (Warren, Jones et al. 2005). B7-H6, which is a membrane protein, was found to be expressed in many tumor cell-lines. NKp30-Ig was found to bind B7-H6, and such interaction had been validated through mouse experiments, antibody structure, and crystal structure analysis to be important for NK cell mediated lysis (Brandt, Baratin et al. 2009, Li, Wang et al. 2011). Like the other NCRs, NKp30 is also involved in pathogen recognition. It was shown that a human cytomegalovirus (HCMV) protein, pp65, interacted with NKp30, preventing its interaction with its adaptor proteins, thus decreasing NK cell activation (Arnon, Achdout et al. 2005).

1.4.4 Killer immunoglobulin-like Receptor (KIR)

KIRs are only found in human. They are encoded by 15 polymorphic genes, and recognize human leukocyte antigen (HLA)-A/B/C. Within KIRs, they can be divided into activating and inhibiting. Such simultaneous expression of activating and inhibiting KIRs allow for the tight surveillance of HLA status. KIRs are highly polymorphic, exhibiting great haplotypic and allelic diversity. KIRs can be divided into two subfamilies based on the number of extracellular Ig-like domains, such that the KIR2D subfamily has two extracellular Ig-like domains, and the KIR3D has three. Furthermore, within these subfamilies, there is great diversity in the length of the intracellular cytoplasmic chain. Generally, KIRs with long cytoplasmic chain (L) contains ITIM, and are inhibitory in nature; while those with short cytoplasmic chain (S) are activating. Further diversity within the KIRs is generated by alternative mRNA splicing and allelic polymorphism (Lanier 1998). Based on gene content, KIR haplotypes can be divided into A and B. The B haplotype consists of one or more of KIR2DL5, KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS5 and KIR3DS1 (Robinson, Waller et al. 2005). Generally, the A haplotype has a lower expression of activating KIRs on NK cells surface. Such varied combinations of KIR genes thus result in haplotypes with differing biases between activating and inhibitory potential, which has a functional consequent in the individual's susceptibility to disease (Khakoo and Carrington 2006). For example, despite all KIR3DL1 recognizing the same

HLA-Bw4, different KIR3DL1 alleles exhibit different inhibitory responses to the same HLA-Bw4 allotype, as well as different recognition potential to different HLA-Bw4 allotypes (Carr, Pando et al. 2005). In another example of the variations in KIR responsiveness between individuals, it is observed that in unrelated donor hematopoietic cell transplantation for treatment of AML, donors with a type B haplotype improved recipient survival (Cooley, Trachtenberg et al. 2009).

1.4.5 NK cells differences between human and murine

There are innate differences between murine and human NK cells. As mentioned before, the biggest difference is the absence of NCAM (CD56) on murine NK cells. This is of considerable significance because based on CD56 expression, human NK cells can be further divided into CD56^{bright} and CD56^{dim}. CD56^{dim} population is the major NK cell population, and are thought to be more cytotoxic. CD56^{bright} NK cells are observed to be major secretors of cytokines (Cooper, Fehniger et al. 2001). In mouse models, it was suggested that murine NK cells could be divided into CD11b^{low}CD27^{high} and CD11b^{high}CD27^{low}, which correlated in functionality to human CD56^{bright} and CD56^{dim} respectively (Hayakawa and Smyth 2006). However, in human, majority of peripheral blood NK cells were CD27⁻. Instead, CD27 was highly expressed in decidua NK cells and cord blood NK cells, prompting the authors to suggest that CD27 in human served as a NK cell maturation marker (Fu, Wang et al. 2011). Additionally, murine NK cells lack KIRs, though their counterparts, the

Ly49 family of molecules, seem to play a similar role (Ravetch and Lanier 2000). Of the 3 identified NCRs, only NKp46 is expressed by murine NK cells. No NKp44 gene is found in the mouse genome (Colucci, Di Santo et al. 2002). The murine NKp30 contained 2 premature stop codon, and is thus a pseudogene, and no expressed sequence tags were detected (Hollyoake, Campbell et al. 2005).

In contrast to mouse NK cells, primary human NK cells can be cultured *in vitro* for months and exhibit effector functions without cytokines maintenance, while murine NK cells often die within days (Bryceson, March et al. 2006). Resting murine NK cells are also observed to be minimally cytotoxic, with non-detectable perforin and granzyme. In contrast, human NK cells constitutively express perforin and granzyme, allowing for rapid response. This was observed to be due to a translation block in murine NK cells, which required cytokines, like IL-2 and IL-15, to overcome (Fehniger, Cai et al. 2007).

1.4.6 NK cells and diseases

From their first discovery, it is evident that NK cells play a critical role against cancer. In fact a defining characteristic of NK cells is their ease of lysing K562 cells, a tumor line derived from chronic myelogenous leukemia (CML), which lacked MHC class I. Alloreactive NK cells generated from hematopoietic stem cells transplant (HST) that are HLA-haplotype and KIR-ligand mismatched in the graft-versus-host (GVH) direction for treatment of acute myeloid leukemia (AML), protected the

host from graft-versus-host-disease (GVHD), and efficiently eliminated tumor cells and prevented AML relapse, thus improving survival (Ruggeri, Capanni et al. 2002). NK cells have also been hypothesized to be involved in cancer immunosurveillance. Support comes from the discovery that the ligands for NKG2D, which are rarely found on healthy cells, are up-regulated in tumor cells, and the interaction of NKG2D with its ligand override the MHC class I-dependent inhibition, culminating in tumor cell lysis (Nausch and Cerwenka 2008).

Much work had been done on the association of KIR and diseases. HIV is one of the more well studied viral infections, where different KIRs had been shown to confer a protective effect. Homozygosity for HLA-Bw4, which is a ligand for KIR3DS1, was associated with reduced HIV viraemia and delayed disease progression (Flores-Villanueva, Yunis et al. 2001). It was then further shown that the association of KIR3DS1 and a subset of HLA-Bw4 was involved in delayed HIV progression, especially in delaying CD4⁺ T cell depletion (Martin, Gao et al. 2002). Unlike HIV, where KIRs were associated with rates of disease progression, HCV infections provided a model for disease resolution. It was observed that in patients homozygous for both KIR2DL3 and HLA-C1, they had higher self-resolution rate of acute disease. The hypothesis was that the weaker inhibitory receptor-ligand interaction between KIR2DL3-HLA-C resulted in a lower threshold required for overriding activation signals, as opposed to the stronger interaction between other KIR possessing two Ig domains

(KIR2D) and HLC-C allotypes (Winter, Gumperz et al. 1998, Khakoo, Thio et al. 2004)

Another important function of NK cells is their role in innate defense against infections, especially from viruses. The protective mechanisms have largely revolved around the role of NK cells in HLA surveillance, as viral infections are often associated with down-regulation of HLA class I in an attempt to evade CD8⁺ T cells (Jost and Altfeld 2013). In humans, the protective effects of NK cells on viral infection have been observed from DNA viruses, such as human cytomegalovirus (HCMV); to RNA viruses, such as influenza A virus, hepatitis C virus (HCV) and dengue virus; and to retroviruses, such as human immunodeficiency virus-1 (HIV-1) (Lodoen and Lanier 2006). Deficiencies in functional NK cells have been associated with repeated bouts of viral infections, as well as with human immunodeficiency syndromes (Jost and Altfeld 2013).

With regards to protozoan disease, apart from malaria, NK cells are known to contribute to early disease resistance in *Toxoplasma gondii*, leishmaniasis, and trypanosomiasis (Korbel, Finney et al. 2004). Like *Plasmodium*, *Toxoplasma* also belong to the apicomplexan phylum, characterized by the presence of an apicoplast. *T. gondii* infection in mice was observed to enhanced murine NK cell cytotoxicity (Hauser, Sharma et al. 1982). Similarly, *T. gondii* lysates were able to enhance human NK cell cytotoxicity (Sharma, Verhoef et al. 1984). Vaccination of β_2m -deficient mice, which do not express MHC-I or CD8⁺ T cells,

demonstrated the importance of murine NK cells in conferring resistance to subsequent lethal challenge (Denkers, Gazzinelli et al. 1993). Perforin knockout mice though able to survive acute *T. gondii* infection, were unable to survive chronic infection (Denkers, Yap et al. 1997). This suggested a role for perforin-cytolysis in long term survival during *T. gondii* infection. Regarding the recognition of *T. gondii*, it was noted that MYD88^{-/-} and IL-12p40^{-/-} mice were unable to survive acute *T. gondii* infection (Scanga, Aliberti et al. 2002). A profilin-like molecule, TgPRF, from *T. gondii* was subsequently found to interact with murine TLR11, becoming the first identified ligand for TLR11 (Yarovinsky, Zhang et al. 2005). TLR11 activation was required by murine NK cells to secrete IFN- γ in response to *T. gondii* (Yarovinsky 2014). Interestingly, though the TLR11 gene is present in the human genome, it contains stop codons within the open reading frame and is nonfunctional (Yarovinsky 2014). Apart from TLR11, NKp46 was also required by murine NK cells for IFN- γ secretion and subsequent protection against *T. gondii* infection (Klose, Flach et al. 2014).

NK cells also play significant roles in controlling *Trypanosoma cruzi* infection. It was first observed that NK cells in *T. cruzi* infected had increased cytotoxicity (Hatcher, Kuhn et al. 1981). Hatcher *et al* later showed that NK cells were able to kill *T. cruzi* epimastigotes and trypomastigotes (Hatcher and Kuhn 1982). Lieke *et al* further showed that such killing occurred in a contact-dependent manner involving cytotoxic

granules (Lieke, Graefe et al. 2004). Importance of NK cells in resistance to *T. cruzi* infection was shown when depletion of NK cells in C57BL/6 mice led to increased parasitemia and higher mortality (Rottenberg, Cardoni et al. 1988, Lieke, Graefe et al. 2004). IFN- γ secretion by NK cells were also shown to be important in conferring resistance to *T. cruzi* infection (Cardillo, Voltarelli et al. 1996). Like in *T. gondii*, MYD88^{-/-} mice were more susceptible to *T. cruzi* infection (Koga, Hamano et al. 2006). The glycosylphosphatidylinositol (GPI) anchors of *T. cruzi* (Campos, Almeida et al. 2001), as well as a protein, Tc52, were shown to be potent activators of TLR2 in macrophages (Ouaissi, Guilvard et al. 2002). Subsequently, TLR9 was shown to be required for *T. cruzi* recognition, and that TLR2^{-/-} and TLR9^{-/-} together accounted for all MYD88-dependent signaling in macrophage (Bafica, Santiago et al. 2006). Whether NK cells utilize similar receptors in *T. cruzi* recognition remains to be elucidated (Tarleton 2007).

In leishmaniasis, NK cells were activated by *L. major* infected cells, and secreted IFN- γ in response (Bogdan 2012). When NK cells were depleted in *L. major* resistant C57BL/6 mice, marked exacerbation of the infection was found observed (Laskay, Rollinghoff et al. 1993). PolyI:C activation of NK cells in *L. major* susceptible BALB/C mice, on the other hand, alleviated symptoms and reduced parasitemia (Laskay, Rollinghoff et al. 1993). Conventional DCs were observed to be essential for enhancing NK cell cytotoxicity and IFN- γ release *in vivo* in a TLR9-, and

IL-12-dependent fashion (Schleicher, Liese et al. 2007). However, NK cell cytotoxicity did not seem to be important in conferring resistance to *L. major* infection as *L. major* infected macrophages were noted to be resistant to NK cell killing. However, TNF- α and IFN- γ secreted by NK cells were observed to stimulate infected macrophages to kill the intracellular *L. major* parasite (Prajeeth, Haeberlein et al. 2011). All in all, it is apparent that NK cells are critical effector cells of the innate immune system, conferring resistance to cancer and a wide range of pathogens, ranging from virus to protozoans.

1.5 ***Humanized mice***

Due to the unique human-tropism of human malarial parasites, different murine models have been developed to study different aspect of malaria pathology. For example, the usage of lethal and non-lethal strains of *P. chabaudi*, is commonly used to dissect host immune response (Schofield and Grau 2005); *P. berghei* ANKA is known to cause experimental cerebral malaria (CM) in rodent models, and thus served as a surrogate for human CM (Craig, Grau et al. 2012); *P. yoelii* is used to study vaccine response (Schofield and Grau 2005). Despite the utility of these murine malaria models, innate differences between murine and human malarial-strains mean that no one murine model can accurately reproduce the full spectrum of *P. falciparum* pathology in human (Druilhe and Barnwell 2007, Craig, Grau et al. 2012). Also, not all aspects of *P. falciparum* malaria can be modelled in murine models. For example, PfEMP1 had been found to mediate a range of pathological processes, however, no homologs exist in murine malaria species. Also, the model of experimental cerebral malaria (ECM) using *P. berghei* ANKA infection in CB57BL/6 mice is controversial. White *et al* noted that instead of iRBC sequestration in the brain as observed in human cerebral malaria (HCM), the ECM model demonstrated marked platelets and leukocytes accumulation in the brain. The authors also noted that therapeutic interventions, such as dexamethasone and anti-TNF, though were able to ameliorate ECM in mice, had totally no effect on HCM (White, Turner et

al. 2010). Immunity against murine malaria is also observed to develop rapidly and often sterilizing, in stark contrast to immunity in human which is slow to develop and often incomplete (Craig, Grau et al. 2012).

Nonhuman primates (NHP), which are susceptible to infection by human malarial species, had previously served as an animal model for human malaria research. The first observation of drug-resistance loci was identified in chimpanzees via genetic crosses between different *P. falciparum* clones (Walliker, Quakyi et al. 1987). Hypnozoites of *P. vivax* was also first discovered in NHP (Krotoski, Collins et al. 1982). NHPs had also served as great model for vaccine candidate identification and testing (Herrera, Perlaza et al. 2002). Nonetheless, due to costs and ethical concerns, their usage had since been under intense scrutiny. In 2011, the US National Institutes of Health (NIH) restricted funding to research utilizing chimpanzees (Wadman 2011, Councils 2013). In 2015, NIH completely ceased all research utilizing chimpanzees (Reardon 2015). This change is slated to be adopted worldwide.

Numerous mouse models had been developed over the years to support human malaria species infection in mice. Early works by Tsuji *et al* using SCID mice infused with human RBC demonstrated low levels (<1%) of *P. falciparum* parasitemia for about 2 weeks (Tsuji, Ishihara et al. 1995). Using NOD^{scid/B2m^{-/-}} mice, Angulo-Barturen *et al* showed that daily intra-peritoneal (IP) injection of human RBC achieved RBC chimerism of 40-50%, and could support *P. falciparum* 3D7 infection, albeit at low

76

parasitemia of 2% (Angulo-Barturen, Jiménez-Díaz et al. 2008). The authors also showed that splenectomy had negligible effects in RBC chimerism or parasitemia (Angulo-Barturen, Jiménez-Díaz et al. 2008). This model was then repeated in NOD-*scid* *IL2Rγ*^{-/-} (NSG) mice and parasitemia as high as 17% could be achieved (Jimenez-Diaz, Mulet et al. 2009). In 2011, Hu *et al* demonstrated that administration of clodronate intraperitoneally, which depleted macrophages, could improve RBC reconstitution in NSG mice (Hu, Van Rooijen et al. 2011). By incorporating clodronate, Arnold *et al* showed that parasitemia in RBC-supplemented NSG mice could be increased to 30-35% (Arnold, Tyagi et al. 2011).

The above models though capable of sustaining *P. falciparum* infection in RBC-supplemented mice, lacked the presence of a human immune system for study of immune response to malaria. To establish a human immune system in NSG mice, Hiramatsu *et al* transplanted human cord blood CD34⁺ cells, thus generating humanized mice. They showed that there was efficient reconstitution of various human immune cell subsets, including functional B, T and NK cells (Hiramatsu, Nishikomori et al. 2003). However, it was noted that NK cells reconstitution was suboptimal, representing only 2.2% of total human lymphocytes in bone marrow, and 1.0% in spleen (Hiramatsu, Nishikomori et al. 2003). By hydrodynamic injection of plasmids encoding IL-15 and Flt3L, Chen *et al*, showed that human NK cells

reconstitution in peripheral blood could be improved to 5-20%, thus attaining levels equivalent to that in humans (Chen, Khoury et al. 2009).

1.6 ***Project Objectives***

I will elucidate the requirements for human NK cells response to iRBC, including the conditions and cellular receptors that NK cells use to interact and recognize iRBC.

Chapter 2. Materials and Methods

2.1 *Generation of RBC-supplemented Immune Cell optimized Humanized (RICH) mice*

RICH mice were generated by incorporating human RBC supplementation on top of the humanized mice model previously published by Chen *et al* (Chen, Khoury et al. 2009). NSG mice were obtained from Jackson Laboratories (Bar Harbor, Maine). New born pups were sub-lethally irradiated with 100 cGy within 48 hrs of birth. 2×10^5 positively selected cord blood CD34⁺ hematopoietic stem cells (HSC) (Stem Cell Technologies) were then intracardially injected into each mouse. Human leukocyte reconstitution was analysed at 10-12 weeks by FACS analysis of mouse CD45.1 (A20), and human CD45 (2D1), CD3 (17A2), CD56 (HCD56), CD14 (HCD14) and CD19 (HIB19) (BioLegend). Mice with more than 20% human leukocyte chimerism, were hydrodynamically injected with plasmids encoding human IL-15 (50 µg) and Flt3L (10 µg) (Chen, Khoury et al. 2009). Thereafter, daily intraperitoneal injection of 1ml human RBC (40% human RBC, 30% heat-inactivated human AB serum, 30% RPMI) was started. Human RBC chimerism was quantified by FACS analysis of human CD235ab (HIR2) (BioLegend). To deplete human NK cells, 50 µg/mouse anti-human CD56 (HCD56) was injected intravenously. Human NK cell reconstitution was then checked by FACS 24 hrs later using another anti-human CD56

antibody (MEM188), which recognize another epitope. Control mice were injected with equivalent volume of PBS intravenously. For *P. falciparum* infection, 5×10^6 ring stage *P. falciparum* 3D7 iRBC at 50% parasitemia was intravenously injected into each mice. Parasitemia was then monitored every 48 hrs by Giemsa staining. Studies involving mice were approved by the institutional animal care and use committee (IACUC) of the National University of Singapore (NUS) and Massachusetts Institute of Technology (MIT).

2.2 *Peripheral blood mononuclear cell (PBMC) purification.*

Whole blood was donated by healthy non-malarial immune adult volunteers at the National University Hospital of Singapore Blood Bank. Informed consents were 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 was collected in Citrate-Phosphate-Dextrose-Adenine-1 (CPDA-1, JMS). PBMC were isolated from whole blood, which was diluted 1:1 with 2 mM EDTA-PBS, over a Ficoll-Paque PLUS (GE Healthcare) density gradient. Pelleted RBC were washed twice in RPMI 1640 (Sigma-aldrich), diluted 1:1 with collected plasma, and stored at 4°C for malaria culture. Remaining RBCs within the PBMC fraction were lysed with ACK lysis buffer (Life Technologies). Purified

PBMC were washed twice with RPMI 1640 and either used fresh or resuspended at a concentration of 1×10^8 cells/ml in PBMC freezing medium (85% fetal bovine serum [FBS, gibco], 15% dimethyl sulfoxide [DMSO, sigma]) and stored in liquid nitrogen.

2.3 *P. falciparum* culture and infection.

P. falciparum strains 3D7 (MRA-102, contributed by Daniel J. Carucci), Dd2 (MRA-150, contributed by David Walliker), HB2 (MRA-767, contributed by ATCC), KAHRP KO (MRA-554, contributed by Alan F. Cowman) (Crabb, Cooke et al. 1997), T994 (MRA-153, contributed by David Walliker), W2mef (MRA-615, contributed by Alan F. Cowman), T996 (Rosario 1981) and A4 (Roberts, Craig et al. 1992) were obtained through BEI Resources Repository, NIAID, NIH. MAHRP KO parasites were gifts from Dr. Hans-Peter Beck (Spycher, Rug et al. 2008). AP2KO6 parasites were gifts from Dr. Artur Scherf (Martins, Macpherson et al. 2017). *P. knowlesi* AIH.1 parasites were gifts from Dr. Blackman (Moon, Hall et al. 2013). Parasites were cultured at 2.5% haematocrit using human RBC in malaria culture media (MCM) (10.43 g RPMI 1640 powder (Gibco), 25 ml 1 M HEPES (Gibco), 2 g NaHCO_3 (Sigma-aldrich), 5 g Albumax (Gibco), 0.05 g of hypoxanthine (Sigma-aldrich) in 1 mL of 1 M NaOH, and 25 mg gentamicin (Gibco) in 1 L milli-Q water) (Moll, Ljungström et al. 2009). Cultures were incubated in a Heracell 150 incubator (Thermo Scientific) at 37°C in an atmosphere of 5% CO_2 , 3% O_2

and 92% N₂. Ring-stage iRBC were synchronized by lysis of later stages using 2.5% D-sorbitol. Schizont-stage iRBC were harvested by centrifugation on a 60% Percoll gradient (Moll, Ljungström et al. 2009).

Surface expression of CD44, CD47, CD147 and ICAM-4 on iRBC was assessed by FACS using the following antibodies from BioLegend CD44 (IM7), CD47 (CC2C6) and CD147 (HIM6); and ICAM4 (ab112554) from Abcam. Surface expression of these markers were performed on late trophozoites-schizont staged-iRBC at a parasitemia of 10-15%. Human recombinant IFN- γ (570202) was obtained from BioLegend to determine effect of IFN- γ on iRBC parasitemia.

2.4 ***In vitro NK cell experiments***

NK cells (CD56⁺CD3⁻) were obtained by negative selection using EasySep™ Human NK Cell Enrichment Kit (Stemcell Technologies). NK cell purity was consistently above 95% (Fig 2.1). For *in vitro* culture, synchronized schizonts-stage iRBC at a parasitemia of 0.2% were incubated with NK cells at a ratio of 1:10 (5×10^4 : 5×10^5).

Fig. 2.1 Purity assessment of NK cells after negative selection. Singlets were first gated using FSC-H against FSC-A. NK cell population was then selected on SSC-A against FSC-A. Next, DAPI⁺ live cells were gated upon. NK cell purity was then assessed on a CD3 against CD56 plot. Shown were plots from 3 different donors. Number indicate percentage of the gated population.

For transwell experiments, Millicell® 0.4 μ m polyethylene terephthalate hanging cell culture inserts (Millipore), which allowed free passage of soluble factors, but prevented passage of iRBC or ruptured merozoites (1.0-2.0 μ m) (Sherman 1998), were used. NK cells (5×10^5) were seeded inside the transwell, while iRBC (5×10^4 , 0.5% parasitemia) were seeded outside of the transwell in a 24 well plate (Cellstar®, Greiner bio-one) containing the insert. In another experiment, both NK cells (5×10^5) and iRBC (5×10^4 , 0.5% parasitemia) were seeded together inside the transwell, while only iRBC (5×10^4 , 0.5% parasitemia) were seeded outside the transwell.

