INVESTIGATION OF A NOVEL HOST-DIRECTED THERAPY FOR MALARIA THROUGH ENU MUTAGENESIS

By

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Summary

Malaria, caused by the *Plasmodium* parasite, remains one of the top deadly infectious diseases worldwide. Currently the only way to treat malaria is with drugs that are directed against the parasite. However, the development of resistant parasites to all these antimalarials is a growing concern. Therefore, new strategies are needed for drugs that are not affected by resistant parasites. A clue has been provided by so called “natural genetic antimalarials”. Over millennia, exposure to the parasite has led to the evolution of several genetic mutations in the human genome that offer stable protection against infection. Unfortunately many of these gene polymorphisms have been associated with other, often lethal, side-effects. Therefore, the aim of this thesis was to identify novel protective genes and biochemical pathways that may be targeted to provide a novel and more lasting host-directed therapy (HDT) for malaria.

We employed a large scale ENU (N-Ethyl-N-Nitrosourea) based gene-driven mutagenesis screen in a mouse model to assist in the discovery of potential new drug targets for an HDT. Several novel single point mutations in a number of gene candidates were identified and experiments were conducted to reveal the associated phenotype highlighting the biological function behind the affected gene. Further, the mode of action of the acquired resistance was examined and demonstrated. Overall, the work presented here contributes to a better understanding of host-parasite interactions in malaria. Further, this thesis offers proof of principle that targeting the host is an effective strategy in the discovery of a new generation of antimalarials that may not be limited by drug resistant parasites.

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21/03/2014

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Statement of ethical conduct

The research associated with this thesis abides by the international and Australian codes on human and animal experimentation and Institutional Biosafety Committees of the University.

Andreas Greth

21/03/2014

Publications and abstracts

The following abstracts of publications in peer-reviewed journals and presentations at conferences resulted from work in this thesis during my time as a PhD student at the Menzies Research Institute (University of Tasmania) and Australian School of Advanced Medicine (Macquarie University).

Publication

Greth, A., Lampkin, S., Mayura-Guru, P., Rodda, F., Drysdale, K., Roberts-Thomson, M., McMorran, B. J., Foote, S.J., Burgio, G.R. 2012, 'A novel ENU-mutation in ankyrin-1 disrupts malaria parasite maturation in red blood cells of mice', *PLoS One*, 7(6)**:** e38999.

Conference abstracts

Greth, A., Rowe, D., Hortle, E., McMorran, B., Burgio, G.R, Foote, S.J. 2010, *Investigation of an ENU-mutation affecting erythrocytes against malaria*, poster, XIIth International Congress of Parasitology Australia (ICOPA), Melbourne, Victoria.

Greth, A., Rowe, D., Hortle, E., McMorran, B.J., Burgio, G.R, Foote, S.J. 2010, *Investigation of an ENU-mutation in red blood cells combating malaria*, oral presentation, Sharing Excellence in Research Conference (SIER), Hobart, Tasmania.

Greth, A., Lampkin, S., Mayura-Guru, P., Rodda, F., Drysdale, K., Roberts-Thomson, M., McMorran, B.J., Foote, S.J., Burgio, G.R. 2012, *Investigation of a novel Ank-1 mutation through ENU-mutagenesis in red blood cells combating malaria*, poster, Molecular Approaches to Malaria Conference, Lorne, Victoria.

Greth, A., Lampkin, S., Mayura-Guru, P., Rodda, F., Drysdale, K., Roberts-Thomson, M., McMorran, B.J., Foote, S.J., Burgio, G.R. 2012, *A novel ENU-mutation in ankyrin-1 disrupts malaria parasite maturation in red blood cells of mice*, oral presentation, Australian Society for Parasitology Annual Conference, Launceston, Tasmania.

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Contributions

In accordance with the "Rules and Regulations" governing the degree of Doctor of Philosophy at Macquarie University, the following statements are made:

I assess my contribution to the work presented in each chapter to be as follows:

Chapter 2: 95%

- Dr Gaetan Burgio and Professor Simon Foote performed the ENU injections.
- Shelley Lampkin and Fleur Rodda were responsible for breeding the mouse colony.
- Preethi Mayura-Guru and Meredith Roberts-Thomson helped to analyse blood samples on the haemocytometer.
- Malarial infection in mice was performed by Shelley Lampkin, Fleur Rodda and Dr Gaetan Burgio.
- Dr Gaetan Burgio performed the statistical analysis that identified the critical interval containing the ankyrin-1 mutation.

Chapter 3: 95%

- Dr Gaetan Burgio and Professor Simon Foote performed the ENU injections.
- Shelley Lampkin, Emmaline Brown and Carlie Crawford were responsible for breeding the mouse colony as well as obtaining blood for full blood analysis.
- Bernardette Schnider, Ning Huang and Dr Gaetan Burgio analysed blood samples on the haemocytometer.
- Malarial infection in mice was performed by Shelley Lampkin and Dr Gaetan Burgio.
- Dr Denis Bauer performed the bioinformatics of the raw data resulting from exome sequencing.

Chapter 4: 90%

- Dr Gaetan Burgio and Professor Simon Foote performed the ENU injections.
- Shelley Lampkin, Emmaline Brown and Carlie Crawford were responsible for breeding the mouse colony as well as obtaining blood for full blood analysis.
- Bernardette Schnider, Ning Huang and Dr Gaetan Burgio analysed blood samples on the haemocytometer.
- Malarial infection in mice was performed by Shelley Lampkin and Dr Gaetan Burgio.
- Dr Denis Bauer performed the bioinformatics of the raw data resulting from exome sequencing.

Therefore, my overall contribution was greater than 90%.

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CONTENTS

Abbreviations

Ahctf1	AT hook containing transcription factor
AMA-1	Apical membrane antigen 1
Ank-1	Ankyrin-1
BSA	Bovine serum albumin
CM	Cerebral malaria
CR1	Complement receptor 1
CSA	chondroitin sulfate A
DNA	Deoxyribonucleic acid
DV	Digestive vacuole
EBA	Erythrocyte binding-like
ENU	N-ethyl-N-nitrosourea
EtOH	Ethanol
g	G-force
G6PD	Glucose 6 phosphate dehydrogenase
Hb	Haemoglobin
HDT	Host-directed therapy
HE	Hereditary elliptocytosis
HIV	Human immunodeficiency virus
HS	Hereditary spherocytosis
ICM	Inner membrane complex
iRBC	Infected red blood cells
ITN	Insecticide-treated mosquito nets
KAHRP	Knob-associated histidine-rich protein
kg	Kilogram
MCV	Mean corpuscular volume
MESA	Mature-parasite-infected erythrocyte surface antigen
mg	Milligram
ml	Millilitre
MSP	Merozoite binding proteins
MTRAP	Merozoite-specific thrombospondin-related anonymous protein
ng	Nanogram
NPP	New permeability pathway
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PECAM-1	Platelet endothelial cell adhesion molecule-1
*Pf*EMP1	*P. falciparum* erythrocyte membrane protein 1
PK	Pyruvate kinase

PSAC	Plasmodial surface anion channel
PV	Parasitophorous vacuole
PVM	Parasitophorous vacuole membrane
Rb	Retinoblastoma protein 1
RBC	Red blood cell
Rbl1	Retinoblastoma-like protein 1
RESA	Ring-infected erythrocyte surface antigen
REX1	Exported protein-1
RH	Reticulocyte binding homologue
RIS	Residual indoor spraying
RON	Rhoptry neck protein
rpm	revolutions per minute
SNP	Single nucleotide polymorphisms
TSP	thrombospondin
Tubb4a	Tubulin Beta 4A Class IVa
TUNEL	Terminal deoxynucleotidyl transferase dUTP Nick End Labelling
WHO	World Health Organisation
WT	Wild-type
µg	Microgram
µl	Microliter
µM	Micromolar

Chapter 1 – A REVIEW OF THE LITERATURE

Prevalence

Briefing

The development of resistant parasites to conventional drugs is a major hurdle in the fight against malaria, one of the most deadly infectious diseases human-kind has encountered. We therefore are in desperate need to explore novel avenues of treatments against the disease.

Numerous examples of red cell and other mutations that confer resistance have pointed to the host as a potential target for the design of resistance-proof antimalarials. This thesis is therefore dedicated to investigating novel genes and their products that may be of interest in the development of a so-called “host-directed therapy”. Through the use of ENU-mutagenesis, several potential genes are described and examined for their role in the pathology of malaria.

Malaria prevalence; the past and the present

Malaria is an infectious disease caused by the protozoan parasite *Plasmodium* that is transmitted through a mosquito vector. The term malaria is derived from the Italian mal'aria, “bad air” influenced by the original belief that the disease was caused by foul swamp odours (Carter and Mendis, 2002). About one third of the world's population currently live in malaria endemic regions, comprising 108 countries that congregate within the tropical and subtropical climate zone. Of these regions the highest prevalence is found in sub-Saharan Africa accounting alone for 85-90% of malaria incidences (White et al., 2014). The World Health Organisation (WHO) estimates an annual mortality rate of around 655,000 people, affecting mostly young children and pregnant women. Consequently the negative aspects of malaria not only affect global health but also have a profound socioeconomic impact in the developing world with limited access to the healthcare system.

Malaria has coexisted with humans for thousands of years. While it is thought that the ancestral form of malaria existed 2-3 million years ago (Rich et al., 2009) the modern parasite strains of *Plasmodium* probably emerged with the transition from hunter-gathered to

agricultural societies: 5,000-10,000 years ago (Harper and Armelagos, 2010). Ancient historical records dating back several thousand years noted that malaria was widespread around the shores of the Mediterranean, in southern Europe, across the Arabian Peninsula and in Central, South, and Southeast Asia, including China, Korea, and Japan. By the end of the 15th century, malaria had reached the New World and three centuries later the disease was common all over the globe, including southern European countries such as Italy and Greece and even in the most modern and industrialised cities of London, Paris, and even New York City, and Washington D.C. By the 19th century, malaria distribution had peaked, leaving over one-half of the world's population at risk of contracting the disease (Carter and Mendis, 2002). The impact of malaria in some parts of the world was exemplified during World War II. The disease claimed the lives of more troops deployed in the Solomons and Papa New Guinea than did bombs and bullets (McCoy, 1944).

The French army doctor, Charles L. A. Laveran, first identified the blood-borne protozoan parasite that caused malaria in the late 1800's, for which he was later awarded the Nobel Prize in Medicine. In the mid-20th century implementation of the first antimalarials, including quinine, and vector control measures using dichlorodiphenyltrichloroethane (DDT) resulted in a turning point of the devastating disease. Global mortality rates decreased to less than one-quarter and disappeared completely in the United States and from almost all of Europe. Thus, in the interim, "complete malaria eradication" became the lofty goal of the WHO (WHO 1946), a task which dismally failed. Despite global efforts, progress in malaria control has since been stagnant and clinical cases have once again been on the rise (Greenwood et al., 2008). Furthermore, Sachs and Malaney in 2002 (Sachs and Malaney, 2002) suggested that malaria incidents may double in the next 20 years. Reasons for that can be partly attributed to a variety of socioeconomic factors but increasing development of drug resistant parasite strains and insecticide resistant mosquitoes exacerbates the situation even more. The supply of new antimalarial compounds in the arduous pipeline of drug discovery and clinical trials remains sparse and the quest for an effective vaccine has resulted in very limited success. Clearly a new strategy is needed for treatment but also to overcome this lethal disease. To win this battle we need a better understanding in the complex nature of host-parasite interaction,

which will assist to explore new avenues for therapeutic intervention that may not be affected by parasite resistance.

Clinical spectrum of malaria

Malaria infections can cause a wide spectrum of clinical symptoms, from asymptomatic to effects that are often lethal (Menendez et al., 2000, Crawley et al., 2010). Factors including parasite species, host age, host genetics, and host immune status of the infected individual influence the severity and the progress of the disease.

The disease symptoms are characterised by periodic fever, chills, malaise, and joint pain that can be resolved into a mild attack and run an uncomplicated course. In certain cases, however, the disease progresses into life threatening severe malaria, including acute renal failure, respiratory distress, and metabolic acidosis (Haldar et al., 2007). Severe anaemia is diagnosed when the haemoglobin level falls under the concentration of < 5g/dl. An inadequate erythropoietic response coupled with peripheral destruction of parasitised erythrocytes and hypersplenism mediates a decrease in the red cell count (Lamikanra et al., 2007, Kai and Roberts, 2008, Haldar and Mohandas, 2009). Increased adherence of infected red blood cells (iRBC) to the vascular endothelium is another characteristic particular to *P. falciparum* infections and results in sequestration of red blood cells (RBCs) in a number of tissues such as the liver, heart, and brain. This erythrocyte sequestration results in the occlusions of the microvascular circulation leading to tissue hypoxia, which causes a syndrome called cerebral malaria; this causes coma from which the chance of recovery is poor (Ponsford et al., 2012).

Parasite biology

Plasmodium species

Malaria is caused by infections of the protozoan parasite *Plasmodium,* which belongs to the phylum Apicomplexa. It was first described in 1885 by Ettore Marchiafava and currently includes around 200 species. *Plasmodium* is known to infect a spectrum of vertebrates, including many birds, reptiles, rodents and primates (Perkins and Schall, 2002). Five species of *Plasmodium* are recognised to infect humans; *P. falciparum, P. vivax*, *P. malariae*, *P. knowlesi*, and *P. ovale. P. knowlesi* is a zoonotic parasite, whose primary vertebrate host is the macaque (a species of monkey). The other four principally infect humans. While all strains have the potential to induce serious and sometimes life-threatening symptoms, *P. falciparum* is the most lethal (Liu et al., 2010). The life cycle of *Plasmodium* is complex and requires two hosts – an insect transmission vector as the primary host, and a vertebrate host.

Life cycle of *Plasmodium* in humans

Infection of a human is initiated when sporozoites are injected underneath the subcutaneous layer by a bite from an infected female *Anopheles* mosquito (Figure 1.1). Within one hour around 50% of the sporozoites will migrate to the blood circulation and eventually to liver sinusoids. The fate of the remaining parasites in the dermis is unclear; they continue however to be capable of producing infective merozoites (Gueirard et al., 2010). Once the sporozoite reaches the liver, it passes from the sinusoid of the liver into the parenchyma, from where it migrates through several hepatocytes before infecting a final one (Mota et al., 2001). Over the next 6 days (in the case of *P. falciparum*) the sporozoite undergoes asexual differentiation and extensive mitotic replication (forming up to 30,000 new parasites per liver cell). The incubation time in the liver varies according to the *Plasmodium* and can range from days to years (as in the case for *P. vivax*). The symptomatically silent liver stage is an obligatory step during infection for the establishment and full development of sporozoites into merozoites. Cell egress from the now called merozoites involves the proteolytic parasite derived enzymes *P. falciparum* subtilisin-like protease 1 (*Pf*SUB1) and serine repeat antigen (SERA) (Yeoh et al., 2007). Additionally this results in the formation of merozoite-filled vesicles of host origin (merosomes). While still enclosed by host merozoite surface proteins (MSP-1) and thereby invisible from the immune system they make their way back into the circulatory system

(Graewe et al., 2011). Through a complex procedure, the extracellular merozoite invades its next target – the red blood cell (RBC). Once inside the RBC, the merozoite grows and undergoes a number of asexual divisions until the cell eventually ruptures and releases new merozoites capable of further rounds of invasion and multiplication. It is this stage of merozoite release that coincides with the characteristic periodic spells of fever and chills (Hill, 2006). At a certain point during the intracellular red cell stage some merozoites will differentiate into either male of female gametocyte forms of the parasites. These cells may be ingested by feeding mosquitoes (Hawking et al., 1971).

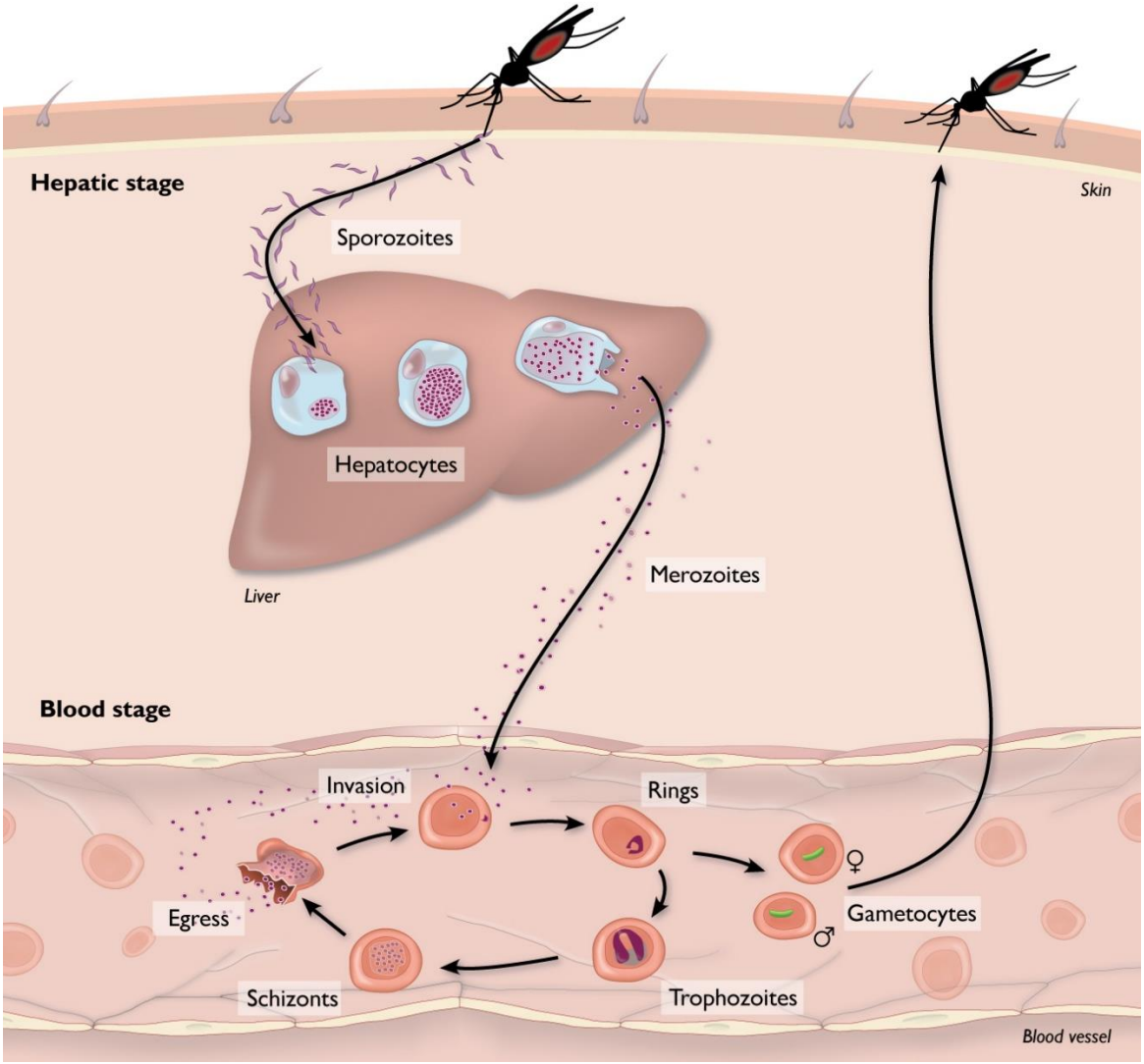


Figure 1.1 – Life cycle of Plasmodium species. The life cycle of *Plasmodium* in humans begins with a bite from an *Anopheles* female mosquito. The following pathological processes can be grossly divided into two stages: An asymptomatic hepatic or liver stage where sporozoites undergo their first asexual round of replication. This is followed by the migration of the now called merozoites to the circulatory system to begin their second, blood stage, which gives clinical symptoms. While the majority of parasites will continue to undergo asexual division a number will differentiate into gametocytes which are taken up by another blood feeding *Anopheles* mosquito. Within the insect vector gametes combine, undergo meiosis and subsequently differentiate into new, genetically unique sporozoites, ready to be injected back into another human individual *(illustrated by A. Greth, ASAM, 2013).*

Once in the mosquito gut gametes fuse to form zygotes, which subsequently develop into ookinetes, which can release up to several thousand new sporozoites (Boisson et al., 2011). From the body cavity the sporozoites migrate to the salivary glands of the mosquito, where they are prepared to be injected back into another human (Ghosh et al., 2011) (Figure 1.2). Genetic recombination, and hence generation of genetically variant parasites, also occurs during the mosquito stage of the life cycle.

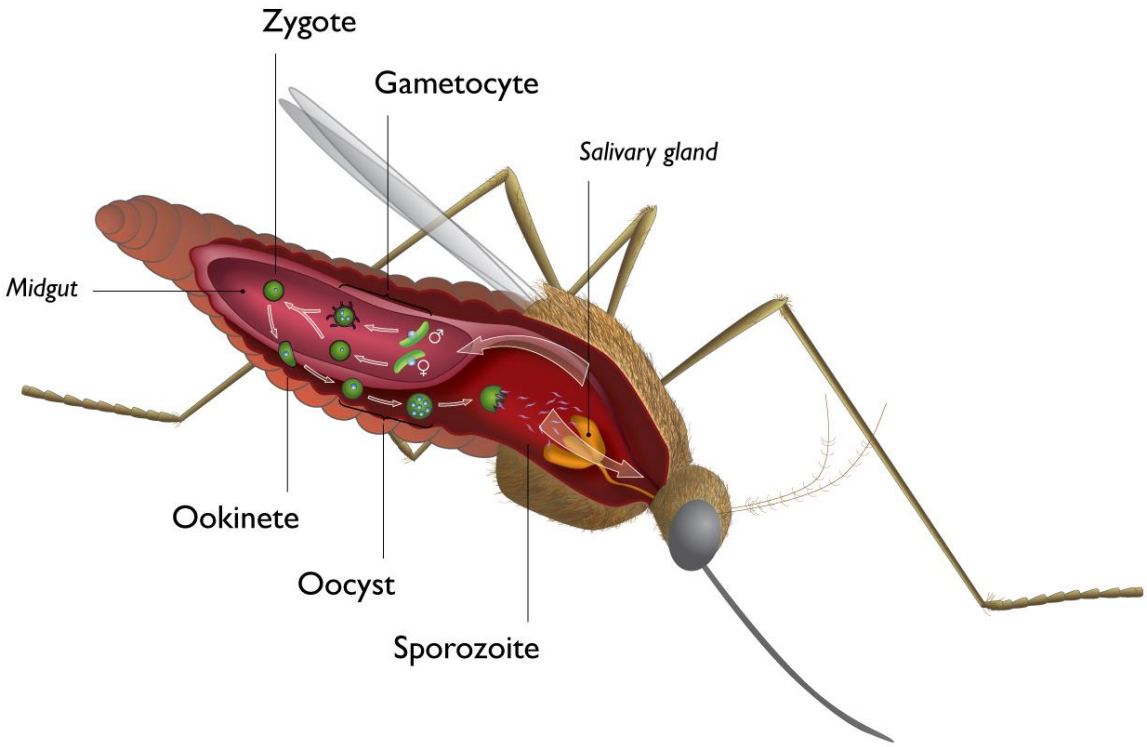


Figure 1.2 – Malaria parasite development in the mosquito. Once the gametocytes, the sexual stage of the malaria parasite, are ingested by a mosquito during a blood meal, they give rise to gametes that emerge and fertilise in the mosquito midgut. The resulting zygote develops into ookinetes that migrate across the epithelial layer to the outer surface of the midgut and develop into oocysts. The oocyst grows in size and divides repeatedly producing thousands of sporozoites, which eventually break out of the cell and invade the salivary glands of the insect. From there they are injected back into another host when the mosquito takes another blood meal (*illustrated by A. Greth, ASAM, 2013*).

The intraerythrocytic parasite and its interaction with the host

Following invasion of the RBC, the parasite undergoes a sequence of morphologically distinct stages (Figure 1.3). Specifically, immature parasites, otherwise known as ring stage parasites, mature into trophozoites, before they start their asexual replication, to become schizonts. A complete cycle of growth and replication in the red cell occurs in highly synchronous manner over a period of either 24, 48 or 72 hours depending on the parasite species. The asexual red cell life cycle of *P. falciparum* is 48 h. The rupture of the cell ends the erythrocytic cycle resulting in the release of 30-40 new merozoites back into the blood stream ready for another round of invasion (Miller et al., 1994). Throughout this cycle the parasite interacts with multiple host cell components, which are detailed below.

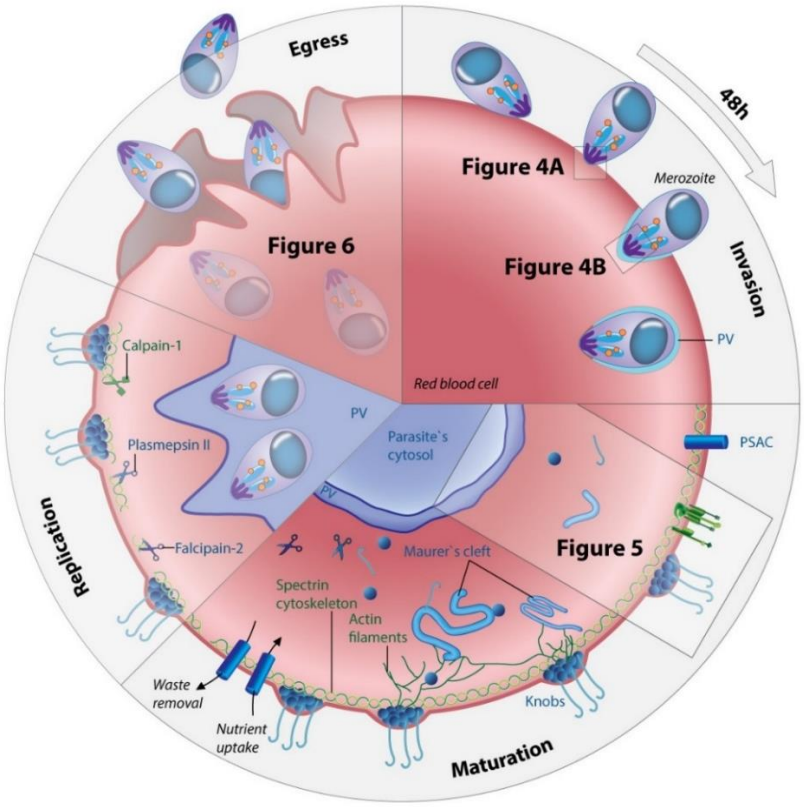


Figure 1.3 - The erythrocytic stage of *P. falciparum*. The erythrocytic stage relies on numerous interactions between parasite-derived (blue/purple), and host proteins (green). Invasion is initiated by irreversible attachment with the red blood cell (RBC) (Figure 1.4A and B). Once inside, the merozoite is surrounded by a parasitophorous vacuole (PV) protecting it from the cytosol. Throughout its development the intracellular parasite remodels the red blood cell (RBC) from the inside out. These include insertion of plasmodial surface anion channels (PSAC) into the cell membrane in order to transport toxic by-products and extracellular ions across the host cell membrane. Alongside this, exportation of numerous proteins across the PV into the cytosol and to the surface of the red cell is observed (Figure 1.5). About 40 hours after invasion the parasite starts to asexually replicate. Simultaneously the RBC cytoskeleton is disassembled assisting the newly formed merozoites to egress out of the cell (*illustrated by A. Greth, ASAM, 2013*).

The role of the red blood cell during parasite invasion

Invasion of RBCs by *P. falciparum* parasites occurs in four distinct phases: Adhesion, reorientation, tight-junction formation, and ingress. Primary contact between the merozoite and the RBC surface is crucial, as the parasite needs to differentiate between erythrocytes suitable for invasion and other cell types. This is mediated at least in part by reversible binding between merozoite binding proteins (MSP) on the parasite and red cell surface-expressed proteins, including Band 3; other unidentified host and parasite receptors are also likely to be involved (Goel et al., 2003). Once attached to the red cell, initiation of invasion requires that the merozoite reorientate so that its apical end points towards the erythrocyte. During this process the parasite derived apical membrane antigen-1 (AMA-1) appears to be crucial as antibodies specific to *P. falciparum* abolish reorientation of the merozoite but do not prevent attachment (Mitchell et al., 2004).