To assess the requirement of selected NK receptors in iRBC recognition, blocking antibodies against human NKp30 (P30-15), NKp44

(P44-8), NKp46 (9E2), NKG2D (1D11), CD2 (RPA-2.10), 2B4 (Cl.7), DNAM-1 (11A8), CD11a (H1111) and CD18 (TS1/18) were acquired from BioLegend and used at a concentration of 1 µg/ml. To block LFA-1, 1 µg/ml of αCD11a and 1 µg/ml of αCD18 was used. To determine the involvement of perforin, concanamycin A (CMA, Sigma-aldrich) was introduced into co-cultures at 1 nM (Kataoka, Shinohara et al. 1996). Blocking of TLR1 (mabg-htlr1), TLR2 (maba2-htlr2), TLR4 (mabg-htlr4), TLR5 (maba2-htlr5), and TLR6 (mabg-htlr6) were achieved using neutralizing antibodies acquired from Invivogen. Blocking of TLR7/8 (ODN2087, ODN2088, ODN20959) and TLR9 (ODN2006) were achieved using antagonistic oligonucleotide sequences synthesized by Integrated DNA Technologies with phosphorothioate bonds. MYD88 inhibition was achieved using a MYD88 homodimerization inhibitor peptide (NBP2-29328) obtained from Novus Biologicals at 100 µM. The following TLR agonists were obtained from Invivogen and used for NK cell stimulation: Pam3CSK4 (tlrl-pms), poly(I:C) (tlrl-pic), MPLA (tlrl-mpls), R848 (tlrl-r848-5) and ODN 2006 (tlrl-2006g5). Bx795 (SML0694, from Sigma-Aldrich) was used to inhibit TBK1/IKKε at a concentration of 10 nM.

FACS, staining was performed for 15 mins at 4°C in flow buffer (0.2% bovine serum albumin [BSA, sigma], 0.05% sodium azide [sigma] in PBS). This was followed by two subsequent washes in flow buffer before data acquisition. For CD107a and intracellular staining, brefeldin A

(final concentration 5.0 µg/ml) was added into the culture 6 hrs before FACS cell surface staining was performed. For intracellular staining, after cell surface staining, cells were fixed in 4% paraformaldehyde in PBS at 4°C for 30 min. Cells were then washed twice in flow buffer followed by permeabilization in perm buffer (0.1% saponin in PBS) at 4°C for 30 min. Thereafter, subsequent antibody staining was performed in perm buffer at 4°C for 30 min. Stained samples were then analyzed on a BD LSR II flow cytometer, and data analyzed in FACS Diva (BD Biosciences) or FlowJo (TreeStar). Isotype-matched control antibodies were used for all fluorochrome-isotype combinations. Statistical significance was quantified by paired T-test.

Quantification of cytokines was performed using LEGENDplex™ Human Inflammation Panel (13-plex) (BioLegend) at 48 hrs timepoint. This was because a change of media at 48 hrs was required to maintain optimal viability of NK cells and iRBC.

Parasitemia was quantified by flow cytometry and Giemsa-staining (Moll, Ljungström et al. 2009). Parasite DNA was stained using Hoechst 33342 (20 µg/ml) (Thermo Scientific).

2.5 *Imaging.*

To allow for the observation of high resolution live cell images, we created customized culture slides. Culture chambers were fabricated from Polydimethylsiloxane (PDMS) using Sylgard 184 Silicone elastomer kit

(Sigma-aldrich) and cured overnight in a 70°C oven. Culture chambers were then mounted onto Menzel-Gläser 22×60 mm #1 glass cover slips at 70°C after interacting surfaces had been plasma cleaned with a plasma cleaner (Harrick Plasma).

NK cells (1×10^5) were co-cultured with 1×10^4 3D7 schizont-stage iRBC at 10% parasitemia in MCM on a culture slide. Live-cell imaging was performed on an inverted Olympus IX71 fitted with an Olympus Planapo 60×/1.4 oil lens. Microscope stage was equipped with a Tokai Hit INU Live Cell Microscope Chamber, where temperature was maintained at 37 °C in an atmosphere of 5% CO₂, 3% O₂ and 92% N₂. Images were acquired using a Hamamatsu ORCA-ER (C4742-80-12AG) CCD camera, and analyzed using ImageJ. For quantification of NK cell and iRBC interaction time, 100 interactions throughout a 12 hrs period were assessed.

2.6 ***Microvesicle (MV) enrichment***

Microvesicles were concentrated using method described by Mantel *et al* (Mantel, Hoang et al. 2013). iRBC or RBC culture supernatant were first centrifuged at 10000 g for 15 mins. The resultant supernatant was then concentrated using a 100k MWCO concentrator (Vivacell 100, Sartorius). Microvesicles within the concentrated supernatant was next spun down at 100000 g for 1 hr using a Ti70 rotor on a Optima L-100K ultracentrifuge (Beckman Coulter). Resultant pellet was resuspended in 10 ml PBS, layered over 5 ml of 60% sucrose cushion and centrifuged at

10000 g for 16 hrs using a SW28Ti rotor. MVs were collected from the interphase. MV concentration was approximated by measuring absorbance at 280 nm using a nanodrop (Thermo Scientific). Yield of MV is about 120ng per million iRBC. Labelling of MV was achieved using either PKH26 or PKH67 (Sigma-Aldrich), as per manufacturer protocol with modification. Post-staining, excess dye was absorbed and washed with 1% BSA solution 3 times. MVs were then concentrated in a 10 kDa Amicon Ultra-0.5 mL (Merck Millipore) filter.

2.7 *mRNA transcriptional analysis*

10^6 NK cells were co-cultured with 10^5 iRBC as described above. After RBCs were lysed using ACK lysis buffer (Thermo Scientific), FACS was then used to sort out live activated NK cells using the following gating strategy – live NK cells were first gated as indicated by DAPI, followed by gating on CD56⁺CD69⁺ NK cells. RNA was then extracted from sorted NK cells using the TRIzol (ThermoFisher) method. DNA was extracted from the interphase using back extraction protocol (Thermo Fisher), and used for KIR genotyping. RNA quality was assessed by electrophoretic assay on the Agilent 2100 Bioanalyzer using a Nano chip (Agilent RNA 6000 Nano chip). RNA with a RIN value of >7.0 was sent to DUKE-NUS Genome Biology Facility for genome-wide transcriptional analysis by microarray using HumanHT-12 v4 BeadChip (Illumina). A total of 6 pairs (NK + iRBC and NK + RBC) of responder and 6 pairs of non-responder samples were analyzed.

Microarray data was analyzed using R (Team 2016). BeadChip data was converted to an expression set, variance stabilized and quantile-normalized using the lumi package (Du, Kibbe et al. 2008). Differentially expression genes (DEG) was identified via linear modelling using the limma package (Ritchie, Phipson et al. 2015), with a false discovery rate (FDR)(Bonferroni correction) of 0.05. Pathways were generated using ConsensusPathDB (<http://cpdb.molgen.mpg.de>), and visualized with PathVisio (van Iersel, Kelder et al. 2008) using WikiPathways (Kelder, van Iersel et al. 2012). Heatmaps were made with GENE-E (<https://software.broadinstitute.org/GENE-E/>).

DEGs involved in the interferon signaling pathway were submitted to www.interferome.org to identify number of genes regulated by each interferon type. Out of 19 genes submitted, 17 were mapped. From the interferome database, using the following search criterias: interferon type II, 24-48 hrs treatment time, *in vitro* data and homo sapiens; the median fold change of these 17 genes in interferon treated cells against controls were obtained for correlation plots.

2.8 *qPCR*

1 µg RNA from the microarray was reverse transcribed to cDNA using SuperScript™ III Reverse Transcriptase (ThermoFisher) as per manufacturer protocol. cDNA volume was then increased to 100 µl, and 1 µl of cDNA was used for each qPCR reaction. qPCR primers were

obtained from Primerbank (<https://pga.mgh.harvard.edu/primerbank/>) (Wang and Seed 2003). qPCR was performed using KAPA SYBR® FAST qPCR Kits (Kapa Biosystems) on a C1000™ Thermal Cycler (Bio-Rad) equipped with a CFX96™ optical reaction module (Bio-Rad). Cycling conditions were as follows: initial enzyme activation at 95°C for 3 mins; 40 cycles of denaturation at 95°C for 5 s and annealing/extension/acquisition at 60°C for 30 s. Melt curve analysis was performed from 55°C-95°C. For qPCR analysis, 3 reference genes – IPO8, B2M and GAPDH were used. qPCR results were analyzed using REST2009 (Qiagen).

Table 2.1 Primers used for qPCR

Gene	Forward	Sequence	Reverse	Sequence
IFIT2	IFIT2-f	AAGCACCTCAAAGGGGCAAAAC	IFIT2-r	TCGGCCCATGTGATAGTAGAC
IFIT1	IFIT1-f	TTGATGACGATGAAATGCCTGA	IFIT1-r	CAGGTCACCAGACTCCTCAC
IFIT3	IFIT3-f	TCAGAAAGTCTAGTCACTTGGGG	IFIT3-r	ACACCTTCGCCCTTTCATTTTC
OAS1	OAS1-f	TGTCCAAGGTGGTAAAGGGTG	OAS1-r	CCGGCGATTAACTGATCCTG
OAS3	OAS3-f	GAAGGAGTTCGTAGAGAAGGCG	OAS3-r	CCCTTGACAGTTTTACGACACC
MX1	MX1-f	GTTTCCGAAGTGGACATCGCA	MX1-r	CTGCACAGTTGTCTCAGC
TRIM25	TRIM25-f	AATCGGCTGCGGGAATTTTTC	TRIM25-r	TCTCACATCATCCAGTGCTCT
IRF7	IRF7-f	GCTGGACGTGACCATCATGTA	IRF7-r	GGGCCGTATAGGAACGTGC
DDX58	DDX58-f	CTGGACCCTACCTACATCCTG	DDX58-r	GGCATCCAAAAGCCACGG
DHX58	DHX58-f	GGGCCTCCAACTCGATGG	DHX58-r	TTCTGGGGTGACATGATGCAC
IFIH1	IFIH1-f	TCGAATGGGTATTCCACAGACG	IFIH1-r	GTGGCGACTGTCTCTGAA
TBK1	TBK1-f	TGGGTGGAATGAATCATCTACGA	TBK1-r	GCTGCACCAAAATCTGTGAGT
ISG15	ISG15-f	CGCAGATCACCCAGAAGATCG	ISG15-r	TTCGTCGCATTGTCCACCA
GAPDH	GAPDH-f	GGAGCGAGATCCCTCCAAAAT	GAPDH-r	GGCTGTTGTCATACTTCTCATGG
B2M	B2M-f	GAGGCTATCCAGCGTACTCCA	B2M-r	CGGCAGGCATACTCATCTTTT
IPO8	IPO8-f	TCCGAATATTATCGACAGGACC	IPO8-r	GTTCAAAGAGCCGAGCTACAA
MYD88	MYD88-f	GGCTGCTCTCAACATGCGA	MYD88-r	CTGTGTCCGCACGTTCAAGA

2.9 KIR genotyping

Genotyping of KIR was performed using polymerase chain reaction with sequence specific primers (PCR-SSP) as per Gomez-Lozano *et al* (Gomez-Lozano and Vilches 2002). Primers used mainly obtained from

Gomez-Lozano *et al* (Gomez-Lozano and Vilches 2002), except for primers for KIR2D3 and KIR3DL1, which were repeated with primers from Vilches *et al* (Vilches, Castano et al. 2007) (Table 2.2). Each 20 µl reaction contained 100 ng DNA, 2 µl 10X *Taq* Buffer with KCl, 3 µl MgCl₂ (25 nM), 1 µl dNTP (10 mM each) and 0.5 µl *Taq* DNA polymerase (5 U/µl) (Thermo Scientific) (Yang, Zhang et al. 2013). Touchdown PCR was performed using conditions optimized by Yang *et al* (Yang, Zhang et al. 2013), and were shown in Table 2.3. PCR products were then analyzed on 2% agarose gel.

Table 2.2 Primers used for KIR PCR-SSP

Gene	Forward Primer			Reverse Primer			length (bp)
	Name	Sequence (5'-3')	Conc (uM)	Name	Sequence (5'-3')	Conc (uM)	
KIR2DL1	Fcg622	ccatcagtcgcatgacg1	0.5	Ra957	ccactcgtatggagagtcac1	0.1	1903
				Ra872	aatgttccgttgaccttggt1	0.2	1818
KIR2DL2	Fa538	acttccttctgcacagagaa1	1	Rt854	gccctgcagagaacctaca	1	1868
KIR2DL3	Fg550	acagagaagggaagttaag1	1	Rcon854	ccctgcagagaacctacg1	1	1858
	Fg1158	ccttcacgctggtgctg	0.3	Rt1375	caggagacaacttggatca	0.3	812
KIR3DL1	Ft624	ccatygttcccatgatgct1	2.5	Rc723	agagagaaggtttctcatatg	2.5	1690
KIR3DL2	Ft627	cgggtcccttgatgcctgt	0.5	Rc962	gaccacacgcagggcag	0.1	1948
KIR2DS1	Fr621	tctccatcagtcgcatgar	2.5	Ra899	agggtccagaggaaagt	2.5	1839
KIR2DS2	Fa546	tgcacagagagggaagta	1	Rt767	cacgtctctcctgccaa	1	1775
KIR2DS3	Ft682	tactccccctatcagttt	2.5	Ra925	gcatctgtaggttcctcct	2.5	1800
KIR2DS4	Fa459	ctggccctcccaggtca	1	Rg876	ggaatgttccgttgatgc	1	1981
KIR2DS5	Fc551	agagaggggacgtttaacc	1	R939	ggaaagagccgaagcatc1	1	1952
KIR3DS1	Fg299	ggcagaatattccaggagg	1	Rt539	aggggtccttagagatcca	1	1765
KIR2DL4	Ftgc157	aggacaagccttctgc1	1	R939	ggaaagagccgaagcatc1	1	1395
KIR2DL5	Ft196	tgcctcaggaggagacat1	1	Ra739	ccggctgggctgagagt1	1	1151
KIR3DL3	F153	tggtcaggacaarccctt1	1	Rta669	ccgacaactcatagggtat1	1	1824
KIR3DP1	Fcon80	ccatgtcgtcatggtcg1	1	Rt369	tgaccaccagtgagga1	1	361/1834
	Fcon113	agggttcttctgtgc1	1				1047
KIR2DP1	FD674	tctgttactcactcccca1	1	R939	ggaaagagccgaagcatc1	1	1825
GH1	GH1F	gccttcccaaccattcccta	0.5	GH1R	tcacggatttctgtgtgttc	0.5	424
KIR2DS3-07	Ft803d	cttgcctcgcagctcct	1.25	Ra925	gcatctgtaggttcctcct	1.25	158
KIR3DL1-07	Ft624	ccatygttcccatgatgct	2.5	Rt697d	ccacgatgtccaggga	2.5	108
	Ftt624d	tccatcggtcccatgatgtt	2.5				109

Table 2.3 Touchdown PCR cycling condition.

Step	Temperature (°C)	Time	Cycles
Initial Denaturation	95	15min	1
Denaturation	95	30s	5
Annealing	65	1min	
Extension	72	10min	
Denaturation	95	30s	30
Annealing	69.5 (-0.5 each cycle)	1min	
Extension	72	1.5min	
Final Extension	72	10min	1

Chapter 3. Results

3.1 *RBC-supplemented Immune Cell optimized*

*Humanized (RICH) mice support *P. falciparum* infection*

Chen *et al* had previously shown that expression of human IL-15 and Flt3L in humanized mice by hydrodynamic injection can improve human immune cell reconstitution (Chen, Khoury et al. 2009). Chen *et al* also showed that by expression of IL-3 and EPO, endogenous generation of human RBC in humanized mice can reach ~3% (Chen, Khoury et al. 2009). This level, however, was unable to sustain productive *P. falciparum* infection *in vivo* (Amaladoss, Chen et al. 2015). To generate a humanized mouse model capable of supporting *P. falciparum* infection, in collaboration with Dr. Qingfeng Chen, we instituted daily intraperitoneal human RBC supplementation in addition to cytokine expression in humanized mice. We termed these RBC-supplemented humanized mice as RICH mice (Fig. 1A).

To generate humanized NSG mice (humice), we sub-lethally irradiated 48hrs old NSG pups, and injected each with 2×10^5 CD34⁺ HSC. Human leukocyte reconstitution was measured as percentage of human CD45⁺ cells in total PBMC at 12 weeks. We then selected mice with >20% human leukocyte reconstitution for hydrodynamic injection of IL-15 and Flt3L plasmids, as well as RBC supplementation (RICH mice). Prior to

treatment, human leukocyte reconstitution was $33.3 \pm 14.7\%$. Of the $CD45^+$ cells, $2.3 \pm 1.6\%$ were $CD14^+$ monocytes, $50.9 \pm 22.6\%$ were $CD19^+$ B cells, $1.4 \pm 0.5\%$ $CD56^+CD3^-$ NK cells, $42.0 \pm 22.2\%$ $CD56^-CD3^+$ T cells and $0.2 \pm 0.2\%$ $CD56^+CD3^+$ NKT cells ($n=26$) (Fig 3.1B and C). 1 week after hydrodynamic injection and RBC supplementation, no significant differences were seen between humice and RICH mice in reconstitution levels of CD45, CD14, CD19, CD3 and NKT cells ($30.0 \pm 9.5\%$, $2.7 \pm 1.8\%$, $23.2 \pm 15.7\%$, $42.0 \pm 22.2\%$ and $0.2 \pm 0.3\%$ respectively). On the other hand, $CD56^+CD3^-$ NK cells levels increased to $4.5 \pm 2.6\%$ ($p < 0.0001$, $n=26$) (Fig. 3.1B and C), as expected from earlier published results (Chen, Khoury et al. 2009). Human RBC reconstitution in RICH mice was $74.9 \pm 26.5\%$ ($n=26$) after 1 week of human RBC supplementation (Fig. 3.1B and C).

Having observed high human RBC chimerism in RICH mice, we next sought to determine if RICH mice can sustain *P. falciparum* infection. 1 week after human RBC supplementation, we intravenously infected RICH mice with 5×10^6 synchronized ring stage *P. falciparum* 3D7 iRBC. Parasitemia was quantified by Giemsa microscopy at 12 hours intervals. All stages (rings, trophozoites and schizonts) of the IDC can be observed in the peripheral blood of RICH mice (Fig. 3.2A-G). Parasitemia steadily increased from 0.01% (0.01 ± 0.001 , $n=10$) at 48 h to 0.13% (0.13 ± 0.02) at 240 h ($n=10$) (Fig. 3.2H). No other symptoms of malaria could be observed, and mice were not observed to be lethargic. Though hepatomegaly and splenomegaly could be observed, this was the

consequence of entrapping of human RBC from daily RBC supplementation. Nonetheless, these results indicated the suitability of RICH mice as an *in vivo* model for evaluating *P. falciparum* infection in the presence of human immune cells.

Fig. 3.1 RICH mice supported robust human leukocyte and RBC reconstitution.

(A) NSG mice were irradiated within 48 hrs of birth and transplanted with human CD34⁺ cells intra-cardially to generate Humice. To make RICH mice, humice with >20% human leukocyte chimerism were hydrodynamically injected with plasmid-encoding human IL-15 and Flt3L at 12 weeks. One day later, daily intraperitoneal injection of human RBC was started. At week 13, RICH mice were infected with 5x10⁶ ring stage Pf-

iRBC. (B) Reconstitutions of human RBCs and leukocytes were assessed at week 12 for humice and week 13 for RICH mice. Human leukocyte chimerism was determined by percentage of CD45⁺ cells in PBMC. Reconstitution of human monocytes (CD14⁺), B cells (CD19⁺), T cells (CD3⁺), NK cells (CD56⁺CD3⁻) and NKT (CD56⁺CD3⁺) cells were assessed via their respective markers, gated on total CD45⁺ cells. Human RBC chimerism was assessed by percentage of CD235ab⁺ cells in whole blood. Numbers represent percentage of the gated population. (C) Quantification of human leukocyte and human RBC chimerism (n=26). Each dot represents an individual mouse. Error bars represented mean $\pm$ SD. ****: $p < 0.0001$

Fig. 3.2 RICH mice supported robust *P. falciparum* infection. (A) Humice were infected with 5×10^6 synchronized ring-staged iRBC. A general microscope field at 100x magnification of humice peripheral blood smear fixed with methanol and stained with giemsa at 12 hrs post-infection (hpi). (B-C) Image of a ring-stage iRBC at 12 hpi. (D) Image of a trophozoite-stage iRBC at 24 hpi. (E) Image of a early schizont-stage iRBC at 36 hpi. (F) Image of a late schizont-stage iRBC at 48 hpi. (G) Image of an early ring-stage iRBC is also observable at 48 hpi. (H) Time course of *P. falciparum* 3D7 infection in RICH mice (n=10). Error bars represented mean $\pm$ SD.

3.2 ***Human NK cells control acute parasitemia in vivo.***

We next investigated whether human NK cells were involved in control of *P. falciparum* iRBC *in vivo*. RICH mice were generated as mentioned before. However, one day before iRBC infection of RICH mice, human NK cells were depleted using a depleting anti-CD56 antibody (50 µg/mouse) (Fig. 3.3A). RICH mice were first split into two groups with similar NK cell reconstitution profiles (Fig. 3.3B) – $5.0 \pm 1.6\%$ (n=6) NK cells in control group; $4.7 \pm 1.6\%$ (n=6) NK cells in treatment group prior to depletion. 24h after antibody depletion, NK cells population fell to $0.6 \pm 0.2\%$ (n=6) (Fig. 3.3C). This was assessed using anti-human CD56 (MEM188), which recognized another epitope. NK cell depletion lasted at least 4 days (Fig. 3.3D).

One day after NK cell depletion, RICH mice were infected with 5×10^6 synchronized 3D7 rings-stage iRBC, and parasitemia assessed every 48 hrs via microscopy. As shown in Fig. 3.3E, at 96 hrs post infection, parasitemia in the PBS-treated mice, was $0.02 \pm 0.02\%$ (n=6). In contrast, parasitemia increased about four-fold to $0.074 \pm 0.02\%$ ($p < 0.05$, n=6) in NK cell-depleted mice. NK cells are major secretors of IFN-γ in response to malaria infection. As such, we measured serum IFN-γ as well. In PBS-treated RICH mice, the level of IFN-γ was 189.1 ± 20.2 pg/ml (n=6), respectively (Fig. 3.3F). In NK cell-depleted RICH mice, the level of IFN-γ was reduced significantly to 42.4 ± 7.0 pg/ml (n=6) ($p < 0.05$). These results

suggested that human NK cells played a significant role in the immediate control of *P. falciparum* infection in RICH mice.