After initial interaction with the RBC, the parasite irreversibly attaches to the cell membrane via the erythrocyte binding-like (EBL) and *P. falciparum* reticulocyte binding protein homolog (*Pf*Rh) proteins, which are expressed at the apical end of the merozoite. Several studies indicate that certain members of parasite-expressed EBL and *Pf*Rh proteins bind to specific receptors on the surface of the RBC (Figure 1.4A). For example, erythrocyte-binding antigen (EBA)-175 binds to glycophorin A (Sim et al., 1994), and EBL-1 (Mayer et al., 2009) and EBA-140 (Lobo et al., 2003, Maier et al., 2003) interact with glycophorin B and C, respectively. Members of the *Pf*Rh protein family have been shown to bind to complement receptor (CR) 1 and basigin (Crosnier et al., 2011, Tham et al., 2010).

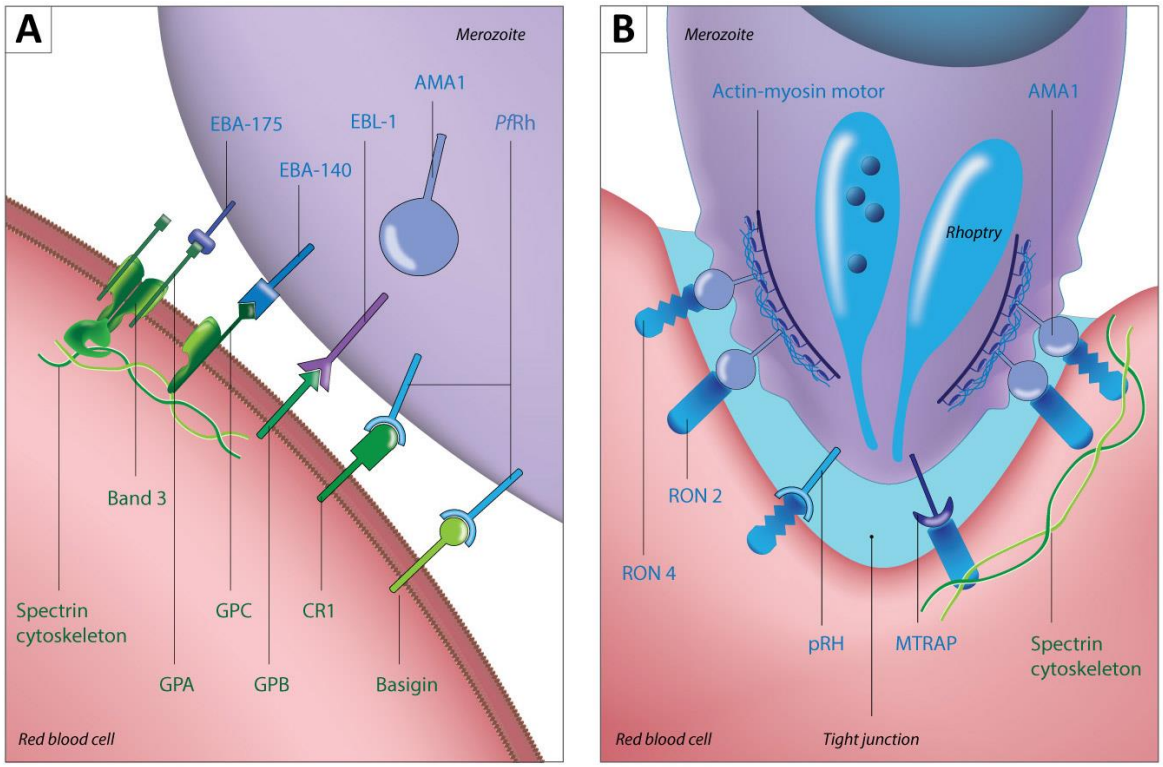


Figure 1.4 – The interaction between red cell and parasite during invasion of *P. falciparum*. The process of merozoite invasion is initiated by the binding between several parasite ligands (blue) to red blood cell (RBC) receptors (green) (Box A). The invading merozoite begins to drive its body into the cytosol of the cell. A set of protein-protein interactions, then mediate the formation of the tight or moving junction (Box B). Parasite derived rhoptry neck proteins (RON2, RON 4) are secreted into the RBC bilayer and bind to erythrocyte membrane proteins, *P. falciparum* reticulocyte binding protein homolog (*Pf*Rh), apical membrane antigen-1 (AMA1), and merozoite-specific thrombospondin-related anonymous protein (MTRAP). AMA1 coupled to an actin-myosin motor complex found within the parasite, generates the force required for parasite movement. As the parasite is propelled inwards, a parasitophorous vacuole (PV) is created by dragging the tight junction and the RBC membrane across the surface of the merozoite. Eventually the PV fuses, thereby completely engulfing the intracellular merozoite and separating it from the RBC cytosol (*illustrated by A. Greth, ASAM, 2013*).

After contact is formed between the apex of the parasite and the red cell surface, major buckling of the erythrocyte membrane is observed, possibly as a result of parasite-induced reorganisation of the RBC cytoskeleton (Zuccala and Baum, 2011). Cell entry commences with the formation of a set of protein-protein interactions that links the parasite and red cell membrane, creating a ring-like structure, otherwise known as the moving or tight junction (Aikawa et al., 1978) (Figure 1.4B). Along this process, secretion and insertion of parasitic proteins into the RBC bilayer is observed, with rhoptry neck proteins (RON) 2 and 4 being the prime candidates that are inserted into the red cell (Besteiro et al., 2011). These proteins provide a substrate onto which the merozoite can bridge across the two cellular membranes

via the proteins AMA-1, merozoite-specific thrombospondin-related anonymous protein (MTRAP) and pRH (Uchime et al., 2012, Thompson et al., 2004, Riglar et al., 2011, Baum et al., 2009). The basic mechanism that drives the cellular invasion process is mediated by an actin-myosin motor complex. A single headed myosin is attached to filamentous actin in the inner membrane complex (IMC) of the merozoite (Baum et al., 2006, Bullen et al., 2009, Opitz and Soldati, 2002), and generates force for movement, thereby driving the merozoite into the intracellular space of the RBC. As the merozoite is propelled inwards, a parasitophorous vacuole (PV) is created by dragging the tight junction and the erythrocyte membrane across the surface of the merozoite. Finally fusion of the PV and the host cell membrane separates the parasite from the red cell cytosol and creates a hospitable environment in which the parasite can develop (Riglar et al., 2011).

Parasite-erythrocyte interactions during intracellular development

Once the parasite has invaded the red cell, it begins to remodel the internal and external structure of the erythrocyte. This is believed to enable the parasite to achieve its basic nutritional requirements, mediate excretion of toxic molecules, as well as accommodate its increasing size as it grows (Figure 1.5). One source of vital amino acids is obtained by digesting host haemoglobin within the digestive vacuole (DV) (Loria et al., 1999). Haemoglobin digestion is accompanied by the polymerisation and subsequent detoxification of haem into an inert crystalline polymer called haemozoin (Loria et al., 1999). However, the parasite remains dependent on other nutrients, ions, and amino acids that are obtained from the serum. In order to obtain these extracellular sources, a so called new permeability pathway (NPP) is created. The NPP significantly increases the passive diffusion of monovalent cations from the red cell membrane to the plasma (Cobbold et al., 2011, Kirk and Horner, 1995, Martin and Kirk, 2007, Saliba and Kirk, 2001). In addition, the NPP serves as a conduit for the import of additional nutrients and exchange of toxic by-products, allowing developing merozoites to condition the intracellular ionic environment. The plasmodial surface anion channel (PSAC) mediates this transport and expression of two genes (*CLAG3.1*/ *3.2*) have recently been identified to be affiliated with PSAC (Nguitragool et al., 2011).

Additionally, the remodelling of the RBC is dependent on the establishment of a trafficking network that allows the distribution of parasitic proteins from within the parasitophorous vacuole membrane (PVM) to subcellular locations of the red cell. A key feature of this protein trafficking network is the formation of several exomembranous structures of various sizes (Trelka et al., 2000). This includes Maurer's clefts (Aikawa et al., 1986) which serve a role as a secretory organelle that concentrates virulence proteins for delivery to the erythrocyte bilayer (Lauer et al., 1997). At early ring-stage development they bud from the parasitophorous vacuole membrane (PVM) (Gruring et al., 2011) and migrate towards the RBC membrane, where they become physically tethered as the intracellular parasite matures (Waterkeyn et al., 2000, Hanssen et al., 2008). It appears that attachment of Maurer's clefts occurs through a network of host-derived actin filaments (Cyrklaff et al., 2011). Along with the establishment of a highly controlled protein transport system (collectively known as the *P. falciparum* translocon of exported proteins - PTEX), the intracellular parasite is able to selectively transport proteins to specific sites in the host cell.

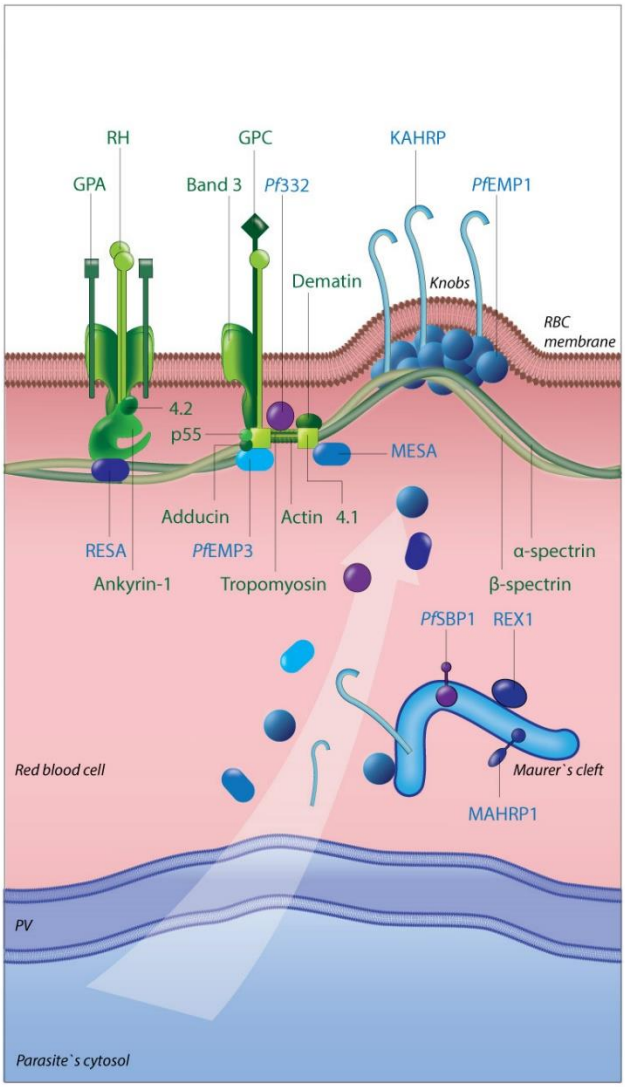


Figure 1.5 – Parasite and red cell interactions during the intraerythrocytic stage of *P. falciparum*. In order to transport merozoite proteins (blue) from the parasite's cytosol to various locations within the red blood cell (RBC), the parasite relies on the interaction with several host proteins (green). Originating from the parasitophorous vacuole (PV), exomembranous structures, known as Maurer's clefts are involved in trafficking of the cytoadherence-mediating knob-associated histidine-rich protein (KAHRP) and *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) to the cell's surface. Thesxe structures are tethered to the cell membrane by host derived actin filaments. The architecture of Maurer's clefts are maintained by resident proteins such as the ring exported protein 1 (REX1) and membrane-associated His-rich protein 1 (MAHRP1). *P. falciparum* skeleton binding protein 1 (PfSBP1) together with Pf332 and PfEMP3 appear to be involved in the trafficking process of PfEMP1. To restore and increase the rigidity of the iRBC ring-infected erythrocyte surface antigen (RESA) and mature-parasite-infected erythrocyte surface antigen (MESA) are associated with a number of cytoskeletal and membrane bound components. Thereby modulating p55 - 4.1 interaction but also replacing the continuous loss of tropomyosin and adducin, important in the stabilisation of the actin-spectrin cytoskeleton (illustrated by A. Greth, ASAM, 2013).

Several of the exported parasite proteins are known to alter the architecture of the RBC membrane, and modify the structural and adhesive properties of the cell. Approximately 16

hours following invasion, knob-like protrusions appear at the surface of iRBCs (Langreth et al., 1978, Gruenberg et al., 1983). These knobs are mainly composed of knob-associated histidine-rich protein (KAHRP), which binds to the host cytoskeleton (Pei et al., 2005, Oh et al., 1997). In the infected erythrocyte, these structures act as a platform for the presentation of an important cytoadhesive membrane-embedded protein called *P. falciparum* erythrocyte membrane protein 1 (*Pf*EMP1) (Maier et al., 2008, Kilejian, 1979). In order to direct *Pf*EMP1 to the RBC membrane a number of proteins localised with Maurer's clefts have been identified, including the ring skeleton-binding protein 1 (*Pf*SBP1) (Cooke et al., 2006) and membrane-associated histidine-rich protein 1 (MAHRP1) (Spycher et al., 2008). Similarly, the exported protein-1 (REX1) (Dixon et al., 2011) and *Pf*EMP3 (Waterkeyn et al., 2000) are thought to indirectly assist *Pf*EMP1 trafficking by stabilising the architecture of Maurer's clefts. Presentation of *Pf*EMP1 on the cell surface is crucial for parasite virulence. It enables the cytoadherence of the iRBC to endothelial and other cell types in the microvasculature (Magowan et al., 1988, Baruch et al., 1996, Rogerson et al., 1995). This property, which is unique to *P. falciparum* in humans but is also observed in some rodent malaria species, is believed to assist the parasite in escaping phagocytic clearance by the spleen. Therefore all the knob-associated proteins are regarded to be important virulence factors *in vivo* (Yamada et al., 1989, Aikawa et al., 1990, Wickham et al., 2001).

As a result of the interactions between parasite and host cytoskeletal and membrane proteins, the mechanistic properties of the RBC membrane are changed. While uninfected red cells are highly deformable, infected erythrocytes lose their elasticity and becoming increasingly spherocytic and rigid. The elastic properties are provided by the spectrin/ actin based cytoskeleton which is tethered to the cell membrane by the ankyrin-1/ band 3 and protein 4.1R/ p55 complex (Nunomura et al., 2000, Mohandas and Gallagher, 2008). Several parasite and host-derived molecules assisting in this process have been identified so far. Early after invasion, phosphorylation of band 3 is observed to promote the disconnection of the protein from the underlying cytoskeleton (Pantaleo et al., 2010). Subsequently plasmodial proteins become integrated into the submembranous protein network to restore and increase the rigidity on the iRBC (Hodder et al., 2009, Maier et al., 2008). Ring-infected erythrocyte surface antigen (RESA) is one such protein that binds to spectrin soon after invasion (Foley et al.,

1991), whereas the mature-parasite-infected erythrocyte surface antigen (MESA) may modulate 4.1R-p55 interaction at a later stage during parasite development (Waller et al., 2003), although this remains to be confirmed. Another protein called *Pf*332 may also function in this process since its deletion results in increased cell rigidity; it has also been associated in Maurer's cleft morphology and *Pf*EMP1 trafficking (Glenister et al., 2009).

Parasites-erythrocyte interactions during cell egress

After 48h inside the red cell, the *P. falciparum* schizont undergoes rupture and releases 30-40 new merozoites, which are each capable of infecting other RBCs. Although still under investigation, it has been suggest that during the event of cell egress the rupture of the PVM precedes the breakdown of the host cell membrane (Figure 1.6). In support for such a mechanism are two studies, which observed prior to the break-down of the RBC membrane, leakage of green fluorescent particles (GFP) into the cytoplasm of RBCs that have been infected with transient GFP-expressing parasites (Glushakova et al., 2010, Millholland et al., 2011b). In addition the destabilisation of the RBC cytoskeleton is another important step preceding cell egress. It appears that 10-15 hours after invasion the cell is primed by weakening of the host cytoskeleton, ready for dismantling by proteases and subsequent rupture. Evidence for the destabilisation of the cytoskeleton comes from work that reported a loss of adducin and tropomyosin in the iRBC results in the de-stabilisation of the spectrin/ actin/ 4.1 junction complex to band 3 (Millholland et al., 2011b). In order to continue the disassembly of the cytoskeleton, the parasite cysteine protease falcipain-2 (Dua et al., 2001, Hanspal et al., 2002) and the aspartic protease plasmepsin II (Le Bonniec et al., 1999) cleave protein 4.1, ankyrin-1, and spectrin. Additionally, *P. falciparum* recruits the host protease, calpain-1. Inhibition of calpain-1 prevents parasite egress *in vitro* (Chandramohanadas et al., 2009).

Overall the parasite interacts with numerous host proteins at the molecular level and many of them have been shown to be crucial for parasite development and propagation. As discussed below, it is therefore not surprising that modifications to certain host proteins that disturb such interactions could (and do so in some cases) impede parasite growth.

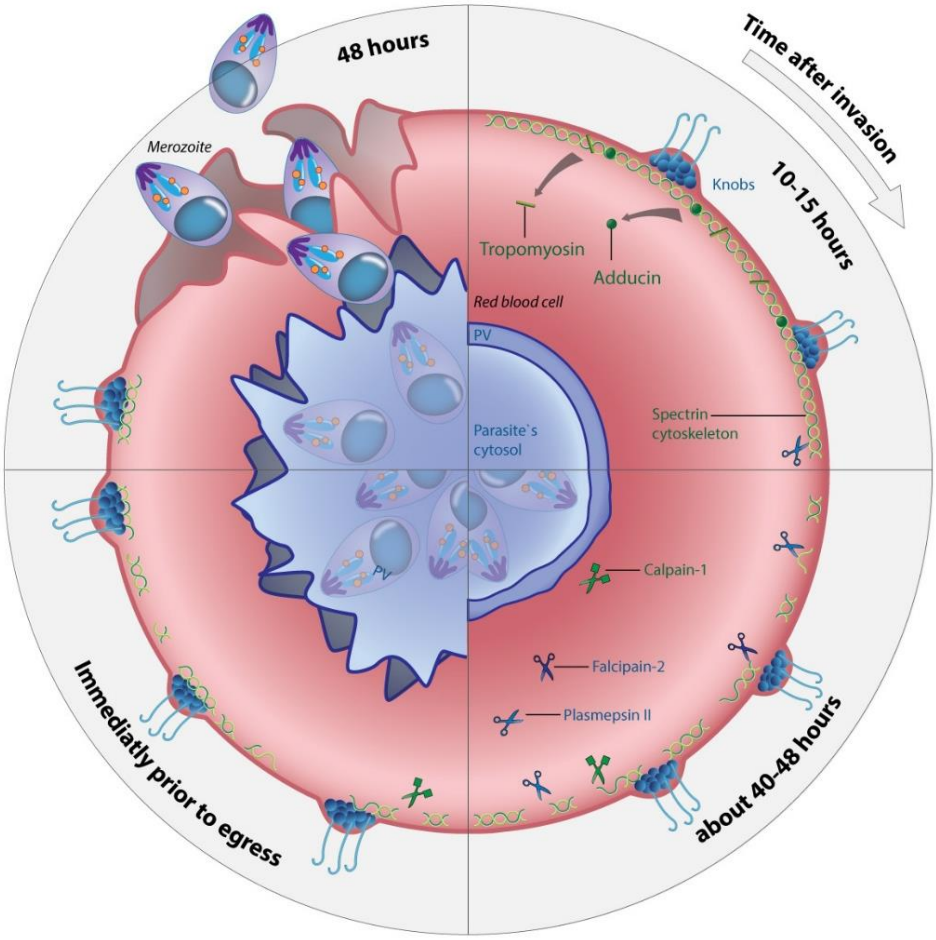


Figure 1.6 –*P. falciparum* and red cell interactions during cell egress. In order to successfully exit the red blood cell (RBC) after replication, the parasite begins to weaken the erythrocyte structure 10-15 hours after invasion. Thereby loss of the host proteins tropomyosin and adducin from the cytoskeleton is observed. This process is thought to assist the later disassembly of the actin/spectrin cytoskeleton by parasitic (blue) and host (green) proteases. Eventual egress probably occurs in a two-step process with rupturing of the parasitophorous vacuole (PV) occurring just prior to the breakdown of the RBC.

Malaria treatment

The origins of the treatment of malaria

The history of antimalarial drug development has its origins in a natural plant called *Chinchona*. It is unclear when it was first used for its medicinal properties, but it is thought to have first been discovered by the Aztecs many hundreds of years ago. However, it was not until the 1630s that *Chinchona* was introduced into European medicine (Dobson, 1998, Ballal et al., 2009) by Robert Talbor, who used the active ingredients from the bark in the treatment of malaria (Toovey, 2004). The active agent of this concoction was later identified as quinine by two French pharmacists, Pierre Joseph Pelletier and Joseph Bienaimé in 1820. For many years quinine was the first (and only) choice in the treatment of severe malaria until the synthesis of chloroquine in 1934 by Hans Andersag at the Bayer laboratories (Krafts et al., 2012). Another example of natural compounds used in the treatment against malaria is Artemisinin the most recently released antimalarial. It is derived from the Chinese plant, *Artemisia annua* L, and several derivatives including dihydroartemisinin, artemether, and artesunate have since been prepared from the plant (Krishna et al., 2008).

Antimalarials and the development of drug resistant parasite strains

The current range of antimalarial drugs are classified into four major groups: aminoquinolines (chloroquine, quinine, mefloquine, halofantrine, primaquine); anti-folate compounds (pyrimethamine, sulfadoxine, proguanil, chlorcycloguanil, dapsone); artemisinins (artemisinin, artesunate, artemether, arteether, dihydroartemisinin); and atovaquone. The control of malaria is hampered by the emergence of drug resistant parasites to all of these classes of antimalarials. In most cases the development of drug resistance has occurred quite soon after the drug has been introduced, and such strains have spread throughout the world with remarkable speed. For example, the first type of resistance to be acknowledged was to chloroquine in Thailand in 1957, which occurred only 12 years after its introduction. Since then resistance to chloroquine has spread throughout South America, Southeast Asia, and all of sub-Saharan Africa, where the drug has been reported to be no longer effective (Hyde, 2007). Similarly, resistance to artemisinin, the latest drug on the market, has been observed in western Cambodia in 2008, only 12 years after its official introduction outside China (Dondorp et al., 2009) and has spread since to neighbouring Thailand, Myanmar, and Vietnam (Phyo et

al., 2012). A list of some antimalarials, when they were introduced on a large-scale, and the year when resistance was first discovered can be found in Table 1. Mode of actions of several antimalarials and how the parasite circumnavigates this are listed below.

Table 1 – Time of antimalarial resistance development.

Drug	Drug class	Introduction	1st resistance reported	Difference (years)	Reference
Quinine	Aminoquinolines	1632	1910	278	(Wongsrichanalai et al., 2002)
Chloroquine	Aminoquinolines	1945	1957	12	(Wongsrichanalai et al., 2002)
Mefloquine	Aminoquinolines	1983	2002?	19	(Wongsrichanalai and Meshnick, 2008)
Halofantrine	Aminoquinolines	1990	1992	2	(Pradines et al., 2006)
Proguanil	Antifolate	1948	1949	1	(Wongsrichanalai et al., 2002)
Sulfadoxine-pyrimethamine	Antifolate	1967	1967	<1	(Wongsrichanalai et al., 2002)
Artesunate	Artemisinins	early 1990	2002?	~12	(Wongsrichanalai and Meshnick, 2008)
Artemisinin	Artemisinins	1996	2008	12	(Dondorp et al., 2009)
Artemether-Lumefantrine	Artemisinins	2008	2009	1	(Mizuno et al., 2009)

The year of introduction presents when the antimalarial was first used on a large-scale base. Adapted from (Wongsrichanalai et al., 2002).

Aminoquinolines: Mechanism of action and resistance

As discussed earlier, haemozoin formation in the DV as a consequence of haemoglobin degradation is essential for the continuing growth of the intracellular parasite. Therefore preventing this process is one mechanism to reduce parasite proliferation. Chloroquine prevents the formation of haemozoin by accumulating in the DV and thus allowing the build-up of toxic by-products that damages the parasite (Slater and Cerami, 1992, Sullivan et al., 1996).

Resistance to chloroquine occurs via the up-regulated expression and/or accumulation of mutations in parasite expressed transporter proteins that act to expel the drug from the parasite. This results in a reduced sensitivity of the parasite to the drug. Mutations have been identified in the ABC transporter *Pf*MDR1 (*P. falciparum* multidrug resistance analogue) (Martin et al., 2009, Vangapandu et al., 2007, Foote et al., 1989) and *P. falciparum* chloroquine resistance transporter (*Pf*CRT) (Reed et al., 2000, Fidock et al., 2000). *Pf*CRT is an integral membrane protein localized to the membrane of the parasite's internal DV. Compared to the wild-type form, mutant *Pf*CRT transports chloroquine at a faster rate, thus resulting in decreased accumulation of the drug at the site of action (Martin et al., 2009). In addition mutations in those transporters have been identified to provide cross-resistance to other antimalarials such as mefloquine (Cowman et al., 1994, Ochong et al., 2003, Reed et al., 2000, Cowman and Foote, 1990), quinine, halofantrine (Reed et al., 2000, Cowman and Foote, 1990), and even artemisinin (Duraisingh et al., 2000).

Antifolate: Mechanism of action and resistance

Folate is essential for a number of basic metabolic processes, in particular the biosynthesis of purines, pyrimidines, and amino acids. The parasite cannot utilise exogenous folic acid and instead employs a unique de novo synthetic pathway to obtain such folic acid derived metabolites (Vangapandu et al., 2007). Antifolate antimalarials are directed against the folate biosynthetic pathway depriving the parasite of essential folate co-factors. They are divided into 2 groups: The first group includes the sulfa drugs, sulfonamides and sulphones, and acts by targeting dihydropteroate synthetase (Zhang and Meshnick, 1991). The second group consists of pyrimethamine and cycloguanil competing with dihydrofolate, thereby inhibiting dihydrofolate reductase (DHFR) (Gregson and Plowe, 2005). Increased drug efficiency was achieved by the combination of pyrimethamine and sulphonamide and the combination has been in widespread use. However, evidence of clinical failure in the mid to late 60's suggested that the combined use might be an ineffective long-term solution (Jacobs et al., 1963, Harinasuta et al., 1967). Single point mutations in dihydropteroate synthetase (Wang et al., 1997) and DHFR (Cowman et al., 1988, Foote et al., 1990) have been found to be responsible for the acquired resistance.

Artemisinin: Mechanism of action and resistance

Artemisinin and its derivatives are a group of drugs that possess the most rapid and effective action of all current antimalarials against *P. falciparum* (White, 1997), and the use of artemisinin-based combination therapies are currently the most effective drug treatment (Fairhurst et al., 2012). The biologically active metabolite dihydroartemisinin targets the parasite during its intraerythrocytic stage. Although the molecular mechanism by which it kills the parasite has not been resolved (Kappe et al., 2010). It is thought that artemisinin exerts its antimalarial action by perturbing the parasite's redox homeostasis. One hypothesis is that it disrupts cellular redox cycling during haemoglobin degradation thereby inhibiting parasite growth (Haynes et al., 2011). Evidence comes from a number of *ex vivo* studies which have shown accumulation of haemoglobin with simultaneous inhibition of haemozoin production and membrane damage of the DV after treatment with artemisinin (Pandey et al., 1999, del Pilar Crespo et al., 2008, Klonis et al., 2011). Another theory is that the drug targets the parasite's own sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump (Eckstein-Ludwig et al., 2003) however this has been questioned by several studies (Cui et al., 2012, Cardi et al., 2010, Abiodun et al., 2013). Drug resistance has emerged in 2008 at the Thai-Cambodian border (Dondorp et al., 2009) and as a result the WHO has advocated the use of artemisinin-based combination therapies (WHO, 2012). Recent findings have identified some novel polymorphisms in *Pf*MDR1 and which have previously been associated with resistance to Aminoquinolines (Veiga et al., 2011). Other work demonstrated the association of mutations in *PF3D7_1343700* kelch propeller domain ('K13-propeller') with artemisinin resistance (Ariey et al., 2013).

The future of antimalarials

Due to the global spread of drug resistance the number of available and effective antimalarial drugs is declining rapidly. Therefore, there is an urgent need to expand our range of antimalarials that are not prone to the development of drug resistant parasite strains. Several strategies that assist in the development of new, more effective drugs have been proposed: 1) changing the strategy in the use of existing antimalarials by either a combinatorial or replacement/ rotation approach; 2) searching for antimalarial activity in drugs currently used to treat other infections or diseases; 3) chemical modifications of existing antimalarial

molecules; 4) exploring natural sources; 5) large-scale screening of chemical libraries; and 6) through parasite genome-based ("targeted") discovery (Grimberg and Mehlotra, 2011). For example, a trial for the combined use of Azithromycin and chloroquine in the treatment of uncomplicated malaria is underway and currently in Phase II/III trials (Chandra et al., 2013). In another case, a number of natural compounds with interesting antimalarial properties have been identified in several screening projects which have entered the early-stage discovery pipeline and are further reviewed by Olliaro et al. (Olliaro and Wells, 2009). Finally several large-scale drug screens have also revealed a large number of potential drug candidates. The chemical structures and associated data have been made publically available for the scientific community to further investigate (Gamo et al., 2010, Guiguemde et al., 2010, McNamara et al., 2013).

However these strategies are all parasite directed, and therefore do not address the problem of drug resistance. A rapid lifecycle in the human host that is under intense selective pressure, as well as sexual replication in the mosquito vector are driving forces for a high genetic variation in *Plasmodium*. This can lead to the development of resistant parasites that rapidly become dominant in a particular geographical region (Vangapandu et al., 2007, Castellini et al., 2011). Further concern is raised, since it has been shown (as discussed above) that a single point mutation is sufficient to render the parasite resistant to specific antimalarial therapies. Consequently, there is a need for new therapeutic strategies less prone to resistance.

Development of a vaccine for malaria

Vaccines have been highly successful in eliminating many infectious diseases such as measles, mumps, rubella and smallpox. An equivalent effective vaccine against malaria is regarded by many as a major alternative to novel drugs. Observations of acquired immunity in individuals exposed to *Plasmodium* suggest there is potential for the development of a successful candidate (Hommel, 1981). Unfortunately, natural acquired immunity is dependent on repeated and persistent infection occurring over the lifetime of an individual (Cohen et al., 1977, Marsh and Kinyanjui, 2006) and is often lost when leaving endemic areas (Taylor-Robinson, 2010). The first promising studies demonstrating the potential for a malaria vaccine were performed in 1967 by immunising mice with live, radiation-attenuated sporozoites

(Nussenzweig et al., 1969, Vanderberg, 2009). Since then several potential vaccine candidates have been evaluated but only one has reached phase III clinical trials (Langhorne et al., 2008). Glaxo-SmithKline's RTS,S/AS01 targets the circumsporozoite protein of *P. falciparum* and recent results showed that the vaccine conferred about 50% protection in children 5-7 month old and about 30% protection in 6-12 week old infants (Agnandji et al., 2011, Rts et al., 2012). However, a four-year study has shown that efficacy is declining over time (Olotu et al., 2013). Despite this limitation, RTS,S continues on the path to licensure due to its ability to reduce severe morbidity in young children at least to some degree.

Vector control and bednets

With the dwindling efficacy of antimalarials and no fully protective vaccine in sight, our primary control of the malaria burden is the use of insecticide-treated mosquito nets (ITN) and residual indoor spraying (RIS). It has been estimated that, when full distribution of ITN is achieved, it could reduce clinical episodes of malaria caused by *P. falciparum* and *P. vivax* infections by 50% on average (Lengeler, 2004). A growing concern is that the majority of ITN are treated with pyrethroids, therefore the success of global malaria control is mainly based on a single class of insecticide. This has resulted in increasing resistance of the mosquito vector to pyrethroids jeopardising malaria control efforts once more (Gu and Novak, 2009, Fane et al., 2012). This underlines again the importance for a novel strategy required to control the disease in the future.

Host responses during malarial infection

The host response plays a pivotal role in the outcome of a malaria infection and is both beneficial and detrimental in the pathology of the disease. The infection outcome can also be further modulated by genetic defects in the parasite and/or host. This section therefore provides a brief overview of two major components of the host response, namely immune and haematological and then focuses on several examples in how natural polymorphisms have induced host resistance to malaria.

Regulation of the immune system during infection

The host immune system is a combination of many biological processes, comprised of various cell types, tissues and molecules. In concert, they provide protection against pathogenic microbes and toxins (antigens). The immune system is divided into two-separately defined, but complimentary systems. They are called the innate and adaptive immune system (de Souza, 2014, Wright and Rayner, 2014). The more primitive, innate immune system, has evolved to provide immediate but non-specific attack at any site of infection. In malaria infection, the innate immune system is involved in controlling the initial wave of parasite replication. This is an immediate but broad attempt to try to reduce the peak in parasitaemia load. It is the first line of defence and provides the host with time to develop the more specific and adaptive responses that will be required for further clearance of the infection. In contrast, the adaptive immune system is highly sophisticated. It becomes activated once antigens have evaded or overcome the innate immune defences and provides a more precise antigen-specific response. Activation of the adaptive immune system also initiates acquired immunity through immunological memory to target specific antigens, leading to a stronger immune response in subsequent encounters. With respect to malaria, the adaptive immune system has been implicated in the development of acquired immunity in repeatedly infected individuals (Bull et al., 1998). Overall, protection against the symptomatic blood stage of malaria is thought to rely on a delicate balance between the two immune response systems, with a predominance of one over the other depending on the particular stage of infection.

Mechanisms of the innate immune response

Activation of the innate immune response occurs when malaria infected cells are recognised by dendritic cells. These cells initiate the production of the pro-inflammatory cytokine interleukin 12 (IL-12), which then promotes the differentiation of $CD4^+$ T helper cells into the $CD4^+$ Th1 subset (O'Garra and Arai, 2000). Early through infection, splenic $CD4^+$ Th1 cells are regarded as the major actors in the fight against malaria infection, manifested by an intense but short lasting appearance. This is followed by elimination of a large proportion of parasites after about a week (Muxel et al., 2011, Li et al., 2001). It has been shown that when $CD4^+$ Th1 cells have been depleted in infected mice, the result is hyper-parasitaemia and an inability to clear iRBCs (Podoba and Stevenson, 1991). Other pro-inflammatory cytokines, such as tumour necrosis factor α (TNF-α) and interferon-γ (IFN-γ), are also involved in the stimulation of acute phase reaction. Delayed production or absence of either cytokine has been implicated with a more severe course of infection and higher peak parasitaemia (Weiss et al., 2009, Butler et al., 2010). It has been proposed that IFN-γ and TNF-α promote early phagocytosis and clearance of iRBCs by macrophages (Li et al., 2003, Langhorne et al., 2004). IFN-γ is released by Th1 and natural killer (NK) cells resulting in the production of more TNF-α as well as several interleukins (IL-1, IL-2, and IL-6), nitric oxide (NO), and reactive oxygen species, all of which have the potential to kill parasites (Stevenson and Riley, 2004). Although NK cells are among the first to produce IFN-γ, their response is heterogenic and thus they are believed to bridge both the innate and adaptive immune responses (Korbel et al., 2005). Finally, platelets have been proposed to play a protective function in the early stage of infection. It has been shown that they preferentially bind to iRBCs over uninfected cells (McMorran et al., 2012). The killing of intraerythrocytic parasites by platelets occurs through the involvement of platelet factor 4 (PF4 or CXCL4) and the erythrocyte Duffy-antigen receptor (Fy).

Mechanisms of the adaptive immune response

A detrimental side effect of the innate immune response is that it often causes excessive inflammation, thus damaging host tissue. However, as the infection progresses, there is a switch from the pro-inflammatory Th1 to an anti-inflammatory Th2 response. This is associated with the adaptive immunes system (Li et al., 2001, Torre et al., 2002). Th2 cells do this by releasing the cytokines IL-4 and IL-10, which in turn also aid in stimulating B cells and

the production of antibodies. Expression of IL-4 by Th2 cells also results in the activation of basophil and mast cells, which mediate $CD4^+/CD8^+$ crosstalk and lead to the development of acquired immunity against malaria (Kumaratilake and Ferrante, 1992). Of the two cytokines, IL-10 is one of the key players in the anti-inflammatory response. Whilst it inhibits IFN-γ production in Th1 cells it does however not affect their proliferation. Moreover, it prevents T-cell priming and thereby decreases TNF-α and IL-6, as well as inducing B-cell proliferation and immunoglobulin production, which are essential for the development and maturation of antimalarial antibodies (Akdis and Blaser, 1999). Its importance has been shown in IL-10 knockout mice. These mice exhibit increased mortality in malaria infection and their mortality can be decreased by the inhibition of IFN-γ (Li et al., 1999a).

Regulation of the haematopoietic system during infection

One of the major causes of morbidity and mortality in severe malaria is anaemia, resulting from the reduction of total RBC mass in the blood stream (Lamikanra et al., 2007). It can be assumed that in the absence of host immune responses, rupture of RBCs by schizonts and the resulting release of merozoites would continue until the availability of uninfected red cells were depleted. However, in reality, parasite burden does not completely account for the degree of anaemia. Severe malarial anaemia (SMA) has been observed in humans with low parasitaemia and even after elimination of detectable parasites in the blood (Phillips et al., 1986). Two mechanisms have been proposed to explain this apparent paradox, (1) increased destruction of uninfected RBCs and (2) impaired erythropoietic responses.

It has been estimated that in acute and late stage malaria infection, about 90% of the RBCs destroyed are uninfected (Jakeman et al., 1999, Evans et al., 2006). In SMA, membrane modifications have been observed in both infected and uninfected RBCs, which is believed to result in increased red cell clearance by the spleen (Dondorp et al., 1997). There is evidence that this increase in destruction of red cells is the primary task of the reticulo-endothelial system (RES). This system consists of monocytes and phagocytic macrophages that accumulate in the spleen. As such, it has been demonstrated that in the acute phase of infection, RES is hyperactivated, involving IL-10 and thereby contributing to increased splenic clearance. Further there is evidence that the host ligand CD36 mediates phagocytosis of *P.*

falciparum-infected red cells (Patel et al., 2004). Thus, removal of uninfected erythrocytes, combined with haemolysis of iRBCs, significantly contributes to the anaemia in malarial infections.

Anaemia is further exacerbated by impaired erythropoietic responses as a result of insufficient or defective erythropoiesis. Dyserythropoiesis – the production of functionally and morphologically abnormal RBCs – has been found to occur in children with chronic infection (Abdalla et al., 1980), but not in those with acute infection (Chang and Stevenson, 2004). Its cause is believed to be the result of an overproduction in host cytokines (Robson and Weatherall, 2009, Miller et al., 1994). Similarly, a variety of host factors have been implicated in erythropoiesis suppression during malaria. Despite this, some studies have reported that levels of erythropoietin (EPO) – the growth factor responsible for inducing erythropoiesis – are normal in infected humans. Although there is some evidence that inadequate EPO production is linked to complicated malaria (Burgmann et al., 1996) and also malaria induced anaemia (Leowattana et al., 2008). Additionally, a recent study reported that host macrophage migration inhibitory factor (MIF) – in synergy with TNF-α and IFN-γ – impairs erythropoietin-dependent erythroid colony formation (McDevitt et al., 2006). A number of parasitic products are also known to have an effect on the deregulation of erythropoiesis. They include, *P. falciparum* haemozoin (*Pf*Hz), glycosylphosphatidylinositols (GPIs) and parasitic antigens released from ruptured iRBCs (Krishnegowda et al., 2005). Recent data shows that *Pf*Hz suppresses RBC production directly and also indirectly via phagocytosis of *Pf*Hz. It does this by affecting circulating monocytes, neutrophils and resident macrophages (Awandare et al., 2011, Shio et al., 2010), thereby altering the inflammatory mediator profile in favour of the parasite. Furthermore, *PfHz* decreases the activity of RANTES (regulated on activation, normal T cell expressed and secreted), which is an important immunoregulatory stimulator of erythropoiesis (Were et al., 2009). It can be assumed that a deregulation in erythropoietic response will likely alter the progression and ultimate outcome of the host to malaria infection.

Natural host resistance

Malaria is known to have placed one of the strongest selective forces on the human genome since the origin of agriculture 10,000 years ago (Kwiatkowski, 2005, Hedrick, 2011). As a result, many genetic mutations in the human genome have arisen because of their advantageous effects in malaria infection. Haldane (1949) originally recognised that genetic resistance to a disease was potentially an important evolutionary force in humans. He postulated that balanced polymorphism is the likely explanation for why thalassaemia had risen to high frequencies in certain populations (Haldane, 1949b, Canali, 2008). Since then several inherited gene mutations have been discovered that have fixed themselves in malaria endemic regions. The number of erythrocyte variants is remarkable, although this may not come as a surprise since the erythrocytic stage of the parasite plays a major part in the pathology of malaria. Host resistance however does not only involve red cells polymorphisms, but also genes involved in regulating the adaptive immune responses (Lopez et al., 2010). Nevertheless, this probably presents only the tip of the iceberg. Our knowledge about the effects of genetic resistance through evolution is rather limited, possibly because of the less profound phenotypic consequences of other polymorphisms, compared to the classic red cell variants. As our arsenal of antimalarials, due to resistant parasite, is rapidly declining could we exploit the insights of host resistance in our favour? For proof of concept, several common mutations that have been associated with malaria resistance are outlined below.

Haemoglobinopathies and thalassaemias

Haemoglobinopathies are structural haemoglobin variants while thalassaemias affect the synthesis of α- and β-globins in adult haemoglobin. Although common worldwide, the genetic disorders of haemoglobin occur most frequently in the malaria burdened countries of Sub-Saharan Africa and Asia (Weatherall, 2008). Examples of some haemoglobin disorders that have been associated with malaria resistance are discussed below.

There are hundreds of Hb variants that have been documented but only three, Haemoglobin C (HbC), Haemoglobin E (HbE), and Haemoglobin S (HbS) have been observed at substantial frequencies. The sickle cell trait (HbS) is regarded as the classical example of balanced polymorphisms (Hedrick, 2004). The allele that gives rise to sickle haemoglobin is maintained

at a frequency of 10-20% in many malaria-endemic regions (Hardison et al., 2002, Flint et al., 1998). The HbS allele is a single glutamic acid for valine substitution in the β-globin chain. It causes red cell deformation (sickling) when deoxygenated in heterozygous carriers (Wood and Granger, 2007). While homozygotes (HbSS) suffer a severe haemolytic syndrome, the heterozygous state is usually not associated with clinical abnormality. However, a beneficial effect of the affected allele is that it protects against severe malaria. It has been documented that the heterozygous allele confers approximately a 10-fold reduced risk of developing severe malaria (Hill et al., 1991). Ferreira et al. demonstrated that the protective mechanism is mainly associated with chronically elevated production of haem oxygenase-1 (HO-1) and decreased expression of inflammatory cerebral chemokines during infection (Ferreira et al., 2011). A more recent study reported that an atypical activity of the microRNAs, miR-451 and let-7i, in HbS and HbSS erythrocytes negatively regulates parasite growth (LaMonte et al., 2012). Similarly Cyrklaff et al. reported that *P. falciparum* parasites are unable of host actin polymerization within erythrocytes containing HbS (Cyrklaff et al., 2011).

Another haemoglobin variant associated with protection against malaria is HbC. Although mostly found in western Africa, it is less common than HbS. The responsible variant was identified in the β-globin chain (β6 Glu -> Lys) and causes a milder haematological phenotype at homozygosity than the HbS variant (Diallo et al., 2004). The rate of protection against malaria has been reported to be around 90% in homozygotes and 30% in heterozygotes (Modiano et al., 2001b). The protective effects of HbC have not been fully elucidated, but are thought to operate by increasing immune clearance of iRBCs due to reduced cytoadherence due to the occurrence of abnormal PfEMP1 expression on the red cell surface (Fairhurst et al., 2005, Cholera et al., 2008, Arie et al., 2005). Another hypothesis postulates that parasite growth is impaired in HbC cells because of an observed increase in hypoxic conditions and altered membrane structure of the infected cell (Fairhurst et al., 2003).

A third haemoglobinopathy that is associated with protection against malarial infection is HbE. It is found most commonly in Southeast Asia with carrier rates of 50% in areas of Cambodia and Thailand (Flatz, 1967). A lysine for glutamic acid substitution in the β-globin chain is responsible for microcytic red cells with decreased haemoglobin concentration in

homozygotes (Bunyaratvej et al., 1992a). In general, carriers with two HbE copies display a mild thalassaemia phenotype with symptomless anaemia whereas heterozygotes appear normal. There is *in vitro* evidence that HbE red cell variants offer innate resistance to malaria by impairing *P. falciparum* invasion (Chotivanich et al., 2002). However the molecular mechanism has not been resolved yet.

Thalassaemia comprises of a group of inherited blood disorders that affect production of either the α- or β-globin chain of haemoglobin. They are among the most common Mendelian diseases in human populations (Weatherall and Clegg, 2001). They include a broad spectrum of clinical phenotypes, reflecting a range of different known mutations (Lopez et al., 2010) and the existence of compound heterozygosity (Fucharoen et al., 2001). Generally, homozygous thalassaemia is often lethal while heterozygotes display only mild anaemia. This differs however if *HBA1* and *HBA2* are disrupted separately allowing some α-globin to be synthesised. This is known as α^+ thalassaemia, and homozygotes are only mildly anaemic (Fowkes and Day, 2008). Occurrence of both α- or β-thalassaemia highly correlates to regions where malaria is currently or was historically endemic (Flint et al., 1986, Weatherall and Clegg, 2001). Overall, it is believed that a reduction in expression of the α-globin chain caused by many different haplotypes induces an advantage in resistance to severe malaria. This is based on several studies that have demonstrated an association between α^+ thalassaemia individuals and protection against severe malaria (Williams et al., 2005, Mockenhaupt et al., 2004, Allen et al., 1997). The protective mechanisms however are unknown. While one *in vitro* study has provided some evidence for impaired parasite growth (Pattanapanyasat et al., 1999) another one demonstrated increased binding of antibodies from malaria-immune plasma (Williams et al., 2002). Another more recent proposal comes from Fowkes and colleagues who suggested that the increased number of microcytic red cells as seen in homozygotes may offset anaemia during severe infections (Fowkes and Day, 2008).

Most β-thalassaemias are caused by single point mutations or small deletions or insertions in the *HBB* gene. In contrast to *HBA* there is only one copy of the *HBB* and β^+ and β^0 thalassaemia refers to either the decrease or loss of the functional protein. Individuals with β-thalassaemia major, the most severe form, are often homozygous for β^0 and suffer from severe anaemia

and will require life-long blood transfusions to survive. Heterozygosity (β-thalassaemia minor) although not life-threatening, causes a mild to moderate anaemic phenotype with a risk of iron overload (Weatherall, 1997, Galanello and Origa, 2010). Several mechanisms for malarial protection have been proposed, including increased rate of phagocytised iRBCs (Ayi et al., 2004), prolonged production of foetal-haemoglobin in infants (Pasvol et al., 1976), elevated rate of antibody binding to affected red cells (Smith et al., 2002), and amplification of oxidative stress caused by the parasite thereby destabilising the host cell membrane (Pantaleo et al., 2012).

Red cell surface variants

The erythrocyte surface is an important site in parasite-host interaction. Erythrocytic invasion by the parasites involves a complex series of events involving multiple receptors found on the outside of the erythrocyte. It therefore comes as no surprise that some red cell surface protein variants have been associated with malaria protection.

One of the best understood and widely distributed examples of a red cell membrane protein variant conferring protection is the Duffy blood group antigen (Duffy). Duffy is a chemokine receptor, also known as the Duffy antigen receptor for chemokines (DARC), encoded by the *FY* gene. Duffy is expressed mostly on post-capillary endothelial cells but is also found on the surface of red cells (Mallinson et al., 1995). Surveys have shown that most populations in sub-Saharan Africa do not possess either of the two allele groups (*FYA** & *FY*B*), but a third known as the Duffy 'null' allele (*FY*0* – reaching near fixation) that results in the absence of the antigen on RBCs (Tournamille et al., 1995a, Hamblin et al., 2002, Howes et al., 2011). Duffy negativity prevents red cell invasion by *P. vivax* and is thought to account for the remarkable absence of that parasite species in most parts of Africa where other parasite strains are very common (Miller et al., 1976). Further, support for selective pressure at this locus comes from observations in Papua New Guinea where another polymorphism has been implicated in the decrease of antigen expression and resistance to *P. vivax (Zimmerman et al., 1999, Michon et al., 2001)*. Despite the importance of a functional Duffy antigen in the pathology of *P. vivax* infections, a recent report identified Duffy negative individuals infected with *P. vivax* in Madagascar (Menard et al., 2010). Although a profound decrease in the invasion efficiency

was still evident, they postulated that the diverse genetic makeup in this area may provide an environment for *P. vivax* to adapt to the *FY*0* host variant.

Unlike *P. vivax*, invasion of erythrocytes by *P. falciparum* merozoites appears to involve several red cell receptors that have an over-lapping redundancy. Therefore, no protective mechanism equivalent to Duffy exists for *P. falciparum*. However, a small number of common red cell surface protein variants have been associated with protection against infection. One example is the sialoglycoproteins glycophorin A and B. In 1983 it was documented that genetic deficiencies in glycophorin A (*GYPA*) and B (*GYPB*) lead to decreased invasion efficiency of *P. falciparum* (Facer, 1983). More recent work provided further evidence for a strong positive selection for either *GYPA* or *GYPB* in the human host possibly to evade malaria parasites (Wang et al., 2003).

The ABO blood group system is the most important blood type system in humans. A large study comprising several different regions in Africa reported an association between the ABO glycosyltransferase and risk of contracting severe malaria (Fry et al., 2008). This has led to the suggestion that the O group (loss of glycosyltransferase) provides a beneficial outcome in malaria attacks. Furthermore, there is evidence that the mechanism of resistance is linked to reduced rosetting of iRBCs, thereby leading to a better clinical outcome (Rowe et al., 2007).

Red cell membrane and cytoskeletal variants

It has been widely accepted that the RBC cytoskeleton plays an important role in the parasite-host interaction during intracellular growth of *P. falciparum* and several host proteins have been associated with increased resistance to malaria (Maier et al., 2009). One example is Band 3, which is encoded by the *SLC4A1* gene and a major transmembrane protein with anion exchange functionality in RBCs. A highly conserved 27 base-pair deletion in *SLC4A1* produces a mutant Band-3 protein which destabilises the cytoskeletal scaffold of the cell and results in a form of hereditary elliptocytosis (HE) known as Southeast Asian ovalocytosis (SAO). It is mainly found in people native to Malaysia and Papua New Guinea (Jarolim et al., 1991, Liu et al., 1990). Although it is homozygous lethal, heterozygotes are highly protected against cerebral malaria (Allen et al., 1999). It also appears that carriers face a reduced risk of infection

by both *P. falciparum* and *P. vivax* infections (Rosanas-Urgell et al., 2012, Kimura et al., 2002a). While the mechanism has yet been determined, it is believed to involve a decrease in cytoadherence between iRBCs and the vascular endothelium (Genton et al., 1995).

The cellular complexity of the red cell membrane and underlying cytoskeleton suggests that polymorphisms in other molecules might also contribute to an abnormal elliptical cell shape. Indeed the most common genetic defects in HE are in genes encoding α- and β-spectrin. The two polypeptides combine to form a heterodimer, which then combines with another dimer to form spectrin tetramers. These spectrin heterodimers play a major part in the horizontal structural linkage of the cytoskeleton (McMullin, 1999). Other mutations causing HE, although less common, are found in protein 4.1. Its purpose is to link the spectrin tetramers with actin to complete the cytoskeletal scaffold. In general, heterozygotes with mutations in either of those proteins are usually asymptomatic or display a mild anaemic phenotype. Severe haemolytic diseases can be seen in the homozygous state. HE is a worldwide disorder but more commonly found in malaria endemic regions of West Africa with a prevalence of around 2% (Mohandas and Gallagher, 2008). Based on what is known about the relationship between these host proteins and the parasite, it has been postulated that some of these mutations may confer resistance to malaria (Chishti et al., 1996, Schulman et al., 1990, Facer, 1995).

Ankyrin-1 is another major structural component in most cells including RBCs. In contrast to the spectrins and protein 4.1, it determines the vertical stability of the cell by linking the membrane with the underlying cytoskeleton. Mutations in ankyrin-1 are regarded as the major cause of hereditary spherocytosis (Eber et al., 1996b). Studies using mice, including one such animal which is a subject of this thesis, have shown protective roles for ankyrin-1 polymorphisms in malaria infections (Rank et al., 2009, Greth et al., 2012). This is discussed in more detail in chapter 3.

Enzymopathies

Glucose-6-phosphate dehydrogenase (G6PD) is an important enzyme that lies at the start of two major metabolic pathways - the Glycolysis and Pentose phosphate pathways - which are required to protect the RBC from oxidative damage by synthesising NADPH (Frank, 2005,

Cappellini and Fiorelli, 2008). As a result, G6PD deficient RBCs are more susceptible to oxidate stress because NADPH is no longer produced in sufficient quantities to protect the cell (Clarke et al., 2001). In general G6PD deficiency is asymptomatic, but certain factors that affect oxidative stress such as particular drugs (including some antimalarials), diet (fava beans), or infections may induce acute haemolytic anaemia (Piomelli, 1981, Cappellini and Fiorelli, 2008). It is encoded by the X-linked gene *G6PD* and deficiency in the enzyme affects over 400 million people worldwide (Nkhoma et al., 2009). The highest frequencies of G6PD deficiency are observed in Asia, Papa New Guinea, Africa, and the Mediterranean (Ruwende et al., 1995). Further it has been shown that the distribution of G6PD deficiency correlates positively with the degree of malaria transmission in these regions (Ganczakowski et al., 1995). In Gambian children, decreased enzyme activity was associated with a 50% reduced risk in contracting severe malaria (Ruwende et al., 1995). Although there is still uncertainty in the exact mechanism of resistance, impaired parasite growth (Wajcman and Galacteros, 2004), early phagocytosis of iRBCs (Cappadoro et al., 1998), and premature lysis of infected G6PD deficient red cells (Ruwende and Hill, 1998) have been proposed.

Another important enzyme in glycolysis is Pyruvate kinase (PK). It catalyses the last step in which pyruvate and ATP are formed, and is therefore required to maintain cellular energy (Chan and Sim, 2005). PK deficiency particularly affects cells that lack mitochondria such as RBCs since they must rely on anaerobic glycolysis as their sole source of energy. This affects the survival of red cells and causes them to distort and form small thorny projections, also known as echinocytes. PK deficiency is responsible for the second most common cause of enzyme-deficient haemolytic anaemia. Recent publications identified a global co-distribution pattern in sub-Saharan Africa between malaria and PK deficiency (Machado et al., 2012, Machado et al., 2010, Alves et al., 2010). Protection against malaria due to PK deficiency was initially demonstrated in a mouse study (Min-Oo et al., 2003) and later confirmed in *in vitro* studies with *P. falciparum* infected RBCs (Ayi et al., 2008, Durand and Coetzer, 2008). So far preferential clearance of iRBCs (Ayi et al., 2008) and impaired invasion (Min-Oo et al., 2003) have been suggested as possible resistance mechanisms.

Immune system variants

Several variants of immune response genes have been associated with protection against malaria infections. One example is the major histocompatibility complex (human leukocyte antigen – HLA), which is a set of cell surface molecules encoded by a large family of genes. It is divided into 3 main groups: class I, II, and III, and is involved in antigen presentation (Janeway and Medzhitov, 2002). Variants in the HLA complex have been shown to induce adaptive immunity to malaria (Hill et al., 1991, Hill et al., 1992). The Class I HLA antigen HLA-Bw53 mutation is more common in West Africa, compared with other parts of the world, and has been associated with decreased susceptibility to severe malaria (Gilbert et al., 1998, Hill et al., 1991, Hill et al., 1992).

Several other mutations were discovered in immunomodulating genes such as interleukins and interferon. Interleukins are important mediators of the inflammatory response to infection and or fever. Experimental studies of murine malaria have shown that disruption of interleukin-1 beta (IL1B) and interleukin-10 (IL10) results in increased disease severity including cerebral pathology (Li et al., 1999b, Touzani et al., 1999). In contrast, haplotypes in *IL1B* (Walley et al., 2004) and *IL10* (Wilson et al., 2005) have been found to be associated with reduction in malaria severity in Gambian natives. Interferon (IFN-y) encoded by the *IFNG* gene is a key immunological modulator and plays a beneficial role in the pathological outcome in malaria. Individuals heterozygous for the $IFNG_{1\text{-}56}$ polymorphism in the Mandinkas population (the largest Gambian ethnic group) were found to be protected against cerebral malaria (Koch et al., 2002). Finally two variants in Interferon-alpha/beta receptor alpha chain (*IFNAR1*) were found to inhibit *P. yoelii* blood-stage malaria by interfering with the production of its target cell, the reticulocyte (Vigario et al., 2001). Additionally there is proof that *IFNAR1* plays a protective determinant of the inflammatory response in CM from *P. falciparum* infection (Ball et al., 2013).