Fig. 3.3 NK cells play critical role in acute control of parasitemia in vivo. (A) RICH mice were generated as before. At week 13, 50 μ g/mouse of CD56 depleting antibody was intravenously injected to deplete human CD56 cells. Control mice were injected with an equivalent volume of PBS. One day later, RICH mice were infected with 5×10^6 ring stage Pf-iRBC, and parasitemia assessed every 48 hrs. (B) NK cell reconstitution before and after NK cell depletion. Shown are representative FACS plot of CD3 against CD56 gated on CD45⁺ cells. (C) NK cells reconstitution in control RICH mice, and in RICH mice before and after NK cell depletion. (D) NK cell depletion last at least 5 days after depleting antibody injection. (E) Comparison of parasitemia between control and NK cell depleted RICH mice at 96 hrs. (F) Comparison of serum IFN- γ between control and

NK cell depleted RICH mice at 96 hrs. Each dot represents an individual mouse. Error bars represented mean $\pm$ SD. *: $p < 0.05$; ****: $p < 0.0001$.

3.3 ***Human NK cells eliminate P.falciparum-iRBC in vitro.***

Having shown that human NK cells are important for *in vivo* control of parasitemia, we next sought to determine the mechanisms involved. To establish an *in vitro* co-culture, 5×10^5 NK cells were purified from human PBMC by magnetic selection and co-cultured with 5×10^4 synchronized 3D7 schizont-stage iRBC at 0.5% parasitemia. We also chose to use a culture media which replaced human serum with AlbuMAX (Gibco), which has $< 0.1\%$ bovine IgG. This removed confounding factors of human immunoglobulins and complement, which can affect iRBC growth.

After two rounds of invasions (96 hrs), parasitemia rose to $20.4 \pm 7.2\%$. However, in the presence of NK cells, parasitemia increase was restricted to $4.98 \pm 2.4\%$ ($p < 0.0001$, $n=12$) (Fig. 3A). We next look at activation status of NK cells in the presence of iRBC. Geometric mean fluorescence intensity for CD69, which is an activation marker for NK cells (Borrego, Pena et al. 1993), increased from 182 ± 53 to 889 ± 257 ($p=0.008$, $n=12$) in the presence of iRBC compared to RBC controls (Fig. 3B). CD25 surface expression increased from $7.52 \pm 4.9\%$ to $25.3 \pm 18\%$ ($p=0.0053$, $n=12$) (Fig. 3C). CD107a, which is a degranulation marker

99

(Alter, Malenfant et al. 2004), also increased when NK cells were co-cultured with iRBC ($18.5 \pm 9.4\%$) compared to RBC ($9.95 \pm 9.1\%$, $n=12$, $p=0.0002$) (Fig 3D). Having observed increased degranulation, we next looked at effector molecules via intracellular FACS staining. Both IFN- γ and granulysin expression were significantly increased from $1.37 \pm 0.6\%$ and $7.78 \pm 4.4\%$ to $4.47 \pm 2.4\%$ ($n=12$, $p=0.0004$) (Fig. 3E) and $17.2 \pm 10\%$ ($n=12$, $p=0.0018$) (Fig. 3F) respectively in the presence of iRBC. We also looked at other NK cell markers like the NCRs and NKG members, but there was no significant change in expression after exposure to iRBC (Table 3.1).

Next, we quantified secreted cytokines within the co-culture supernatant at 48 hrs. A total of 13 cytokines (IFN- α , IFN- γ , IL-1 β , IL-6, MCP-1, TNF- α , IL-10, IL-8, IL-12p70, IL-17A, IL-18, IL-23 and IL-33) were assayed by multiplex assay. IFN- γ , IL-1 β , IL-6, MCP-1 and IL-8 were consistently being detected at >100 pg/ml in more than 80% of the samples tested. Noticeably, TNF- α was only detected in 3 out of 10 sample pairs. As shown in Fig. 3.4G, only IFN- γ was significantly upregulated in the supernatant of NK cells co-cultured with iRBC (1283 ± 300 pg/ml) compared to those cultured with RBC (275 ± 84 pg/ml, $p=0.002$, $n=10$). Concentrations of IFN- α , TNF- α , IL-10, IL-12, IL-17A, IL-18, IL-23 and IL-33 were below the 2.5 pg/ml detection limit

As shown in Fig. 3.4E, there was an absence of IFN- α , IL-12 and IL-18 in our co-culture system, indicating that iRBC alone was sufficient to

100

activate NK cells. This contrasted with earlier reports where IL-12 and IL-18 were shown to be required for NK cell response to iRBC (Artavanis-Tsakonas and Riley 2002). A few differences were notable between our methodology and the earlier report. Firstly, Artavanis-Tsakonas *et al* terminated their experiment after 24 hrs, as opposed to ours, which lasted either 48 hrs or 96 hrs. Secondly, Artavanis-Tsakonas *et al* used 10^6 PBMCs in their experiments, while we used 10^6 purified NK cells. As such in terms of absolute numbers of NK cells and duration of co-culture, our methodology was both higher and longer, and this might explain why cytokine activation of NK cells was not required.

Fig. 3.4 iRBC activated NK cells, induced granulysin and increased IFN- γ secretion. (A) iRBC were either cultured alone or co-cultured with NK cells. Parasitemia was quantified at 96 hrs by FACS, gating only on total RBCs. Representative Hoechst vs CD56 FACS plot was shown. Number indicate parasitemia gated on total RBC. NK cell control of parasitemia was quantified by paired t-test plots. (B) Representative CD69 vs CD56 FACS plot gated on total CD45⁺ cells were shown. CD69 upregulation on NK cells was quantified by geometric mean fluorescence intensity on paired t-test plots. (C-F) Representative FACS plot for the indicated marker against CD56 gated on total CD45⁺ cells were shown, together with paired t-test plots of percentage of NK cells positive for the indicated marker. (E) Amount of the indicated cytokine present in either NK + RBC or NK + iRBC co-culture supernatant was quantified by multiplex immunoassay. Each dot represents an independent repeat. Error bars represented mean $\pm$ SD. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.005$; ****: $p < 0.001$.

Table 3.1 NK cells upregulated activation markers and effector molecules when exposed to iRBC.

	Fold Change ^a	P val ^b	Significant
CD69	5.12	0.0014	*
CD25	3.08	0.0053	*
CD107a	2.36	0.0097	*
Granulysin	2.20	0.0018	*
IFNγ	3.05	0.0004	*
Perforin	1.00	0.8170	
NKp30	1.09	0.8270	
NKp44	0.61	0.1130	
NKp46	1.03	0.2500	
NKG2A	1.17	0.2420	
NKG2C	1.08	0.5750	
NKG2D	0.99	0.8680	
CD94	1.18	0.1200	
2B4	1.00	0.8920	
CD36	1.52	0.3490	
CD18	0.92	0.4500	
CD11a	0.92	0.5050	
DNAM1	0.99	0.8980	
CD2	1.03	0.1080	
CD44	0.97	0.2100	
TLR2	1.27	0.8910	
TLR4	1.21	0.2290	
TLR6	1.36	0.1510	
TLR9	1.22	0.2980	

^a Mean fold change of either MFI or % positive cells of NK co-cultured with iRBC over RBC controls. ^b p. value of paired sample t-test, n=12. * = p<0.05.

3.4 ***Human NK cells specifically eliminate late stage *P.falciparum*-iRBC in vitro.***

Having observed that NK cells can control *P. falciparum*-iRBC parasitemia, we next sought to determine which stage of iRBC was responsible for NK cell activation and response. NK cells were co-cultured with ring-stage-iRBC at a starting parasitemia of 2% and sampled at various timepoints through the 48 hrs IDC. As the amount of DNA content in iRBC increased as it progressed through the IDC, Hoechst 33342 staining of DNA can crudely separate iRBC into early and late stages (Grimberg, Erickson et al. 2008). As shown in Fig 3.5A, at 12 hours post infection (hpi), about 2% of RBC are Hoechst^{low}, indicating that they were still in the ring-stage. There was no difference in parasitemia between iRBC cultured alone or in the presence of NK cells. At 24hpi (Fig 3.5A second column), a Hoechst^{high} population emerged, corresponding to the progression of ring-stage into late-stage iRBC (trophozoites and schizonts). In the absence of NK cells, $1.7 \pm 0.4\%$ of RBC were late-stages (Hoechst^{high}), and $0.8 \pm 0.4\%$ were ring-stage (Hoechst^{low}). However, in the presence of NK cells, late-stage iRBC (Hoechst^{high}) was significantly reduced to $0.3 \pm 0.1\%$ ($p=0.0001$, $n=5$) (Fig 3.5B). No significant difference was observed for the ring-stage (Hoechst^{low}) parasitemia. By 48 hpi, the IDC was completed, and newly invaded iRBC began as ring-stage (Hoechst^{low}) again. As late-stage iRBC were responsible for the production of merozoites and subsequent propagation of parasitemia, the

104

loss of late-stage iRBC when co-cultured with NK cells led to a significant reduction in ring-stage (Hoechst^{low}) RBC ($1.65 \pm 0.6\%$ vs $5.26 \pm 1.5\%$, $p=0.0001$, $n=5$) (Fig 3.5B).

We next investigated the activation status of NK cells in response to iRBC. NK cells were co-cultured with either RBC or ring-stage iRBC, and CD69 surface expression was determined at various timepoints through the iRBC IDC. As quantified by flow cytometry, no significant differences in the percentages of CD69 positive NK cells were observed within the first 12 hrs, which corresponded with ring-stage iRBC. However, at 24 hrs, where ring-stage iRBC transition to late-stage iRBC, CD69 expression on NK cells co-cultured with iRBC was significantly higher than RBC controls ($60.7 \pm 14\%$ vs $8.69 \pm 1.8\%$, $p=0.0001$, $n=5$) (Fig. 3.5C, D). CD69 expression on NK cells exposed to iRBC persisted through 48 hrs, even though majority of iRBC had become rings. All in all, these data showed that NK cells responded specifically to late stage iRBC resulting in NK cell activation and iRBC elimination.

Fig. 3.5 NK cells specifically eliminated late stage iRBC. (A) NK cells were co-cultured with synchronized ring-stage iRBC and sampled at the indicated time point. Representative FACS plots of Hoechst against FSC gated on total RBC at the indicated time point. Numbers indicate percentage of the indicated gate. (B) Stacked bar-chart showing the contribution of different stages of *P. falciparum* iRBC to total parasitemia, as well as the stage-specific reduction of trophozoites/schizonts when iRBC were co-cultured with NK cells (n=5). (C) Histogram of CD69 changes over the indicated time point. (D) Graph depicting changes in percentage of CD69+ NK cells over time. Error bars represented mean $\pm$ SD. *: $p < 0.05$; ***: $p < 0.005$.

3.5 ***Over-expression of surface variant-antigen protects iRBC from NK cells.***

To determine if specific parasite ligands were involved in NK cell recognition, various strains/isolates of parasites (Table 3.2) were used to determine their susceptibility to NK cell elimination.

Table 3.2 Characteristics of parasites used for NK cell co-culture studies.

Strain	Characteristics (parental strain/isolate location)	Species
3D7	cloned from NF54 (NF54)	<i>P. falciparum</i>
AP2KO6	Over-expression of surface variant antigens on iRBC (3D7) (Martins, Macpherson et al. 2017)	<i>P. falciparum</i>
A4	Doesn't express STEVOR on surface of iRBC (ITO4) (Roberts, Craig et al. 1992)	<i>P. falciparum</i>
KAHRP KO	Loss of knobs on surface of iRBC (3D7) (Crabb, Cooke et al. 1997)	<i>P. falciparum</i>
MAHRP KO	Doesn't express PfEMP1 on surface of iRBC (3D7) (Spycher, Rug et al. 2008)	<i>P. falciparum</i>
DD2	mefloquine, chloroquine resistant (W2mef)	<i>P. falciparum</i>
HB2	non-gametocyte producing (Honduras)	<i>P. falciparum</i>
T994	RH1 biased invasion (T9)	<i>P. falciparum</i>
T996	Non-RH biased (T9)	<i>P. falciparum</i>
W2Mef	Sialic acid dependent invasion (Indochina)	<i>P. falciparum</i>
A1H.1	Adapted to grow in human RBC	<i>P. knowlesi</i>

As shown in Fig. 3.6A and Table 3.3, compared to 3D7 parasites, AP2KO6 parasites were less susceptible to NK cell elimination. Over 96 hrs, NK cells reduced 3D7 parasitemia by $77.7 \pm 4.2\%$ (n=8) compared to AP2KO6 parasites which were only reduced by $42.8 \pm 22.7\%$ (n=8) (p=0.0008). Interestingly, no defect in iRBC parasitemia reduction was observed for MAHRP KO iRBC, which do not express PfEMP1 on iRBC surface. Similarly, no defect was observed for *P. knowlesi*-iRBC, where NK

cells reduced parasitemia by $83.4 \pm 2.9\%$ (n=3). No significant differences were observed with the other parasite strains/isolates tested.

In response to 3D7-iRBC, MFI of CD69 expression on NK cell was significantly increased from 192 ± 71 to 855 ± 359 (n=10, $p < 0.0001$). In contrast, CD69 expression on NK cells when exposed to AP2KO6-iRBC increased only to 475 ± 328 , which was significantly lower than when exposed to 3D7-iRBC ($p = 0.02$, n=10). Nonetheless, CD69 expression on NK cells exposed to AP2KO6-iRBC were still significantly increased compared to uninfected RBC controls ($p = 0.02$, n=10). CD69 expression on NK cells were not significantly different between 3D7-iRBC and A4-iRBC or MAHRP-KO-iRBC (Fig 3.6B and Table 3.3). No significant differences in secretion of IFN- γ by NK cells were observed across the different strains tested (Fig. 3.6C and Table 3.3). IFN- γ secretion by NK cells was significantly increased by 3D7-, A4-, AP2KO6- and MAHRP-KO-iRBC compared to uninfected-RBC controls ($p < 0.05$).

Together, these observations suggested that 1) PfEMP1 was not required for NK cell response to iRBC; 2) over expression of variant surface antigen impaired NK cell response to iRBC, but didn't seem to influence IFN- γ secretion; 3) human NK cells responded equally well to *P. knowlesi*-iRBC, thereby demonstrating species-transcending recognition and response.

Fig. 3.6 Over-expression of surface variant antigens confer protection to NK cell elimination. (A) NK cells were co-cultured with the indicated strain/species of parasite for 96 hrs, and parasitemia was assessed FACS. (B) MFI of CD69 expression on NK cells when co-cultured with the indicated parasite. (C) IFN- γ in supernatant of co-culture between NK cells and the indicated parasite. Each dot represents an independent repeat. Error bars represented mean $\pm$ SD. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$; ns: not significant.

Table 3.3 NK cell response to different parasite strains/lines

	Parasitemia Reduction (%)	CD69 (MFI)	IFNγ (pg/ml)
3D7	77.7 $\pm$ 4.2	854.7 $\pm$ 358.8	1986 $\pm$ 939.7
AP2KO6	42.8 $\pm$ 22.7	475.1 $\pm$ 327.7	2262 $\pm$ 577.5
A4	72.4 $\pm$ 16.6	713.8 $\pm$ 141.7	2051 $\pm$ 229.5
MAHRP KO	76.2 $\pm$ 8	595.1 $\pm$ 123.8	1962 $\pm$ 540.6
KAHRP KO	86.3 $\pm$ 1.9	n.d.	n.d.
DD2	71.6 $\pm$ 0.6	n.d.	n.d.
HB2	68.5 $\pm$ 10.1	n.d.	n.d.
T994	72.8 $\pm$ 15.6	n.d.	n.d.
T996	77.1 $\pm$ 9.6	n.d.	n.d.
W2mef	77.3 $\pm$ 5.4	n.d.	n.d.
Knowlesi	82.4 $\pm$ 2.9	n.d.	n.d.

n.d: Not done

3.6 ***Increased ICAM-4 expression on iRBC***

It had been reported that certain host proteins were upregulated in iRBC, such as hsp70 (Böttger, Multhoff et al. 2012). Hence, we looked at the expression levels of some markers (CD44, CD47, CD48, CD147) on iRBC. In a screen of targets required for iRBC invasion, CD44 was one of the highest hit (Egan, Jiang et al. 2015). CD147 (basigin), had been shown to be the binding partner of *Pf*RH5, and the interaction was necessary for merozoite invasion (Zenonos, Dummler et al. 2015). RBC lacked MHC class I molecules, and to evade destruction by NK cells, an interaction between SIRP α on NK cells, and CD47 on RBC had been shown to be critical (Oldenborg, Zheleznyak et al. 2000). Reduction in CD47 expression on RBC had been linked to RBC senescence, and served as a trigger for macrophage phagocytosis (Khandelwal, van Rooijen et al. 2007). We also chose to look at expression of ICAM-4 which is an erythrocyte ligand for LFA-1, which we subsequently showed to be involved in NK cell and iRBC interaction (Chapter 3.8).

To control for the effects of RBC senescence, the uninfected RBC within the same culture was used as control (Fig 3.7A). Of the markers examined, no significant difference between normal and infected RBC could be detected for CD47, CD44 and CD147. Instead, ICAM-4 was significantly different between iRBC and RBC as quantified by paired sample t-test ($p < 0.001$, $n = 7$). The MFI of ICAM-4 expression on iRBC was 1502 ± 432 ($n = 7$) compared to 685 ± 195 ($n = 7$) on RBC (Fig. 3.7B).

Representative FACS plot of Hoechst against ICAM-4 and histogram depicting ICAM-4 expression of RBC and iRBC population was shown in Fig. 3.7C.

Our observations thus suggested that there was an increase in ICAM-4 expression on iRBC. However, it was recently observed that *P. falciparum* merozoites bound to ICAM-4, and ICAM-4 was required for efficient erythrocyte invasion (Bhalla, Chugh et al. 2015). Given the inability of erythrocytes to synthesize proteins, it is possible that what we had observed was a preferential invasion of high ICAM-4 expressing RBC by merozoites.

Fig. 3.7 Increase ICAM-4 expression on iRBC. (A) Representative FACS plot of Hoechst-stained iRBC, demonstrating the gating strategy for iRBC and uninfected control RBC within the same culture to control for effect of RBC senescence during culture. (B) MFI of the indicated marker was quantified by FACS. (C) Representative FACS plot of Hoechst against ICAM-4, as well as histogram of ICAM-4 intensity. Each dot represents an independent repeat. Error bars represented mean $\pm$ SD. **:p<0.01.

3.7 ***NK cell activation and iRBC elimination required cell-to-cell contact.***

To determine the nature of NK cell and iRBC interaction, live-cell video microscopy was performed on NK-iRBC co-cultures. Video microscopy showed that despite the random nature of NK cells and iRBCs interactions, once NK cells contacted iRBCs, stable interactions were established. Quantification of 100 interactions between NK cells and iRBCs or RBCs over a period of 12 hrs, demonstrated that the average length of time of NK cell interaction with iRBC was about 10 times longer for iRBC vs RBC (4075 ± 617 s vs 359 ± 62 s, $n=100$, $P<0.0001$, Fig. 3.8A). During video microscopy (Fig. 3.8B), we observed that NK cells (arrow) form transient attachments with normal RBC (green block arrow). However, upon contact with iRBC (blue arrowhead), the attachment via pseudopod-like projections between NK cell and iRBC became stronger and long-lasting, thereby forming a NK-iRBC conjugate. After about 15 mins of constant contact between NK cell and iRBC, a slight increase in iRBC volume was observed. This was followed by appearance of numerous blebs on iRBC (black empty arrows). Subsequently, there was membrane rupture, loss of iRBC cell content coupled with rapid iRBC cell swelling and flattening, reminiscent of osmotic cell lysis. The process from migration, attachment, conjugation and target cell lysis took about 1 hr (Fig. 3.8B) (Sl.avi).

Given the observed cytolytic effect of NK cells on iRBC as well as the increase in granulysin expression when exposed to iRBC, we next tested the hypothesis that NK cells utilized lytic granules in controlling iRBC parasitemia. Concanamycin A (CMA), which is a vacuolar-type ATPase inhibitor, prevented the acidification of lysosomes, thereby accelerating the degradation of contents within lytic granules of NK cells, such as perforin, granzymes and granulysins (Kataoka, Shinohara et al. 1996). As shown in Fig 3.8C, starting with an initial parasitemia of 0.5%, parasitemia rose to $7.3 \pm 1.1\%$ after 96 hrs. In the presence of NK cells, parasitemia declined to $4.1 \pm 1.3\%$. However, when CMA, at a concentration of 1 nM, was added into the co-culture, parasitemia significantly increased to $7.2 \pm 0.2\%$ ($n=3$, $p<0.05$), suggesting that the control of parasitemia by NK cells was mediated through cytotoxic granules. At a concentration of 1 nM, CMA did not affect the growth of iRBC.

Having determined the requirement of cytotoxic granules and observed contact dependent lysis of iRBC, we further validated such contact-dependent killing by transwell experiments. NK cells were magnetically enriched and co-cultured with iRBC either in the same well, or separated by a 0.4 μm membrane transwell (Millipore). When cultured alone in the absence of NK cells, parasitemia consistently rose to $7.3 \pm 1.1\%$ ($n=5$). In the presence of NK cells, parasitemia declined to $2.4 \pm 1.3\%$ ($n=5$).

When NK cells were separated from iRBC by a transwell, parasitemia was not significantly affected ($7.5 \pm 0.8\%$, $n=5$, $p>0.05$, Fig. 3.9A).

Fig. 3.8 NK cells used lytic granules to kill iRBC. (A) Video microscopy was performed on either NK+iRBC or NK+RBC co-culture and the interaction time between 100 NK cells and iRBC or RBC was quantified. ****: $p<0.001$. (B) Still images captured at the indicated timepoint demonstrating the lysis of iRBC by NK cells. NK cell (red arrow) was first observed attaching to an uninfected RBC (green block arrow). Such attachment was transient, and the NK cell quickly detached. Upon contact with iRBC (blue arrowhead), it attached stably. Over time, blebs (black empty arrows) were observed on the iRBC. This was followed subsequently by rupture of iRBC, increased in iRBC cell volume and loss of cell content. (C) Representative FACS plot of Hoechst against CD56 of iRBC in the presence of InM concanamycin A (CMA), NK co-cultured with iRBC (NK+iRBC) and NK co-cultured with iRBC in the presence of CMA (NK+iRBC+CMA).