The enzyme nitric oxide synthase type 2 (NOS2) plays an important role in immune regulation by producing nitric oxide. Studies have demonstrated that increased levels of the free radicals provides the host with some protection against malaria (Perkins et al., 1999). However, protective polymorphisms in NOS2 are not that well defined. While studies in Gabonese (Kun

et al., 2001, Kun et al., 1998), Tanzania, and Kenyan children (Hobbs et al., 2002) have associated a number of allele variants with protection against severe malaria, other studies have failed to detect such associations (Levesque et al., 1999, Burgner et al., 2003). This has been further underpinned by recent work in Tanzanian children, where no link has been observed between polymorphisms in the NOS2 promoter and severity in the outcome of the malarial infection (Levesque et al., 2010).

The monocyte-derived cytokine, tumour necrosis factor-α (TNF-α), is a potent immunomodulator and proinflammatory cytokine that has been implicated in the pathogenesis of severe and cerebral malaria (Wilson et al., 1997). Linkage analysis of 34 families from Burkina Faso suggested that various single point polymorphisms (SNPs) within the TNF-promoter help to control parasitaemia during infection (Flori et al., 2005). Several other SNPs in the TNF-α gene have been independently associated with increased susceptibility and re-infection rates (Meyer et al., 2002, McGuire et al., 1999, Aidoo et al., 2001).

Localised on the surface of leukocytes and red cells, complement receptor 1 (CR-1) is responsible for complement-regulatory processes and for the removal of immune complexes (Reinagel et al., 1997). In *P. falciparum* infections CR1 has been shown to mediate the binding of iRBC to uninfected red cells to form rosettes, a parasite-induced mechanism believed to avoid the immune response and splenic clearance (Rowe et al., 1997). A study by Cockburn et al. demonstrated that polymorphisms in CR1 is associated with resistance to severe malaria (Cockburn et al., 2004). Further, there is evidence that decreased expression of CR1 protects against cerebral malaria (Thathy et al., 2005). However, these results were contradicted by several other studies reporting that there is no association between decreased CR1 levels and reduced severe malaria (Sinha et al., 2009, Zimmerman et al., 2003a, Nagayasu et al., 2001). Aside from this, there has been some evidence that CR1 interacts with *P. falciparum* reticulocyte-binding like homolog 4 (PfRh4) suggesting a possible role for the molecule in *P. falciparum* invasion (Tham et al., 2010).

Malaria in a mouse model

Due to the complex nature of disease susceptibility in humans, many aspects of our understanding of malaria pathogenesis, immunology and genetics have been derived by using mouse models. In comparison, genetic analysis in humans are often complicated by population heterogeneity, environmental factors and parasite polymorphisms (Manske et al., 2012). Although merely a model of the human disease, using the mouse in combination with rodent-specific *Plasmodium* parasites to study malaria and the role of host genetics presents several advantages. Mice have a close genetic resemblence to humans. The use of mice in a controlled environment allows the control over pathogen-derived variables such as parasite dose, route of infection, virulence status and strain of parasite (Fortin et al., 2002). Similarly, by using the many available inbred mouse strains, they provide the opportunity to study pathogenesis in a well defined genetic background (Bedell et al., 1997, Justice, 2000). Mice can be used in breeding programmes to facilitate the isolation of single gene variants by mutagenesis - either randomly or by gene targeting – to investigate genotype/ phenotype correlations. Mice are inexpensive and easy to maintain and the short gestation time of about 3 weeks allows the generation of informative progenies in large numbers for linkage analysis and positional cloning (Guenet, 2005). With the availability of the full genome sequence of the mouse (Mouse Genome Sequencing et al., 2002) and the use of freely accessible databases (i.e. Mouse Genome Informatics, Ensemble, NCBI, and UCSC Genome browser), the researcher has access to a vast amount of resources concerning polymorphisms, gene and protein expression. Together all these advantages render the mouse model a powerful and valuable tool for discovering novel host genetic factors that underlie susceptibility to malaria infection.

Utilising *P. chabaudi* to model human blood-stage infections

Four species of rodent *Plasmodium* have been used in mice to model malaria infection; *P. chabaudi*, *P. berghei*, *P. yoelii*, and *P. vinckei*. *P. berghei* is widely used as a model for CM although some scepticism exists as to whether the pathology in the mouse model is comparable to human CM (Langhorne et al., 2011, White et al., 2010, Stevenson et al., 2010). Further, *P. berghei* together with *P. yoelii* are also used to replicate *P. vivax* infections in people due to their preferential invasion of reticulocytes (Lamikanra et al., 2007). Another useful rodent-specific parasite species is *P. chabaudi*, which was isolated between the 1940s and

1960s from the thicket rat in Central Sub-Saharan Africa. The isolate has proved in many elegant experiments to be a worthy contender to model the blood stage in *P. falciparum* infections. A neat example is a study by McMorran et al. who identified a comparable innate response in regards to platelets during *P. chabaudi* and *P. falciparum* infections (McMorran et al., 2009). Indeed the model shares many similarities to its human counterpart, including a variety of analogous blood-stage antigens (Langhorne et al., 1989) and comparable innate and adaptive immune responses (Stevenson and Riley, 2004). Sequestration of iRBC with *P. chabaudi* is another phenomenon that is comparable with *P. falciparum*, although in the mouse this happens mainly in the spleen and liver and not in the brain as seen in humans (Cox et al., 1987). The parasite preferentially invades mature red cells but reticulocyte invasion is possible at high parasitaemia (Jarra and Brown, 1989). Finally, depending on the genetic origin of the host, *P. chabaudi* infections can result in either non-lethal or lethal infections (Eugui and Allison, 1980, Stevenson et al., 1982). To study the blood stage of infection, *P. chabaudi* is inoculated into mice by administration of a predetermined dose of iRBC from infected donor animals, either intra-venously or intra-peritoneally. However, it is important to note that there are some marked differences in the pathological dynamics between *P. chabaudi* infections in the laboratory mouse and *P. falciparum* infections in humans. For example, in the mouse a higher level of circulating parasites is required to observe pathogenic symptoms. In blood-stage infections with *P. chabaudi* a rapid proliferation of iRBC is evident and usually peaks within 7-9 days with 30% iRBC (Jarra and Brown, 1985) or higher depending on the dose, route of infection, and genetic background of the mouse. At this point 'resistant' mice usually develop a strong immune, inflammatory and erythropoietic response resulting in the elimination of parasites by days 21-28. In susceptible mice, however, parasite proliferation continues leading to death due to severe anaemia by day 10-13 (Fortin et al., 2002). In contrast, maximum parasitaemia in humans with *P. falciparum* typically does not exceed 5% (Collins and Jeffery, 1999) and all-cause mortality rates are around 0.1-1% (Snow et al., 1999, Alles et al., 1998). Nevertheless much of our understanding on the intricacies of host responses to *P. chabaudi* can be extrapolated to *P. falciparum* infections and vice versa; it is therefore a good model to study human malaria.

Genetics of host resistance in inbred mouse strains

Inbred strains of mice vary in their susceptibility to malaria. This includes differences in both the rate of iRBC proliferation (Figure 1.7) and whether they survive the infection or not. A study in 2004 by Hernandez-Valladares and colleagues highlighted this by infecting susceptible A/J and resistant C57BL/6J mice with several strains of *P. chabaudi* differing in their virulence. They found that survival was generally dependent on the genetic background of the mouse strain and not parasite isolate (Hernandez-Valladares et al., 2004b). Similarly, Fortin et al. as well as previous work in our laboratory demonstrated a reduced resistance in C3H/HeJ and SJL mice compared to C57BL/6J (Fortin et al., 2002, Foote et al., 1997b).

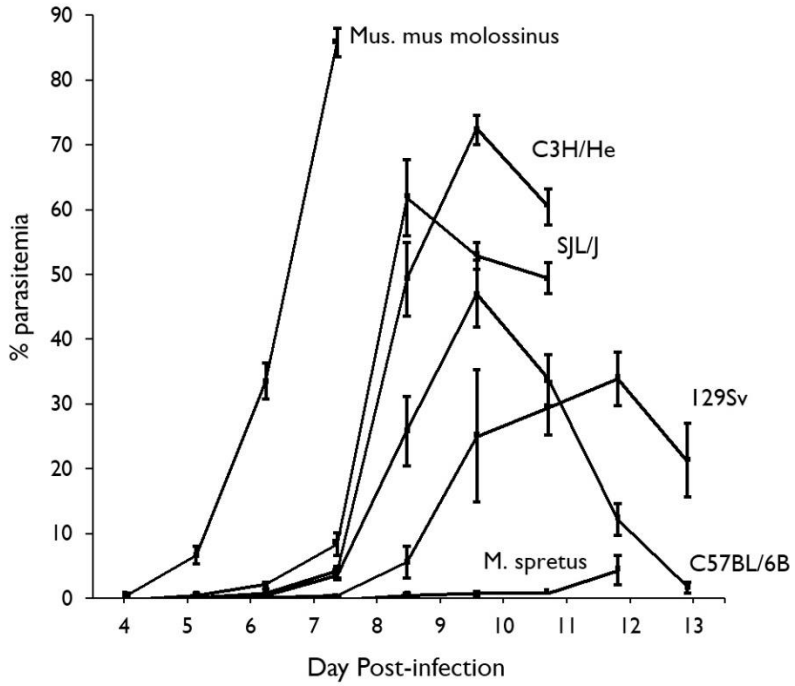


Figure 1.7 – Comparison of malaria susceptibility in different mouse strains. Several inbred mouse strains were infected with *P. chabaudi* parasitised erythrocytes and parasitaemia was monitored by daily blood smears. A difference in susceptibility to malaria is evident by the varying levels of parasitaemia and survival depending on the genetic background of the mouse (Unpublished data by Vikki Marshall and Simon Foote, WEHI).

These observations imply that the susceptibility to infection is governed by the genetic make-up of the host. The genetic basis is rather complex, and although not fully resolved, crosses between resistant and susceptible mouse strains have led to the identification of several

chromosomal regions associated with resistance to malaria (Table 2). These regions are known as quantitative trait loci (QTL) and are termed *chabaudi* resistance (*char*) loci, and *berghei* resistance loci (*berr*) depending on which parasite strain resistance is observed for. For example, genome-wide linkage analyses on the F2 progenies of crosses between the two susceptible strains, SJL and C3H/He, and the resistant strain C57BL/6 assisted in the discovery of *char1*. The locus which determined the ultimate outcome of infected mice was mapped to chromosome 9 (Foote et al., 1997a). Similarly, *char2*, controlling parasite density, was mapped to chromosome 8 (Foote et al., 1997a, Fortin et al., 1997b). In another instance, linkage analyses of mice that have inherited one allele from susceptible A/J and one from resistant C57BL/6J mice revealed a region on chromosome 3, termed *char4*. Sequencing of candidate genes in that locus pointed to the underlying gene responsible for the associated resistance (Min-Oo et al., 2003). The gene was identified as pyruvate kinase (PK) and led to the proposal that PK deficiency manifests malaria resistance by inducing haemolytic anaemia that in turn up-regulates compensatory erythropoiesis (Min-Oo et al., 2004). The existence of genetic differences between inbred strains is a crucial factor allowing the use of mouse models to uncover novel genetic host components in malaria.

Table 2 – Genetic loci linked to resistance and their associated phenotype in murine malaria infections.

Parasite strain	Locus	Phenotype	Chromosome	LOD or χ^2	Reference
P. chabaudi	*Char1*	Parasitaemia	9	6.6/ 9.1	(Foote et al., 1997a)
	Char2	Parasitaemia	8	8.83	(Foote et al., 1997a, Fortin et al., 1997b)
	Char3	Parasitaemia	17	5	(Burt, 1999)
	Char4	Parasitaemia	3	6.57	(Fortin et al., 2001)
	Char5	Parasitaemia	5	2.16	(Hernandez-Valladares et al., 2004a)
	Char6	Parasitaemia	5	2.16	(Hernandez-Valladares et al., 2004a)
	Char7	Parasitaemia	17	5.75	(Hernandez-Valladares et al., 2004a)
	Char8	Parasitaemia	11	1.9	(Hernandez-Valladares et al., 2004c)
	Char9	Parasitaemia	10	4.74	(Min-Oo et al., 2007)
	Char10	Parasitaemia	9	7.24	(Min-Oo et al., 2010)
	Char11	Parasitaemia	9	4.26	(Laroque et al., 2012)
P. berghei	*Berr1*	Survival	1	$\chi^2 = 18.98$	(Bagot et al., 2002)
	Berr2	Survival	11	$\chi^2 = 16.51$	(Bagot et al., 2002)
	cmsc	Survival	17	$\chi^2 = 26.18$	(Ohno and Nishimura, 2004)
	Berr3	Parasitaemia	9	$\chi^2 = 4.9$	(Campino et al., 2005)
	Berr4	Parasitaemia	14	$\chi^2 = 3.42$	(Campino et al., 2005)
	Berr5	Survival	19	$\chi^2 = 4.69$	(Berghout et al., 2010a)
	Berr6	Survival	4	6.7	(Torre et al., 2013)
	Berr7	Survival	1	4.03	(Torre et al., 2013)
	Berr9	Survival	9	4.9	(Bopp et al., 2013)

Adapted from (Longley et al., 2011).

Host-directed therapy

Therapeutic interventions to combat infectious diseases such as malaria have focused on targeting parasite derived components. Based on evolutionary strategies an alternative way is to target host components that are required by the parasite to fulfil its life-cycle, also known as host-directed therapy (HDT). The rationale for this approach in prohibiting drug resistant parasite development is highlighted by highly effective examples of genetically acquired host resistance to malaria (see section "Natural host resistance"). Additionally, major advancements in HDT have been made in other types of infectious diseases that added proof of concept for the feasibility of such an approach against malarial infections. Some examples are outlined below.

Host-directed anti-viral therapy

An HDT approach has been successfully used to combat several different types of pathogenic viruses. Gleevec (Imatinib mesylate) is an Ab1 tyrosine kinase inhibitor (TKI) and exemplifies the benefits of a host-targeting anti-viral strategy. Originally designed to treat multiple cancers, recent studies pointed to an additional anti-viral effect of the drug (Kee and Zalcberg, 2012). Knowledge that the viral protein A36R of the vaccinia virus, requires phosphorylation by Ab1 receptor tyrosine kinase led to the discovery of Gleevec as a possible treatment against the virus. Its high efficacy has been demonstrated in a *in vivo* study which showed that it not only decreases virus titer but also increased survival in infected mice (Reeves et al., 2005). Another host-directed anti-viral drug is Celgosivir. It prevents the folding of Hepatitis C virus (HCV) envelope proteins through inhibiting of host endoplasmic reticulum glycosidase. This results in reduced morphogenesis and infectivity. Encouraging phase II data have recently been disclosed (Durantel et al., 2007).

Human immunodeficiency virus (HIV) is a lentivirus that causes acquired immunodeficiency syndrome (AIDS), a condition that results in progressive failure of the immune system (Weiss, 1993). HIV is unique in the way that it expresses high genetic variability (Rambaut et al., 2004). Consequently, rapid emergence of resistance to conventional drugs is a common phenomenon similar to what is seen in malaria (Pingen et al., 2011). However, a number of host-cell targets required by the virus have been identified including HMG-CoA reductase (del

Real et al., 2004), an enzyme involved in the cholesterol biosynthetic pathway. Currently there exists a whole class of licensed drugs, known as statins that inhibit the activity of this enzyme. Although originally designed to prevent cardio-vascular disease (Linninger et al., 2009), up-regulation of several genes encoding substrates of the enzyme in HIV patients suggested a putative anti-HIV effect in statins. In a ground-breaking study, del Real et al. presented data where 6 patients were treated with statins for one month and all showed more than three-fold reduction in viral load (del Real et al., 2004). They proposed that inhibition of HMG-CoA reductase disrupts Rho activity resulting in the prohibition of gp120 induced cell entry by the virus. Lovastatin is one member of the statins and currently in phase II clinical trials (Montoya et al., 2012). In a similar fashion CCR5 chemokine receptors interact with the viral protein gp120 (Ferain et al., 2011) enabling cell invasion of HIV (Lalani et al., 1999). Several CCR5 antagonists have been designed to inhibit enzyme function, of which Maraviroc received full approval by the US Food and Drug Administration in 2007 (Krauskopf, 2007). However, a drawback of CCR5 inhibitors is that different HIV strains uses other means of cell entry and resistance to Maraviroc has already been observed in some patients (Lederman et al., 2006). Nevertheless, other host targets are at various stages through pre-clinical and clinical trials.

Host-directed therapy in bacterial infections

Antibiotic resistant bacterial infections are perhaps the best-known example of drug resistance. Today the problem has reached heights that have prompted the WHO to call this one of the biggest threats to human health today (WHO 2012). Several host targets are currently being exploited in attempt to combat drug resistant pathogenic bacteria. This includes treatment of *Pseudomonas aeruginosa* (Calfee et al., 2001), *Listeria monocytogenes* (O'Riordan et al., 2003), and *Myobacterium tuberculosis*. Particularly in the treatment of Tuberculosis, HDT is receiving increasing attention. For example, it has been proposed that *M. tuberculosis* requires host isocitrate lyase to catabolise fatty acids as a carbon source for intracellular growth and virulence. Successfully prohibiting *in vivo* growth of the bacterium with an isocitrate lyase inhibitor (Munoz-Elias and McKinney, 2005) points to a possible drug target for an HDT. Another approach was taken by Kuijl et al. who examined host kinase AKT1 as a potential target. By using several AKT1 inhibitors they were able to prevent the growth of multidrug resistant *Myobacterium tuberculosis* (Kuijl et al., 2007). In an effort to assist

conventional therapies Maiga and colleagues used successfully inhibitors of host phosphodiesterase Pd3 (Maiga et al., 2012).

Another host factor identified to have a wide therapeutic use is iron. The general hypothesis is that microorganisms will be less able to multiply and cause infections if iron is restricted in the host. The underlying concept is based on the almost universal requirement of iron in microorganisms (Weinberg, 1978, Lankford, 1973). Therapeutic strategies with iron chelators have been studied in animal models of *M. tuberculosis*, but also *in vitro* with *Escherichia coli*, *Salmonella*, *Pseudomonas*, and several other microbes (van Asbeck et al., 1983). Additionally, iron chelating agents have been examined for their antimalarial capacity, although the protective effect in humans was rather transient (Golenser et al., 2006).

In summary, the examples described above show that targeting host-cell processes has great potential in designing next generation therapies for the treatment of infectious diseases. Combined with advances in our understanding of host-pathogen interactions, the coordinated use of unbiased large-scale genomic and proteomic methods will likely further unlock the large potential of unidentified host targets.

Is host-directed therapy the answer to a resistance-proof therapeutic strategy?

With the increasing development of drug-resistant parasites, the battle against malaria is becoming progressively problematic. The need for a novel resistance-proof strategy is more essential than ever before. *Plasmodium* parasites rely on a number of host factors in order to be infectious in an otherwise hostile environment. As such, more creative treatment solutions that limit host resources needed by the parasite may be one possible way to circumvent the emergence of resistant parasites. While the high genetic variability in parasites can render useless the efficacy of a particular drug target, the need to significantly alter its entire infection strategy in order to compensate for a missing host factor through natural selective variation is a much bigger obstacle. Thus, since host factors lie outside the genetic control of the parasite, HDT may provide a novel way to address the resistance problem. Evidence for this is also shown by the striking difference in the speed at which parasites have become resistant to conventional drugs and the time host genetic mutations have offered resistance to the

disease (discussed in “Natural host resistance”). Nevertheless, it should be acknowledged that disruption of host elements is more prone to interfere with important biological functions in the host leading to increased side effects. This paradigm is evident in many of the identified host variants influencing susceptibility to malaria. However, there may be other mutations that provide protection, but leave no pathological ‘footprint’ and therefore remain undiscovered. In addition, several present successes in other infectious diseases (Durantel et al., 2007, Montoya et al., 2012) show proof of concept that an HDT can be a potent tool in the fight against malaria. The discovery of novel host factors essential to the *Plasmodium* life cycle will not only deepen our understanding of host-parasite interactions but also bring us a step closer in the development of a next generation HDT against the disease.

ENU mutagenesis; a way forward in the search for novel host targets

As discussed above, targeting the host instead of the parasite, could be a way to avoid the problem of parasite resistance that has reduced the efficacy of the classical antimalarials. However, many of the protecting host genetics identified so far come at a cost by inducing other, often life-threatening side effects and are therefore not ideal to mimic by an HDT. In order to further investigate the genetic control of host responses to malarial infection, N-ethyl-N-nitrosourea (ENU) mutagenesis can be used as a tool to induce random genetic mutations into mice. ENU treated mice may be screened for malaria susceptibility or another phenotype of interest. Animals that demonstrate the relevant trait can then be sequenced to identify the responsible mutation.

Ideal host targets for HDT should be selected on the basis that they don`t interfere with crucial biological processes, but still alleviate parasitaemia by positive host modulation. While genome-wide association studies using a variety of crosses have set the baseline in uncovering loci associated with a malaria resistant phenotype, most studies have been unsuccessful in identifying causative genes. Moreover, these types of studies are limited by the number of mouse strains available and limited amount of genetic heterogeneity between inbred mouse lines (Richer et al., 2008). Another approach is to conduct a reverse genetic screen. This strategy relies on previous knowledge about the genetic sequence of a given phenotype and is therefore not optimal for the search of novel host factors. In contrast, forward genetics -

otherwise known as a phenotype based screen - is an approach used to identify genes responsible for a particular phenotype of an organism. The underlying principle it is that there is no preconception about the genetic control of the disease. It allows the unbiased screening of an entire genome for the gene causing the trait of interest (Justice et al., 1999, Richer et al., 2008) and is therefore an efficient method for revealing novel genes. Many centres around the globe are engaged in using forward genetics screens in mice and other model organisms to investigate various biological processes such as male infertility (Weiss et al., 2012), lung disease (Yates et al., 2009b), craniofacial development (Sandell et al., 2011), and the host response to infection (Casrouge et al., 2006, Zhang et al., 2007, Divangahi et al., 2008). Since spontaneous mutations occur at a low frequency ($\sim 5x10^{-6}$ per locus), mouse geneticists have searched for mutagens to generate novel mutations efficiently.

ENU was discovered as a chemical mutagen in the late 1950s by William Russell (Russell et al., 1979). It acts as an alkylating agent of DNA, transferring its ethyl group to nucleophilic nitrogen or oxygen species found on any of the four deoxyribonucleoides (Justice et al., 1999). The added ethyl group creates a DNA adduct that, during replication, causes mistaken identity resulting in heritable point mutations (Bielas and Heddle, 2000, O'Neill, 2000). It induces specific nucleotide substitutions, the most commonly reported mutations are A->T base transversions or A->G transitions occurring at a frequency of 70-85%, and to a lesser extent G->C transversions (Noveroske et al., 2000b, Takahasi et al., 2007).

ENU mutagenesis can cause loss or gain of function of a given protein through the introduction of new amino acids in the coding sequence, introduction of new stop codons, or conversion of existing stop codons into residue codons (Noveroske et al., 2000a). Mutant proteins may also occur by alteration of sequences involved in intro-exon splicing. Induced mutations at promoter sites and untranslated regions that determine transcript stability may also affect gene and protein expression. ENU is administered to young adult male mice and targets the spermatogonial cells. Following a period of infertility, male mice are then mated with normal female mice to produce so-called G1 offspring. The G1 inherits one set of normal (wild type; WT) chromosomes from the mother and an ENU-affected (mutant) set from the father. The later chromosomes will carry a variable and unique set of ENU-induced mutations. The rate of

random single-base pair mutations is largely dependent on gene size and dose of ENU, but it has been estimated that the frequency of functional mutations is every 1-1.5 Mb, or 30-50 mutations in each G1 animal (Justice et al., 1999). This translates to approximately one damaging mutation in a specific gene for every 700 gametes (Hitotsumachi et al., 1985).

Breeding schemes for the propagation of the G1 animals are structured according to the allelic characteristics required (dominant vs. recessive screen), and in some cases the need for backcrossing to other inbred mouse lines to assist in the mapping of the mutation (Figure 1.8). The goal of using a dominant screen is to identify mutations that display a phenotype in the heterozygous condition, whereas a recessive screen detects traits that require the mutations on both alleles.

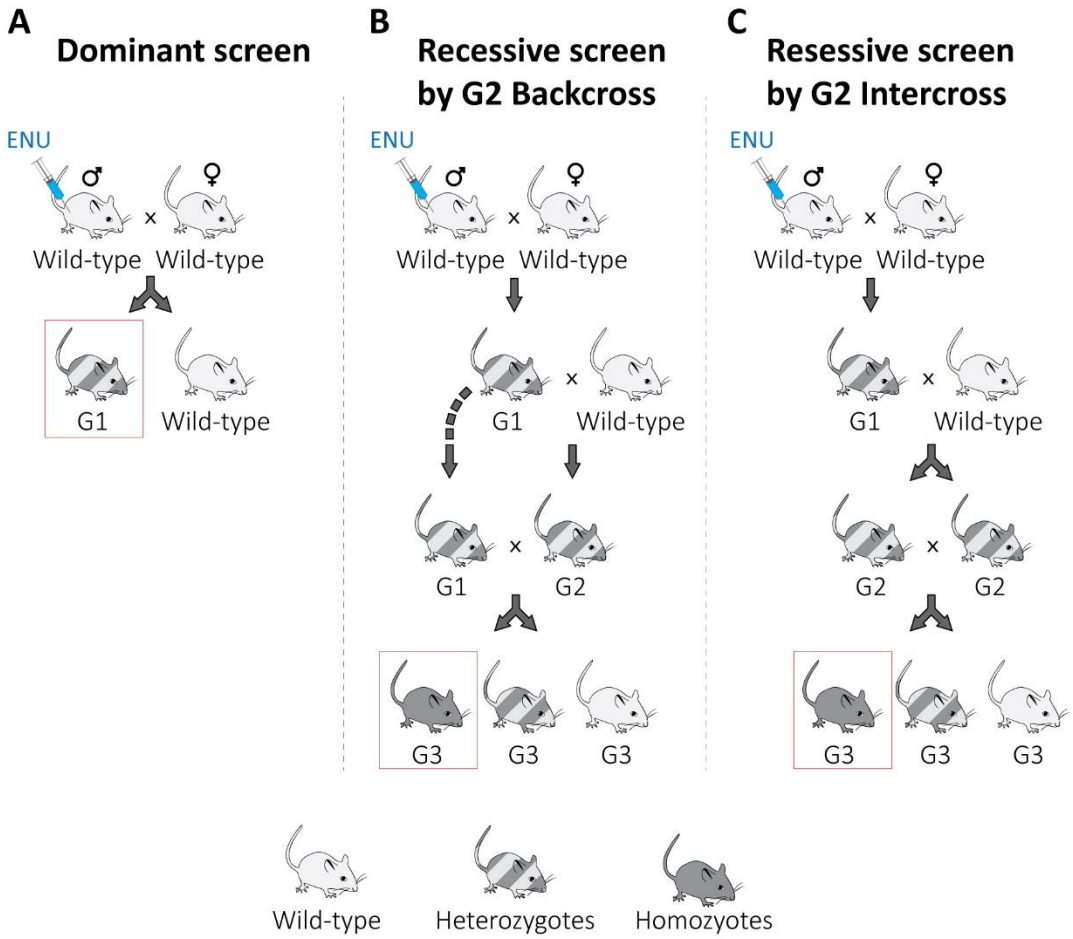


Figure 1.8 – Schematic diagram of basic dominant and recessive breeding scheme in the mouse. A male G0 mouse of a given strain is treated with ENU and bred with wild-type females. The resulting G1 mice are then screened for a dominant phenotype of interest (A). To perform recessive screens, G3 animals can be produced in several ways. (B) In a G2 backcross breeding scheme, affected G1 mice are bred with wild-type partners to produce G2 animals. The original G1 parent is then crossed with several G2 progenies, and the resulting G3 mice are then examined for the phenotype of interest. (C) In the G2 intercross scheme, G2 mice are bred with each other to produce G3 progenies.

The efficacy of the phenotype screening strategy is an important determinant in the ability to find the correct trait of interest. This has led to the proposal of several standardised sets of protocols to phenotype genetically modified mice, such as SHIRPA (SmithKline Beecham, Harwell, Imperial College, Royal London Hospital, phenotype assessment) (Nolan et al., 2008, Rogers et al., 1997) and EMPReSS (European Mouse Phenotyping Resource for Standardized Screens) (Brown et al., 2005). They have been used to identify a range of mutant mice

including dystrophin-deficient mutants, mice that display Alzheimer's disease, and mice that differ in their response to infectious diseases. Screens to identify host responses to infectious diseases, such as malaria, often incorporate a survival screen. Combined with the benefits of using a mouse model, the use of such a screen with ENU-mutagenised mice provides the opportunity to dissect the genetic and molecular mechanisms of the host response to infections. This approach has been used previously in various studies. For example, Philippe Gros at McGill University, infected C56BL/10J mice with a lethal dose of *P. berghei* parasites. Screening for survival identified an ENU-induced dominant negative mutation in the JAK3 kinase which was proposed to protect against cerebral malaria (Bongfen et al., 2012). Another type of screen that has previously been employed is the identification of an abnormal blood count. Dr Benjamin Kile and Prof Doug Hilton from the Walter and Eliza Hall Institute for Medical Research set up a dominant ENU screen for investigating the genetic control of erythropoiesis. Their screening procedure included a full blood count analysis of progenies from founder mice. This led to several discoveries providing closer insights into the molecular mechanism in platelet response (Mason et al., 2007, Carpinelli et al., 2004, Josefsson et al., 2011). These examples emphasise the benefits of an unbiased phenotype-driven screen. Similarly, our laboratory uses a haematological analyser together with a survival screen as routine tests in the identification of malaria resistant G1 and G2 progenies. This thesis investigates several identified mutants resulting from this screen.

Thesis aim

The main aims of this thesis were two-fold. The first was to identify novel host genes that influence the ability of the *Plasmodium* parasite to invade, grow, replicate, and otherwise survive in a mouse. The second was to understand the biological and molecular role for the gene and its product during malarial infection. This information was used to ascertain the suitability of the genes under investigation as targets for antimalarial HDT.

Previous work in the S. Foote, G. Burgio, and B. McMorran laboratory had generated over 80 mutant strains that resulted from a large-scale ENU mutagenesis screen. These strains were subject to dominant screening for an abnormal RBC phenotype - assessed by an automated haematological analyser (Siemens, Advia 120) - and/ or increased survival to *P. chabaudi*

infection. The basis of this work lies upon the investigation of several of these mutant lines. The lines that are reported on in this thesis are called MRI23420, MRI47455, and MRI47495. Each of the lines was derived from G1 founder mice that displayed a phenotype where RBCs size deviated in size from WT. This is defined as mean corpuscular volume (Table 3).

Table 3 – Mutant strains investigated in this thesis.

Mutant strain	Selected for	MCV		Chapter No.
		Affected	Normal	
MRI23420	Abnormal blood phenotype	45.3 ±0.1	53.4 ±0.2	3
MRI47455	Abnormal blood phenotype	49.7 ±0.4	53.3 ±0.3	4
MRI47495	Abnormal blood phenotype	58.0 ±0.4	52.5 ±0.6	5

Values for mean corpuscular volume (MCV) present the affected and normal population of 1st generation (G1) of mice after ENU mutagenesis.

Aim 1: Identification of ENU mutations

The first step in characterising a mutant line was to identify the causative gene responsible for the phenotype the mutants were selected for. Depending on the availability of genetic tools, two methods were employed to reveal potential gene candidates:

- An “affected only” mapping strategy, involving a backcross strategy genome-wide SNP genotyping, followed by sequencing of candidate genes.
- Next-generation gene sequencing bioinformatics analysis of G1 mice and their affected progeny, followed by sequencing of candidate genes.

Aim 2: Phenotype characterisation

To investigate how the mutation affected the function of the gene and physiology of the animal, as well as the role of the gene in malarial infection. This primarily included assessing the expression of the affected gene, measuring a number of haematological parameters, parasitology kinetics and the host response during infection, as well as other assays determined by the specific function of the causative gene.

Chapter 2 – MATERIALS & METHODS

Protocols

Mice and Ethics Statement

All mice from the line MRI23420 were housed under controlled temperature (21 ± 1°C) with a 12:12 hour light-dark cycle. All procedures were conducted in accordance with the policies of the University of Tasmania and conformed to the National Health and Medical Research Council (NHMRC) Australian code of practice. The work was performed under the agreement Ethics No. A0104070, approved and obtained from the Animal Ethics Committee at the University of Tasmania (Appendix A).

Similarly, mice from MRI47455 and MRI47495 were maintained in a temperature controlled room with a 12:12 hour light-dark cycle. However, animal handling and experimental procedures were conducted according to the policy guidelines of Macquarie University and in reconciliation with the National Health and Medical Research Council (NHMRC) Australian code of practice. This work was approved under the agreement Ethics No. 2012019, obtained from the Animal Ethics Committee at Macquarie University (Appendix B).

ENU mutagenesis and dominant phenotype screening

Generation 0 (G0) SJL/J male mice were mutagenized with two intraperitoneal injections of 150 mg/kg ENU (Sigma-Aldrich, Oakville, ON, Canada) at one week intervals. These mice were mated to untreated SJL/J females to create G1 progeny. To screen for a dominant blood phenotype, blood samples obtained by mandibular bleeding of G1 litters at seven weeks of age were tested on an automated blood analyser (Siemens Advia 120 Automated haematological analyser). All mutant lines described in this thesis were identified and selected on the basis of a reduced or increased mean corpuscular volume (MCV) that was 3 standard deviations higher when compared to the normal population. To test the validity of heritability of the ENU mutation, G1 phenodeviants were test-mated with WT SJL/J to produce G2 progenies.

Gene mapping in MRI23420

For genetic mapping in MRI23420 G2 animals were backcrossed to C57BL/6 mice. F1 mice were screened for their abnormal MCV phenotype and affected F1 were mated with C57BL/6J strain to produce N2 generation mice that were also screened for their MCV. Genomic DNA was then purified from the tail of 20 affected N2 mice using the phenol chloroform extraction procedure described by Kochl et al. (Kochl et al., 2005). Genome-wide SNP analysis on a MASSarray platform using IPlex GLOD technology (Sequenom Inc, San Diego, CA) was conducted on the N2 mice using a mouse linkage marker set of over 600 SNPs, including 300 polymorphic markers between C57BL/6 and SJL/J evenly distributed throughout the genome. The logarithm of odds (LOD) score was determined by using a chi square test between observed values (number of heterozygous genotype mice per SNP) and theoretical values. Fine mapping of the critical interval was conducted by microsatellite genotyping of recombinant mice for chromosome 8, between 22 and 44Mb (D8Mit170a, D8Mit95, D8Mit190, D8Mit65). Sequencing of the *Ank-1* gene was conducted by amplification of all exon and intron/exon boundaries using the polymerase chain reaction (PCR). For a single PCR 12.5µl of GoTaq Green Mastermix containing Taq polymerase, magnesium chloride, DNTPs, and the loading buffer (Promega Corporation, Madison, WI), 5.5µl of distilled H_20, 1µl of each specific primer (concentration 10µmol/L) and 5µl of the corresponding DNA sample (concentration of 20ng/µl) were used. PCR products were purified using a DNA clean up kit (Promega Corporation, Madison, WI) before being sent for Sanger sequencing at the Australian Genome Research Facility (AGRF).

SDS Page and Western blot analysis

For Western blotting, whole blood lysate samples were separated by non-denaturing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) using 8% gradient gels. For immunoblotting, samples were transferred to nitrocellulose membranes. The membrane was then incubated with either mouse monoclonal GAPDH (Milliporo) or N-terminal *Ank-1* "p89" (kindly provided by Connie Birkenmeier, Jackson Laboratory, USA) and then washed extensively prior to incubation with peroxidase-conjugated secondary antibodies. After further washes, the blots were visualized using enhanced chemiluminescence (ECL) reagents (Amersham Biosciences, Piscataway, NJ).

Ensuring that equal protein levels were loaded for either *Ank-1*$^{MRI23420/+}$ or WT, bands were quantified by densitometry and adjusted to the calculated intensity of bands from GAPDH control. Such analysis was done in Photoshop CS4 (Adobe) by masking each band containing equal numbers of pixels and the mean from the histogram recorded. Values were subtracted from the background and divided by the signal from corresponding GAPDH bands.

Quantitative PCR

RNA was isolated from the kidneys, spleens, brains and livers of uninfected SJL and *Ank-1*^{MRI23420} heterozygous mice using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. RNA was cleaned up using a Qiagen midi kit (Qiagen, Valencia, CA) and reverse transcribed with a cDNA synthesis kit (Roche Applied Science, Basel, Switzerland) with Oligo-p(dt)$_{15}$ primers . Quantitative PCR of cDNA was conducted using SYBR green fluorescent dye (Roche Applied Science, Basel, Switzerland) at a serial concentration from 1:10 to 1: 160. The gene expression study was conducted on a Light Cycler 480 (Roche Applied Science, Basel, Switzerland). Expression of the gene *Ank-1* was normalised to ß-Actin using the $2^{-\Delta\Delta Ct}$ formula and expressed as a fold change of the wild-type mice. Primers for *Ank-1* gene were spanning throughout the gene on exons 6 and 17. The primer sequences are: *Ank-1*_1, 5'-CTACAGCAGGGTCACGAGAA-3'; *Ank-1*_1, 5'-GTCCGTGTGTCATCGTTGC-3'; *Ank-1*_2, 5'-TGCCAAGCAGAACCAGATAG-3'; *Ank-1*_2, 5'-AGTGGGGTCACGCCTTGTA-3'; ß-Actin, 5'-TTCTTTGCAGCTCCTTCGTTGCCG-3'; and ß-Actin, 5'- TGGATGCGTACGTACATGGCTGGG-3'.

Histology

Spleens and livers from uninfected SJL and *Ank-1*$^{MRI23420/+}$ mice were collected and fixed in 10% formalin for 24 hours, dehydrated on a Leica ASP200 S Tissue Processor (Leica Microsystems) and embedded in paraffin wax. Sections were cut on a microtome at 5 µM and fixed to glass slides and then stained with either hematoxylin-eosin or Perl's Prussian Blue staining.

Total non-haem iron quantification in mouse tissue

Liver and spleen tissue were dissected from uninfected animals. Between 50 and 100mg of each tissue was dried at 45°C for 48 hours and then placed in a 10% hydrochloric acid / 10% trichloroacetic acid solution to digest for 48 hours at 65°C. Samples were then centrifuged at

13000rpm for five minutes. 200µl of supernatant was then added to 1ml of 1,10- Phenanthroline monohydrate solution (Sigma-Aldrich) and incubated for 15 minutes at room temperature. After incubation 300µl of sample was transferred to a flat bottom plate (Corning®) and absorbance measured at 508nm. All samples were analysed in duplicates and compared for statistical significance to WT.

Scanning electron microscopy

One to two drops of tail venous blood were sampled from uninfected and infected mice at low parasitaemia (3-8%) displaying synchronised ring stage parasites. The blood was collected in 1ml of 1x MT-PBS (pH 7.2) and centrifuged at 500rpm for five minutes at room temperature. The pellet was then fixed in 1ml of 2.5% glutaraldehyde for one hour under constant agitation before being stored at 4°C for up to three days until further processing. Cells were placed on THERMANOX coverslips (ProSciTech, Queensland) and postfixed with 2.5% osmium tetroxide (OsO_4) for 30 minutes at room temperature. Samples were rinsed 2 to 3 times in 10% EtOH before being dehydrated through a number of incubations with increasing concentrations of ethanol (10 minute changes in each of 30, 50, 70, 80, 90, 95 and 100% EtOH, 100% dry EtOH, and 100% dry acetone). Thereafter, samples were critical point dried with a 30 minute purge time (tousinis autosanwdri-815) and mounted on metal studs with a carbon disk underneath. Edges were sealed with carbon paint for better conductivity and samples were sputtercoated with a 6mm platinum layer (Cressington sputter coater 208HR with Cressington thickness controller mtm20). Imaging was carried out using a JEOL JSM-6701F scanning electron microscope.

Cells were quantified by counting morphologically similar cells according to a reference image. Normal RBCs (Figure 2.1A) were those displaying a classic round donut shape with an obvious centre depression and no blebs visible on the surface. Abnormal RBCs (Figure 2.1B) were specified if they possessed distinct blebbing of the surface responsible for a complete loss of round cell structure and a centre depression.

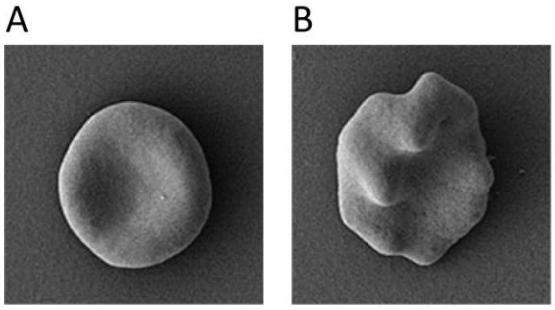


Figure 2.1 - Examples of the defined morphology of RBCs in the quantification of misshaped *Ank-1*^{MRI23420} RBC with SEM. (A) normal RBC and (B) abnormal RBC.

Osmotic fragility assay

Mutants and SJL/J or WT mice seven weeks or older were mandibular bled and blood incubated in various salt concentrations ranging from zero to 160mmol (physiological concentration - 1x MT-PBS). After 30min at 37°C, samples were gently centrifuged (500g) and supernatant collected to measure absorbance at 540nm. The percentage of lysis per sample was calculated assuming 100% lysis occurs in water.

Erythropoietin Immunoassay

Blood was collected from adult uninfected WT and *Ank-1*$^{MRI23420/+}$ mice and left to clot at room temperature for two hours. Samples were centrifuged at 2000g for 20min and serum removed. A Quantikine Mouse/Rat Immunoassay (R & D Systems, Minneapolis) was used to measure the concentration of erythropoietin (EPO) according to the manufacturer's instructions.

Malaria infection with *P. chabaudi*

The rodent malarial parasite species *P.chabaudi adami* DS was used for all experimental infections. C57BL/6 donor mice were infected by intraperitoneal injections with 250µl of thawed parasite stock. Once donors reached 5-15% parasitaemia they were anesthetized with isofluorane and bled by cardiac puncture before being sacrificed. The following procedure differed to the mutant line examined. In MRI23420 an appropriate amount of blood was diluted into 25ml of HEM (HEPES-buffered minimum Essential Media) which consisted of Mini

Essential Media (MEM) + 2% of 1M HEPES and 1% Penicillin from total volume and pre-heated to 37 °C to give a final parasite concentration of $1x10^4$ parasitised RBCs. In MRI47455 and MRI47495 parasitized blood was diluted in Krebs buffered saline containing 0.2% glucose, according to Jarra and Brown (Jarra and Brown, 1985), and $1x10^4$ parasitized RBCs (if not otherwise mentioned) were injected into the intraperitoneal cavity of recipient mice. To record survival and levels of infected RBCs, mice were checked daily including taking blood smears on a glass slide from tail. Smears were fixed for 1 minute in methanol and stained with 10% Giemsa (Sigma-Aldrich) for another 10 minutes. Quantification of parasitaemia was assessed under a light microscopy at 1000x magnification and the percentage of infected and uninfected RBCs calculated by counting between 300-500 cells.

Survival challenges were split by gender as males are known for their increased susceptibility to *P. chabaudi* infections (Stevenson et al., 1982, Stevenson et al., 1990). Statistical analysis for malarial survival was determined by a Mantel-Cox log rank test on a Kaplan-Meier survival curve, using Prism 5.04 (Graphpad) or R.2.15.0 (http://cran.r-project.org) and Excel (Microsoft Office 2013).

In vivo invasion and growth assay in MRI23420

Fresh blood was collected from uninfected WT and *Ank-1*$^{MRI23420/+}$ mice of at least seven weeks of age. RBCs were concentrated by centrifugation. WT and *Ank-1*$^{MRI23420/+}$ RBCs were stained with the fluorescent dyes, ATTO 633 and 495 (Sigma-Aldrich, St Louis, Missouri) in all possible combinations (WT-ATTO 633, WT-ATTO 495, *Ank-1*$^{MR23420/+}$ATTO 633 and *Ank-1*$^{MRI23420/+}$ATTO 495). The blood was stained with the dyes according to the manufacturer's instructions. Stained cells were mixed into the following combinations: WT-ATTO 633 with *Ank-1*$^{MRI23420/+}$ ATTO 495; WT-ATTO 495 with *Ank-1*$^{MRI23420/+}$ATTO 633 at a ratio of 1:1; and diluted with 1x MT-PBS to a cell concentration of $1x10^9$ cells/mL. 0.1mL from each blood combination was injected intraperitoneally into separated groups of infected WT and *Ank-1*$^{MRI23420/+}$ mice in the evening. The day of cell labelling and injection was chosen when parasitaemia of infected mice was still similar between both cohorts at day six or seven post inoculation (2-4%) as observed on giemsa stained blood smears. The next day, early morning, one drop of blood from each mouse tail was collected into FACS buffer (1% BSA, 0.1% Sodium Azide in 1x MT-PBS) and

stained for TER119 +ve cells with the marker TER119-PE (BD Biosciences PharMingen) and Hoechst 23580 for DNA. Samples were analysed using flow cytometry (FACS Canto II, BD Biosciences PharMingen). This involved gating all events for TER119 +ve. From this gate the rate of double positive (ATTO 633 + Hoescht23580) out of the total ATTO 633 and the rate of the double positive (ATTO 495 + Hoescht23580) to the total number of total ATTO 495 for each recipient strain (SJL and *Ank-1*$^{23420/+}$) was calculated. In WT mice the combined means of WT (double positive for either TER119 + ATTO 633 + Hoescht23580 or TER119 + ATTO 495 + Hoescht23580) and *Ank-1*$^{MRI23420/+}$ (double positive for either TER119 + ATTO 633 + Hoescht23580 or TER119 + ATTO 495 + Hoescht23580) infected RBCs were expressed as 100% and the proportion of infected RBCs for each cohort was calculated. The same comparison was repeated separately in infected *Ank-1*$^{MRI23420/+}$ mice.

Quantification of pyknotic intracellular parasites

The percentage of pyknotic vs healthy looking intracellular parasites was calculated under a light microscope at 1000x magnification. In contrast to healthy ring parasites (6-8 hour post-invasion) displaying a big (1/4-1/5 of RBC size) ring body form, ring stage parasites expressing a pyknotic phenotype were defined if their body was either ≤ 1/5 the size of an RBC or lacked a classic ring form. Pyknotic parasites 18-20 hour post-invasion were identified if they exhibited a similar small (≤ 1/4 the size of an RBC), dense and irregular body shape lacking a clear cross-hatch or similar line pattern, as opposed to healthy trophozoites.

Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) in MRI23420

Blood smears from the mouse tail were fixed in 100% MeOH. For all TUNEL staining, an APO-BrdU TUNEL assay kit was used (Invitrogen, Carlsbad, CA). Slides were initially washed three times with 1ml of wash buffer solution before being incubated with 50µl of DNA labelling solution mix (according to manufacturer's instructions) overnight at room temperature. The next day slides were rinsed three times with 1ml rinse solution with two minutes incubation time each. Stained sections were then incubated with 100µl of BrdU-antibody for one hour in the dark at room temperature with subsequent washes in 1%BSA/ 1x MT-PBS (three times with a two minute soak each). Slides were then further labelled with 100µl of nuclear yellow (Invitrogen, Carlsbad, CA) (1:5000 in 1% BSA/ 1 x MT-PBS) for one minute in the dark. After further washes with 1ml MT-PBS (three times with a two minute soak each) sections were affixed with Fluorescent Mounting Medium (DakoCytomation). Once the medium dried, slides were examined on an upright epifluorescence microscope (Olympus BX50) between 600x and 1000x magnification. At least 100 nuclear yellow positive parasites present inside red blood cells were counted per slide. They were identified as having DNA fragmentation if they also stained positive for TUNEL.

Labile iron quantification in red blood cells

The procedure of quantifying labile iron in RBCs is based on a previously published protocol (Prus and Fibach, 2008). Whole blood was obtained from tails of mice and washed twice with FACS buffer before being incubated at a density of 1 x 10^6 cells/ml with the iron stain calcein-AM (0.5µM) and RBC marker TER119 (0.5µl/ 100µl) for 30min at room temperature. Then, cells were washed twice and treated with the iron chelator Deferiprone (Sigma-Aldrich) in 0.5ml FACS buffer at a concentration of 100µM. Following staining, cells were resuspended in 0.7ml of FACS buffer and analysed on a flow cytometer (Influx, BD). TER119+ cells were gated for Calcein-AM (FITC) and the total fluorescence intensity of this population was calculated by multiplying the mean of the gate's fluorescence by the event count of this gate (Figure 2.2).

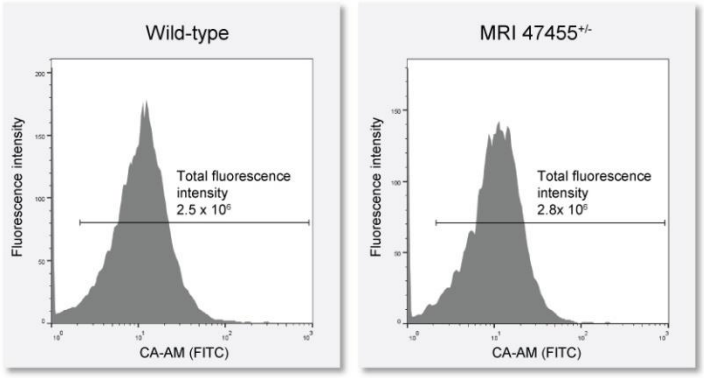


Figure 2.2 - Example of flow cytometry plots corresponding to labile iron quantification in RBCs. Flow cytometric study showing normal proportion of labile iron in MRI47455$^{+/-}$ RBCs. Analysed with FACSDiva^{TM} V8.0 (BD Bioscience) software.

In vivo invasion and growth assay in MRI47455

A number of uninfected MRI47455$^{+/-}$ and WT mice were anesthetized with isofluorane and bled by cardiac puncture in order to obtain enough blood for subsequent RBC labelling with ATTO 633 (Sigma-Aldrich) and biotin in all possible combinations (WT-biotin, WT-ATTO 633, MRI47455$^{+/-}$-biotin, MRI47455$^{+/-}$-ATTO 633). This involved incubating equal amounts of blood in 2µl dye/ 100µl 1x MT-PBS for 30-60 minutes at 4°C in the dark. Once stained, samples were washed 2-3 times and mixed in the following combinations in a 1:1 ratio: WT-biotin/ MRI47455$^{+/-}$-ATTO 633 and WT-ATTO 633/ MRI47455$^{+/-}$-biotin. 0.2ml (3x10^9 cells per mouse) from each combination was then IV injected into separate groups of infected mutant and WT mice with low parasitaemia. The time point of IV injection was chosen at a late stage of parasite maturation, as determined prior by Giemsa thin blood smears. 0.5µl of blood from tail was sampled 30 minutes, 16 hours and 30 hours after into a 50µl cocktail containing 0.25µl of fluorescently labelled antibodies specific for CD45 APC-Cy7, 0.25µl CD71 PE-Cy5 and Streptavidin (0.5µl) in 1%BSA/ MT-PBS buffer and left for 30-60 minutes on ice. This was followed by adding another 400µl of MT-PBS including Hoechst 33342 (1:5000) and 1µl of Thiazole orange (800µg/ml). After 2-5 minutes incubation, samples were concentrated by centrifugation and resuspended in 700µl of MT-PBS, in order to be analysed on an Influx cell sorter. This included gating out all CD45+ and CD71+ cells with the remaining population representing mature erythrocytes. From this, the proportion of triple positive cells for biotin,

Hoechst 33342 and Thiazole orange out of the total biotin+, and the rate of triple positive for ATTO 633, Hoechst 33342 and Thiazole orange to the total number of ATTO 633+ cells for each recipient cohort (MRI47455$^{+/-}$ and WT) was determined. Separate for each background of infected mice, the combined means of MRI47455$^{+/-}$ and WT infected RBCs (positive for either biotin/ Hoechst 33342/ Thiazole orange or ATTO 633/ Hoechst 33342/ Thiazole orange) were presented as 100% and the percentage of infected RBCs for each recipient strain calculated.

In vivo red blood cell half-life assay

In uninfected mice blood from adult MRI47455$^{+/-}$ and WT animals was labelled *in vivo* by IV injection of 1mg/ml sulfobiotin-LC-NHS (biotin) (Thermo Scientific). Starting 24 hours post-injection, 1µl of blood from tail was sampled at regular intervals and stained with fluorescently labelled antibodies for Streptavidin PE-Cy7, TER119 FITC, and CD71 PE-Cy5 (BD Biosciences PharMingen) at a concentration of 0.5µl/100µl FACS buffer for 30-60 minutes at 4°C in the dark. Thereafter, samples were washed twice and resuspended in 0.7ml FACS for analysis on a flow cytometer (BD FACSAria II). Cells of interest were gated as double positive for TER119 and Streptavidin minus CD71+. Values for all samples were adjusted to the 24 hour time point representing 100%.

For assessing the half-life of RBCs in infected mice, blood from MRI47455$^{+/-}$ and WT was individually labelled with either ATTO 633 or biotin (see protocol for '*In vivo* invasion and growth assay in MRI23420

Fresh blood was collected from uninfected WT and Ank-1MRI23420/+ ^{mice} of at least seven weeks of age. RBCs were concentrated by centrifugation. WT and Ank-1MRI23420/+ ^{RBCs} were stained with the fluorescent dyes, ATTO 633 and 495 (Sigma-Aldrich, St Louis, Missouri) in all possible combinations (WT-ATTO 633, WT-ATTO 495, Ank-1MR23420/$^{+\text{-ATTO }633}$ and Ank-1MRI23420/+-ATTO $^{495).}$ The blood was stained with the dyes according to the manufacturer's instructions. Stained cells were mixed into the following combinations: WT-ATTO 633 with Ank-1MRI23420/+-ATTO $^{495;}$ WT-ATTO 495 with Ank-1MRI23420/$^{+\text{-ATTO }633}$ at a ratio of 1:1; and diluted with 1x MT-PBS to a cell concentration of 1x109 cells/mL. 0.1mL from each blood combination was injected intraperitoneally into separated groups of infected WT

and Ank-1MRI23420/+ mice in the evening. The day of cell labelling and injection was chosen when parasitaemia of infected mice was still similar between both cohorts at day six or seven post inoculation (2-4%) as observed on giemsa stained blood smears. The next day, early morning, one drop of blood from each mouse tail was collected into FACS buffer (1% BSA, 0.1% Sodium Azide in 1x MT-PBS) and stained for TER119 +ve cells with the marker TER119-PE (BD Biosciences PharMingen) and Hoechst 23580 for DNA. Samples were analysed using flow cytometry (FACS Canto II, BD Biosciences PharMingen). This involved gating all events for TER119 +ve. From this gate the rate of double positive (ATTO 633 + Hoescht23580) out of the total ATTO 633 and the rate of the double positive (ATTO 495 + Hoescht23580) to the total number of total ATTO 495 for each recipient strain (SJL and Ank-123420/+) was calculated. In WT mice the combined means of WT (double positive for either TER119 + ATTO 633 + Hoescht23580 or TER119 + ATTO 495 + Hoescht23580) and Ank-1MRI23420/+ (double positive for either TER119 + ATTO 633 + Hoescht23580 or TER119 + ATTO 495 + Hoescht23580) infected RBCs were expressed as 100% and the proportion of infected RBCs for each cohort was calculated. The same comparison was repeated separately in infected Ank-1MRI23420/+ mice.

TUNEL labelling in MRI47455

This experiment was conducted in line with the *in vivo* invasion assay, and methods for initial labelling and staining of MRI47455$^{+/-}$ and WT RBCs are described in more detail below (see '*In vivo* invasion and growth assay in MRI23420

Fresh blood was collected from uninfected WT and Ank-1MRI23420/+ mice of at least seven weeks of age. RBCs were concentrated by centrifugation. WT and Ank-1MRI23420/+ RBCs were stained with the fluorescent dyes, ATTO 633 and 495 (Sigma-Aldrich, St Louis, Missouri) in all possible combinations (WT-ATTO 633, WT-ATTO 495, Ank-1MR23420/+-ATTO 633 and Ank-1MRI23420/+-ATTO 495). The blood was stained with the dyes according to the manufacturer's instructions. Stained cells were mixed into the following combinations: WT-ATTO 633 with Ank-1MRI23420/+-ATTO 495; WT-ATTO 495 with Ank-1MRI23420/+-ATTO 633 at a ratio of 1:1; and diluted with 1x MT-PBS to a cell concentration of 1x109 cells/mL. 0.1mL from each blood combination was injected intraperitoneally into separated groups of infected WT and Ank-1MRI23420/+ mice in the evening. The day of cell labelling and injection was

chosen when parasitaemia of infected mice was still similar between both cohorts at day six or seven post inoculation (2-4%) as observed on giemsa stained blood smears. The next day, early morning, one drop of blood from each mouse tail was collected into FACS buffer (1% BSA, 0.1% Sodium Azide in 1x MT-PBS) and stained for TER119 +ve cells with the marker TER119-PE (BD Biosciences PharMingen) and Hoechst 23580 for DNA. Samples were analysed using flow cytometry (FACS Canto II, BD Biosciences PharMingen). This involved gating all events for TER119 +ve. From this gate the rate of double positive (ATTO 633 + Hoescht23580) out of the total ATTO 633 and the rate of the double positive (ATTO 495 + Hoescht23580) to the total number of total ATTO 495 for each recipient strain (SJL and *Ank-123420*$^{-/+}$) was calculated. In WT mice the combined means of WT (double positive for either TER119 + ATTO 633 + Hoescht23580 or TER119 + ATTO 495 + Hoescht23580) and *Ank-1MRI23420*$^{-/+}$ (double positive for either TER119 + ATTO 633 + Hoescht23580 or TER119 + ATTO 495 + Hoescht23580) infected RBCs were expressed as 100% and the proportion of infected RBCs for each cohort was calculated. The same comparison was repeated separately in infected *Ank-1MRI23420*$^{-/+}$ mice.