Number indicated percentage of events in gated region. Each dot represents an independent repeat *: $p < 0.05$

Looking at NK cell activation status, when NK cells were co-cultured in contact with iRBC, percentage of CD69 positive NK cells significantly increased to $52.2 \pm 6.6\%$ ($p < 0.001$, $n=5$) (Fig. 3.9C). In contrast, when NK cells were separated from iRBC by a transwell, preventing direct cell-to-cell contact, CD69 expression was significantly reduced ($5.1 \pm 0.6\%$, $p < 0.001$, $n=5$). This was similar to expression levels observed when NK cells were cultured with uninfected RBC (Fig. 3.9B). This further demonstrated that NK cell activation and control of parasitemia was contact-dependent.

In addition to cell cytotoxicity, NK cells are also important cytokine secretors. For example, in response to dengue-infected DCs, NK cells secrete copious amount of IFN- γ , which prevented viraemia increase (Costa, Ye et al. 2017). We had also shown that a plethora of cytokines were secreted by NK cells in response to iRBC (Fig. 3.4G). To determine the contribution of NK cell secreted soluble mediators in control of parasitemia, NK cells and iRBC were co-cultured inside the transwell, while another group of iRBC was cultured outside the transwell without NK cells (Fig. 3.9C). As expected, when in direct contact with NK cells, parasitemia inside the transwell was reduced to $1.5 \pm 0.4\%$ ($n=5$), as compared to iRBC only controls ($7.3 \pm 1.1\%$, $n=5$). Interestingly, parasitemia outside the transwell, was not significantly different from that of iRBC

controls ($7.2 \pm 0.2\%$, $n=5$, $p>0.05$). As one of the most abundant cytokine secreted by NK cells in response to iRBC was IFN- γ , we further validated that IFN- γ , up to $1.5 \mu\text{g/ml}$, which was up to 5000 times the amount secreted by NK cells into co-culture supernatant, have no direct effect on parasitemia (Fig. 3.9D). These results, thus suggest that NK cells control iRBC in a contact-dependent manner, and NK cell secreted soluble mediators have no direct effect on *P. falciparum* parasitemia, *in vitro*.

Fig. 3.9 NK cell activation and iRBC elimination required cell-to-cell contact. (A) iRBCs were cultured in the indicated conditions. Representative Hoechst vs CD56 FACS

plots of cultured cells were shown. Numbers indicate parasitemia as determined by percentage of Hoechst positive cells gated on total RBCs. **(B)** CD69 activation profile of NK cells in the indicated condition. NK + RBC: NK cells co-cultured with RBC; NK + iRBC: NK cells co-cultured with iRBC; NK inside: NK cells co-cultured with iRBC but separated by a transwell. **(C)** iRBCs cultured in the indicated conditions. Representative Hoechst vs CD56 FACS plots of cultured cells were shown. Numbers indicate parasitemia as determined by percentage of Hoechst positive cells gated on total RBCs. **(D)** iRBC were cultured with indicated concentration of IFN- γ for 96 hrs and parasitemia was quantified by FACS. Each dot represents an independent repeat. Error bars represented mean $\pm$ SD. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.005$; ****: $p < 0.001$; ns: not significant.

3.8 *LFA-1 is involved in NK cell interaction and elimination of iRBC.*

We had shown that NK cells get activated upon direct contact with iRBC and upregulate effector molecules such as granulysin and IFN- γ in response. Secretion of IFN- γ by NK cells is a highly complex and tightly regulated process involving the coordination of at least LFA-1, 2B4 and NKG2D (Fauriat, Long et al. 2010). The NCRs have also been implicated in the recognition of pathogenic molecules, and though NKp30- and NKp46-immunoglobulin fusion peptide had been shown to bind PfEMP1 (Mavoungou, Held et al. 2007), their exact involvement in human NK cell interaction with iRBC was not shown. As such, to elucidate receptors involved in NK cell interaction with iRBC, a panel of blocking antibodies against NK cell surface receptors were tested.

Parasitemia in the absence of NK cells was $7.3 \pm 1.1\%$ (n=5), while that in the presence of NK cells was $2.4 \pm 1.3\%$ (n=5). As shown in Fig. 3.10A and B, parasitemia remained around 2% when NK cells were co-cultured with iRBC in the presence of NKG2D, NKp30, NKp44, NKp46, 2B4 and CD2 blocking antibodies. In contrast, parasitemia significantly rose to $6.8 \pm 1.8\%$ (n=5, $p < 0.05$) and $4.5 \pm 1.0\%$ (n=5, $p < 0.05$) when antibodies against LFA-1 (CD11a and CD18) and DNAM-1 were used respectively. Given that LFA-1 is an adhesion molecule, video microscopy was performed to determine if anti-LFA-1 (α CD11a + α CD18) affected the interaction time between NK cells and iRBC. Indeed, as shown in Fig 119

3.10C, the average length of time that NK cells interacted with iRBC was reduced from 4236 ± 718 s to 428 ± 109 s ($n=100$) in the presence of α CD11a + α CD18. Hence, it was possible that LFA-1 was involved in the elimination of iRBC by NK cells by mediating the cell-to-cell interaction. In the presence of anti-LFA-1 (α CD11a + α CD18), the percentage of CD69⁺ NK cells declined significantly to $16 \pm 3.7\%$ from $66 \pm 16\%$ ($n=5$, $p<0.001$) as observed without the blocking antibody (Fig. 3.10D). All in all, we showed through the usage of neutralizing antibodies against NK cell surface receptors, that NCRs were dispensable in NK cells' response to iRBC. Instead, NK cell surface adhesion molecules, like LFA-1 and DNAM-1, played critical roles in mediating NK-iRBC interactions.

Fig. 3.10 Involvement of LFA-1 in NK cell interaction and elimination of iRBC. (A)

To determine the role of different activating NK cell surface receptor and adhesion molecules in the control of parasitemia observed, NK cells were co-culture with iRBC in the presence of the indicated blocking antibodies (1 μ g/ml). Parasitemia was quantified 96 hrs later. Representative Hoechst vs CD56 staining profiles of cells were shown. The numbers indicate the percentage of iRBC among total erythrocytes. **(B)** Quantification of the NK+iRBC co-culture with the indicated blocking antibody at 96 hrs. *: $p < 0.05$. **(C)** NK cells were co-cultured with iRBC in the presence or absence of α LFA-1 (α CD11a +

α CD18) blocking antibody, and video microscopy was performed. 100 interaction times between NK cells and iRBC with and without α LFA-1 (α CD11a + α CD18) blocking antibodies were quantified. Box plot indicates mean $\pm$ SD. **(D)** Activation of NK cells, as indicated by CD69 expression level, in the presence or absence of the α LFA-1 (α CD11a + α CD18) blocking antibody was performed. Representative Hoechst vs CD56 staining profiles of cells were shown. The numbers indicate percentages of CD69⁺ cells gated on CD56⁺ cells. Each dot represents an independent repeat. Error bars represented mean $\pm$ SD. *: p<0.05; **: p<0.01; ***: p<0.005; ****: p<0.001.

3.9 ***Heterogenous Donor NK cell response to iRBC.***

In vitro malarial studies had shown that NK cells from different donor differed in their ability to secrete IFN- γ in response to iRBC exposure, even in the presence of IL-12 and IL-18. The percentage of such non- to low-responders ranged from 30-60% (Artavanis-Tsakonas, Eleme et al. 2003, Korbel, Newman et al. 2005). Similarly, we also observed that NK cells in ~30% of our donors not only secreted lower levels of IFN- γ , but also failed to eliminate iRBC during co-culture. We henceforth term NK cells from responding donors as responder NK cells, and NK cells from non-responding donors as non-responder NK cells.

As shown in Fig 3.11A, in the absence of NK cells, parasitemia rose to 12% after 96 hrs. As expected from previous results, in the presence of responder NK cells, parasitemia was controlled at 2%. However, when non-responder NK cells were used, parasitemia rose to 11%. Due to variations in baseline parasitemia across experiments, percentage reduction in parasitemia ($\% \text{ parasitemia reduction} = \frac{\text{parasitemia}_{iRBC} - \text{parasitemia}_{NK}}{\text{parasitemia}_{iRBC}} \%$) was calculated to better capture the efficiency of NK cells in controlling parasitemia. To categorize NK cells as either responder or non-responder, a 50% parasitemia reduction was used as an arbitrary cutoff. Responder NK cells could reduce parasitemia by $65.2 \pm 3.8\%$, compared to non-responder NK cells ($22.0 \pm 14\%$), demonstrating a difference of $43.2 \pm 4.5\%$ ($p=0.0001$, $n=10$, Fig. 3.11A). This

demonstrated that in our sampled population of PBMC donors, there existed a group of donors whose NK cells were not able to control *P. falciparum* parasitemia. These results also showed that the observed reduction in parasitemia was indeed due to NK cells' activity, and not simply an effect of nutrient starvation by having more cells in the co-culture.

Next, we looked at levels of various surface and intracellular markers to determine phenotypic differences between responder and non-responder NK cells when co-cultured with iRBC. Due to variation in baseline between donors, fold change was calculated as a ratio of marker expression when NK was co-cultured with iRBC over uninfected control ($FC = \frac{NK_{iRBC}}{NK_{RBC}}$). Fold change was subsequently log-2 transformed to obtain a normal distribution for testing the hypothesis that responder NK cells were significantly different from non-responder NK cells using independent sample Student's t-test.

Non-responder NK cells were observed to be non-responsive to iRBC. When co-cultured with iRBC, CD69 expression on responder NK cells was significantly higher than non-responder NK cells. Responder NK cells increased CD69 expression by about 3-fold ($\log_2FC: 1.8 \pm 0.9$). In contrast, non-responder NK cells showed minimal fold change over RBC controls ($\log_2FC: 0.5 \pm 0.3$, $p=0.0007$, $n=10$, Fig. 3.11B and F). Similarly, CD25 expression between responder and non-responder NK cells was also

significantly different. Non-responder NK cells showed a log₂FC of 0.5±0.2 over RBC controls, compared to responder NK cells, which had almost 5-fold increase in expression (log₂FC: 2.2±1.1, p=0.02, n=10, Fig 3.11C and F).

Looking at the effector molecules, responder NK cells had significantly more granulysin and IFN-γ compared to non-responder NK cells. Compared to RBC controls, non-responder NK cells had less than 1-fold change in granulysin (log₂FC: 0.2±0.5) and IFN-γ (log₂FC: 0.5±1.0). In contrast, responder NK cells showed at least 2-fold increase in granulysin (log₂FC: 1.0±0.6, p=0.008, n=10, Fig 3.11D and F) and IFN-γ (log₂FC: 1.5±0.2, p=0.0006, n=10, Fig 3.11E and F). As such, CD107a expression was also significantly different between responder (log₂FC: 1.2±0.6) and non-responder NK cells (log₂FC: 0.3±0.4, p=0.003, n=10, Fig. 3.11F).

Interestingly, the inhibitory NKG2A receptor was significantly upregulated in non-responder NK cells (log₂FC: 1.0±0.7) compared to responder NK cells, where NKG2A was down-regulated (log₂FC: -0.3±0.6, p=0.0001, n=10, Fig. 3.11F). No significant differences between responder and non-responder NK cells were observed for adhesion molecules (CD2, DNAM-1, 2B4, CD18 and CD11a), NCRs (NKp30, NKp44 and NKp36), CD36, CD94, NKG2C, NKG2D, TLR2, TLR4 and TLR6 (Fig. 3.11F).

We then quantified levels of 13 cytokines (IFN- α , IFN- γ , IL-1 β , IL-6, MCP-1, TNF- α , IL-10, IL-8, IL-12p70, IL-17A, IL-18, IL-23 and IL-33) within the co-culture supernatant at 48h by multiplex immunoassay. IFN- γ , IL-1 β , IL-6, MCP-1 and IL-8 were consistently being detected at >100 pg/ml, while IFN- α , TNF- α , IL-10, IL-12, IL-17A, IL-18, IL-23 and IL-33 were below the 2.5 pg/ml detection limit. Cytokine profiles were then log₂ fold change transformed. As shown in Fig. 3.11G, only IFN- γ secretion profile between responder and non-responder NK cells were significantly different ($p < 0.0001$, $n = 10$). When responder NK cells were co-cultured with iRBC, there was a 5.6-fold (log₂FC: 2.5 ± 0.7) increase in IFN- γ secretion compared to RBC controls. In contrast, IFN- γ secretion in non-responder NK cells was not changed by exposure to iRBC (log₂FC: 0.1 ± 1.5).

In corroboration with others (Artavanis-Tsakonas, Eleme et al. 2003, Korbel, Newman et al. 2005), we too observed heterogeneity in IFN- γ secretion by NK cell towards iRBC amongst different donors. In addition, we demonstrated that non-responding NK cells failed to become activated by iRBC, and consequently were unable to control iRBC parasitemia.

Fig 3.II. Heterogenous response donor NK cell response to *P. falciparum* iRBC.

(A) Representative FACS plot of Hoechst against CD56 of iRBC cultured alone, iRBC co-cultured with responder NK cells or with non-responder NK cells. 50% reduction in parasitemia was used to separate responder from non-responder NK cells. Number represent parasitemia gated on total RBC. (B-E) Representative FACS plot of the indicated marker against CD56 gated on total CD45⁺ cells demonstrating difference in marker upregulation between responder and non-responder NK cells when co-cultured with iRBC or RBC. Numbers indicate the percentage of positive cells for the indicated marker. (F) Changes in the indicated marker as determined by FACS was converted to log₂FC over uninfected controls. (G) Cytokines secreted into co-culture supernatant was quantified by multiplex immunoassay and concentration was converted to log₂FC over uninfected controls. IFN- α , TNF- α , IL-10, IL-12, IL-17A, IL-18, IL-23 and IL-33 were below the 2.5 pg/ml. Each dot represents an independent repeat. Error bars represented mean $\pm$ SD. *: p<0.05; **: p<0.01; ***: p<0.005; ****: p<0.001.

3.10 *Involvement of pathogen recognition pathway in iRBC response.*

Having observed a heterogenous response in donor NK cells' response to iRBC, we next asked if transcriptionally comparing responder and non-responder NK cells could yield insights on how NK cells responded to iRBC. As such, we repeated the co-culture experiment as before, and FACS sorted DAPI⁻CD56⁺CD69⁺ NK cells from the co-culture for RNA extraction (Fig. 3.12A and B). We transcriptionally profiled 6 responders and 6 non-responders (Table 3.4) iRBC-RBC pairs of NK cells. After initial sample clustering by dendrogram (Fig. 3.13A) and visualization of sample intensity by histogram (Fig. 3.13B), 2 samples pairs were removed. After removal of these samples, no further outliers were detected by dendrogram sample clustering (Fig. 3.13C) and sample intensity histograms (Fig. 3.13D). This yielded 5 responders and 5 non-responders sample pairs.

Fig. 3.12 Gating strategy for FACS sorting of activated NK cells from iRBC co-culture. (A) Representative FACS plot of DAPI against CD45 after lysis of RBCs by ACK

lysis buffer. DAPI-CD45+ fraction was selected for subsequent gating. **(B)** Representative FACS plot of CD69 against CD56 gated on DAPI-CD45+ fraction. Gated region indicated final gate for FACS sort.

Table 3.4 Donor profile of samples submitted for mRNA microarray.

Donor	Status	Age	Gender
R2	Responder	27	Male
R3	Responder	23	Female
R7	Responder	35	Male
D2	Responder	35	Male
D6	Responder	27	Male
D8	Responder	27	Male
NR1	Non- Responder	26	Male
NR2	Non- Responder	35	Male
NR3	Non- Responder	21	Female
D1	Non- Responder	28	Male
D3	Non- Responder	29	Male
D5	Non- Responder	21	Male

Fig. 3.1B Microarray data quality check. (A) Initial dendrogram clustering of samples demonstrated 2 outlier samples – R3i and D5i. (B) Expression intensity histogram of all probes in each sample revealed R3i and D5i had lower than average intensity indicated issues with probe hybridization and were removed from the analysis. (C) Dendrogram clustering after removal of the 2 outlier sample pairs. (D) Expression intensity histogram after removal of 2 outlier sample pairs.

First, we asked if there was a correlation between protein expression of CD69, CD25, CD107a, granulysin and IFN- γ observed upon NK cell co-culture with iRBC (Fig. 3.11F) and their mRNA transcripts. As such, for each NK cell pair, we computed a $\log_2FC$ score as follows:

$$\log_2FC \text{ score} = \log_2Expression_{iRBC} - \log_2Expression_{RBC}. \text{ A heatmap of}$$

such a score was shown in Fig. 3.14A. Independent sample t-test was performed to determine if the log2FC score of responder was significantly different from non-responders. Indeed, apart from CD69, CD107A (LAMP1)($p < 0.02$), CD25 (IL2RA)($p < 0.01$), IFN- γ (IFNG)($p < 0.001$) and granulysin (GNLY)($p < 0.05$) were significantly different. For responder NK cell samples, the differential change in gene transcript levels of CD25, CD107a, IFNG and GNLY also correlated with the change observed in expression levels observed on FACS (Fig. 3.14 B-E), spearman correlation coefficient was given in Fig. 3.14F. CD69 was not significantly different, and this was mostly likely because our NK cell samples were all sorted on CD69⁺ cells (Fig. 3.12B).

Fig. 3.14 Transcriptomic changes correlated with protein changes observed on FACS. (A) To determine if changes in the markers observed by FACS could be replicated in the transcriptional analysis, gene expression was first converted to log₂FC over uninfected controls. Heatmap of markers observed to be significantly changed between responders and non-responders by FACS. (B-E) Correlation plots of log₂FC observed in transcriptional analysis against log₂FC observed by FACS for the indicated marker. (F) Spearman correlation coefficient for the indicated marker. Each dot represents a sample of the indicated group.

Next, to determine genes involved in NK cell response to iRBC, we contrasted responder NK cells co-cultured with iRBC to non-responder NK cells co-cultured with iRBC. Gene expression changes were computed by linear modelling to obtain sets of differentially expressed genes (DEG) using a FDR of 0.05. 412 DEGs were identified using these criteria. Expression levels of DEGs were visualized using a heatmap. As shown in Fig. 3.15A, hierarchical clustering revealed two dominant clusters of gene expression. Over-representation analysis revealed that genes upregulated in responder group were mainly involved in immune responses and pathogen recognition (Fig. 3.15B). The top pathway identified was interferon signaling ($p < 1.5 \times 10^{-10}$), followed by RIG-I/MDA5 signaling pathway ($p < 2.1 \times 10^{-7}$). Other notable pathways identified, include type II interferon signaling ($p < 4.6 \times 10^{-7}$), activation of interferon regulatory factor (IRF) by cytosolic pattern recognition receptors (PRR) ($p < 6.2 \times 10^{-4}$), and TLR signaling ($p < 0.02$). On the other hand, downregulated genes in responder group fell into pathways involved in metabolism, such as superpathway of inositol phosphate compounds ($p = 3.0 \times 10^{-4}$), folate metabolism ($p < 0.001$), and phospholipid metabolism ($p < 0.002$), as well as cell growth and differentiation, such as SMAD2/3 signaling ($p < 0.004$) and hematopoietic cell lineage differentiation ($p < 0.007$) (Fig. 3.15C).

Additionally, when responder NK cells co-cultured with uninfected RBC were contrasted to non-responder NK cells in similar conditions, no DEGs were identified. This suggested that these NK cells were not

transcriptionally different to begin with, and that the changes in DEGs observed were indeed due to a response to iRBC. All in all, these results showed that in contrast to non-responder NK cells, where genes involved in cell growth and metabolism were more highly expressed, responder NK cells responded to *P. falciparum* iRBC by upregulating pathogen recognition and response genes.

Fig. 3.15 Involvement of pathogen recognition receptors in response to iRBC. (A) Heatmap of expression levels of DEGs identified in Responder vs Non-responder group. Each column represents a donor of the indicated group. (B) Over-representation analysis of genes upregulated in responder group. (C) Over-representation analysis of genes downregulated in responder group.

3.10.1 RIG-I/MDA signaling pathway

One of the top pathogen recognition pathway enriched with 12 DEGs was the retinoic acid-inducible gene-I (RIG-I) receptor signaling pathway (Fig. 3.16A and C). In our analysis, we observed that gene expression of MDA5 (IFIH1) and LGP2 (DHX58) in the responder group increased 2.2-fold ($p < 0.008$) and 2.4-fold ($p < 8.3 \times 10^{-4}$) respectively over the non-responder group. RIG-I (DDX58), though significantly increased ($p < 0.04$), only increased by 1.3-fold. As such, RIG-I might have less biological significance in the response to iRBC than other members of the family. This result corroborated with the findings of Liehl *et al*, that RIG-I was dispensable in response to *Plasmodium* (Liehl, Zuzarte-Luis et al. 2014). Another critical member of the RIG-I pathway, is TRIM25, which had been shown to interact directly with RIG-I/MDA5 to potentiate downstream signal transduction. This was achieved by stabilizing the interaction of RIG-I/MDA5 and its adaptor molecule, MAVS, leading to downstream activation of NF κ B and IRF7 (Kawai, Takahashi et al. 2005, Gack, Shin et al. 2007). TRIM25 expression increased 1.8-fold ($p < 0.004$). In contrast, PCBP2, which is involved in degradation of MAVS (You, Sun et al. 2009), was repressed (0.7-fold, $p < 0.03$). IRF7 was upregulated by 2.6-fold ($p < 0.001$) in responder NK cells upon exposure to iRBC.

Microarray results were validated by qPCR with primers from PrimerBank (Wang and Seed 2003). qPCR of seven genes (DDX58, DHX58, IFIH7, IRF7, TRIM25, TBK1, ISG15) and three housekeeping

genes (B2M, IPO8, GAPDH) were performed, and results analyzed using REST 2009 (Qiagen) (Fig. 3.16B and Table 3.5). Of the seven genes tested, apart from TBK1, all the remaining six genes were significantly up-regulated in responder NK cells compared to non-responder NK cells.

Fig 3.16 RIG-I Like Receptor Signaling Pathway. (A) Heatmap of DEGs involved in RIG-I-like receptor signaling. (B) qPCR validation of selected DEGs. 3 housekeeping genes (B2M, IPO8, and GAPDH) were used, and qPCR results were analyzed using REST 2009. Expression ratios of responder NK cells to non-responder NK cells for the indicated gene were shown. *: p<0.05. (C) Genes were represented as HGNC symbol and color coded according to the Log₂FC of responder/non-responder. *: indicate significant DEGs.