Six and 18 hours after, a minimum of 100,000 labelled RBCs (2µl blood) were collected in FACS buffer and sorted separately for each dye and genetic background of the animal on an Influx cell sorter (BD Biosciences) into 1ml of 2% paraformaldehyde (Sigma Aldrich) containing MT-PBS and left for 30 minutes at 4°C. After gentle centrifugation the majority of supernatant was aspirated and cells resuspended in the remaining buffer. The suspension was then transferred onto pre-coated glass sides with 0.1% polyethyleneimine (Sigma-Aldrich) and allowed to adhere for 5-10 minutes. Thereafter slides were washed with MT-PBS and fixed in 100% methanol for 1 minute. Slides were then TUNEL stained using an APO-BrdU TUNEL assay kit (Invitrogen, Carlsbad, CA) according to manufacturer instructions and described in detail above. The slides were examined on an upright epifluorescence microscope (Zeiss Axio Imager Z2) at 600x magnification, counting at least 100 nuclear yellow positive infected cells per sample. The proportion of double positive cells for TUNEL and nuclear yellow out of the total number of counted infected RBCs was determined for each RBC group and expressed as combined means (WT-ATTO 633 and WT-biotin versus MRI47455$^{+/-}$-ATTO 633 and MRI47455$^{+/-}$-biotin).

Ex vivo invasion assay

Mice on a WT background were infected at high dose ($9x10^9$ infected RBCs) with *P. chabaudi* allowing for better synchronised parasites at high parasitaemia. In the morning on day 3 post-infection, when mice reached parasitaemia levels of around 20-30%, uninfected mice of both strains were labelled *in vivo* by IV injection of 0.2ml with either biotin (0.5 mg/ml saline) or ATTO 633 (0.5mg/ml saline) in the same combination pattern as done for the *in vivo* invasion assay. After 12-14 hours (1-2am) at schizont rupture, infected and uninfected mice from both groups were bled by cardiac puncture with heparin-lined syringes and blood collected into 3ml flat bottom tubes (Corning®). From this step forward, cells were kept at a constant 37°C. Samples were combined in equal proportions (WT-biotin / MRI47455$^{+/-}$-ATTO 633 and WT-ATTO 633 / MRI47455$^{+/-}$-biotin) and 50µl of each combination was mixed separately with 50µl of blood from infected MRI47455$^{+/-}$ or WT mice. The blood mixture was washed with a pre-warmed culture media (pH 7.3) containing RPMI without indicator (Gibco® by Life technologies™), 25% foetal calf serum, 2% glutamine (Sigma-Aldrich), 0.5% gentamycin (Sigma-Aldrich), 0.6% w/v HEPES (Sigma-Aldrich), and 0.2% w/v sodium bicarbonate (Sigma-Aldrich), and then resuspended in a 1:1 volume ratio. Samples were gassed (10 sec with 1% O_2, 5% CO_2, in N_2) and incubated for 3-4 hours at 37°C with constant agitation and then placed on ice to stop invasion. Finally, cells were stained in 100µl 1%BSA/ MT-PBS containing 1.0µl of fluorescently labelled antibodies for Streptavidin 0.25µl PE-Cy7, 0.25µl CD71 PE-Cy5 and 0.25µl CD45 APC-Cy7 for 30-60 minutes in the dark. After another 2-5 minutes incubation in 400µl of 1%BSA/ MT-PBS including Hoechst 33342 (1:5000) and 1µl of Thiazole orange (800µg/ml), cells were diluted into 700µl of MT-PBS and analysed by flow cytometry (BD Influx). The mature RBC population was defined as CD71-, CD45- population which then was gated in the same manner as described in the *in vivo* assay (see '*In vivo* invasion and growth assay in MRI23420

Fresh blood was collected from uninfected WT and *Ank-1MRI23420*$^{/+}$ mice of at least seven weeks of age. RBCs were concentrated by centrifugation. WT and *Ank-1MRI23420*$^{/+}$ RBCs were stained with the fluorescent dyes, ATTO 633 and 495 (Sigma-Aldrich, St Louis, Missouri) in all possible combinations (WT-ATTO 633, WT-ATTO 495, *Ank-1MR23420*$^{/+\text{-ATTO}}$ 633 and *Ank-1MRI23420*$^{/+\text{-ATTO}}$ 495). The blood was stained with the dyes according to the manufacturer's instructions. Stained cells were mixed into the following combinations: WT-

ATTO 633 with *Ank-1MRI23420*$^{/+\text{-ATTO}}$ 495; WT-ATTO 495 with *Ank-1MRI23420*$^{/+\text{-ATTO}}$ 633 at a ratio of 1:1; and diluted with 1x MT-PBS to a cell concentration of 1x109 cells/mL. 0.1mL from each blood combination was injected intraperitoneally into separated groups of infected WT and *Ank-1MRI23420*$^{/+}$ mice in the evening. The day of cell labelling and injection was chosen when parasitaemia of infected mice was still similar between both cohorts at day six or seven post inoculation (2-4%) as observed on giemsa stained blood smears. The next day, early morning, one drop of blood from each mouse tail was collected into FACS buffer (1% BSA, 0.1% Sodium Azide in 1x MT-PBS) and stained for TER119 +ve cells with the marker TER119-PE (BD Biosciences PharMingen) and Hoechst 23580 for DNA. Samples were analysed using flow cytometry (FACS Canto II, BD Biosciences PharMingen). This involved gating all events for TER119 +ve. From this gate the rate of double positive (ATTO 633 + Hoescht23580) out of the total ATTO 633 and the rate of the double positive (ATTO 495 + Hoescht23580) to the total number of total ATTO 495 for each recipient strain (SJL and *Ank-123420*$^{/+}$) was calculated. In WT mice the combined means of WT (double positive for either TER119 + ATTO 633 + Hoescht23580 or TER119 + ATTO 495 + Hoescht23580) and *Ank-1MRI23420*$^{/+}$ (double positive for either TER119 + ATTO 633 + Hoescht23580 or TER119 + ATTO 495 + Hoescht23580) infected RBCs were expressed as 100% and the proportion of infected RBCs for each cohort was calculated. The same comparison was repeated separately in infected *Ank-1MRI23420*$^{/+}$ mice.

Cell cycle assay

During cell proliferation, intracellular DNA content is approximately doubled during the transition from the G1 to G2/M phase. Therefore DNA content was quantified from cells of excised spleens in *Rbl1*$^{MRI47495/MRI47495}$ as follows. Whole spleen tissue was mashed through 70µm nylon sieves (Falcon®) with the addition of 1ml of 1x MT-PBS buffer to produce a single cell suspension. Cells were then washed twice, resuspended in 1ml of MT-PBS at a concentration of 2 x 10^6 cells/ml and fixed for 2 hours at 4°C by slowly adding 9ml of ice-cold 70% ethanol. Fixed cells where then stained at 37°C for 15 minutes in freshly prepared 500µl propidium iodide (PI) / Triton X-100 staining solution containing 0.1% Triton X-100 (Biorad, NSW, AU), 0.2mg/ml DNAase-free RNA A (Sigma-Aldrich) and 200µg/ml PI (Sigma-Aldrich) in MT-PBS. After staining, cells were washed twice and analysed by flow cytometry (Aria II – BD

Biosciences). This involved gaiting for PI (Texas Red) positive cells and calculating the mean fluorescence intensity (MIF) of this population by multiplying the mean of the gate`s fluorescence by the event count of this gate. From this, the percentage of each cell cycle stage was determined by gating for each population and calculating the percentage from the total MIF.

Identification of ENU mutations by whole exome sequencing

Next Generation sequencing

DNA was extracted from the ear tissue of four affected animals in MRI47455 and two in MRI47495. DNA was extracted using a Qiagen DNeasy extraction kit following the manufacturer's protocol. 10µg of DNA was prepared for paired-end genomic libraries using a paired-end preparation kit from Illumina, following the manufacturer's instructions. Exome enrichment was performed using an Agilent Sure select mouse exome kit according to the manufacturer's protocol. Enriched libraries were tested for enrichment using quantitative PCR. The samples were then sequenced on an Illumina Hiseq 2000 platform, which generated paired-end reads of 100 nucleotides. The libraries were multiplexed and barcoded. The exome coverage was 50x on target.

Sequencing alignment and variant detection pipeline

The sequencing alignment and variant detection pipeline was performed by Dr Denis Bauer (CSIRO). The offspring genomes were grouped by founder and the identified mutations compared to those from other founder-groups previously sequenced. Only investigating mutations that occur in all offspring from one founder (complete) and not in those from other founders (exclusive) will robustly separate segregating ENU-triggered mutations from strain-specific variants or sequencing errors. The subsequent section describes the mapping variant-calling and filtering process. Where not stated otherwise, the default settings were used.

Calling mutations from raw sequencing data (fastq) combines the result from four separate mapping and variant calling pipelines (P1-P4), which make use of different combinations of mappers and variant callers to avoid the loss of variants due to algorithm bias and were triggered with NGSANE (Buske et al., under review). P1 and P2 uses Burrow-Wheeler Aligner

(BWA V0.6.1) (Li and Durbin, 2010) and BOWTIE2 V2.1.0 (Langmead and Salzberg, 2012) to the mouse genome reference sequence (GRCm38 or mm10) in conjunction with SAMTOOLS V0.1.19 (Li et al., 2009) to call variants (mpileup -q1 -D) in each of the offspring groups individually after removal of duplicates (rmdup) and subsequent filtering. P3 and P4 also map with BWA, but in addition use GATK 2.5 (McKenna et al., 2010) to realign reads and recalibrate the quality score to improve the read location and base-pair call-accuracy. While P3 again uses SAMTOOLS, P4 uses GATK's UnifiedGenotyper with subsequent hard filtering of variants to call variants over all mouse genomes in the study simultaneously. In all cases, reads were mapped against Genome Reference Consortium Mouse Build 38 (equivalent to UCSC mm10). The known mouse variants used during realignment, recalibration and GATK variant calling were downloaded from UCSC (dbSNPv128, mm9) and lifted over to mm10. Only variants from exons, exon-intron borders, and promoter were considered due to low coverage from the capture.

After variants are available from P1-P4 the information from all founder-groups is combined using GATK and annotated using GATK with dbSNPv128 and variants from Sanger's The Mouse Genomes Project (lifted over from mm9). Then, only compete and exclusive variants that have not been annotated previously are retained (dbSNP, Sanger) using in-house python scripts. These scripts also filter out variants that are only supported by one method (P1-P4) well as specific genotypes (homo- or heterozygous mutation), that can be ruled out based on prior genetic information. The resulting high-confidence variants are then functionally annotated using ANNOVAR (Wang et al., 2010) (Ensembl, MGI, dbSNP137, phastConsElements60way).

Variant validation analysis

Resulting variants from Next Generation gene sequencing were then filtered for non-synonymous variants that were shared between all four samples. Further verification involved SANGER sequencing mice that were selected for their abnormal RBC phenotype of interest. In particular, the region around each mutation was amplified using the polymerase chain reaction (PCR). For a single PCR 2.5µl of the DNA sample (concentration 20-40ng/µl) was added to 4.5µl of distilled H_20, 1.0µl specific primer (concentration 10µM), and 5.5µl of Amplitaq Gold (LifeTechnology^{TM}) containing GeneAmp 10x PCR buffer I and 15 mM $MgCl_2$.

PCR products were purified by employing a DNA clean up kit (Promega Corporation, Madison, WI) according to the manufacurer's instructions, and Sanger sequencing was conducted at the Australian Genome Research Facility (AGRF). Electropherograms were analysed using CodonCode aligner software (CodonCode Corporation) on a PC computer running Windows 7 (Microsoft, 2009).

Statistical analysis

If not otherwise mentioned, all statistical analyses for MRI23420 were evaluated with a Mann-Whitney-Wilcoxon test under R.2.15.0 (http://cran.r-project.org/). For MRI47455 and MRI47495 statistical analyses were performed by a student`s t-test. Linkage analysis was evaluated by a chi-square goodness of fit test. A p-value of 0.05 or less was considered significant and all analyses were performed using Excel software (Microsoft Office, 2013).

General recipes

MT-PBS buffer (10x)

87.6g sodium chloride, 22.7g sodium phosphate dibasic anhydrous, 6.2 sodium phosphate monobasic monohydrate made up in 1L deionised H_20 and autoclaved, pH 7.2. Lower concentrations of MT-PBS were diluted accordingly with deionised H_20.

FACS buffer

1% BSA, 0.1% sodium azide diluted in 1x MT-PBS, filter sterilised

SDS PAGE electrophoresis buffer (10x)

30g Tris base, 144g glycine, 10g SDS made up in 1L deionised H_20, pH 8.3

Required volume of 10x stock was diluted to 1x with deionised H_20 for each electrophoresis

Western transfer buffer

25mM Tris, 182mM glycine made up in 1L deionised H_20, pH 8.3

Chapter 3 – ENU MUTANT MOUSE LINE MRI23420

This chapter has been published in: Greth, A., Lampkin, S., Mayura-Guru, P., Rodda, F., Drysdale, K., Roberts-Thomson, M., McMorran, B. J., Foote, S.J., Burgio, G.R. 2012, 'A novel ENU-mutation in ankyrin-1 disrupts malaria parasite maturation in red blood cells of mice', *PLoS One*, 7(6)**:** e38999. The main body of this study is therefore a direct replica of the published version.

Introduction

Malaria is caused by transmission of the protozoan parasite *Plasmodium*. This disease kills almost 1 million people annually and affects a further 300-500 million (WHO 2010). Multiple epidemiological studies in human populations have shown that host genetics is a major determinant of susceptibility to malaria infection. (Verra et al., 2009, Sabeti et al., 2006, Haldane, 1949a). Numerous variants and mutations have been identified that are associated with survival and impaired parasite growth, including several polymorphisms in genes encoding erythrocyte-expressed proteins (Modiano et al., 2001a, Louicharoen et al., 2009, Ko et al., 2011, Jallow et al., 2009, Garcia et al., 1998). For example, the erythrocyte Duffy antigen receptor for chemokines (DARC), encoded by the *FY* gene, is necessary for the invasion of RBCs by *P. vivax* (Wertheimer and Barnwell, 1989, Miller et al., 1976, Horuk et al., 1993). A mutation in the GATA motif of the *FY* promoter prevents erythrocyte expression of FY. This mutation is common in African populations and confers protection against *P. vivax* infection (Tournamille et al., 1995b).

The cytoskeleton of erythrocytes has been of major interest in red cell biology for decades as multiple disorders arise from mutations in cytoskeletal components (Mohandas and Gallagher, 2008). Several such red cell disorders are also associated with resistance to malarial infection (Rank et al., 2009, Schulman et al., 1990, Shear, 1993, Shear et al., 1991, Zimmerman et al., 2003b). For example, individuals with hereditary elliptocytosis have deficiencies in either protein 4.1 or glycophorin C and have been reported to show resistance towards malaria infections (Chishti et al., 1996). Other RBC disorders such as hereditary spherocytosis

(HS) (Gallagher, 2005, Mohandas and Gallagher, 2008) have also shed light into the host-parasite interaction due to their involvement in malaria pathogenesis (Cooke et al., 2004, Maier et al., 2009). However, the elucidation of the mechanisms of resistance mediated by changes to these host erythrocytic proteins remains incomplete.

The murine host response to a malarial infection is an appropriate model for the human response to malaria (Berghout et al., 2010b, Hernandez-Valladares et al., 2005, Longley et al., 2011, Ohno et al., 2001). Mouse models of malaria have been used to study genetic factors that determine the host response to infection and to identify novel mechanisms that confer protection against Plasmodium (Carvalho, 2010, Hunt et al., 2010). Similar to humans, there is considerable variability in either the rate of development of blood parasitaemia or outcome to infection in different inbred mouse strains (Yap and Stevenson, 1992, Fortin et al., 1997a, Foote et al., 1997b). In addition to the identification of several quantitative trait loci that determine susceptibility towards rodent malarial infection, investigations of various mouse mutants have revealed novel genes associated with malaria pathogenesis (Burt et al., 2002, Foote et al., 1997b, Fortin et al., 1997a, Hernandez-Valladares et al., 2005, Lin et al., 2006). Given the difficulty identifying genes underpinning quantitative trait loci, we have employed a complementary approach using N-ethyl-N-nitrosourea (ENU) to generate random point mutations in mice to identify novel genes and mechanisms that underlie susceptibility to malaria infection. This strategy has been previously employed to decipher the genetic architecture determining several phenotypes (Yates et al., 2009a, Aigner et al., 2008), including the host response to other infectious diseases (Crozat et al., 2006, Hoebe and Beutler, 2008, Richer et al., 2010). In our ENU mutagenesis screen, we aimed to identify novel mutations that increase the resistance of an otherwise susceptible mouse strain to a malarial infection. Here we describe an ENU-mutant mouse line with increased survival to *P. chabaudi adami* DS infection, carrying a novel hypomorphic mutation in the *Ank-1* gene (*Ank-1*^{MRI23420}).

Ankyrin-1 is a large (210 kDa) cytoskeletal protein encoded by the *Ank-1* gene, found predominantly in the erythrocyte membrane. Mutations in human *Ank-1* have been studied for their role in the inherited hemolytic anaemia disorder, HS (Gallagher, 2005). Over 50% of HS cases are caused by mutations in this gene (Eber et al., 1996a). Recently, *Ank-1* has been

investigated for its role in malaria infections using a naturally occurring mutant (*Ank-1*^{nb}) (Shear et al., 1991) and an ENU generated mutant (*Ank-1*^{1674}) mouse line (Rank et al., 2009). Both of these mutations result in an abnormal ankyrin-1 protein in the erythrocyte. Mice carrying these mutations are more likely to survive infection from *P. chabaudi* and *P. berghei* (Shear et al., 1991, Rank et al., 2009). However, the role played by mutant ankyrin-1 in conferring increased resistance remains a point of conjecture. Studies have variously suggested that merozoite release, (Dhawan et al., 2003, Hanspal et al., 2002, Raphael et al., 2000) parasite invasion (Shear et al., 1991) and intraerythrocytic development (Shear et al., 1991) may be affected, although the evidence for these is not conclusive.

In this study we aim to further characterise the mechanistic basis of malarial resistance imparted by ankyrin-1 deficiency through the analysis of the *Ank-1*^{MRI23420} mouse line. We present novel evidence that maturation of intracellular *P. chabaudi* parasites is impaired in ankyrin-1 deficient RBCs. Although we cannot exclude other contributions, such as splenic clearance, we conclude that parasite growth retardation is the major mechanism contributing to the resistant phenotype observed in *Ank-1*$^{MRI23420/+}$ mice.

Results

Identification of a novel ENU *Ank-1*^{MRI23420} mutation

The ENU mutant line MRI23420 was generated during a screen for dominant ENU-induced mutations that affect RBCs in inbred SJL/J mice. Full automated blood analysis was conducted on samples collected from G1 animals at seven to eight weeks of age. The line MRI23420 was identified based on a mean corpuscular volume (MCV) that was three standard deviations less than the normal population (MCV = 45.2 ± 0.2 fl in mutant versus MCV = 52.0 ± 0.4 fl in wild type mice; p-value <0.001) (Table 4).

Table 4 – Haematological parameters on wild-type and Ank-1$^{MRI23420/+}$ mice

	WBC	*RBC*	*HGB*	*HCT*	*MCV*	*MCH*	*MCHC*	*RDW*	*PLT*	*%Retic*
Wild-type	6.1 ± 0.1	5.1 ± 0.1	70.4 ± 1.7	0.25 ± 0.01	53.4 ± 0.2	15.7 ± 0.5	293.9 ± 8.3	15.4 ± 0.1	498.3 ± 7.8	2.5 ± 0.2
Ank-1 $^{MRI23420/+}$	6.1 ± 0.2	**5.5±0.1****	72.0 ± 1.4	0.26 ± 0.02	**45.3 ± 0.1****	**13.6 ±0.3****	285.7 ± 5.9	15.5 ± 0.1	**540.6 ± 13.4***	3.0 ± 0.2

Automated full blood analyses were obtained on 69 WT and 72 *Ank-1*$^{MRI23420/+}$ mice at 7 weeks of age. Values are represented as mean value ± SEM. Statistical differences performed by a student`s t-test are indicated as (*) p-value <0.05 and (**) p-value <0.001. WBC indicates white blood cell count (n x 10^9/L), RBC, red blood cell count (n x 10^{12}/L); HGB, haemoglobin (g/L); HCT, haematocrit (ratio); MCV, mean corpuscular volume (fl); MCH, mean corpuscular haemoglobin (pg); MCHC, mean corpuscular haemoglobin concentration (g/L); RDW, red cell distribution width (%); PLT, platelet count (n x 10^9/L); and %Retic, %Reticulocytosis.

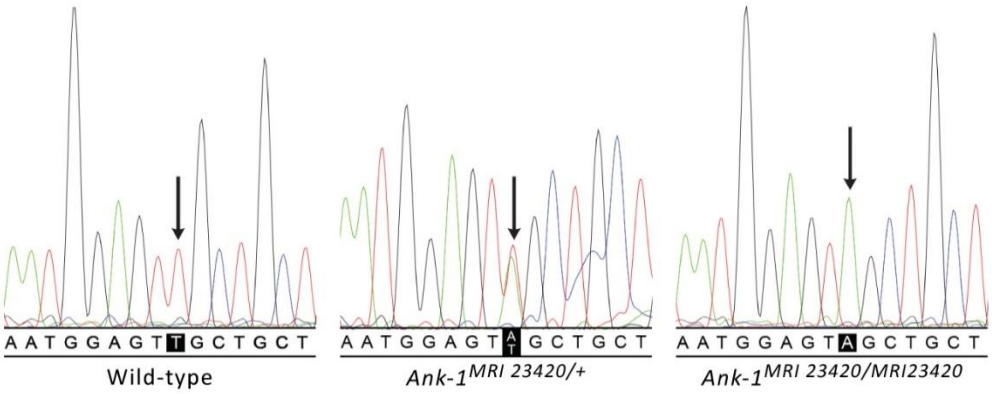
The mutation was mapped by backcrossing SJL/J mice exhibiting the mutant phenotype to C57BL/6 animals. Using an affected-only mapping strategy, progeny (F1s & N2s) were selected on the basis of an MCV <50.0 fl and DNA analysed using genome-wide SNP genotyping (20 affected N2 mice using 300 polymorphic SNPs). Linkage analysis identified a region on chromosome 8 shared by all affected N2 mice (LOD = 5.11), and the critical interval was further defined using microsatellite mapping to a region between 22 - 25Mb from the centromere (Figure 3.1A). Within this interval, the *Ank-1* gene was selected as a candidate and sequencing the affected G1 founder mouse revealed a single heterozygous mutation, which was also present in the affected N2 animals. The mutation, a single point transversion (T → A) in exon 11 at nucleotide position 1265 of *Ank-1* (Figure 3.1B), creates a premature termination codon

at position 422 predicted to produce an ANK-1 protein truncated within the band 3 binding domain (Figure 3.1C-D).

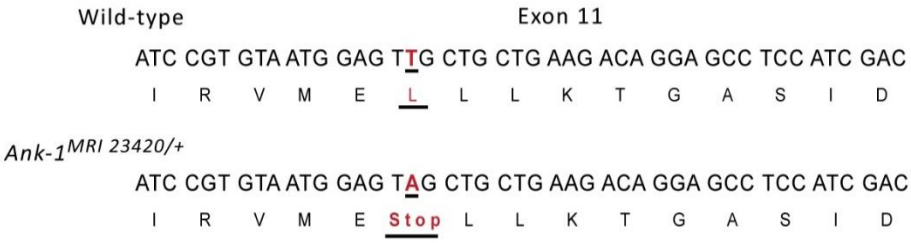
A

Marker	Position (Mb)	Affected				Unaffected	
D8Mit170a	22						
D8Mit95	25						
D8Mit190	37						
D8Mit65	44						
Number of mice		1	7	2	3	2	3

B



C



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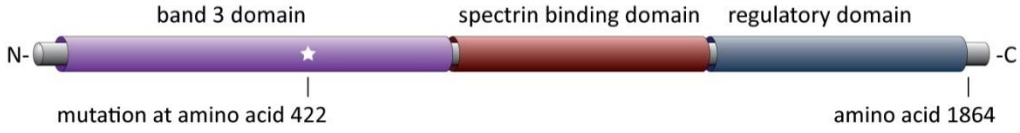

Figure 3.1 - Identification of the *Ank-1*^{MRI23420} allele. (A) Haplotypes of 2nd generation offspring. Narrowing down critical interval using microsatellites on chromosome 8. Grey boxes represent heterozygous and white boxes homozygous mice. The candidate interval for *Ank-1*^{MRI23420} was refined to 22–25 Mb on Chr. 8. (B) DNA sequence electropherograms showing the T to A transversion in exon 11 for WT, *Ank-1*$^{MRI23420/+}$ and *Ank-1*$^{MRI23420/MRI23420}$ mice. (C) Schematic view of the amino acid change. (D) Representation of the position of the mutation within the protein.

To investigate the effect of the mutation on *Ank-1* expression, we conducted immuno-blotting on whole blood lysates from WT, *Ank-1*$^{MRI23420/+}$ and *Ank-1*$^{MRI23420/MRI23420}$ mice. A 210 kDa anti-mouse ANK-1 immunoreactive band, corresponding to the full-length protein, was observed in samples from the WT and heterozygous mice, while this band was not detected in the homozygote mice (Figure 3.2A-B). A smaller band (~50 kDa) was also present in the heterozygous and homozygous mutant samples, closely matching the theoretical size of the truncated form of ankyrin-1 (49 kDa; Figure 3.2A). Quantification of these immunoreactive bands revealed reduced levels of the full-length protein in *Ank-1*$^{MRI23420/+}$ blood cells compared to WT cells, while *Ank-1*$^{MRI23420/MRI23420}$ cells displayed a greater amount of the truncated form compared to their heterozygous relatives. To assess if a compensatory mechanism substitutes for the lack of functional *Ank-1* protein, a quantitative PCR was conducted using two *Ank-1* primers spanning before and closely after the ENU mutation (Figure S3.1A-B). Relative quantification of *Ank-1* mRNA expression in different tissues found significantly elevated levels of transcript in some *Ank-1*$^{MRI23420/+}$ tissues. In the spleen and the brain the transcript level of *Ank-1*$^{MRI23420/+}$ is respectively 4 and 2.5 fold over-expressed and 0.7 fold under-expressed in the kidney. No differences were found on transcript levels in the liver.

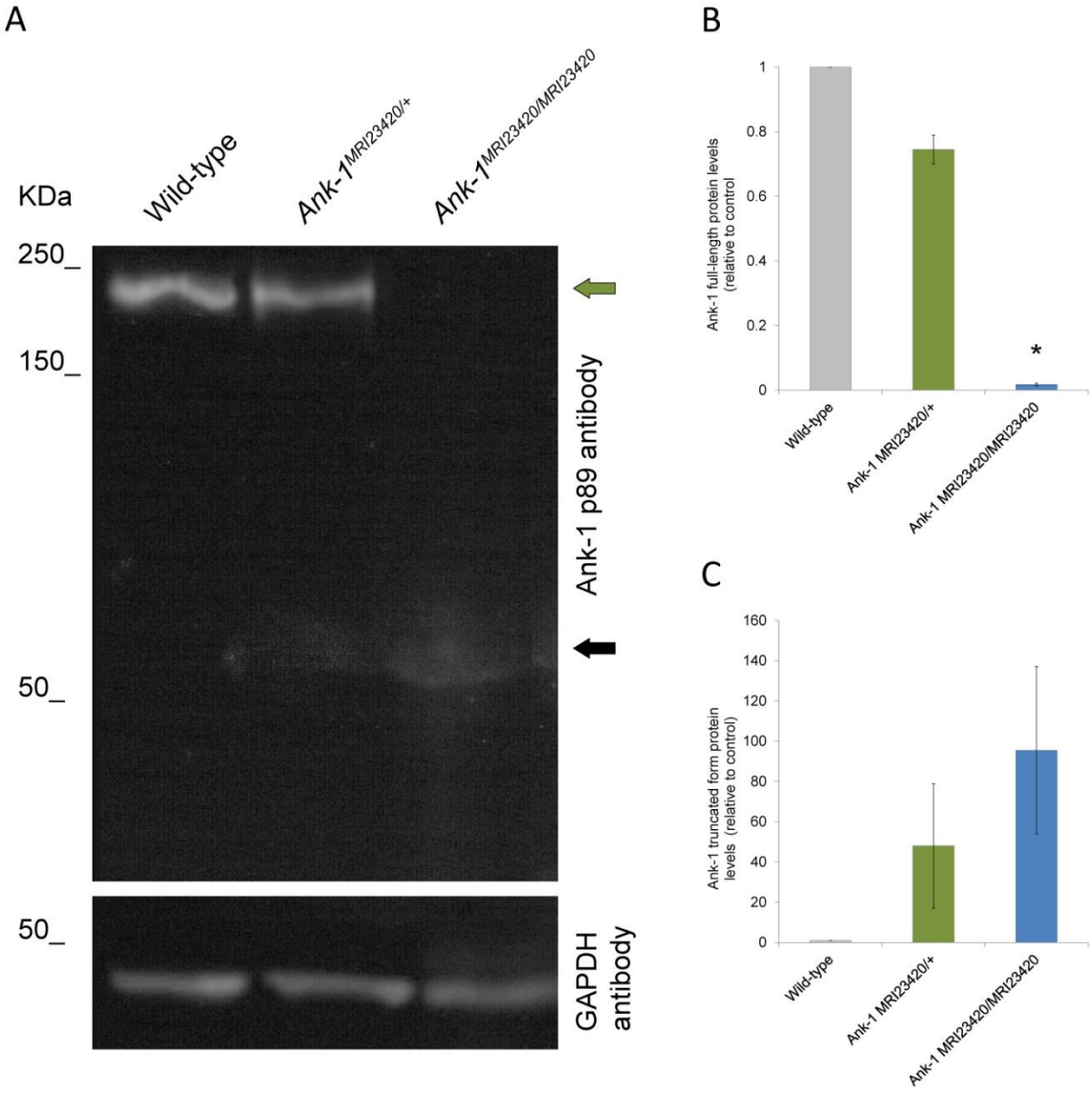

Figure 3.2 - Protein levels of ankyrin-1 analysis. (A) Immunoblot of erythrocytic ankyrin-1 in WT, *Ank-1*$^{MRI23420/+}$, and *Ank-1*$^{MRI23420/23420}$ mice with the N-terminal antibody ANK-1 p89. Bands for full-size (green arrow) and the truncated species (back arrow) are indicated. Quantification of the intensity of bands by densitometry for full-size ankyrin-1 (210 kDa) (B) and the truncated species (C) (calculated size 49 kDa) with n=2 mice per group. Error bars indicate SEM and statistical differences (student`s t-test) are presented by * (p-value <0.05).

The ENU blood mutant MRI23420 displays an hereditary spherocytosis phenotype

The observed frequency of homozygous live births was 10%, compared to an expected 25%, suggesting that the *Ank-1* mutation is embryonic or neonatal lethal in the homozygous condition. *Ank-1*$^{MRI23420/MRI23420}$ mice displayed severe jaundice and had a life expectancy of only 12-48 hours (Figure 3.3A). Autopsy revealed distinct splenomegaly (spleen weight divided

by body weight in WT mice 5.5 ± 0.4g versus 7.1 ± 0.6g in *Ank-1*$^{MRI23420/MRI23420}$, p-value <0.05) and Giemsa stained blood smears showed distinct amorphous poikilocytosis with marked spherocytosis, severe RBC fragmentation and increased immature RBCs (Figure 3.3B). Due to the severity of their phenotype, homozygous mice were not subjected to malarial studies.

Heterozygous *Ank-1*$^{MRI23420/+}$ mice displayed a slight splenomegaly (0.11 ± 0.03g for WT mice versus 0.16 ± 0.01g for heterozygous mice; p-value <0.05). Histological examination revealed changes in the splenic architecture, with a reduced medulla and proliferation of the extramedullary compartment (Figure S3.2A-B). In addition, Perl's Prussian Blue staining revealed extramedullary iron deposition (Figure S3.3A-B). Quantification of non-haem iron (Figure S3.3C) demonstrated an almost two-fold increase in iron deposition in the spleen of heterozygous mice consistent with an iron overload phenotype. Haematological analysis of the heterozygous mutant mice revealed a number of red cell abnormalities (Table 4) including marked microcytosis and mild polycythemia. This was confirmed in peripheral blood smears, where small, hyperchromic red blood cells were observed (Figure 3.3C-D). Scanning electron microscopy revealed that over half (68.9 ± 17.4%) of the red cells in *Ank-1*$^{MRI23420/+}$ mice were severely deformed, with obvious membrane blebbing, although no fragmentation was seen. By comparison only 6.25 ± 3.95% of WT RBCs were abnormal (Figure 3.4A-C). To investigate this further, we conducted osmotic fragility assays and found that cells from heterozygous mice lysed at significantly higher salt concentrations than WT (Figure 3.4D). In concert with the observed anaemic phenotype we observed an almost two-fold increase in serum levels of the erythropoietin, the hormone controlling erythropoiesis, in *Ank-1*$^{MRI23420/+}$ mice (530.7 ± 47.7pg/ml; p-value <0.001) compared to WT (278.3 ± 24.9pg/ml). Several *Ank-1* mutations in both humans and mice have been reported in the literature, and all of them have been associated with a HS phenotype exhibiting anaemia, splenomegaly, and a higher osmotic fragility in RBCs (Eber et al., 1996a, Hughes et al., 2011, Randon et al., 1997, Rank et al., 2009).

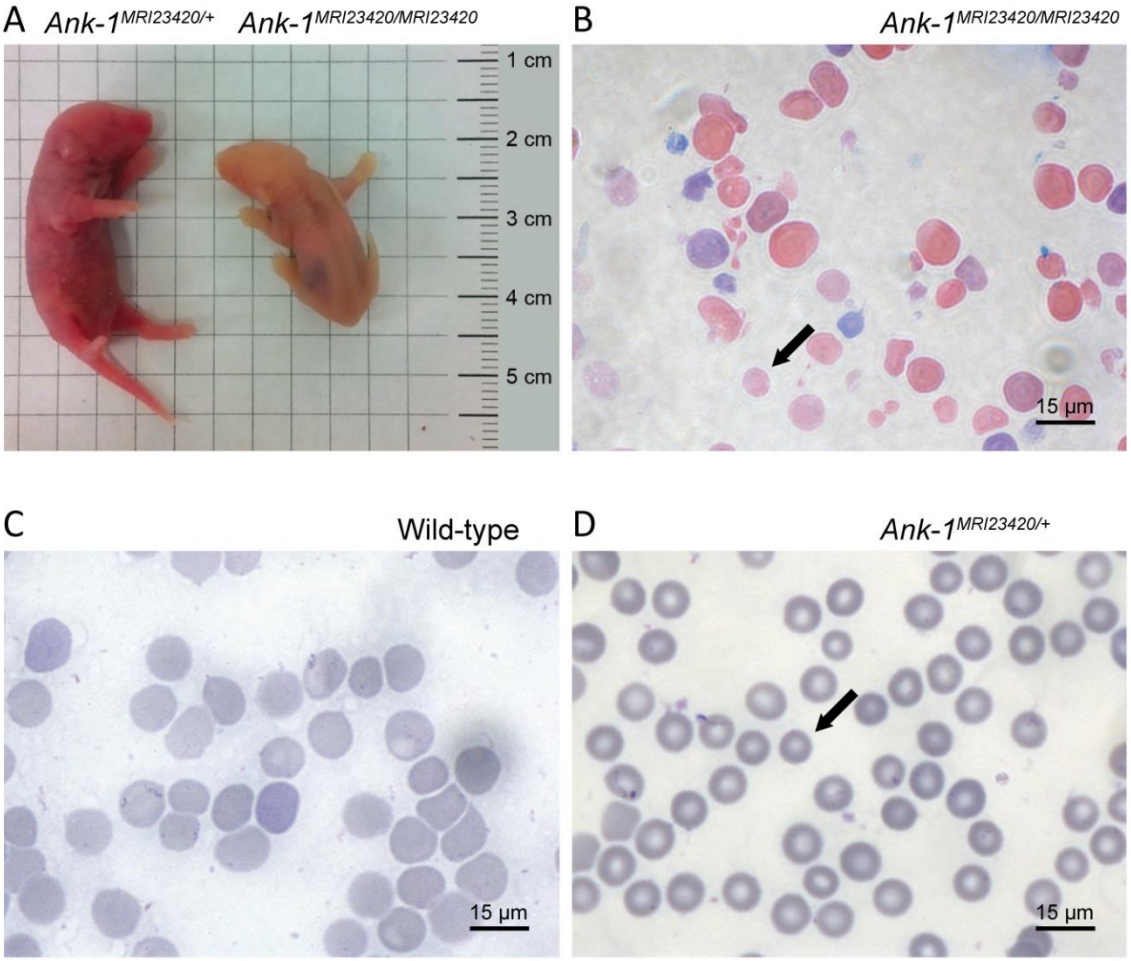

Figure 3.3 - Identification of *Ank-1*$^{MRI23420/+}$ and *Ank-1*$^{MRI23420/23420}$ mice. Jaundiced postnatal day 1 *Ank-1*$^{MRI23420/MRI23420}$ pup and an *Ank-1*$^{MRI23420/+}$ control littermate. Giemsa stained peripheral blood smears from (B) *Ank-1*$^{MRI23420/MRI23420}$ mice, (C) WT, and (D) *Ank-1*$^{MRI23420/+}$ mice. Black arrows indicate microcytic RBC in *Ank-1*$^{MRI23420/+}$ and spherocytes in *Ank-1*$^{MRI23420/23420}$.

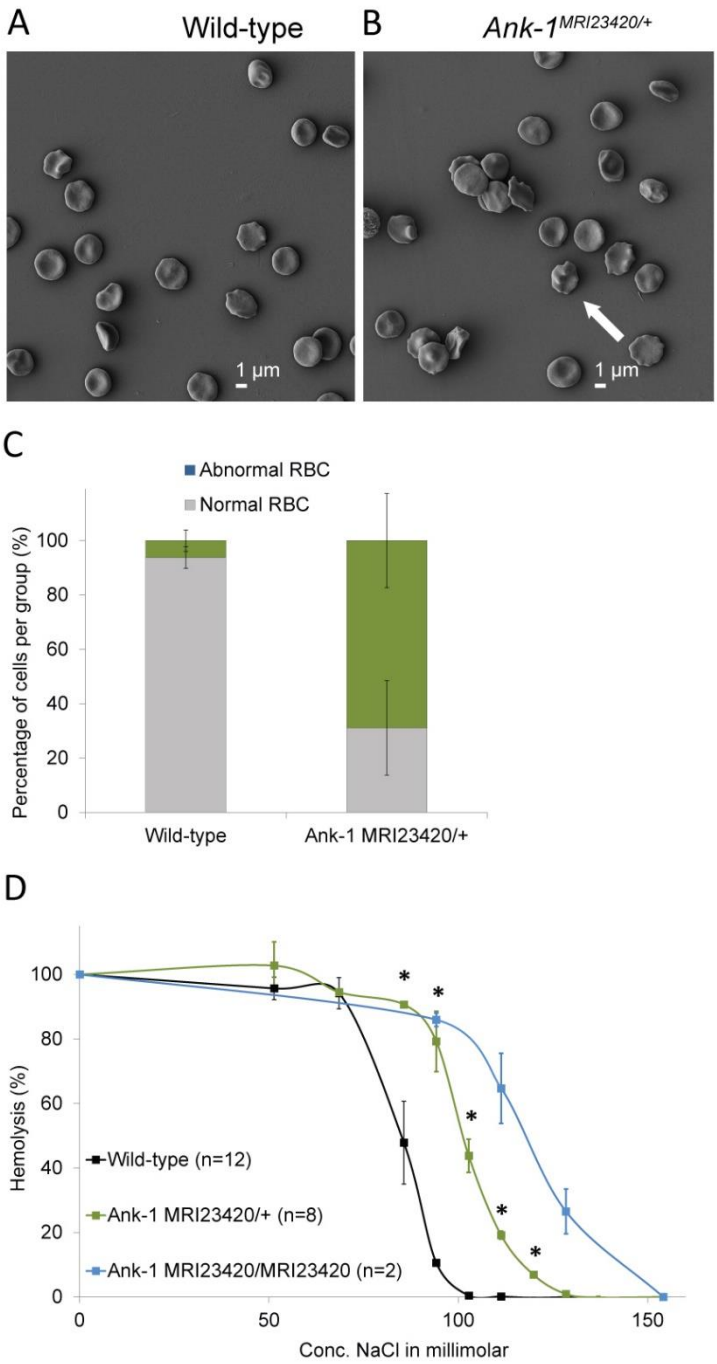

Figure 3.4 - Haematological phenotype of *Ank-1*$^{MRI23420/+}$ RBCs. Scanning electron microscope imaging of peripheral blood from uninfected (A) WT and (B) *Ank-1*$^{MRI23420/+}$ mice. The white arrow indicates erythrocyte membrane blebbing and deformity. (C) Enumeration of the proportion of normal and abnormal RBCs observed from 5 mice per group. (D) Osmotic fragility plot of WT, heterozygous and homozygous mice. Error bars indicate SEM and statistical differences are marked with a student's t-test p-value <0.01 (*).

Ank-1$^{MRI23420/+}$ mice are resistant to malaria infection

We sought to characterise the effect of the *Ank-1*^{MRI23420} mutation in a malarial infection. Heterozygous and WT mice of both genders were challenged with a normally lethal dose of *P. chabaudi adami* DS. A dramatic increase in survival of the *Ank-1*$^{MRI23420/+}$ mice compared to WT SJL was observed in both males and females ($p < 0.001$ and $p < 0.0001$, respectively, using a Mantel-Cox test); the respective mortality rates for each sex were 6% (females) and 38% (males) for *Ank-1*$^{MRI23420/+}$ and 90% (females) and 100% (males) for SJL mice (Figure 3.5A-B). Further, we monitored the development of blood parasitaemia. The proportions of infected RBCs in mutant animals of both sexes peaked at a significantly lower level and later time (one or two days) compared to WT (Figure 3.5C-D). Therefore, as well as acting dominantly to cause an HS-like phenotype, a single copy of the *Ank-1*^{MRI23420} mutation resulted in dramatically enhanced resistance to malaria infection.

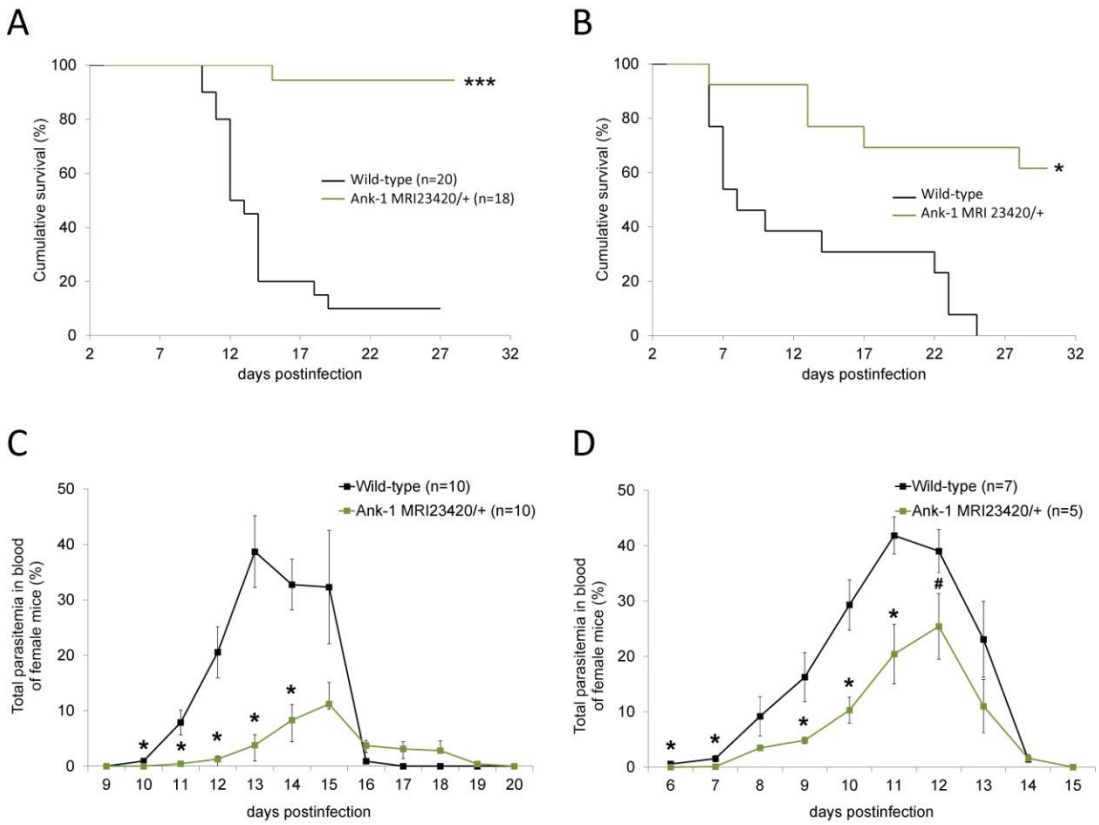

Figure 3.5 - *Ank-1*$^{MRI23420/+}$ mice display resistance towards malarial infections. Kaplan Meier survival curve for (A) female and (B) male animals. Number of infected RBCs in (C) female and (D) male mice. Infection dose was $4.6x10^4$ and $2.6x10^4$ for female and males respectively. Error bars indicate SEM and statistical differences are marked with a student's t-test p-value <0.05 (#), p-value <0.01 (*), and Mantel Cox Log rank with p-value <0.001 (**) and p-value <0.0001 (***).

The *Ank-1*^{MRI23420} malaria resistance phenotype is due to a red cell autonomous effect

To assess the contribution of abnormal red cells and possible secondary effects of the mutation (eg altered splenic function) to the malaria resistance phenotype, we developed an *in vivo* parasite invasion and growth assay. RBCs isolated from uninfected WT and *Ank-1*$^{MRI23420/+}$ mice were labelled with individual fluorescent markers (ATTO-495 and ATTO-633), mixed in equal proportions, and injected back into the bloodstream of infected recipient mice of both genotypes (WT and *Ank-1*$^{MRI23420/+}$). Following a period to allow for parasite reinvasion, samples were collected and examined for the proportion of infected cells of each labelled population growing in animals of each genotype (Figures S3.4A-B). The results revealed a significant decrease in the proportion of infected cells from the *Ank-1*$^{MRI23420/+}$ line compared to WT (p-value<0.0001; Figure 3.6). This difference was observed irrespective of the dye used to label each type of cell, and occurred independently of the genotype of the recipient animal. Therefore, the *Ank-1*^{MRI23420} mutation appears to confer the resistance phenotype by directly affecting the ability of the host red cell to support either invasion, egress and/or growth of the parasite. However, we also observed a modest but significant increase in spleen weight in infected mutant mice around the time of peak parasitaemia (0.30 ± 0.03g for WT versus 0.82 ± 0.10g for *Ank-1*$^{MRI23420/+}$, p-value <0.05) consistent with increased splenic clearance of RBCs. We believe that this does not significantly affect the clearance of abnormal cells from the circulation as the proportion of infected cells of either genotype was not dependent on the background. However, it is possible that infected cells are cleared more in mutant mice, but this would be similar for both mutant and WT cells. This observation may also be related to the underlying different baseline splenomegaly present in these mice (see above).

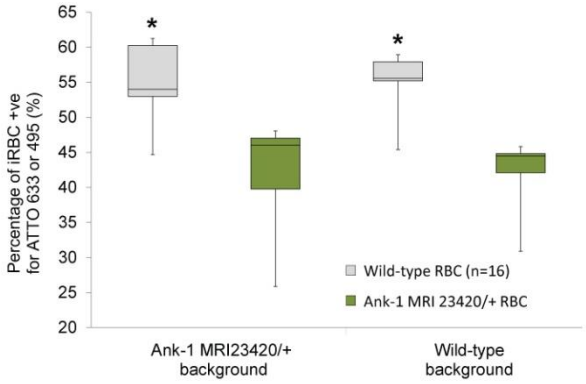

Figure 3.6 - Infection rate of P. chabaudi in *Ank-1*$^{MRI23420/+}$ compared to WT RBCs is impaired. Uninfected RBCs from WT and *Ank-1*$^{MRI23420/+}$ mice were labelled individually with different fluorescent dyes, mixed at a 1:1 ratio and injected into 16 infected animals of each group. 10 hours later tail blood was taken and analysed by flow cytometry, gating for infected RBCs +ve for either dye (ATTO 633 & 495). Results were calculated as the mean of normal and reversible labelled RBCs from each group for each background separately. Error bars are presented as SEM and (*) indicate statistical differences with a student`s t-test p-value <0.0001.

Intraerythrocytic parasites die during infection in *Ank-1*$^{MRI23420/+}$ mice

After merozoites invade RBCs, malaria parasites grow into ring forms and subsequently develop into trophozoites that undergo nuclear divisions, becoming mature schizonts. In the case of *P. chabaudi* the parasites' intraerythrocytic life cycle lasts 24 hours (Chimanuka et al., 1999) and the parasites show a synchronous growth cycle at low parasitaemia.

During our observation of the parasites' asexual life cycle under a light microscope it became noticeable that when compared to WT RBCs, a significantly larger proportion of parasites in mutant RBCs were smaller and pyknotic. This phenomenon was visible 6-8 hours (37.4% ± 6.3 in *Ank-1*$^{MRI23420/+}$ vs. 17.6% ± 1.1 in WT RBCs; p-value <0.05) (Figure 3.7A-B) and 18-20 hours (40.7% 8.0 in *Ank-1*$^{MRI23420/+}$ vs. 16.3% ± 2.2 in WT RBCs; p-value <0.05 (Figure 3.7C-D) post-invasion. From this observation we speculated that parasites are less healthy while growing in mutant RBCs and hypothesised that *P. chabaudi* may suffer from retarded development during the erythrocytic life cycle in *Ank-1*$^{MRI23420/+}$ mice. Further, we observed that in *Ank-1*$^{MRI23420/+}$ compared to WT RBCs, parasite replication was noticeably lower after 18-20 hours post-invasion (Figure S3.5).

To objectively quantify these observations we used the TUNEL stain procedure, adapted to detect sheared or fragmented DNA, indicative of dying or dead intraerythrocytic parasites (Figure 3.8A-C) (McMorran et al., 2009). Compared to WT, a significant increase in dead parasites of almost two-fold (<0.05 p-value) was apparent in blood taken from mutant mice approximately six to eight hours after parasite invasion. The proportion of dead parasites in mutant compared to WT mice remained significantly higher 12 hours later (18-20 hours post-invasion, p-value <0.05) (Figure 3.8D). This is consistent with our observation of morphologically compromised parasites in mutant cells at both time points, and the lower parasite replication rate noticed after 18-20 hours post-invasion. Together these observations suggest an increased death rate of parasites during their development within *Ank-1*$^{MRI23420/+}$ RBCs.

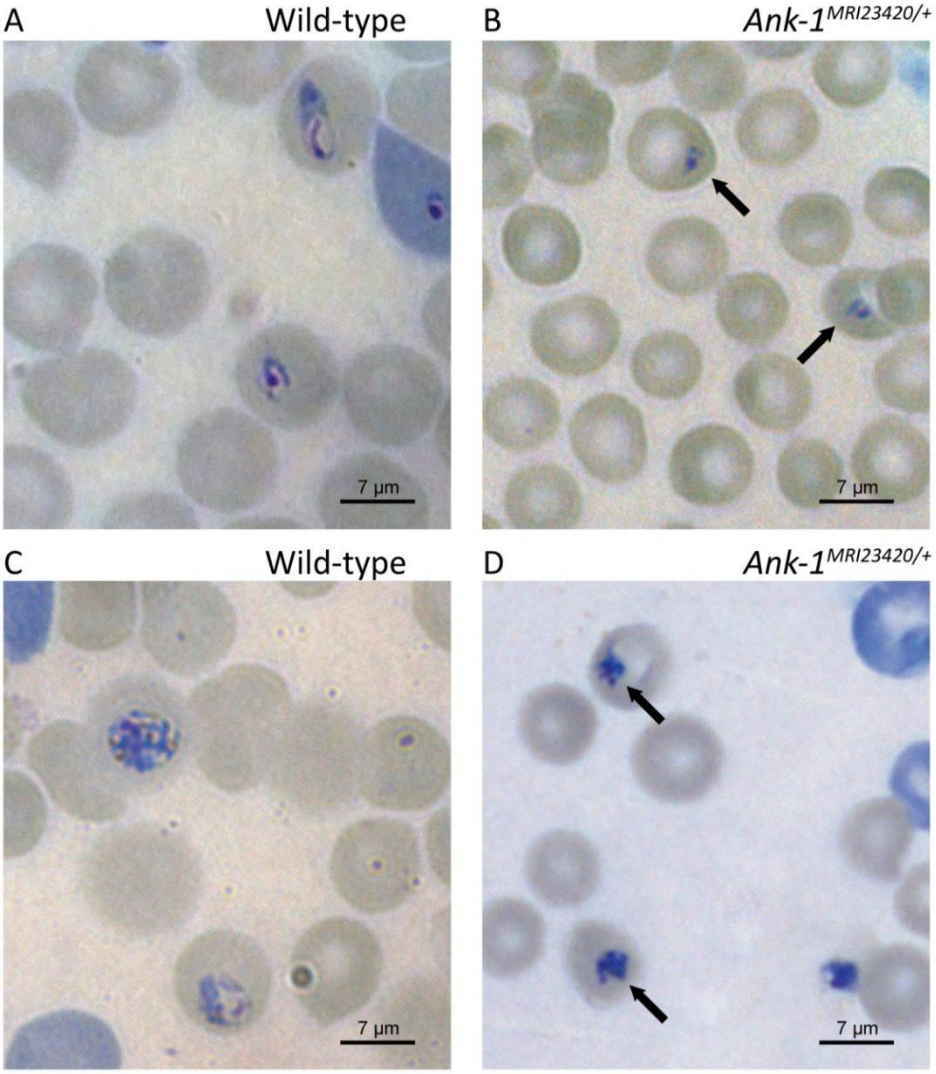

Figure 3.7 - Impaired growth phenotype of P. chabaudi parasites in *Ank-1*$^{MRI23420/+}$ RBCs. Giemsa stained blood smears showing the morphology of parasites in WT and *Ank-1*$^{MRI23420/+}$ mice 6–8 hours (A-B) and 18–20 hours (C-D) post-invasion. Black arrows indicate condensed phenotype of intraerythrocytic parasites observed in heterozygous mutant mice

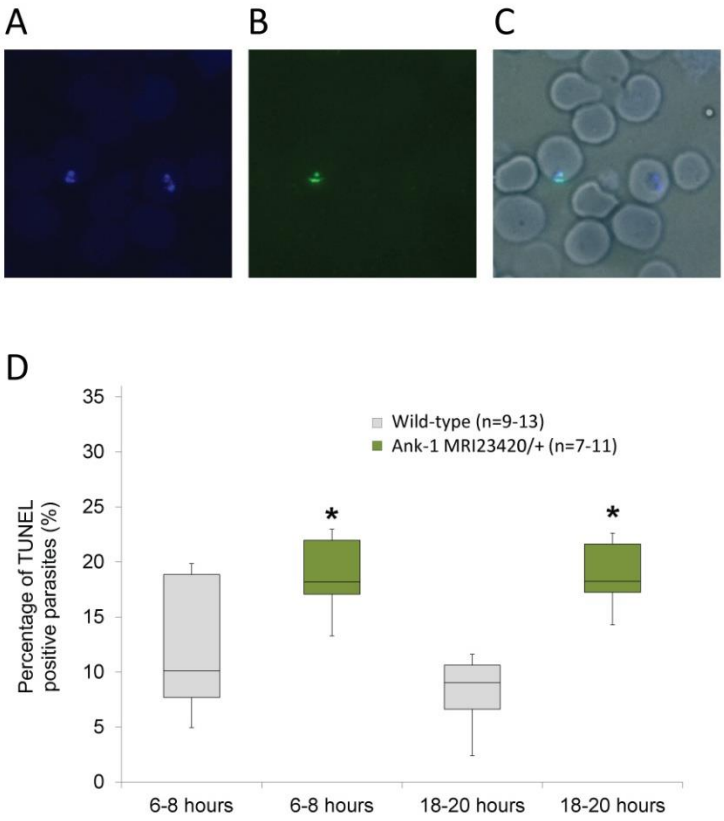

Figure 3.8 - Impaired maturation of P. chabaudi in *Ank-1*$^{MRI23420/+}$ RBCs. (A) Nuclear yellow staining of parasite DNA. (B) TUNEL staining of fragmented parasite DNA, and (C) merged TUNEL and nuclear yellow staining superimposed on brightfield showing infected RBC. (D) Percentage of double positive for nuclear yellow and TUNEL stained parasites in RBCs of both cohorts after counting at least 100 nuclear yellow RBCs per slide. Blood was collected 6–8 hours (ring stage) and 18–20 hours after invasion (trophozoite stage). Error bars indicate SEM and statistical differences with a student`s t-test p-value <0.05 are indicated by (*).