All in all, our analysis showed that there was an over-representation of transcripts involved in RIG-I receptor signaling pathway, and the most of the transcripts were upregulated, suggesting an activation of this pathway.

Table 3.5 qPCR results of selected genes involved in RIG-I like signalling pathway

Gene	Type ^a	Reaction		Std. Error	95% C.I.	P.val	Result
		Efficiency	Expression				
B2M	REF	1	1.02				
GAPDH	REF	1	1.213				
IPO8	REF	1	0.808				
DDX58	TRG	1	2.353	1.481 - 4.419	1.170 - 6.154	0.006	UP
DHX58	TRG	1	3.577	2.240 - 5.795	1.591 - 9.937	0.008	UP
IFIH1	TRG	1	3.81	1.425 - 14.221	0.471 - 33.268	0.045	UP
IRF7	TRG	1	3.471	2.043 - 5.815	1.246 - 11.002	0.006	UP
TRIM25	TRG	1	4.217	2.213 - 7.814	1.198 - 13.057	0.003	UP
TBK1	TRG	1	1.198	0.600 - 2.379	0.255 - 3.283	0.621	
ISG15	TRG	1	8.252	4.307 - 13.439	3.385 - 23.563	0.006	UP

a: REF – reference gene; TRG – target gene.

Recently, Wu *et al*, showed that MDA5 and MAVS were required for peak parasitemia control of *P. yoelii* infection in mice (Wu, Tian et al. 2014). Similarly, Liehl *et al*, showed that murine hepatocytes and DCs responded to plasmodial RNA in a MDA5-dependent manner (Liehl, Zuzarte-Luis et al. 2014). These reports, though performed using murine cells with rodent malarial species, indeed supported our observation. To further investigate the role of RIG-I receptor signaling pathway in NK cell response to iRBC, we sought out to block the Rig-I like receptors (RLR). However, given the lack of inhibitors for RLR, we decided to use a TBK1/IKK ϵ inhibitor (Bx795) (Clark, Plater et al. 2009), which had been shown to inhibit RLR-mediated response in human NK cells (Sutlu, Nyström et al. 2012). As shown in the cartoon in Fig. 3.17, TBK1/IKK ϵ

served as a convergence point to the STING, RLR and TLR3/TRIF pathway. Interestingly, these three pathways are involved in foreign nucleic acid sensing.

Fig. 3.17 Involvement of TBK1/IKKε in sensing of foreign nucleic acids. Cytosolic foreign dsDNA and dsRNA are recognized by cGAS and RLR respectively. Endosomal dsRNA is recognized by TLR3. cGAS, RLR and TLR3, then phosphorylate their respective adaptor molecule – STING, MAVS and TRIF. These pathways then converge on the activation of TBK1/IKKε (Liu, Cai et al. 2015). TBK1/IKKε can then activate the IRF3 and NFκB pathway. Bx795 prevents TBK1/IKKε activation, hence blocking signal transduction induced by foreign dsDNA/dsRNA.

At 10 nM, Bx795 effectively prevented NK cell control of iRBC parasitemia. As shown in Fig. 3.18A, in the presence of NK cells, parasitemia of iRBC was $2.5 \pm 1.0\%$ ($n=8$). However, upon addition of Bx798 at 10 nM, parasitemia significantly increased to $6.0 \pm 2.2\%$ ($n=8$, $p<0.001$). Bx795 also prevented NK cell activation by iRBC. CD69 expression on NK cells when co-cultured with iRBC was significantly reduced from a MFI of 850 ± 491 ($n=6$) to 285 ± 176 ($n=6$, $p=0.02$) in the presence of Bx795 (Fig. 3.18B). Similarly, percentage of CD25 positive NK cells was also significantly reduced from $38.2 \pm 19.6\%$ to $17.3 \pm 11.3\%$ ($n=8$,

$p < 0.001$) in the presence of Bx795 (Fig. 3.18C). Bx795 also abolished IFN- γ secretion by NK cells in response to iRBC. Amount of IFN- γ in the culture supernatant was significantly reduced from 1764 ± 535 pg/ml to 670 ± 815 pg/ml ($n=4$, $p=0.01$) in the presence of Bx795 (Fig. 3.18D).

This suggested that TBK1/IKK ϵ activation was required for NK cell response to iRBC, and that one of the important trigger for NK cell response was malarial DNA/RNA. Indeed, Sharma *et al* identified a malarial AT-rich motif (ATTTTAC) that induced cytokine release from macrophages and DCs (Sharma, DeOliveira et al. 2011). The authors further showed that the recognition of these motifs involved STING and TBK1 (Sharma, DeOliveira et al. 2011).

One question then arises, which is how do parasitic DNA/RNA enter NK cells? One possible way in which parasitic DNA/RNA could be introduced into NK cells, is via the fusion of iRBC microvesicles (MV) with NK cell membranes. It had been shown that iRBC actively released MVs, especially late during schizont-stages (Mantel, Hoang et al. 2013). MVs were noted to carry not only parasite-derived antigens (Mantel, Hoang et al. 2013), but also served as vehicles to transfer DNA and mRNA. Regev-Rudzki *et al* demonstrated that MVs from iRBC were able to transfer drug resistance from resistant iRBC to susceptible iRBC (Regev-Rudzki, Wilson et al.). These MVs were also able to directly activate macrophage inducing release of TNF- α (Mantel, Hoang et al. 2013).

Fig. 3.18 Bx795 prevented NK cell control of parasitemia. (A) iRBC were cultured alone, together with NK cells, and with NK cells in the presence of Bx795 (10 nM). Parasitemia was quantified at 96 hrs by FACS, gating only on total RBCs. Representative Hoechst vs CD56 FACS plot was shown. Numbers indicate parasitemia gated on total RBC. Paired sample t-test (n=8) was performed to determine effect of Bx795 on NK cell control of parasitemia. (B) Histogram of CD69 expression changes in the indicated conditions. Paired sample t-test was performed on geometric mean fluorescence intensity (MFI) of CD69 (n=6). (C) Percentage of CD25 expressing NK cells in the indicated condition was quantified by FACS (n=8). (D) IFN-γ in the supernatant of the indicated condition was quantified by immunoplex (n=4). Each dot represents an independent repeat. *: p<0.05; ***: p<0.005.

As such we purified MVs from iRBC culture as described by Mantel *et al* (Mantel, Hoang et al. 2013). To visualize MVs, we labelled MVs with a lipid dye, PKH26. NK cells were cultured with labelled MVs (1 $\mu\text{g}/\text{ml}$) for 24 hrs, and MVs uptake by NK cells were quantified by FACS after 3 thorough washes. After 24 hrs, $18.0 \pm 2.3\%$ of NK cells stain positive for PKH26. MFI of PKH26 in NK cells also increased significantly to 598 ± 55 over isotype controls ($p < 0.0001$, $n=4$) (Fig. 3.19A). Next, using confocal microscopy to generate z-stack images, we showed that PKH26-dyed MVs were indeed incorporated in NK cells (Fig. 3.19B).

Next, to further obtain evidence of MV uptake/fusion with NK cells, we stained NK cell membranes with the red lipid dye, PKH26, and MVs with the green lipid dye, PKH67, and performed time-lapse microscopy. As early as 2 hrs into the culture of NK cells with MVs, we could see a MV (block arrow) attaching to NK cell surface. The MV was then slowly fused with the NK cell as we observed the green-labelled MV gradually becoming yellow as it fused with the red-labelled NK cell membrane (Fig. 3.19C) (S2.avi).

As MVs had been shown to activate macrophages, we sought to determine if MVs could activate NK cells as well. As shown in Fig. 3.19D, MV at a concentration of 1 $\mu\text{g}/\text{ml}$ were unable to significantly increase CD69 expression on NK cells (CD69 MFI: 295 ± 205 , $n=8$) over RBC controls (CD69 MFI: 310 ± 184 , $n=8$). However, at a MV concentration of 10 $\mu\text{g}/\text{ml}$, MFI of CD69 was significantly increased to 651 ± 383 ($n=8$, 142

$p < 0.05$) over RBC controls. Such expression of CD69 was comparable to when NK cells were co-cultured with iRBC (CD69 MFI: 902 ± 487 , $n=8$). Interestingly, MVs at a concentration of $10 \mu\text{g/ml}$, were unable to induce IFN- γ secretion in NK cells (Fig. 3.19E). Consequently, supernatant IFN- γ levels when NK cells were stimulated with MVs (527 ± 301 , $n=4$) were not significantly different from RBC controls (310 ± 120 , $n=4$). To put the concentration of MVs into perspective, $600 \mu\text{g}$ of MVs could be enriched from 1 L of iRBC culture maintained at 2-5% parasitemia, giving a culture concentration of $0.6 \mu\text{g/ml}$. As such our data showed that at normal culture concentration of MVs, MVs alone were insufficient to activate NK cells. However, at higher concentrations, MVs were able to activate NK cells, though not induce IFN- γ secretion.

Through transcriptional analysis, we showed that DEGs identified by contrasting responder NK cells to non-responder NK cells mapped to the RIG-I receptor pathway, and majority of which were upregulated, suggesting involvement of the pathway. Due to a lack of specific inhibitor for RLRs, we chose to use the TBK1/IKK ϵ inhibitor, Bx795, to investigate the involvement of nucleic acid sensors in NK cells' response to iRBC. Bx795 not only inhibited NK cell activation and IFN- γ response to iRBC, but also prevented NK cell from controlling iRBC parasitemia, suggesting a requirement of TBK1/IKK ϵ in NK cell response to iRBC. We further demonstrated that MV could potentially serve to deliver malarial nucleic acids into NK cells to activate the cytosolic nucleic acid PRRs like the

RIG-I family. At high concentration MV could activate NK cells, but not induce IFN- γ secretion.

Fig. 3.19. Uptake of MVs by NK cells through membrane fusion. (A) MVs (1 μ g/ml) were labelled with a lipid dye (PKH26) and added into NK cell culture for 24 hrs. Uptake of MVs were then quantified by FACS. Shown was an FACS plot of PKH26 against CD56. Number indicate the % of positive cells. Plots of % PKH26 positive NK cells and MFI plot of PKH26 intensity gated on NK cells were shown to quantify MV uptake by NK cells (n=4). (B) Confocal microscopy of NK cells after 24 hrs incubation with PKH26-MV. Representative image of an NK Cell with an internalized MV was shown. Z-stack at of 1 μ m slices were captured and 3D volume rendering was performed to further

demonstrate location of MV. (C) Video microscopy of PKH67-labelled NK cells (red) and PKH26-labelled MV (green) demonstrating fusion of MV and NK cell membrane. (D) CD69 expression on NK cells when co-cultured with RBC (NK+RBC), iRBC (NK+iRBC), MV (1 µg/ml)(NK+MV1), and MV (10 µg/ml)(NK+MV10). CD69 expression was determined by FACS, and expressed as geometric MFI. (E) IFN-γ in the supernatant of the indicated condition was assayed by immunoassay. Each point represented an independent experiment. Error bars show mean ± SD. *: p<0.05; **: p<0.01; ns: not significant.

3.10.2 TLR signaling pathway

Another pathogen recognition pathway involved in iRBC recognition was the toll-like receptor pathway (Fig. 3.20A and C). Toll-like receptors had long been implicated in the activation of immune cells by iRBC. TLR2 and TLR4 had been shown to recognize *Pf*GPI (Krishnegowda, Hajjar et al. 2005), while TLR9 interacts with *Plasmodium* DNA complexed to proteins (Wu, Gowda et al. 2010). MYD88, a convergent point of all TLRs, except TLR3, had also been shown to be required for immune cell response to iRBC (Pichyangkul, Yongvanitchit et al. 2004).

Unlike transcriptional analysis performed on PBMCs of patients after exposure to malaria, we did not observe an increase in transcripts of TLR1, TLR2, TLR4 and TLR8 (Ockenhouse, Hu et al. 2006). As shown in Fig. 3.20A, TLR7 (p<0.05) was the only member of the TLR family that was significantly upregulated in responder NK cells co-cultured with iRBC. MYD88 was also increased by 1.7-fold (p<0.001) in responder NK cells. Activation of MYD88 leads to downstream recruitment and

phosphorylation of IKBKB, TANK and TBK1 (Clark, Takeuchi et al. 2011), which activates the master regulator, IRF7 (Honda and Taniguchi 2006). Phosphorylated IRF7, either as a homodimer or heterodimer with other IRFs, then migrates into the nucleus and promotes transcription of pro-inflammatory genes and cytokines (Ning, Pagano et al. 2011). In our analysis, IRF7 was upregulated by 2.6-fold ($p < 0.001$) in responder NK cells upon exposure to iRBC.

Fig 3.20 TLR signaling pathway. (A) Heatmap of DEGs involved in TLR signaling. (B) qPCR validation of selected DEGs. 3 housekeeping genes (B2M, IPO8, and GAPDH) were used, and qPCR results were analyzed using REST 2009. Expression ratios of responder

NK cells to non-responder NK cells for the indicated gene were shown. *: $p < 0.05$. (C) Genes were represented as HGNC symbol and color coded according to the Log2FC of responder/non-responder *: indicate significant DEGs.

In corroboration with transcriptomic data, qPCR validation of microarray results demonstrated that TLR1-6, and TLR8-10 were not significantly different between responder and non-responder NK cells; while MYD88 and IRF7 were significantly upregulated in responder NK cells (Fig. 3.20B and Table 3.6). Notably, qPCR failed to demonstrate upregulation of TLR7.

Table 3.6 qPCR results of selected genes involved in TLR signalling pathway

Gene	Type ^a	Reaction		Std. Error	95% C.I.	P.val	Result
		Efficiency	Expression				
B2M	REF	1	1.517				
GAPDH2	REF	1	1.472				
IPO8	REF	1	1.224				
TLR1	TRG	1	0.532	0.165 - 1.885	0.072 - 3.217	0.416	
TLR2	TRG	1	2.389	0.260 - 51.210	0.012 - 63.027	0.659	
TLR3	TRG	1	0.524	0.213 - 1.692	0.164 - 3.873	0.326	
TLR4	TRG	1	2.142	0.212 - 59.469	0.010 - 87.851	0.735	
TLR5	TRG	1	2.135	0.180 - 35.672	0.025 - 66.801	0.624	
TLR6	TRG	1	0.495	0.144 - 1.550	0.052 - 2.584	0.477	
TLR7	TRG	1	2.36	0.278 - 38.611	0.013 - 73.968	0.74	
TLR8	TRG	1	1.323	0.089 - 37.063	0.011 - 62.792	0.847	
TLR9	TRG	1	0.771	0.183 - 3.278	0.077 - 4.400	0.733	
TLR10	TRG	1	0.964	0.154 - 11.209	0.016 - 16.504	0.987	
MYD88	TRG	1	1.937	1.303 - 2.818	1.034 - 3.888	0.042	UP
IRF7	TRG	1	4.658	2.835 - 8.605	2.550 - 12.123	0.015	UP

a: REF – reference gene; TRG – target gene.

Given the involvement of the TLR pathway and malarial parasite response (Gazzinelli, Kalantari et al. 2014), as well as the possible involvement of TLR- signaling pathway revealed in our transcriptional analysis, we next tested the requirement of MYD88 in NK cells response

to iRBC. To inhibit MYD88 signaling, we used a MYD88 dimerization inhibitor (MYD88INH). As shown in Fig. 3.21A, in the absence of NK cells, parasitemia rose to $8.1 \pm 1.3\%$ after 2 IDC cycles. In the presence of NK cells, parasitemia declined to $2.5 \pm 0.4\%$. Upon addition of MYD88INH ($100 \mu\text{M}$), the decline of parasitemia was reversed, and parasitemia was significantly increased to $6.0 \pm 1.9\%$ ($p=0.009$, $n=6$). Not only did MYD88INH abolish control of parasitemia by NK cells, it also blocked NK cell activation by iRBC. Expression of CD69 on NK cells was significantly reduced from a MFI of 495 ± 203 to 332 ± 193 ($p=0.005$, $n=6$) (Fig. 3.21B).

MYD88 is a common adaptor molecule for most of the TLR, except TLR3 (Takeda and Akira 2004). In human, there are 10 known TLR members, and human NK cells have been shown to express all of them, albeit at varying levels, with TLR1 having the highest expression levels, and TLR10 the least (Chalifour, Jeannin et al. 2004, Lauzon, Mian et al. 2007). As such, we further investigated the involvement of individual TLRs. Monoclonal antibodies against TLR1, 2, 4, 5 and 6 were used at a final concentration of $1 \mu\text{g/ml}$, while oligonucleotides against TLR 7,8 and 9 were used at a final concentration of $1 \mu\text{M}$. As shown in Fig. 3.21C, neutralization of TLR2 and TLR4 significantly reduced the control of parasitemia by NK cells from $63.3 \pm 9.8\%$ to $32.2 \pm 7.0\%$ ($p=0.001$, $n=5$) and $30.0 \pm 11\%$ ($p=0.01$, $n=5$) respectively.

We next determined if TLR agonists could further boost the control of parasitemia by NK cells. Pam3CSK4 (TLR1/2)(100 ng/ml), Poly(I:C) (TLR3)(100 ng/ml), MPLA (TLR4)(100 ng/ml), R848 (TLR7)(1 µg/ml) and ODN2006 (TLR9)(1 µM) were tested at the indicated concentration. As shown in Fig.3.21D, none of the tested TLR agonists significantly improved parasitemia control. Instead, Pam3CSK4 even had deleterious effect on NK cell response to iRBC. At 100 ng/ml, Pam3CSK4 reduced NK cell control of parasitemia from $77.4 \pm 3.8\%$ to $59.2 \pm 6.4\%$ ($n=4$, $p<0.05$).

Next, we asked if the TLR agonists could improve non-responder NK cells' response to iRBC. As such, we repeated the previous experiment but with non-responder NK cells instead. At 48 hrs, responder NK cells could reduce parasitemia by $43.6 \pm 3.2\%$ ($n=3$), while non-responder NK cells barely reduced parasitemia by $6.6 \pm 2.9\%$ ($n=3$) (Fig. 3.21E). No significant improvement in parasitemia control could be observed for the TLR agonists Pam3CSK4, poly(I:C) and ODN2006, as well as IFN- γ (1 µg/ml). Poly(I:C), ODN2006 and IFN- γ by themselves, could reduce parasitemia by $11.9 \pm 4.9\%$, $36.5 \pm 0.9\%$ and $6.7 \pm 4.3\%$ respectively. However, a significant improvement in parasitemia was observed for the TLR7/8 agonist, R848. In the presence of R848, non-responder NK cells' control of parasitemia ($30.2 \pm 4.1\%$, $n=3$, $p=0.0012$) was significantly improved over PBS controls.

Thus, we showed that NK cells responded to iRBC through TLR2/4 in a MYD88-dependent manner. Additionally, though TLR agonists generally had no effect on responder NK cells, the TLR7 agonist, R848, could convert non-responding NK cells to responders.

Fig. 3.21 MYD88-dependent control of parasitemia. (A) iRBC were cultured alone, together with NK cells, and with NK cells in the presence of MYD88 dimerization inhibitor (MYD88INH). Parasitemia was quantified at 96 hrs by FACS, gating only on total RBCs. Representative Hoechst vs CD56 FACS plot was shown. Numbers indicate parasitemia gated on total RBC. Paired sample t-test (n=5) was performed to determine

effect of MYD88INH on NK cell control of parasitemia. **(B)** Histogram of CD69 expression changes in the indicated conditions. Paired sample t-test was performed on geometric mean fluorescence intensity (MFI) of CD69 (n=5). **(C)** Paired sample t-test plots of NK cells + iRBC co-cultured in the presence of the indicated condition (blue dots) or PBS (red dots) as a control. Blocking of TLR 1, 2, 4, 5, and 6 was achieved using neutralizing antibodies. 2087: oligonucleotide 2087 blocks TLR7 and 8. 2088: oligonucleotide 2088 blocks TLR7, 8 and 9. 20959: oligonucleotide 20959 blocks TLR 7 and 8. **(D)** NK cells and iRBC were co-cultured in the presence of the indicated TLR agonist for 96 hrs, and parasitemia after 96 hrs was quantified by FACS. **(E)** Non-responder NK cells (blue) and iRBC were co-cultured with the indicated TLR agonist for 48 hrs. Responder NK cells (green) were shown as a control. iRBC (red) were cultured with the indicated TLR agonist as a control to the effect the compound might have on iRBC. Each dot represents an independent repeat. Error bars represented mean $\pm$ SD. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.005$; ns: not significant.

3.10.3 IFN signaling pathway

Activation of PRRs often lead to activation of downstream signaling cascade, culminating in the production of interferons and other pro-inflammatory mediators. There are three classes of interferons, namely IFN-I, IFN-II and IFN-III. Traditionally, interferons are known for their anti-viral properties, but this is slowly changing and much focus had been directed to their roles in non-viral infections, such as bacterial and protozoan (Bogdan, Mattner et al. 2004). The prototypical interferon involved in malaria, is IFN- γ . In both human and rodent malaria, an early IFN- γ response has been shown to be critical in host resistance to severe disease (King and Lamb 2015).

In our transcriptional analysis, 22 DEGs (Fig. 3.22A) were mapped to the IFN signaling pathway maintained at the Reactome database

(Croft, Mundo et al. 2014). Amongst them were several interferon-response genes, like MX1, OAS1, OAS3, IFIT1, IFIT2 and IFIT3. These genes are typically activated by nucleic acid PRRs, such as TLR7, TLR8, TLR9, MDA5 and RIG-I (Diamond and Farzan 2013). Given the involvement of malarial DNA/RNA, it was thus not surprising an increase in expression of these genes were observed. The upregulation of these genes, as well as others, were confirmed by qPCR (Fig. 3.22B and Table 3.7).

Fig. 3.22 Interferon signaling. (A) Heatmap of DEGs involved in interferon signaling. (B) qPCR validation of selected DEGs. 3 housekeeping genes (B2M, IPO8, and GAPDH) were used, and qPCR results were analyzed using REST 2009. Expression ratios of responder NK cells to non-responder NK cells for the indicated gene were shown. *: $p < 0.05$. (C) Venn diagram showing number genes regulated by each interferon type. (D) Fold change of DEGs in our transcriptional analysis correlated with experimental data obtained from www.interferome.org. (E) Genes were represented as HGNC symbol and color coded according to the Log2FC of responder/non-responder *: indicate significant DEGs.

The list of DEGs involved in interferon signaling was then submitted to www.interferome.org. Out of the 22 DEGs, 17 were mapped. A Venn diagram was then generated to show the number of genes regulated by each interferon type (Fig. 3.22B). As shown, all 17 genes could be regulated by both Type I and II interferons. Next, we correlated the fold change of DEGs in our transcriptional analysis to that obtained from the interferome database (Fig. 3.22C). The magnitude of change of the indicated DEGs in responder against non-responder was comparable to those observed in IFN- γ treated cells over control cells (Pearson $r=0.943$). Though type I and type II interferons signal through different receptors and transcription factors, significant overlap exists in the genes they regulate (Hertzog, Forster et al. 2011). Together with the finding that there was no type I IFN present in our system (Fig. 3.11G), it was thus possible to assume that the transcriptional changes observed be due to the effect of IFN- γ (Fig. 3.22D).

Table 3.7 qPCR results of selected genes involved in IFN signalling pathway

Gene	Type ^a	Reaction		Std. Error	95% C.I.	P.val	Result
		Efficiency	Expression				
B2M	REF	1	1.02				
GAPDH	REF	1	1.213				
IPO8	REF	1	0.808				
IFIT1	TRG	1	22.367	3.645 - 120.632	0.868 - 224.715	0.012	UP
IFIT2	TRG	1	4.225	1.214 - 11.783	0.550 - 26.774	0.033	UP
IFIT3	TRG	1	12.689	2.610 - 89.981	1.809 - 133.331	0.006	UP
ISG15	TRG	1	8.252	4.307 - 13.439	3.385 - 23.563	0.005	UP
MX1	TRG	1	9.806	5.278 - 21.751	2.928 - 44.640	0.007	UP
OAS1	TRG	1	18.134	9.220 - 41.782	4.395 - 74.473	0.004	UP
OAS3	TRG	1	7.154	1.756 - 21.589	0.611 - 37.403	0.017	UP
TRIM25	TRG	1	4.217	2.213 - 7.814	1.198 - 13.057	0.002	UP
IRF7	TRG	1	3.471	2.043 - 5.815	1.246 - 11.002	0.006	UP

a: REF – reference gene; TRG – target gene.

NK cells are the earliest cells to secrete IFN- γ in response to iRBC (Artavanis-Tsakonas and Riley 2002). Although IFN- γ had no direct anti-parasitic effect on *P. falciparum* iRBC (Fig. 3.6E), it had indirect immune-modulation effects on other cell types and directly affects host survival (De Souza, Williamson et al. 1997). As shown in Fig. 3.15B, we observed upregulation of genes involved in RIG-I/MDA5 and TLR pathways. In the preceding chapters, through neutralizing antibody or chemical inhibition, we had also shown that these pathways were involved in NK cell response to iRBC. These pathways are known to activate NF κ B (Kawai and Akira 2007, Loo and Gale 2011), which is a key regulator of IFN- γ transcription (Sica, Dorman et al. 1997). As shown in Fig 3.21A (and Table S1), IFN- γ gene expression (IFNG) was significantly upregulated in responder NK cells compared to non-responders ($\log_2$ FC: 3.6, $p < 0.004$). Consequently, genes involved in IFN- γ -signaling pathway were also found to be enriched (Fig. 3.22A). This could be a result of autocrine/paracrine signaling by

IFN- γ secreted by NK cells in response to iRBC. This potentially could provide a positive feedback loop like IFN- γ -priming of macrophages (Hu and Ivashkiv 2009).

In this chapter, through transcriptional analysis of responding NK cells and non-responding NK cells, we identified 412 DEGs. Of which, the upregulated ones were enriched in pathogen recognition pathways like the RIG-I receptor signaling pathway and TLR signaling pathway. Through chemical inhibition or neutralizing antibodies, we further demonstrated that NK cell response to iRBC requires a combination of signals involving TLR2/4, MYD88 and TBK1/IKK ϵ . The activation of these pathways ultimately led to IFN- γ secretion, and consequently a plethora of interferon-response genes were upregulated in our transcriptional analysis.

3.11 ***KIRs and NK cell response to iRBC***

KIR genes have been associated with a variety of diseases ranging from HIV and HCV susceptibility, to IFN- γ response in malaria (Khakoo and Carrington 2006). It was shown that although KIR haplotypes had no effect on NK cell IFN- γ positivity, it was observed that donors with a *002 allele of 3DL2 were more likely to be responders (Artavanis-Tsakonas, Eleme et al. 2003). Exactly how KIRs, which typically recognize HLA class I, have an impact on malaria which infects RBC is intriguing. It might be possible that KIRs have non-HLA ligands. For example, it is demonstrated

that the activating KIR2DS4 receptor recognize a protein ligand on MHC class I-negative cell (Katz, Gazit et al. 2004). Likewise, in murine models, the activating Ly49H, a mouse analogue of KIR, is shown to bind a murine cytomegalovirus (MCMV)-derived protein (Arase, Mocarski et al. 2002). This emerging evidence is gradually pointing towards the role of the activating KIR receptors as potential receptors that recognize pathogen-derived antigens as well as 'altered self' molecules. As such, we decided to genotype our donors via PCR with sequence specific primers (Gomez-Lozano and Vilches 2002).

As shown in Fig. 3.23A, each PCR reaction consisted of 2 sets of primers – one set for a 464bp internal control; and another set for a >1.5 kbp product specific to the targeted KIR gene. The presence of only the internal control band would indicate the absence of the targeted KIR gene. Using the original set of primers verified by Gomez-Lozano *et al* (Gomez-Lozano and Vilches 2002), we could consistently obtain products of 14 out of 16 KIR genes, with KIR3DL1 and KIR2DS3 being particularly problematic. As such, a different primer set for KIR3DL1 (Fig. 3.23B) and KIR2DS3 (Fig. 3.23C) was used (Vilches, Castano et al. 2007), and we were able to complete the KIR genotypes for 9 non-responder and 10 responder donors (Table 3.8). We then compared our sequenced KIR genotype with the KIR genotype database available at <http://www.allelefrequencys.net>, to obtain a haplotype and genotype ID.

Fig. 3.23 KIR genotyping by PCR-SSP. (A) Agarose gels from 3 representative donors. Each lane consisted of a 464 bp internal control product and a >1.5 kb product if the indicated gene was present. **(B)** PCR amplification of a 108 bp product of KIR3DL1 from all donors using alternate primer pairs. **(C)** PCR amplification of 158 bp product of KIR2DS3 from all donors using alternate primer pairs.

Fig. 3.24 showed the carrier frequency for each KIR gene in the responder and non-responder groups. All our donors belonged to KIR B haplotype group, indicating that all had at least one of the activating KIR genes (KIR3DS1, KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS5). All the framework genes, KIR2DL4, KIR3DL2, KIR3DL3 and KIR3DP1, as well as 2 other KIR genes, KIR2DL1 and KIR2DP1 were present in all donors.

Comparing the frequency of occurrence of each KIR gene in our two

groups, revealed that the frequencies of 2 activating KIR genes – KIR2DS1 and KIR2DS2 were significantly increased in non-responder group ($p<0.01$). The parasitemia reduction and IFN- γ secretion profiles of the donors were shown in Fig. 3.24B and C respectively. These data suggested that in our sampled population, frequencies of KIR2DS1 and KIR2DS2 were reduced in responder donors.

Table 3.8 KIR genotype profile of responders and non-responders.

Donor ID	Status*	3DL1	2DL1	2DL3	2DS4	2DL2	2DL5	3DS1 ^a	2DS1 ^a	2DS2 ^a	2DS3 ^a	2DS5 ^a	2DL4 ^b	3DL2 ^b	3DL3 ^b	2DP1	3DP1 ^b	Genotype [#]	Parasitemia Reduction (%)	IFN- γ (pg/ml)
#090114	NR																	Bx-9	0	891.0
#041115	NR																	Bx-6	0	1321.3
#030315	NR																	Bx-3	22.5	323.4
#040314	NR																	Bx-93	9	1515.5
#100316	NR																	Bx-233	37	479.6
#Unk	NR																	Bx-3	30	625.1
#220216	NR																	Bx-6	30	1120.6
#210314	NR																	BX-260	11	1120.0
#040615	NR																	Bx-6	21	328.8
#120116	R																	Bx-20	76	566.3
#081214	R																	Bx-81	70	1495.3
#161214	R																	Bx-6	69.9	961.9
#050216	R																	Bx-19	65	605.0
#180815	R																	Bx-18	66	2986.9
#040215	R																	Bx-200	87	4947.6
#290415	R																	Bx-200	85	2343.0
#240615	R																	Bx-188	75	1073.3
#250116	R																	Bx-19	77	2266.2
#251213	R																	Bx-19	79	2010.8
#210915	R																	Bx-30	60	2476.5

Presence of indicated gene was denoted by shaded squares. *: NR: Non-responder; R: Responder. a: Activating KIRs. b: Framework KIRs. #: Genotype is obtained by comparing PCR results with KIR database at <http://www.allelefreuencies.net>. Genotype is written as Haplotype-Genotype ID.

Fig. 3.24 KIR frequency in non-responder and responder donors. (A) Shown are frequencies of each KIR gene in the population of non-responder (n=9) and responder (n=11). Mann–Whitney–Wilcoxon test was performed to determine if frequencies of KIR gene was significantly different between non-responder and responder donors. (B) Parasitemia reduction profile of donors (C) IFN-γ secretion profile of donors. Each dot represents an independent repeat. Error bars represented mean $\pm$ SD. *: $p < 0.05$. ****: $p < 0.001$.

Chapter 4. Discussion

4.1 *RICH mice support P. falciparum growth*

By combining hydrodynamic injection of Flt3L and IL-15 plasmids (Chen, Khoury et al. 2009), and supplementation of human RBC, we developed RICH mice. After one week of cytokine expression and human RBC supplementation, human leukocyte chimerism reached ~35%, with 2-10% human NK cells, and ~75% human RBCs. We then showed that RICH mice could support *P. falciparum* infection with non-rodent-adapted 3D7 strain. Parasitemia gradually increased from 0.01% at 48 hrs post-infection to 0.1% after 10 days. Compared to prior NSG mice model, parasitemia observed in our RICH mice were 100x less (Jimenez-Diaz, Mulet et al. 2009). However, there are two main differences between our system and the authors' system. Firstly, RICH mice possess a functional human immune system, which restricted parasitemia increase. Secondly, the strain of 3D7 used by Jimenez-Diaz *et al* had been rodent adapted, thus explaining their better survivability. In fact, when compared to the non-adapted 3D7 strain used by Angulo-Barturen *et al* in NOD^{scid}/β2m^{-/-} mice, parasitemia in RICH mice are highly similar to parasitemia (~0.1% at day 10 post-infection) reported by the authors (Angulo-Barturen, Jiménez-Díaz et al. 2008). Additionally, using rodent-adapted *P. falciparum*, Dr. Dummeler from our lab demonstrated that RICH mice could support parasitemia in the range of 9-20%, similar to levels

reported by Jimenez-Diaz *et al* (Zenonos, Dummler et al. 2015). Hence, RICH mice present as a superior *in vivo* model to investigate the effects of human immune system on control of *P. falciparum* parasitemia.

4.2 ***NK cells are involved in parasitemia control in P. falciparum infected-RICH mice***

As alluded to before, one of the reason for the low parasitemia observed in RICH mice is the presence of a functional human immune system, especially human NK cells (Chen, Khoury et al. 2009). Indeed, when human NK cells were antibody-depleted, parasitemia in RICH mice rose four-fold from 0.015% to 0.07% at 48 hrs post-infection. These results agree with murine malaria models, where antibody depletion of NK cells in mice showed increased parasitemia (Mohan, Moulin et al. 1997, Choudhury, Sheikh et al. 2000), and show that NK cell are involved in control of acute malaria parasitemia.

As NK cells are major secretors of IFN- γ , it is not surprising that depletion of NK cells, also reduced IFN- γ secretion in RICH mice. It may be tempting to suggest that IFN- γ might have a role in controlling parasitemia. However, we later showed that at up to 1.5 $\mu\text{g/ml}$ of IFN- γ , which is 10000x the amount observed in the serum of RICH mice, no inhibition of parasitemia was observed (Fig. 3.6E). As such the role of IFN- γ is most likely to prime other immune cell subsets, like macrophages and T cells, and activate the adaptive immune response. Indeed, in IFN- γ

deficient mice infected with *P. c. chabaudi*, minimal difference in parasitemia was observed during the acute-phase of disease (<10 days). The effect of IFN- γ deficiency was only visible after 10 days, and manifested itself as prolonged duration of parasitemia (van der Heyde, Pepper et al. 1997).

4.3 ***In vitro control of parasitemia by NK cells***

Having observed the control of *P. falciparum* by NK cells *in vivo*, we next sought to determine the mechanism of how NK cells responded to iRBC. Orago *et al* first showed that NK cells could lyse iRBC by chromium release assay (Orago and Facer 1991). However, later, Mavoungou *et al* showed that purified NK cells can inhibit iRBC parasitemia, but only in the presence of serum from malaria-immune people (Mavoungou, Luty et al. 2003). Artavanis-Tsakonas *et al* then showed, that PBMCs responded to iRBC by secreting IFN- γ , and majority of IFN- γ ⁺ cells were NK cells. However, such response was dependent on the presence of IL-12 and IL-18 (Artavanis-Tsakonas and Riley 2002). Baratin *et al*, further suggested that NK cells secretion of IFN- γ required macrophage help (Baratin, Roetynck et al. 2005). All in all, the requirements for NK cell control of parasitemia isn't very conclusive.

We demonstrated that purified NK cells can control *P. falciparum* parasitemia by 50-80% after 96 hrs of *in vitro* co-culture. However, consistent with prior observations, NK cells alone are unable to totally

eradicate iRBC (Mohan, Moulin et al. 1997, Choudhury, Sheikh et al. 2000). In response to iRBC, NK cells upregulated activation markers, such as CD69 and CD25. CD69 had been shown to be associated with activation of NK cell cytolytic functions (Moretta, Poggi et al. 1991), while CD25 better correlated with NK cell survival and proliferation capacity in response to activation (Clausen, Vergeiner et al. 2003). iRBCs also induce CD107a upregulation on NK cells, and it serve as an indicator of NK cell functional activation, as it is only expressed on NK cell's surface upon cytokine secretion and target cell lysis (Alter, Malenfant et al. 2004). Apart from increased intracellular IFN- γ staining in NK cells, as shown by others (Artavanis-Tsakonas and Riley 2002, Artavanis-Tsakonas, Eleme et al. 2003, Korbel, Newman et al. 2005); we also observed increased intracellular granulysin staining in NK cells in response to iRBC. Farouek *et al* had previously observed that anti-granulysin antibody abrogated $\gamma\delta$ T cells inhibition of *Pf*iRBC growth (Farouk, Mincheva-Nilsson et al. 2004). Granulysin had also been shown to be required for the killing of intracellular bacteria such as *Mycobacteria tuberculosis* (Lu, Wu et al. 2014). Walch *et al* suggested a two-step mechanism where perforin first delivers granzyme and granulysin into an intracellular pathogen-infected host cell, followed by granulysin introducing granzyme into the intracellular bacteria (Walch, Dotiwala et al. 2014). Whether granulysin disrupt the parasitic plasma membrane remains to be elucidated. Similar to previous reports, no significant differences in expression of NKp30,

NKp44, NKp46, NKG2A, NKG2C and NKG2D are observed upon exposure to iRBC (Artavanis-Tsakonas, Eleme et al. 2003).

Of the cytokines secreted by NK cells in response to iRBC, only IFN- γ is significantly increased over RBC controls. Interestingly, secretion of TNF- α is not increased by iRBC exposure. NK cells are known to secrete IFN- γ and TNF- α in response to K562 cells (Fauriat, Long et al. 2010), as well as when stimulated by cytokines such as IL-12, IL-18 and IL-2 (Smith, Maurin et al. 2010). As such it appears that cytokine release in NK cells is a very specific process that is unique to different stimulation.

In our system of NK cell and iRBC co-culture, albumax was used in replacement of human serum, thus removing confounding factors of human complements and immunoglobulins. NK cells were magnetically purified to high purity (Fig. 2.1) to eliminate contaminating immune cell types. Supernatant of the co-culture demonstrated absence of cytokines such as IFN- α , IL-12 and IL-18 (Fig. 3.4G). As such, in contrast to reports that NK cells required immune serum (Mavoungou, Luty et al. 2003), macrophage help (Baratin, Roetyneck et al. 2005), or presence of IL-12 or IL-18 for IFN- γ secretion in response to iRBC (Artavanis-Tsakonas and Riley 2002), our results are in line with what Orago *et al* first observed (Orago and Facer 1991), that iRBC alone is sufficient to fully activate NK cells and induce iRBC lysis.

4.4 *NK cells specifically eliminate late-stage iRBC*

We next show that NK cells get activated by late stage iRBC and specifically eliminate these iRBCs. Upon exposure to late stage iRBC, NK cells get maximally activated within 12 hrs. This agrees with peak IFN- γ secretion observed upon 15 hrs co-culture with late stage iRBC (Artavanis-Tsakonas and Riley 2002). Macrophages are also known to exhibit stage-dependent preference in the phagocytosis of iRBC (Turrini, Ginsburg et al. 1992), suggesting the increased expression of neo-antigens during these stages. This comes as no surprise as extensive remodeling of iRBC occurs during these stages. As shown in Fig. 4.1, minimal morphological changes can be seen during the first half of the IDC (ring-stage). Most changes become apparent towards the late stage, or trophozoite and schizont stage, where one can distinctly observe structures like the hemozoin. During the schizont stage, the changes are even more obvious, as the parasite takes up almost the entire volume of the RBC, and merozoites are easily apparent. Molecularly, extensive remodeling of the iRBC occurs especially towards the late stages, where the various families of variant antigens, such as PfEMP1, STEVOR and RIFIN are inserted into the RBC membrane. The presence of these variant antigens also mediates the phenomenon of sequestration, where mainly ring-stage iRBC are observed in peripheral blood, while more mature forms of the asexual blood stages are sequestered in postcapillary venules (Urban, Ing et al. 2005).

Fig. 4.1 *P. falciparum* development in RBC. Shown are Giemsa images of iRBC throughout the IDC. The IDC commence with the ring stage, so called due to the signet-ring structure it resembles, when a merozoite invades a RBC. This stage last about 24 hrs, and morphologically-wise minimal changes occurred. At 24 hrs, late rings transition towards trophozoite stage with the appearance of a hemozoin focus (red arrow). Hemoglobin from the RBC gets digested, and the cell becomes pale. The parasitophorous vacuole, in which the parasite resides, gets larger and larger, ultimately consuming the entire volume of the RBC. Numerous merozoites are apparent during the late schizont stage.

4.5 ***Variant surface antigen protects iRBC from NK cell elimination***

Previously Mavoungou *et al* through Nkp30-Ig fusion peptide show that Nkp30 bound a DBL domain present on PfEMP1 (Mavoungou, Held *et al.* 2007). However, it should be noted that the concentration of DBL peptides used was at least a million times higher than the natural ligand density on iRBC surface. Through the usage of MAHRP KO parasite strains that do not express surface PfEMP1 (Spycher, Rug *et al.* 2008), we showed that PfEMP1 was not essential for NK cell recognition of iRBC. This corroborated with findings from D’Ombrain *et al* using another PfEMP1⁻ parasite line, that PfEMP1 is not a ligand for NK cell (D’Ombrain, Voss *et al.* 2007).

The observation that KAHRP KO parasites were equally eliminated by NK cells, indirectly added more support to the above conclusion. KAHRP is a key component of knobs. Knobs on iRBC surface serve as platforms for the presentation of PfEMP1 on iRBC surface. Functional knobs are required for adhesion to CD36 under physiological flow conditions (Crabb, Cooke et al. 1997, Rug, Prescott et al. 2006). The loss of these knobs on the iRBC surface thus modified the availability of ligands required for NK cell activation.

Interestingly, NK cell elimination of iRBC was impaired when the AP2KO6 parasite strain was used. This strain of parasite had been shown to over-express all classes of surface variant antigen, including PfEMP1, RIFIN and STEVOR. As such, it appears that surface variant antigens, instead of contributing as a neo-antigen for immune cell recognition, might have a protective role in immune-evasion. Indeed, PfEMP1 had been shown to inhibit IFN- γ release from NK cells (D'Ombra, Voss et al. 2007). Dr. Sampaio, in her thesis, also observed that PfEMP1 impaired NF- κ B signaling in human macrophages and monocytes, possibly through interfering with TLR signalling (Sampaio 2016). However, the exact mechanism of how surface variant antigens protect iRBC remains elusive. It is possible that variant antigens mask potential immune targets, such as PfGPI, the ligand for TLR2 and TLR4, reducing their availability for immune cell recognition.

Using various geographical strains of *P. falciparum*, we demonstrated strain-transcending parasitemia control by NK cells. Additionally, we also showed that human NK cells effectively eliminated zoonotic *P. knowlesi* iRBC, thus demonstrating species-transcending parasitemia control.

4.6 ***Increased ICAM-4 expression on iRBC***

NK cell activation is a fine balance of activating and inhibiting signals. Phagocytosis of RBC by macrophage is inhibited by the presence of CD47 on the RBC surface and its interaction with the signal regulatory protein alpha (SIRP α) on macrophages (Oldenberg, Zheleznyak et al. 2000). Despite not possessing MHC class I on RBC surface, through a similar interaction with yet unidentified NK cell inhibitory receptor, healthy RBC are protected by NK cell lysis (Lanier 2005). Such an inhibitory signal thus prevents the constitutive destruction of RBC by NK cells. However, when the intensity of such activating signal increase, as when Hsp70 is increased on iRBC surface, NK cell activation can be triggered (Böttger, Multhoff et al. 2012). However, in this case Hsp70 is also up-regulated on senescent RBC, which marks them for degradation. Hence the mere up-regulation of Hsp70 alone cannot account for the elimination of iRBC by NK cells.

In our screen of markers on iRBC, of the markers tested, CD44, CD47, CD147 and ICAM-4, only ICAM-4 showed significant differences

between uninfected and infected RBC. Although decreased CD47 had been associated with macrophage phagocytosis observed in murine RBC (Khandelwal, van Rooijen et al. 2007), the expression of CD47 on human RBC had been observed to be relatively static (Oldenburg, Zheleznyak et al. 2000). Similarly, CD44 and CD147 expression on RBCs were also noted to be stable throughout RBC aging (Sparrow, Healey et al. 2006).

The natural host ligands for LFA-1 (CD11a/CD18) belong to the ICAM family, and comprise of five members, namely ICAM-1, ICAM-2, ICAM-3, ICAM-4 and telencephalin (TLN) (Gahmberg 1997). ICAM-4 is RBC-specific and binds CD11c/CD18 on macrophages, where such interactions are postulated to be involved in the clearance of senescent RBC by splenic macrophages (Ihanus, Uotila et al. 2007). NK cells have also been shown to bind purified ICAM-4 in a CD18-dependent manner (Bailly, Tontti et al. 1995). Having demonstrated the importance of LFA-1 in NK cell interaction with iRBC, we looked at ICAM-4 expression on iRBC. Interestingly, ICAM-4 expression on iRBC is increased compared to uninfected RBC. As mature RBC lacks nuclei, and are unable to synthesize new protein, it is unclear how such increase in ICAM-4 expression comes about. One possible explanation comes from the observation that soluble ICAM-4 bound merozoites, and blocking of ICAM-4 inhibited *P. falciparum* merozoites invasion by ~80% (Bhalla, Chugh et al. 2015). As such, it is possible that *P. falciparum* merozoites preferentially invaded RBC with high ICAM-4 expression.

4.7 ***Contact-dependent lysis of iRBC by NK cells***

Via live cell video microscopy, we showed that despite the random nature of cell-to-cell interaction, NK cells migrate, and preferentially bind to iRBCs, leading ultimately to their lysis. Such lysis occurs in the absence of other immune cells, and is contact-dependent. The process of iRBC lysis involves membrane blebbing, volume increase, and leakage of iRBC content, a process like osmotic cell lysis. In support of this, the degradation of cytolytic granules by CMA decreased the parasitemia-control efficiency of NK cells. By preventing cytolytic granule acidification, CMA, effectively promotes the breakdown of effector molecules like perforin, granzymes and granulysin. This is supportive of our observation of a role of granulysin in iRBC lysis. Additionally, Korbelt *et al* had previously observed up-regulation of perforin and granzyme in NK cells upon iRBC contact (Korbelt, Newman et al. 2005).

The requirement for contact-dependent control of iRBC by NK cells is further explored by separating NK cells from iRBC by a 0.4 μm transwell. As expected, parasitemia control is abolished. The requirement for direct contact between NK cells and iRBCs had previously been suggested by Artavanis-Tsakonas *et al*, although whole PBMCs were used instead. The authors showed that PBMC required direct contact with iRBC for NK cells to stain positive for IFN- γ (Artavanis-Tsakonas, Eleme et al. 2003). Such observations thus lead to the hypothesis that iRBC

must have either lost inhibitory ligands for NK cells, or expressed neoantigens that activate NK cells.

4.8 *LFA-1 is required for NK cell response to iRBC*

To identify the receptors on NK cells that are responsible for the recognition of iRBC, we evaluate a vast panel of blocking antibodies against activating NK cell receptors, like the NCRs Nkp30, Nkp44, and Nkp46, NKG2D, 2B4 as well as adhesion molecules like CD2, DNAM-1 and LFA-1. Of these, anti-LFA-1 completely and anti-DNAM-1 partially abrogated the NK cell control of parasitemia (Fig. 4.2). It is shown that LFA-1 and DNAM-1 physically associate within NK cells, and activation via DNAM-1 is dependent on its functional interaction with LFA-1 (Shibuya, Lanier et al. 1999). This lends support to our observation that LFA-1 is the dominant receptor involved in NK cell interaction and elimination of iRBC. We further demonstrate by video microscopy that the preferential interaction of NK cells and iRBC is completely abolished upon addition of anti-LFA-1, and blocked activation of NK cells. Apart from its role as an adhesion molecule, LFA-1 is also known to mediate early signals for cytotoxicity in NK cells (Barber, Faure et al. 2004). One ligand of LFA-1 present on the RBC surface is ICAM-4 (Gahmberg 1997).

Apart from DNAM-1 and LFA-1, blocking of NCRs (Nkp30, Nkp44, Nkp46), 2B4, CD2, and NKG2D have no effect on NK cell response to iRBC. Although Nkp30-Ig fusion proteins had been shown to interact

with PfEMP1 (Mavoungou, Held et al. 2007), their exact contribution to NK cell cytotoxicity towards iRBC is unknown. As mentioned earlier, the concentration of DBL peptides used by Mavoungou *et al* was at least a million times higher than the natural ligand density on iRBC surface. On a similar note, though NKp44 was shown to bind cell wall components of MTB or BCG, anti-NKp44 was unable to inhibit NK cell activation to live BCG (Esin, Batoni et al. 2008, Esin, Counoupas et al. 2013). Similarly, despite reports of recombinant NKp44-Ig fusion peptide being able to bind flavivirus E proteins of West Nile Virus (WNV) and dengue virus (HersHKovitz, Rosental et al. 2009), blocking of Nkp44 by neutralizing antibody do not inhibit NK cell control of dengue viremia in infected DCs (Costa, Ye et al.)

Fig. 4.2 LFA-1 (CD11a/CD18) and DNAM-1 mediates NK cell-iRBC attachment.

Through antibody blocking experiments, we showed that LFA-1 (CD11a/CD18) and to a lesser extent DNAM-1 were required for NK cell attachment and killing of iRBC. Additionally, blocking of NKp30/NKp44/NKp46 have no effect on NK cell killing of iRBC.

4.9 *Heterogenous donor response to iRBC*

In response to iRBC, about 30% of NK cells from malaria naïve donors do not respond to iRBC. By comparing responding and non-

responding NK cells, we show that non-responding NK cells are not activated, and do not secrete IFN- γ . Interestingly, non-responding NK cells also have higher expression levels of the inhibitory NKG2A receptor, which could partly explain their observed inhibited state (Houchins, Lanier et al. 1997).

Within the general population, NK cell cytotoxic activity exists as a spectrum ranging from low to high. Such variations may be attributed to various lifestyle choices, such as smoking (Phillips, Marshall et al. 1985), alcohol consumption (Meadows, Blank et al. 1989) and obesity (Laue, Wrann et al. 2015). Individuals with low NK cell cytotoxic potential had been shown to have higher risk for cancer (Imai, Matsuyama et al. 2000). With regard to malaria, NK cells from patients with acute malaria were noted to have reduced cytotoxicity potential (Ojo-Amaize, Salimonu et al. 1981, Orago and Facer 1991). Low NK cell cytotoxicity in pregnant women have also been linked to higher chance of a positive blood smear for malaria parasites (Bouyou-Akotet, Issifou et al. 2004).

We next performed transcriptional analysis of responder and non-responder NK cells. Three pathways are significantly enriched with differentially expressed genes (DEGs) – interferon signaling pathway, RIG-I like signaling pathway and Toll-like receptor signaling pathway. In comparison to NK cell transcriptional data obtained from IL-2 (Dybkaer, Iqbal et al. 2007), IL-12 or IL-18 activation (Smith, Maurin et al. 2010), there is notably an absence of genes of the TNF superfamily, though this

173

corroborated with our data that iRBC induced minimal TNF- α from NK cells (Fig. 3.4G). In a recent transcriptomic analysis of PBMCs of volunteers vaccinated with the malaria vaccine, RTS,S/AS02A, it is shown that DEGs are mainly induced in IFN- γ pathway, and genes involved in NK cell mediated cytotoxicity are associated with delay parasitemia onset upon malaria sporozoite challenge (Dunachie, Berthoud et al. 2015).

One of the results of IFN- γ signaling is the induction of interferon-induced proteins, like interferon-stimulated gene 15 (ISG15) (Korant, Blomstrom et al. 1984), although ISG15 could also be induced by activation of cytosolic PRRs, like RIG-I (Liu, Jamaluddin et al. 2007). We show that ISG15 is upregulated by 5-fold in responder NK Cells. ISG15 served to potentiate IFN- γ production, as well as augment NK cell cytotoxicity (D'Cunha, Knight et al. 1996). Other functions of ISG15 involves ISGylation, a process similar to ubiquitination. HERC5 and TRIM25 are E3 ubiquitin ligases and are involved in conjugating ISG15 to protein substrates (Sadler and Williams 2008) The function of ISGylation remains to be refined, but ISGylation has been associated with protection against viral and bacterial infection, protein degradation and tumorigenesis (Zhang and Zhang 2011).

When malaria-naïve volunteers were challenged with malaria, genes involved in IFN- γ signaling were notably upregulated at the time of the first positive blood smear (day 9-12) (Ockenhouse, Hu et al. 2006).

When PBMC of children were transcriptionally profiled before and after

malaria-infection, genes involved in TLR signaling as well as IFN- γ signaling were also significantly changed (Portugal, Moebius et al. 2014). Surprisingly, the PBMC transcriptomics of children whom had two episodes of *P. falciparum* malaria, were surprisingly similar to our transcriptomic data (Fig. 4.3). It was observed that in contrast to children experiencing severe malaria, children with mild malaria had higher expression of genes involved in IFN signaling (Krupka, Seydel et al. 2012).

Fig. 4.3 Comparison of DEGs in children with mild and severe malaria against responder and non-responder. (A) Heatmap of DEGs associated with mild and severe malaria obtained from Krupka *et al* (Krupka, Seydel et al. 2012). (B) Heatmap of DEGs between responder and non-responder involved in IFN signaling (from Fig. 3.22A). Highlighted genes were similar with analysis from Krupka *et al*.

There are currently three RIG-I-like receptors (RLR) identified, namely RIG-I (DDX58), MDA5 (IFIH1) and LGP2 (DHX58). Typically, they served as cytosolic sensors for viral RNA (Loo, Fornek et al. 2008). However, since then, the RIG-I pathway have also been shown to be involved in foreign DNA recognition in conjunction with the RNA Pol-III,

which converts poly(dA-dT) DNA to 5'-ppp RNA (Chiu, MacMillan et al. 2009). The origins of foreign RNA have also been expanded to include intracellular bacteria, like *Legionella pneumophila* (Monroe, McWhirter et al. 2009). MDA5, but not RIG-I, had also been shown to be required for murine hepatocytes and DCs responses to *Plasmodium* RNA (Liehl, Zuzarte-Luis et al. 2014). In addition, MDA5 and MAVS were required for parasitemia control in C57BL/6 mice infected with *P. yoelii*, though their presence or absence had no effect on disease outcome (Wu, Tian et al. 2014). This corroborated with our observation that transcripts for MDA5 are significantly increased in response to iRBC, but not RIG-I.

TLR7 and MYD88 transcripts are significantly increased in responder NK cells compared to non-responder. This suggested an involvement of a TLR7-MYD88 pathway in NK cell response to iRBC. Indeed, it was shown that MYD88 was required for IFN- γ secretion by NK cells in response to iRBC (Baratin, Roetynck et al. 2005). Also, both TLR7 and MYD88 were required for type I and II interferon secretion in C57BL/6 mice in response to *P. chabaudi* infection (Baccarella, Fontana et al. 2013). In a mRNA microarray analysis of PBMC from patients before and after treatment for malaria, it was found that TLR7 was the most significant TLR upregulated (Franklin, Parroche et al. 2009).

Previous studies analyzing PBMCs of malaria-infected patients showed increased transcripts of Fc receptors, such as CD16, CD32 and CD64, as well as upregulation of genes involved in MHC class II

presentation (Ockenhouse, Hu et al. 2006). In our analysis, none of these transcripts were significantly different between responders and non-responders. This was most probably in our system, no immune serum was present, and as shown in Fig. 3.6B, the mode of NK cell lysis of iRBC didn't involve phagocytosis.

4.10 ***MYD88, TLR2 and TLR4 are required by NK cells to control parasitemia.***

Numerous pieces of evidence point to the involvement of MYD88 in the response against iRBC. MYD88^{-/-} mice were found to have impaired cytokine responses against *P. chabaudi* (Franklin, Rodrigues et al. 2007, Baccarella, Fontana et al. 2013). MYD88^{-/-} mice also succumbed to *P. yoelii* infection compared to wild-type mice (Gowda, Wu et al. 2012). MYD88 was also required for murine DCs and macrophage response to parasitic components (Pichyangkul, Yongvanitchit et al. 2004, Couper, Barnes et al. 2010). We further add to this pool of knowledge by showing that human NK cells response to iRBC is MYD88-dependent.

Apart from TLR3, signaling of almost all other members of the TLR family is through MYD88. In human, there are 10 known TLR members, and human NK cells have been shown to express all of them, albeit at varying levels, with TLR1 having the highest expression levels, and TLR10 the least (Chalifour, Jeannin et al. 2004, Lauzon, Mian et al. 2007). One of the earliest evidence that NK cells can recognize foreign pathogens

comes from the observation that lipophosphoglycan (LPG) produced by the obligate intracellular protozoan parasites of the genus *Leishmania* activates NK cell by binding to TLR2 (Becker, Salaiza et al. 2003).

We show that TLR2 and TLR4 are involved in NK cell recognition and control of *P. falciparum* parasitemia (Fig. 4.4). TLR2 is one of the earliest TLRs shown to be involved in parasite recognition. Campos *et al*, showed in 2001, that TLR2 was activated by the GPI of *Trypanosoma cruzi* (Campos, Almeida *et al*. 2001). Later, it was shown that secretion of TNF- α by human monocytes was mediated by TLR2 and TLR4 through their interaction with *Pf*GPI (Krishnegowda, Hajjar et al. 2005). Although Baratin *et al* previously showed that anti-TLR2 mAb had no effect on intracellular IFN- γ production in NK cells, the authors' experiments are very different from ours (Baratin, Roetynck et al. 2005). The authors co-cultured human PBMC with *Pf*-iRBC, instead of purified NK cells. By using PBMC, it is not possible to delineate involvement of TLR2 in NK cells' interaction with iRBC, due to presence of other immune cells. This is because NK cells can be activated by monocytes and DCs, which get activated via a TLR2-independent pathway. In fact, DCs had been shown to be activated by iRBC via a TLR9-dependent pathway, and in response secrete IFN- α and IL-12 (Pichyangkul, Yongvanitchit et al. 2004), which activate NK cells.

Fig. 4.4 TLR2 and TLR4 are required for NK cell-iRBC response. Through antibody blocking experiments, we showed that TLR2 and to a lesser extent TLR4 were required for NK cell killing of iRBC. TLR2/4 have been shown to recognize *PfGPI* on iRBC.

PfGPI is known to serve as anchor for a variety of schizont/merozoite proteins. Interestingly, *PfGPI* is exclusively synthesized and expressed at 28-40 hrs post-infection, corresponding to the trophozoite stage of the IDC (Naik, Davidson et al. 2000). Through radioactive-labelling of GPI, it was further confirmed that GPI was most strongly expressed towards the schizont stage (Gilson, Nebl et al. 2006). Majority of GPI-anchored proteins are merozoite proteins, of which the most abundant are MSP-1 and MSP-2. Based on a *P. falciparum* GPI-anchored proteome predictor, apart from merozoite proteins, some trophozoite-stage and schizont-stage proteins are also predicted to be GPI-anchored (Gilson, Nebl et al. 2006). Additionally, a large pool of *PfGPI* also exist free of protein attachment (Gerold, Dieckmann-Schuppert et al. 1994). It remains to be determine whether it is the bound or soluble form of *PfGPI* that activates NK cells. Nonetheless, these findings are in line with our observation that NK cells are specifically activated by late-stage iRBC.

Though TLR2/4 are required by NK cell for control of parasitemia, augmenting NK cells with TLR agonists like the TLR2 agonist, Pam3CSK4, failed to further improve NK cell control of iRBC parasitemia. Remarkably, R848, a TLR7/8 agonist, which had no effect on responder NK cells, improved non-responder NK cells' control of parasitemia. Interestingly, blocking of TLR7/8 had no effect on NK cells response to iRBC. How R848 serves to enhance non-responder NK cells' response remains to be determine. As we observed that non-responder NK cells had increased NKG2A expression in response to iRBC (Fig. 3.IIF), it may be possible that R848 provides increased stimulation to overcome possible increased inhibitory pressure. As such, it might provide a mechanistic explanation to the use of R848 as an adjuvant against malaria. Through immunization of mice with a *P. vivax* antigen (PvRII) and IgG variable-chain sequencing, it was shown that greatest diversity in IgG repertoire were obtained when both TLR4 (GLA) and TLR7/8 (R848) adjuvant was used (Wiley, Raman et al. 2011). TLR4 agonist (GLA) also increased vaccine-specific IgG titers when used as an adjuvant in vaccination of mice with a malaria vaccine candidate, GMZ2 (Lousada-Dietrich, Jogdand et al. 2011). The TLR9 agonist, CpG, had also been shown to improve acquisition and longevity of memory B cells in response to vaccination with the malaria vaccine, AMA1, in malaria-naïve adults (Crompton, Mircetic et al. 2009). Interestingly, although TLR5 hadn't been shown to be involved in parasite response, the addition of

flagellin, which is a TLR5 agonist, could improve intra-nasal vaccination of mice with *PfCSP* protein (Carapau, Mitchell et al. 2013).

4.11 ***Involvement of TBK1/IKK ϵ in NK cell response to iRBC.***

Apart from the TLR signaling pathway, the other pathogen recognition pathway identified in our transcriptional analysis was the RLR signaling pathway. As there were no efficient inhibitors of RIG-I or MDA-5, we used Bx795 to inhibit TBK1/IKK ϵ . Inhibition of TBK1/IKK ϵ led to an inhibition of parasitemia control by NK cells, leading us to identify another activation signal required by NK cell to lyse iRBC (Fig. 4.5). As TBK1/IKK ϵ is a common gatekeeper for endosomal and cytosolic nucleic acid sensors (Desmet and Ishii 2012), it is likely that *P. falciparum* nucleic acid material is important for NK cell activation. In fact, malaria DNA/RNA had been shown to activate macrophages, DCs and even hepatocytes (Sharma, DeOliveira et al. 2011, Liehl, Zuzarte-Luis et al. 2014). Unlike human DNA, *P. falciparum*'s DNA is highly AT-rich (~80%). Sharma *et al* demonstrated that one of such AT-rich motifs (ATTTTAC) stimulated macrophages in a TBK1-dependent pathway.

One way in which *P. falciparum* nucleic acids can be introduced into NK cells, is through microvesicles (MVs). iRBC had been shown to actively release MVs, especially late during schizont-stages (Mantel, Hoang et al. 2013). MVs were noted to carry not only parasite-derived

antigens (Mantel, Hoang et al. 2013), but also served as vehicles to transfer DNA and mRNA. Regev-Rudzki *et al* demonstrated that MVs from iRBC were able to transfer drug resistance from resistant iRBC to susceptible iRBC (Regev-Rudzki, Wilson et al.). These MVs were also able to directly activate macrophage inducing release of TNF- α (Mantel, Hoang et al. 2013). Through video microscopy, FACS and lipid-tagging of iRBC MVs, we showed that iRBC MVs get incorporated into NK cells (Fig. 4.5). This can potentially serve to transfer iRBC DNA/RNA into NK cells and activate cytosolic nucleic acid PRRs. Indeed, we further showed that MVs activated NK cells in a dose-dependent manner, thus corroborating with results from Mantel *et al* (Mantel, Hoang et al. 2013). Interestingly, though MVs at high concentration can increase CD69 expression on NK cell surface, IFN- γ secretion by NK cells were not induced. This reinforced the notion that NK IFN- γ secretion has a high activation threshold and requires engagement of multiple receptors (Fauriat, Long et al. 2010).

Fig. 4.5 Control of parasitemia by NK cells require TBK1/IKK ϵ pathway. Inhibition of the TBK1/IKK ϵ complex by the small molecule, Bx795, abolished NK cell control of parasitemia, suggesting the involvement of the TBK1/IKK ϵ pathway in NK cell response to iRBC. One set of potential receptors upstream of the TBK1/IKK ϵ pathway are the RIG-I like receptors, which are cytosolic dsRNA sensors. One way in which iRBC could activate this pathway, is through shedding RNA-containing microvesicles. It has been

shown that iRBC actively shed microvesicles containing parasite RNA/DNA. Here we also showed that NK cells take up iRBC microvesicles, and at high concentration get activated as indicated by CD69 upregulation. Hence, we hypothesize that iRBC microvesicles have a role in priming NK cell activation through the RIG-I/MDA-5 TBK1/IKK ϵ pathway.

4.12 *Under-representation of KIR2DS1 and KIR2DS2 in genotype of donors whose NK cells respond to iRBC*

In our analysis of KIR genes frequencies within responder and non-responder donors, non-responder donors generally have more activating KIRs than responders. Furthermore, it was revealed that there was a significant increase in frequencies of KIR2DS1 and KIR2DS2 in non-responder donors (Fig. 3.22A). Interestingly, it was observed that the frequencies of activating KIRs – KIR2DS2, KIR3DS1 and KIR2DS5 were increased in critically ill children presenting with severe malaria (Yindom, Forbes et al. 2012). In psoriatic arthritis, it was also observed that the presence of KIR2DS1 and/or KIR2DS2 increased disease predisposition (Martin, Nelson et al. 2002).

KIRs are highly polymorphic, and within a population, there is great variation in gene content. Even within an individual, the number of expressed KIR per cell is highly variable, varying from 2-9 (Valiante, Uhrberg et al. 1997). In fact, having more activating KIRs might not translate to increase activation potential. In an analysis of 2 individuals – one with only 1 activating KIR (KIR2DS4), and the other with all 5 activating KIRs; 90% of NK cells from the first donor expressed the single

activating KIR, while the remaining 10% had no activating KIRs; In the latter case, NK cells could express up to 5 activating KIRs per NK cells. However, this increased activation potential is balanced by increased inhibitory potential from either inhibitory KIRs or the inhibitory CD94:NKG2A receptor (Valiante, Uhrberg et al. 1997).

In response to iRBC, we observe that NKG2A expression in non-responder NK cells is increased about 2-fold (Fig. 3.IIF). Together with the observation that non-responder NK cells have more activating KIRs, it is possible to hypothesize that in the absence of HLA-ligands for the inhibitory KIRs, non-responder NK upregulates NKG2A to compensate this loss of inhibitory potential.

Previously, Artavanis-Tsakonas *et al* showed that there was an increased representation of an allele of KIR3DL2 in low IFN- γ responders towards iRBC stimulation (Artavanis-Tsakonas, Eleme et al. 2003).

KIR3DL2 is a framework KIR gene, hence is present in genotype sequenced. Since, PCR-SSP sequencing of KIR genotype, was unable to provide information on KIR gene copy number and allelic polymorphism, we were unable to determine if these factors contributed to NK cell iRBC response. Further SNP sequencing might be able to yield more insight on this aspect.

Chapter 5. Future Directions

5.1 *NK cell-iRBC responsiveness / KIR and malaria severity association*

We showed that the response of NK cells from malaria-naïve volunteers to iRBC could be categorized into responders and non-responders. It will be interesting to determine if this observation have any bearing on susceptibility towards clinical malaria and disease severity. Additionally, we would also like to answer the question on why NK cells from certain donors are responsive to iRBC, while others don't. In our transcriptional analysis, no DEGs were identified between responder NK cells and non-responder NK cells when co-cultured with uninfected RBC, suggesting they were transcriptionally similar. As such, it is possible that gene polymorphisms of critical PRRs can be responsible for the heterogenous response to iRBC observed. In fact, numerous TLR polymorphisms had been associated with malarial disease outcomes (Gun, Claser et al. 2014).

Regarding KIRs and malaria, we found increased frequencies of the activating KIRs – KIR2DS1 and KIR2DS2 in non-responding NK cells. However, there are several limitations in our study. Firstly, our method of KIR sequencing, i.e. by PCR-SSP, is unable to determine allelic differences between donors. Secondly, PCR-SSP is unable to determine gene copy number, and serve only to indicate presence/absence of gene.

To answer the above two questions, bioinformatic analysis could be carried out using data available from the Malaria Genomic Epidemiology Network (MalariaGEN) (Malaria Genomic Epidemiology 2015). MalariaGEN is an international collaborative effort involved in understanding how genetic variation affects biology and epidemiology of malaria. Among the various projects MalariaGEN are involved in, the data from Consortium Project 1, which investigate the genetic determinants of resistance to malaria, will be a good starting point. The restricted-access dataset “Genome-wide study of resistance to severe malaria in eleven populations” published in March 2016 looks at genetic data and their association with severe malaria in eleven populations. Bioinformatic analysis of this data should be able to reveal certain critical information that can help us answer the following questions: 1) Are there polymorphisms in molecules of the TLR or RLR pathways that are associated with severe malaria? 2) Is there over-representation of any KIRs in patients with severe malaria? This would directly help us validate if any KIRs are associated with severe malaria.

Nonetheless, to address the correlation of NK cell-iRBC responder status and malaria severity, it is necessary to perform NK cell-iRBC assay using NK cells from volunteers and determine if they develop severe malaria. Given the complexity of field studies, this should be performed in collaboration with groups with existing teams in the field. The study design can either be in the form of a prospective or retrospective study.

As a prospective study, PBMCs from children in an at-risk population can be obtained prior to a malaria-outbreak season. The population will then be tracked for onset of clinical malaria and their severity through the malaria-outbreak season. Thereafter correlation studies between NK cells' responsiveness and disease status can be performed. As a retrospective study, PBMCs will be obtained from a malaria-exposed population, and NK cells' responsiveness will then be correlated with prior malaria disease severity. However, this approach will be affected by survival bias, such that only patients that survived will be sampled. Children will be the main population sampled as malaria-immunity is less likely. The outline presented here is indeed drastically over-simplified, and further consultation with researchers versed in field studies will be required before embarkation of the project.

5.2 ***Validation of NK cell receptors involved in iRBC recognition***

In this work, through antibody neutralization or chemical inhibition studies, we identified the involvement of LFA-1, TLR2 and TLR4 in iRBC response through a Myd88- and TBK1/IKK ϵ -dependent pathway. However, TBK1/IKK ϵ is downstream of a few PRRs, such as RIG-I, MDA5, STING and TLR3. To further dissect the requirements of these PRRs, we can perform gene knockouts of the targeted gene. Given the divergence of these PRRs in murine, as well as differences in their

signaling pathways (Bryant and Monie 2012, Schroder, Irvine et al. 2012), it will be better to perform such genetic manipulations using human cells instead. Also, expression and regulation of PRRs, such as MDA5 and RIG-I, in murine innate cells are also different from human cells (Lech, Avila-Ferrufino et al. 2010).

Fig. 5.1 NK92 cells can control iRBC parasitemia. NK92 cells were co-cultured with iRBC and parasitemia was assessed by FACS. Each point represented an independent experiment. Error bars show mean $\pm$ SD.

Two approaches can be adopted to address the above issue. Firstly, instead of primary NK cells, NK92 cell lines can be used, which had been shown to be amenable to various manipulation techniques (Grund and Muise-Helmericks 2005). Preliminary results show that NK92 cells responded to iRBC, and controlled iRBC parasitemia (Fig. 5.1). One drawback of this approach is that NK92 requires IL-2 for growth and maintenance. IL-2 is a strong activator of NK cells and drastically changes its phenotype, such as distribution of surface activation receptor (Huenecke, Zimmermann et al. 2010). Hence, the usage of CD69, CD25, or IFN- γ secretion as surrogate for NK cell response to iRBC will be

impeded. Also, results might not be representative of resting primary NK cells. The other approach would be to use primary NK cells. However, unless stimulated to expand via cytokines, such as IL-2 or IL-15, primary NK cells remain refractory to various transfection techniques (Carlsten and Childs 2015). The alternative would be to work with HSCs. Others and we have shown that HSCs can readily be differentiated into functional NK cells (Chen, Ye et al. 2015). HSCs are rapidly dividing cells and serve as good targets for genetic manipulation. There is also the added benefit that by using these genetically manipulated HSCs to generate humice, the experiment can be expanded into an *in vivo* study. To introduce targeted gene disruption, CRISPR/Cas9 will be the ideal choice, due to its simplicity and permanent nature. The Alt-R™ CRISPR-Cas9 System (IDT) of IDT can be utilized due to its ease and improved efficiency, as well as no plasmids will be left within edited cell.

5.3 ***Verification of TLR2/4 involvement***

We have shown that TLR2/4 are involved in NK cell response to iRBC. One possible ligand on iRBC involved for such interaction is *Pf*GPI (Krishnegowda, Hajjar et al. 2005). In response to *Pf*GPI, macrophages have been shown to secrete TNF- α and nitric oxide (NO) (Schofield and Hackett 1993, Tachado, Gerold et al. 1996). To determine if *Pf*GPI is similarly involved in NK cell activation, either synthetic *Pf*GPI or natural *Pf*GPI enriched from iRBC can be used to stimulate NK cells. One way in

which *Pf*GPI can be enriched from iRBC is via radiolabeling followed by HPLC purification, as shown by Naik *et al* (Naik, Branch et al. 2000).

5.4 ***Priorities in malaria research***

In a recent survey of priorities of malaria research, the need to understand the mechanism of artemisinin-resistance, as well as development of new drugs to combat artemisinin-resistance are ranked as top priorities. Interestingly, with the development of RTS,S, the priority of developing a malaria vaccine has declined (Mulligan and Conteh 2016). In developed nations, one of the top priorities is the development of rapid and accurate malaria detection and surveillance kits (CDC 2017). As the vector for malaria transmissions is present in most countries of the tropics, a single asymptotically infected patient can potentially spread the disease into ‘malaria-free’ countries. Additionally, surveillance, as opposed to prevention, will start to play a key role once transmission has reduced below certain threshold, where preventive measures become economically unfavorable. Such surveillance kits will allow identification of at risk populations, where aid can be targeted to prevent outbreaks.

As an alternative to developing drugs against the malaria parasite, it is also possible to modulate host immunity against the parasite? By targeting the host instead of the pathogen, selective pressure for resistance development in the pathogen is avoided. Already, immune-modulating antibodies, such as anti-PD-1 (Page, Postow et al. 2014), and

small molecules, such as resiquimod (R848), a TLR7 selective agonist, have seen successes in cancer-therapy (Schon and Schon 2008, Hennessy, Parker et al. 2010). Immune-modulating molecules have also seen applications in microbial infections. For example, another TLR7 agonist, Imiquimod, is commonly used for topical treatment HPV-associated warts (A, Tying et al. 2009). Luivac, which comprise of various bacterial lysate, is used for recurring respiratory tract infections, by enhancing TLR responses (Arandjus, Black et al. 2006). Muropeptides, that work on the nod-like receptor pathway, enhances antibiotic responses, and reduces antibiotic dosage (Hancock, Nijnik et al. 2012). However, the application of immune-modulating molecules for parasitic diseases remained to be explored. In my opinion, I feel that one of the reason is that the exact mechanisms and signalling pathways involved in immune recognition of parasites have yet to be fully characterized. Sadly, in a survey of most important developments in malaria research, molecular biology and basic science ranked the lowest (Mulligan and Conteh 2016). As such, on top of actively screening panels of drugs for new drugs against malaria, it is also imperative to fully characterize the pathways involved in iRBC recognition by immune cells. Putting my work into perspective, verifying the contribution of TLR signalling pathway and RLR signalling pathways to the overall immune response towards malaria infected RBC is the first step in identifying immune-modulating molecules that can potentially improve anti-malaria therapy.

Chapter 6. Conclusion

In conclusion, we demarcated the response of NK cells to *P. falciparum*-iRBC. We show that NK cells survey and attach to RBC/iRBC population using LFA-1, forming transient interactions in the case of normal RBCs (Fig. 6.1A). In the presence of additional activating signals, the interaction between NK cell and iRBC become stronger and long-lasting, forming a NK cell-iRBC conjugate. Additional activating signals include 1) interaction between TLR2/4 with *Pf*GPI, which signal through MYD88-dependent pathway; 2) iRBCs actively shed microvesicles containing *Pf*RNA species. Such MVs fuse with NK cells, delivering *Pf*RNA into NK cells, and activate cytosolic nucleic acid sensors like MDA-5. Signaling through MDA-5 involve TRIM25, TBK1/IKK ϵ , and leads to increased IRF7. IRF7 and NF κ B translocate to the nucleus leading to NK cell activation and pro-inflammatory cytokine synthesis. Ultimately, this leads to NK cell activation, with increased expression of CD69 and CD25, as well as increased granulysin and IFN- γ synthesis. As *Pf*GPI synthesis and MV shedding occur mostly during trophozoite to schizont stages, NK cells specifically get activated by late-stage iRBC. (Fig. 6.1B). Armed with its repertoire of lytic granules, through the formation of a lytic synapse (Topham and Hewitt 2009), NK cells discharge the contents of lytic granules into iRBC, leading to membrane bleb formation, cell swelling, and rupture (Fig. 6.1C).

Fig. 6.1 Proposed mechanism of NK cell response to iRBC. (A) NK cells survey and attach to RBCs/iRBCs through interactions between LFA-1 on NK cells and ICAMs on RBC. Such interactions are transient if no other activation signals become available. (B) 1. Upon contact with iRBC the LFA-1 mediated attachment to iRBC becomes stronger forming NK-iRBC conjugates; 2. Interaction between TLR2/4 with PfGPI leads to NK cell activation via MYD88-dependent signaling; 3. Through possible use of cytosolic RNA sensors, such as MDA-5, PfRNA, which are delivered through shedding microvesicles (MVs) result in activation of TBK1/IKKε and signal transduction through IRF7. These signals lead to NK cell activation with upregulation of CD25, CD69 and increase expression of granulysin and IFN-γ. (C) During the lytic execution phase, discharge of lytic granules into iRBC led to membrane bleb formation, cell rupture, and leakage of cell contents. Degranulation of lytic granules and release IFN-γ led to surface expression of CD107a. IFN-γ can signal back to NK cells in an autocrine/paracrine fashion to further increase NK cell activation.

Transcriptional analysis demonstrates that NK cells that responded to iRBC have a similar transcriptomic profile as children with mild malaria disease, while transcriptomic profile of NK cells from donors that don't respond to iRBC are more similar to children with severe malaria (Krupka, Seydel et al. 2012). KIR Genotyping of NK cells revealed that NK cells that don't respond to iRBC, generally have more activating

KIRs, and the frequencies of KIR2DS1 and KIR2DS2 were significantly higher. Is it thus possible to adopt a system biology approach, employing a combination of indices incorporating KIR genotype, *in vitro* NK cell's iRBC response and transcriptomics to predict the probability of severe malaria? As opposed to a reductionist view of disease (i.e. manifestation of a disease is the result of a few aberrant molecules), systems biology attempts to view disease in a comprehensive manner incorporating information from various biological systems, such as genomics, metabolomics and proteomics. For example, in vaccine immunogenicity studies, a system biology approach incorporating cytokine profile, FACS and transcriptomic profiles have been used to predict immune response in human in response to yellow fever vaccination (Querec, Akondy et al. 2009).

On the parasite side of the equation, we show that PfEMP1 is not necessary for NK cell response to iRBC. Instead, over-expression of parasite surface variant antigen reduced iRBC's susceptibility to NK cell elimination, hinting at a possible immune-evasive role. Additionally, we observed increased surface expression of ICAM-4 on iRBC, which is a ligand for LFA-1 on NK cells. Given the lack of translation machinery in mature RBC, the increased in ICAM-4 observed could simply be an artefact of preferential invasion of high ICAM-4 expressing RBCs.

All in all, we identified three layers of interactions between NK cells and iRBC, demonstrating the complexities of NK cell control of

parasitemia. Being key innate immune cells, and one of the earliest cells involved in iRBC response, unravelling the mechanisms involved in NK cell activation by iRBC could aid in discoveries of therapeutics against malaria. For example, we have shown that non-responding NK cells could be converted to responders by R848.

Supplementary Materials

Table S1. List of DEGs between R_iRBC and NR_iRBC.

SYMBOL	logFC ^a	adj.P.Val ^b	ENTREZ	Description
HERC6	2.095	0.006865	55008	hect domain and RLD 6 (HERC6), transcript variant 1, mRNA.
RSAD2	2.363	2.97E-07	91543	radical S-adenosyl methionine domain containing 2 (RSAD2), mRNA.
OASI	1.811	3.85E-08	4938	2',5'-oligoadenylate synthetase 1, 40/46 kDa (OASI), transcript variant 2, mRNA.
MX1	2.592	0.000949	4599	myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse) (MX1), mRNA.
IFIT3	1.460	2.14E-08	3437	interferon-induced protein with tetratricopeptide repeats 3 (IFIT3), mRNA.
OAS2	1.455	0.011292	4939	2'-5'-oligoadenylate synthetase 2, 69/71 kDa (OAS2), transcript variant 1, mRNA.
OASL	1.153	0.256963	8638	2'-5'-oligoadenylate synthetase-like (OASL), transcript variant 2, mRNA.
OASI	2.052	2.40E-05	4938	2',5'-oligoadenylate synthetase 1, 40/46 kDa (OASI), transcript variant 3, mRNA.
ZNF683	-1.299	0.023116	257101	zinc finger protein 683 (ZNF683), transcript variant 2, mRNA.
DHX58	1.261	0.000838	79132	DEXH (Asp-Glu-X-His) box polypeptide 58 (DHX58), mRNA.
SPATS2L	1.449	0.00052	26010	spermatogenesis associated, serine-rich 2-like (SPATS2L), transcript variant 2, mRNA.
MT2A	1.079	0.171797	4502	metallothionein 2A (MT2A), mRNA.
IFI6	2.180	0.029543	2537	interferon, alpha-inducible protein 6 (IFI6), transcript variant 3, mRNA.
LY6E	1.167	0.062096	4061	lymphocyte antigen 6 complex, locus E (LY6E), mRNA.
RPS26L	-1.004	0.018837	400156	PREDICTED: 40S ribosomal protein S26-like (RPS26L), misc RNA.
IFIT1	1.130	5.21E-07	3434	interferon-induced protein with tetratricopeptide repeats 1 (IFIT1), transcript variant 2, mRNA.
IFIT3	2.884	0.014625	3437	interferon-induced protein with tetratricopeptide repeats 3 (IFIT3), mRNA.
EIF2AK2	1.658	0.017302	5610	eukaryotic translation initiation factor 2-alpha kinase 2 (EIF2AK2), mRNA.
IFIT1	3.825	1.58E-05	3434	interferon-induced protein with tetratricopeptide repeats 1 (IFIT1), transcript variant 2, mRNA.

LOC653820	-1.273	0.037886	653820	PREDICTED: similar to family with sequence similarity 72, member A, transcript variant 2 (LOC653820), mRNA.
IFI44L	3.723	0.071147	10964	interferon-induced protein 44-like (IFI44L), mRNA.
LOC650646	-1.131	0.012111	650646	PREDICTED: similar to 40S ribosomal protein S26 (LOC650646), mRNA.
HERC5	1.640	6.46E-05	51191	hect domain and RLD 5 (HERC5), mRNA.
PARP9	1.226	0.183704	83666	poly (ADP-ribose) polymerase family, member 9 (PARP9), mRNA.
OAS2	1.424	0.000255	4939	2'-5'-oligoadenylate synthetase 2, 69/71 kDa (OAS2), transcript variant 2, mRNA.
IFIT2	1.966	0.000311	3433	interferon-induced protein with tetratricopeptide repeats 2 (IFIT2), mRNA.
XAF1	1.788	0.035369	54739	XIAP associated factor 1 (XAF1), transcript variant 2, mRNA.
PLSCR1	1.177	4.28E-05	5359	phospholipid scramblase 1 (PLSCR1), mRNA.
OAS3	2.589	0.000555	4940	2'-5'-oligoadenylate synthetase 3, 100 kDa (OAS3), mRNA.
RPS26L	-1.167	0.020606	400156	PREDICTED: 40S ribosomal protein S26-like (RPS26L), misc RNA.
IFI44	2.941	0.02969	10561	interferon-induced protein 44 (IFI44), mRNA.
IFIH1	1.087	0.085412	64135	interferon induced with helicase C domain 1 (IFIH1), mRNA.
DTX3L	1.093	0.027229	151636	deltex 3-like (Drosophila) (DTX3L), mRNA.
PRIC285	1.414	0.00166	85441	peroxisomal proliferator-activated receptor A interacting complex 285 (PRIC285), transcript variant 2, mRNA.
HIST1H2AC	1.053	0.013469	8334	histone cluster 1, H2ac (HIST1H2AC), mRNA.
DDX60	1.403	0.029543	55601	DEAD (Asp-Glu-Ala-Asp) box polypeptide 60 (DDX60), mRNA.
IRF7	1.472	0.018358	3665	interferon regulatory factor 7 (IRF7), transcript variant b, mRNA.
	1.997	3.41E-06	NA	AGENCOURT_7914287 NIH_MGC_71 cDNA clone IMAGE:6156595 5, mRNA sequence
PARP9	1.066	0.178915	83666	poly (ADP-ribose) polymerase family, member 9 (PARP9), mRNA.
ISG15	2.344	8.87E-06	9636	ISG15 ubiquitin-like modifier (ISG15), mRNA.
HLA-A29.1	3.244	0.094143	649853	major histocompatibility complex class I HLA-A29.1 (HLA-A29.1), mRNA.
LAMP3	1.030	0.239744	27074	lysosomal-associated membrane protein 3 (LAMP3), mRNA.

RPS26P11	-1.030	0.029679	441502	ribosomal protein S26 pseudogene II (RPS26P11), non-coding RNA.
RPS4Y2	-1.837	0.193351	140032	ribosomal protein S4, Y-linked 2 (RPS4Y2), mRNA.
IFNG	3.593	0.004303	3458	interferon, gamma (IFNG), mRNA.
MX2	1.658	2.05E-05	4600	myxovirus (influenza virus) resistance 2 (mouse) (MX2), mRNA.
IFIT3	2.946	3.10E-06	3437	interferon-induced protein with tetratricopeptide repeats 3 (IFIT3), mRNA.
OAS2	1.167	0.003024	4939	2'-5'-oligoadenylate synthetase 2, 69/71 kDa (OAS2), transcript variant 3, mRNA.
RPS26L	-1.083	0.018358	400156	40S ribosomal protein S26-like (RPS26L), non-coding RNA.
IFI6	2.721	0.003798	2537	interferon, alpha-inducible protein 6 (IFI6), transcript variant 2, mRNA.
IRF7	1.338	0.009441	3665	interferon regulatory factor 7 (IRF7), transcript variant b, mRNA.
XAF1	1.672	0.014625	54739	XIAP associated factor 1 (XAF1), transcript variant 2, mRNA.
EPSTI1	1.800	0.168081	94240	epithelial stromal interaction 1 (breast) (EPSTI1), transcript variant 2, mRNA.
OAS1	2.253	6.50E-06	4938	2',5'-oligoadenylate synthetase 1, 40/46 kDa (OAS1), transcript variant 3, mRNA.
KIR3DS1	-1.887	0.033742	3813	killer cell immunoglobulin-like receptor, three domains, short cytoplasmic tail, 1 (KIR3DS1), mRNA.
LOC641768	-1.003	0.018482	641768	PREDICTED: similar to ribosomal protein S26, transcript variant 2 (LOC641768), mRNA.
LOC645979	-1.067	0.020944	645979	PREDICTED: similar to ribosomal protein S26 (LOC645979), mRNA.
LOC728823	-1.083	0.033742	728823	PREDICTED: misc_RNA (LOC728823), miscRNA.

^a logFC: log₂ R_{iRBC}/NR_{iRBC}. ^b adj.P.Val: Bonferroni corrected p. value.

Author's Publications

1. Costa, V. V., W. Ye, Q. Chen, P. Preiser, E. E. Ooi and J. Chen (2017). "Adhesion molecules on natural killer cell surface are essential for NK-mediated IFN- γ production and control of Dengue virus infection in humanized mice." Manuscript in Preparation. (Co-first author)
2. Chen, Q., W. Ye, W. Jian Tan, K. S. Mei Yong, M. Liu, S. Qi Tan, E. Loh, K. Te Chang, T. Chye Tan, P. R. Preiser and J. Chen (2015). "Delineation of Natural Killer Cell Differentiation from Myeloid Progenitors in Human." *Sci Rep* 5: 15118. (Co-first author)
3. Kong, T. F., W. Ye, W. K. Peng, H. W. Hou, Marcos, P. R. Preiser, N. T. Nguyen and J. Han (2015). "Enhancing malaria diagnosis through microfluidic cell enrichment and magnetic resonance relaxometry detection." *Sci Rep* 5: 11425. (Co-first author)
4. Chen, Q., A. Amaladoss, W. Ye, M. Liu, S. Dummer, F. Kong, L. H. Wong, H. L. Loo, E. Loh, S. Q. Tan, T. C. Tan, K. T. E. Chang, M. Dao, S. Suresh, P. R. Preiser and J. Chen (2014). "Human natural killer cells control *Plasmodium falciparum* infection by eliminating infected red blood cells." *Proc. Natl. Acad. Sci. U.S.A.* 111(4): 1479-1484.

Posters Presentation

1. 27th Annual Molecular Parasitology Meeting. Woods Hole, MA (USA). September 2016. Poster presentation: "Human NK cell variation and their control of *Plasmodium falciparum* infected RBC".
2. 8th International Singapore symposium of Immunology, Singapore, May 2016. Poster presentation: "NK cell variation and their control of *Plasmodium falciparum* infected RBC".
3. The fourth Singapore Malaria Network (SingMalNet) meeting. Singapore. February 2016. Poster presentation: "Yeast Surface Display of *Plasmodium falciparum* cDNA for vaccine candidates identification".
4. 25th Annual Molecular Parasitology Meeting. Woods Hole, MA (USA). September 2014. Poster presentation: "Control of *Plasmodium falciparum* Infection by Human NK Cells".
5. 7th APOCB congress, Singapore. Feb 2014. Poster presentation: "Control of *Plasmodium falciparum* Infection by Human NK Cells".

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