Discussion

In this study, we identified a novel ankyrin-1 mutation and characterized several associated haematological phenotypes. Furthermore, we investigated the mechanism of malaria resistance seen in these ankyrin-1 deficient mice. For the first time, we demonstrated that a mutation in the *Ank-1* gene decreases susceptibility to malaria by reducing survival of *P. chabaudi* in affected RBCs.

Ankyrin-1 protein is a crucial component of the RBC cytoskeletal complex and its deficiency is responsible for the most common type of human hereditary spherocytosis (HS) (Eber et al., 1996a, Gallagher, 2005, Gallagher and Forget, 1998). Likewise, mice lacking a functional form of the ankyrin-1 protein also share most of the signs and symptoms of human HS (Birkenmeier et al., 2003, Hughes et al., 2011, Rank et al., 2009). In this report, we demonstrate that our novel ENU-induced mutation in *Ank-1*^{MRI23420} mice displays a clinical phenotype most consistent with the HS *Ank-1*^{E924X} mouse model (Hughes et al., 2011). It has more pronounced symptoms than either the recessively inherited *Ank-1*^{nb} mutation (Birkenmeier et al., 2003) or the null mutant *Ank-1*^{1674} (Rank et al., 2009). Notably homozygous *Ank-1*^{MRI23420} mice exhibit a 100% postnatal death rate with severe signs of jaundice due to massive haemolysis. Heterozygous *Ank-1*$^{MRI23420/+}$ mice display a regenerative anaemia and splenomegaly with iron overload.

The closest human counterpart to the *Ank-1*^{MRI23420} murine mutation is the *Ank-1*^{Bari} mutation, which has a frame-shifting deletion in exon 12 leading to a truncated protein. Interestingly, and similar to the *Ank-1*^{MRI23420} phenotype, humans with the *Ank-1*^{Bari} mutation have a more severe phenotype than other human ankyrin-1 mutations (Randon et al., 1997). Despite the differences in phenotypic severity between the murine HS models, all share several haematological symptoms. These include an increased RBC count, increased osmotic fragility and increased red cell deformity. Therefore, this novel ENU-induced mutation represents another model for the trial of preclinical therapeutic interventions for the treatment of affected individuals with dominant inherited HS.

Importantly, heterozygous *Ank-1*^{MRI23420} mice exhibited a dramatic increase in resistance to malaria. Previous studies focused on two different *Ank-1* mice carrying different mutations in the regulatory domain of the protein: the normoblastosis mutation (*Ank-1*^{nb}) (Birkenmeier et al., 2003) and *Ank-1*^{1674} (Rank et al. 2009). Both of these strains exhibit HS and also display a survival advantage to *Plasmodium chabaudi* infection (Rank et al., 2009, Shear et al., 1991). However, the ENU-induced *Ank-1*$^{MRI23420/+}$ mice exhibited a delay in the rise of peak parasitaemia and a more dramatic reduction in the height of the peak (at least 20 to 40%) compared to heterozygous *Ank-1*^{nb} mice (10%). Increased malaria resistance was initially observed 20 years ago in *Ank-1*^{nb} mice; however, the mechanism underpinning malaria resistance in mice carrying ankyrin-1 mutations remains elusive. Rank et al. (Rank et al., 2009) examined several potential mechanisms including a hypothesised impaired invasion mechanism of the parasite into RBCs. Despite their efforts no differences in invasion efficiency or altered RBC survival between mutant and WT was found. The only possible functional difference between *Ank-1*^{1674} and WT mice was a significant increase in osmotic fragility in affected RBCs. This could possibly result in an increased clearance of infected mutant cells from the circulation by the spleen.

A postulated impairment of parasite invasion into ankyrin-1 deficient RBCs has received considerable attention. This is not surprising considering that the observed increase in osmotic fragility and deformity in ankyrin-1 deficient RBCs is likely to disrupt an association between the cytoskeleton and invading parasite. In support of this hypothesis, elliptocytic RBCs with protein 4.1 and glycophorin deficiencies have been reported to inhibit *P. chabaudi* entry. In contrast, the study conducted by Rank et al. (Rank et al., 2009) did not find evidence for this mechanism using an *in-vitro* invasion assay system with *P. berghei* as a parasite model. Nevertheless, by injecting a 1:1 mix of both host fluorescently labelled RBCs into WT and *Ank-1*$^{MRI23420/+}$ mice in an *in vivo* assay system we demonstrated that *Ank-1*$^{MRI23420/+}$ RBCs were significantly less likely to be infected than WT in a cell-autonomous manner. Thus far, we cannot conclude from our results if the decrease in infectivity is due to impaired invasion as we cannot rule out other contributors such as impaired schizogony due to growth retardation or clearance from the circulation. However, the latter explanation is unlikely given that it

would have to be young ring-stage parasites that were being cleared. Therefore, it remains unclear if a deficiency of ankyrin-1 in RBCs perturbs invasion of malaria parasites.

However, we noticed that intraerythrocytic parasites appeared morphologically abnormal in *Ank-1*$^{MRI23420/+}$ RBCs. The parasites growing in mutant cells were noticeably condensed and smaller 6-8 hours after invasion. The same phenomenon was observed at trophozoite stage (18-20 hours post-invasion). Given that this may represent parasite death, we investigated the health of *P. chabaudi* parasites *in vivo* using TUNEL staining. The proportion of TUNEL positive dead parasites was significantly greater at ring and trophozoite stage in *Ank-1*$^{MRI23420/+}$ RBCs compared to WT. From these results we can conclude that deficiency in ankyrin-1 results in increased death of the intraerythrocytic parasites, which inversely affects the course of parasitaemia resulting in resistance to malarial infection.

A previous study on *P. falciparum* and human RBCs suggested that ankyrin-1 plays a role during the late stages of parasite development (Hanspal, 2002). In this process, falcipain-2 of the cysteine protease family cathepsin (Hanspal, 2000) was shown to cleave ankyrin-1 and protein 4.1 in RBCs (Hanspal et al., 2002). In support, another study found that several proteins associated with the RBC cytoskeleton including ankyrin-1 are proteolysed during merozoite release (Millholland et al., 2011a). To demonstrate a functional role of ankyrin-1 in merozoite release, a synthetic peptide spanning the identified cleavage site of the cytoskeletal protein (amino acid 1210), prevented falcipain-2 activity and subsequent development of *P. falciparum* trophozoites into segmented schizonts and subsequent rings. Our results demonstrating a growth arrest of parasites at trophozoite stage provide *in vivo* support to the hypothesis that ankyrin-1 is important for trophozoite development.

In addition, we noticed that survival of intraerythrocytic parasites was reduced at ring stage. The observation from both the TUNEL experiments and microscopic, morphological changes, that parasites begin dying as early as six hours after invasion, indicates that survival of parasites in mutant RBCs is affected before trophozoite development. This contrasts with the study by Dhawan et al. (Dhawan et al., 2003), showing that ring stage parasites incubated with the anti-ANK-1 peptide matured to trophozoites comparably to their control group. Our

observation would suggest that decreased ankyrin-1 levels affect the parasite`s survival in RBCs via mechanisms additional to falcipain-2 cleavage.

Several studies have suggested that abnormal cytoskeletal proteins in RBCs may disrupt survival of intraerythrocytic parasites (Shear et al., 1991, Shear, 1993, Schulman et al., 1990). Pantaleo et al. (Pantaleo et al., 2010) have reported that phosphorylation of several RBC membrane proteins, including band 3 and ankyrin-1, are influenced by the parasite during maturation. They speculated that the observed phosphorylative changes of cytoskeletal proteins including ankyrin-1 may be a response to perturbation of RBC homeostasis during parasite growth. However, these studies did not address any functional consequences of these changes. To our knowledge this is therefore the first evidence that the increased resistance of *Ank-1* impaired mice is most closely related to intraerythrocytic parasite growth rates during the initial expansion in circulating parasite mass. This is consistent with a red cell autonomous effect.

In summary, we generated a novel ENU-mutation in *Ank-1* resulting in an HS phenotype in mice. We demonstrated that a decrease in ankyrin-1 protein levels reduced susceptibility to malaria infections. Furthermore, we revealed that *P. chabaudi* parasites display reduced growth and probably invasion and/or egress capacity in RBCs exhibiting ankyrin-1 abnormalities. The importance of ankyrin-1 during the parasites maturation stage gives us further insights into the understanding of the molecular interactions between the parasite and its host cell. This study provides the first direct evidence that a cytoskeletal host protein plays a crucial role in parasite maturation. Further, these findings highlight the importance of host cytoskeletal protein abnormalities in malaria resistance.

Acknowledgments

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the mouse colony. We would also like to thank two anonymous reviewers for helpful comments and excellent suggestions on this manuscript to improve its quality.

Supporting figures

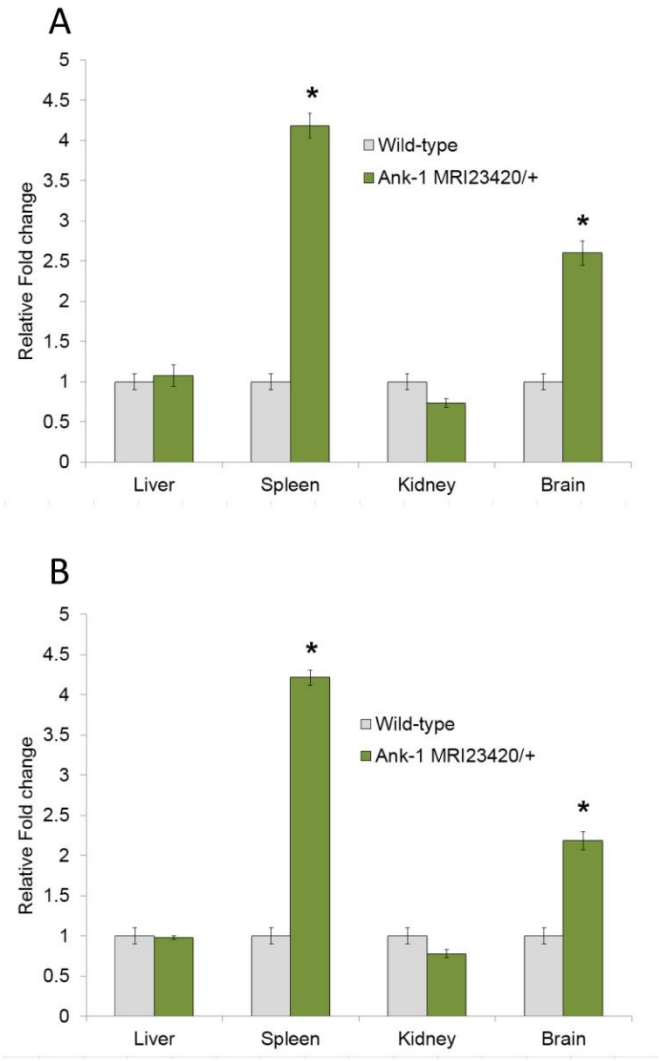

Figure S3.1. Gene expression level of *Ank-1*$^{MRI23420/+}$ and wild type mice in the spleen, liver, kidney and brain. (A) *Ank-1* primers spanning the exons 6 and 7 upstream and (B) downstream (exons 17 and 18) of the mutation. Error bars indicate SEM and statistical differences were indicated by * with a student`s t-test p-value <0.05 and n = 2 per group.

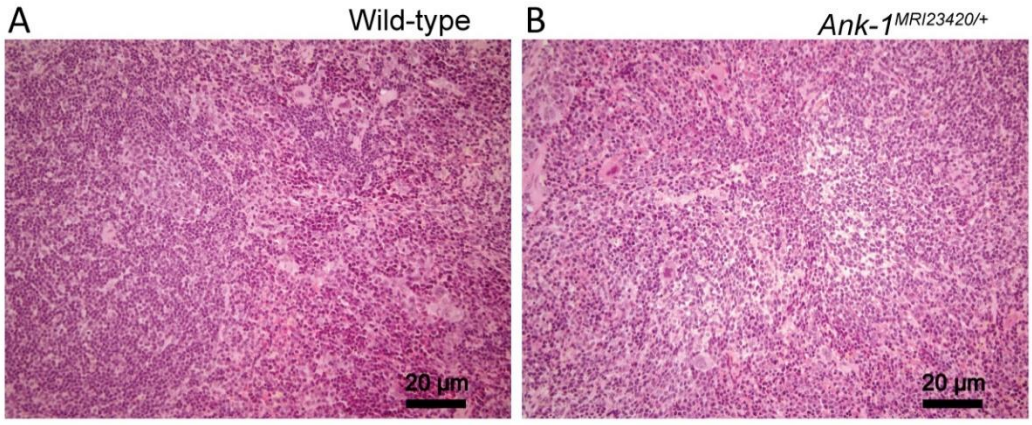

Figure S3.2. H & E stained spleen tissue from uninfected WT and *Ank-1*$^{MRI23420/+}$ mice. Spleen section from uninfected (A) WT and (B) heterozygous mice with a reduced medulla and a proliferation of the extramedullar compartment.

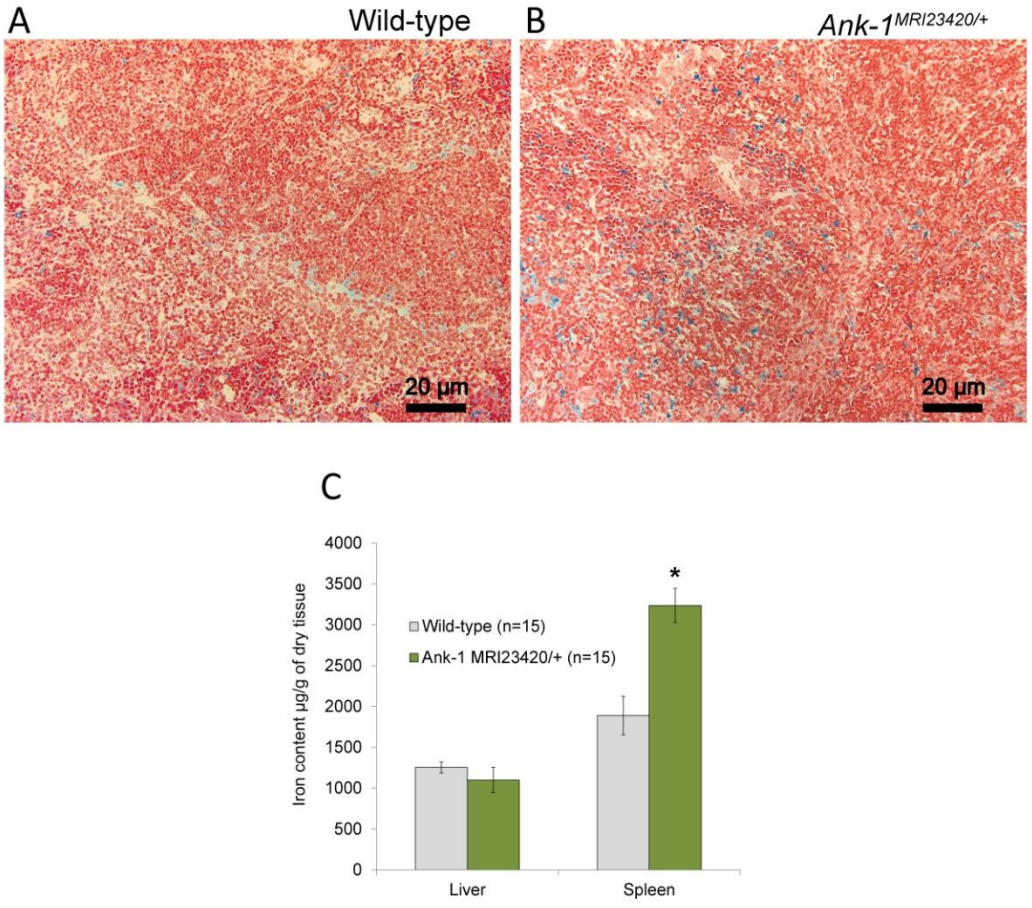

Figure S3.3. Uninfected *Ank-1*$^{MRI23420/+}$ mice exhibit an iron overload phenotype in the spleen. Histology and Perl`s blue Prussian staining for iron in (A) WT and (B) *Ank-1*$^{MRI23420/+}$ spleens. (C) Colourimetric analysis of non-haem iron in spleen and liver from uninfected *Ank-1*$^{MRI23420/+}$ and WT mice. Error bars are presented as SEM and (*) indicate statistical differences with a p-value <0.05.

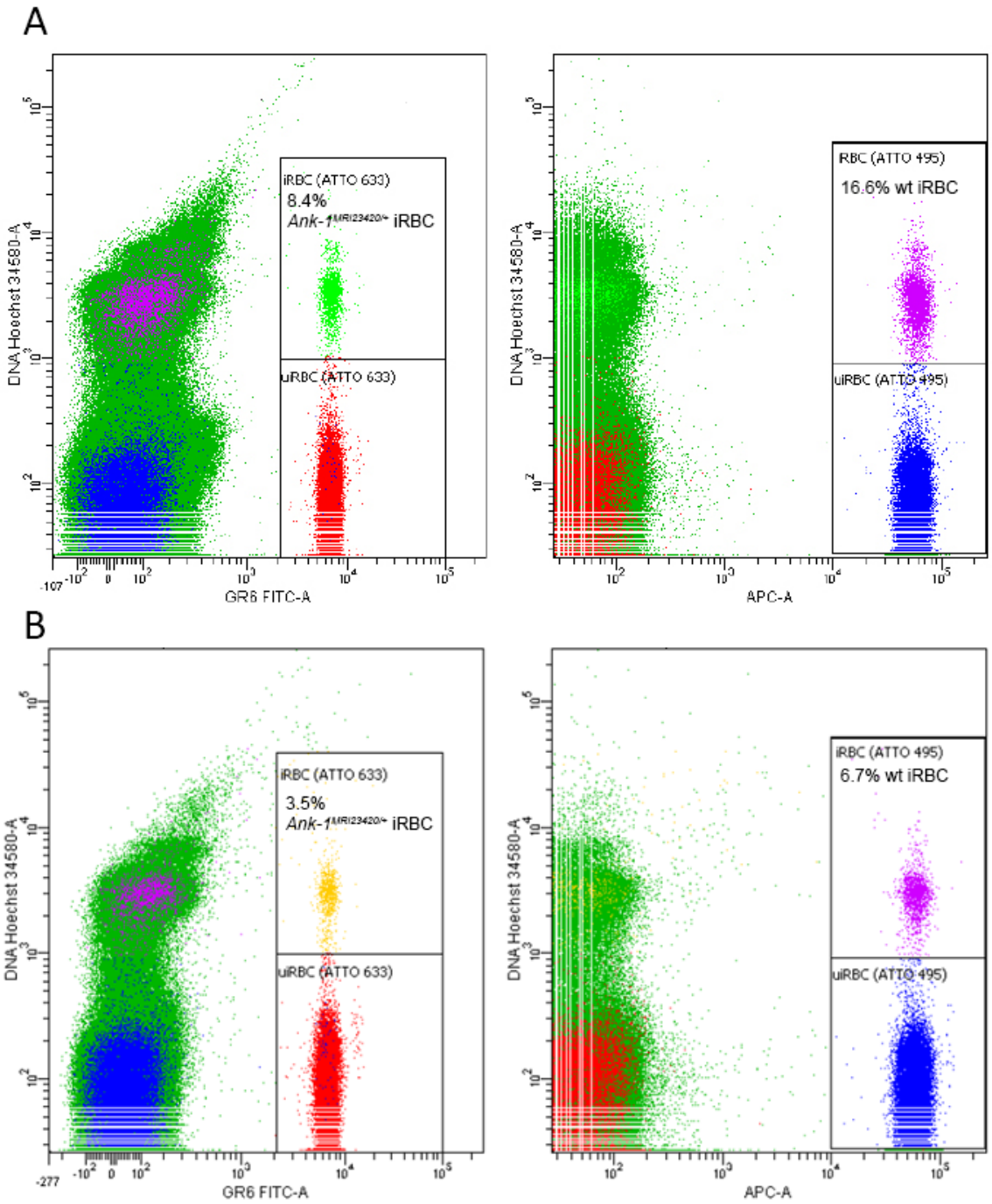

Figure S3.4. Example of flow cytometry plots corresponding to invasion/ growth *in vivo* assay. (A) Quantification of infected *Ank-1*$^{MRI23420/+}$ (ATTO 633 & Hoechst 34580 +ve) and WT (ATTO 495 & Hoechst 34580 +ve) RBCs in (A) *Ank-1*$^{MRI23420/+}$ and (B) WT host mice.

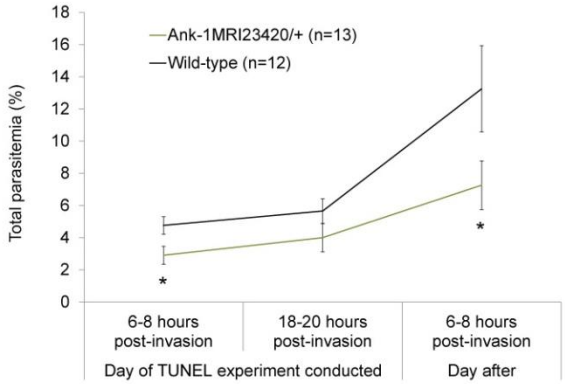

Figure S3.5. Parasitaemia curve between *Ank-1*$^{MRI23420/+}$ and WT mice corresponding to the TUNEL experiment. Blood was collected from infected mice (dose 4x10^4 iRBC) at the same time as for the time points used in the TUNEL experiment including a day after. Error bars are presented as SEM and (*) indicate statistical differences with a student`s t-test p-value <0.05.

Overall conclusion and future perspectives

In this chapter we identified that the mutant line MRI 23420 harbours a novel nonsense mutation in the *Ank-1* gene. Evidence is provided that the mutation is responsible for the underlying microcytic RBC phenotype observed in *Ank-1*$^{MRI23420+/-}$ mice. Further characterisation of the associated phenotype revealed that affected mice display classically similar symptoms to those observed in mice and humans with HS (Birkenmeier et al., 2003, Hughes et al., 2011, Randon et al., 1997). Most importantly, we have provided initial insight into the cellular mechanism in how ankyrin-1 deficiency causes malaria resistance.

This chapter identified that *Ank-1*$^{MRI23420/+}$ are resistant, due to an RBC autonomous effect resulting in impaired intracellular growth of *P. chabaudi*. We have also demonstrated that RBCs from heterozygous mutants exhibit reduced proteins levels of major cytoskeletal and membrane proteins, including band 3. Multiple studies have shown that the parasite interacts with various host factors during all facets of its RBC stage; for example, a study by Kariuki et al. (2005) identified band 3 as an invasion receptor for *P. falciparum* parasites. Additionally, it was demonstrated that peptides derived from the receptor region of band 3 impaired parasite invasion (Goel et al., 2003). Similarly, results from our *in vivo* invasion and growth assay have pointed to additional impairment of the parasite to invade affected RBCs. Due to the limited control conditions associated with this assay, evidence for impaired invasion could not be provided, however. Future work will therefore continue to repeat the experiment under more controlled conditions, for example through the use of an *ex vivo* invasion assay similar to the one employed in chapter 4. This will offer direct evidence if parasite entry into ankyrin-1 deficient RBCs is disrupted or not.

Interestingly, multiple studies in other *Ank-1* mutations in mice revealed a large spectrum of clinical symptoms (Birkenmeier et al., 2003, Huang et al., 2013, Hughes et al., 2011, Rank et al., 2009). This prompts the question as to whether mice with mild HS symptoms still exhibit increased protection against malarial infections. Another important aspect is to verify if *Ank-1* deficient humans show similar resistance to what been observed so far in mouse models. If this is true, then *Ank-1* can be examined as a potential drug candidate in order to develop a novel host-directed therapy. One strategy is to disrupt the binding between ankyrin-1 and its

interaction partner. This however requires the identification of a well-defined binding site, where designed small molecules can compete, at a required level of potency, with the natural molecule in order to modulate the function of the target. It has been shown that mutations in α- and β-spectrin are another cause of HS as well as resulting in malaria resistance (unpublished data from our laboratory, Shear et al., 1991). In this manner, Ipsaro and Mondragon (Ipsaro and Mondragon, 2010) identified, through target mutagenesis, several small key regions that are crucial for ankyrin-1-β-spectrin binding. Therefore, this may be possible by designing small molecules that disrupt the ankyrin-1-β-spectrin interaction. We hypothesise that such molecules may have antimalarial activity by impairing parasite growth and possibly invasion as well. Work in the S Foote, B McMorran and G Burgio laboratory (performed by Hong Huang - PhD student) is currently being undertaken to identify such inhibitors. Additionally, it will be investigated as to whether targeting these regions with such inhibitors at various concentrations evokes a similar type of protection against human malarial parasites, while keeping the severity of clinical symptoms low.

Furthermore it should be noted that deficiency in ankyrin-1 have also been associated with increased susceptibility to *Salmonella* infections. A study by Yuki et al. demonstrated that the ENU-mutant mouse line *Ank1*^{lty16} display an increased mortality rate, when infected with *Salmonella* Typhimurium strain Keller (Yuki et al., 2013). Further it was shown that iron overload and suppression of hepcidin (*Hamp*) expressionn contribute to the increase in susceptibility. However, if ankyrin-1 deficiency predispose mice and possibly humans as well, to other bacterial infections is currently not known. Similarly it remains to be investigated if limited exposure to an *Ank-1* targeting antimalarial has the same effect in regards to *Salmonella* susceptibility as life-long deficiency of the protein.

Overall, this chapter revealed novel evidence that parasite maturation of *P. chabaudi* is impaired in ankyrin-1 deficient RBCs. This verifies previous reports that mutations in the gene *Ank-1* result in a classical HS phenotype, as well as increase resistance to *P. chabaudi* infections. Our data further supports the hypothesis that parasites are dependent on ankyrin-1 and an intact RBC cytoskeleton for survival, and that disruption of such host-parasite interactions will ultimately result in increased protection.

Chapter 4 – ENU MUTANT MOUSE LINE MRI47455

Introduction

The ENU mutant mouse line MRI47455, was identified by screening 1st generation mice after mutagenesis for an abnormal blood phenotype. It was characterised by a microcytic anaemia and increased resistance to *P. chabaudi adami* DS infection. This chapter aimed to identify the causative ENU mutation that underlies the microcytic phenotype. Additionally, work in this chapter was directed to interrogate how the mutation affects the physiology of the animal and its role during malarial infection.

Results

Identification of the line MRI47455

The mutant mouse line MRI47455 was identified in a large-scale ENU mutagenesis screen, by screening for mutant mice that display an abnormal RBC phenotype. For this screen, male wild-type (WT) mice on inbred SJL/J background were treated with ENU and mated with female SJL/J animals. Full automated blood analysis of G1 progenies at 7 weeks of age led to the identification of a G1 outlier that displayed a mean corpuscular volume (MCV) that was three standard deviations smaller than the normal population (Table 5). It should be noted, due to recalibration of the used haematology system (Advia 120), values for WT differ from those in chapter 3. Inheritance testing by crossing the G1 founder to wild-type SJL mice resulted in 50% of G2 progenies exhibiting a comparably low MCV (p-value <0.0001) while the other half displayed an MCV that was similar to wild-type mice. Additionally, affected G2 progenies displayed reduced mean cellular haemoglobin levels (MHC; p-value <0.01), mean cellular haemoglobin levels (MCHC; p-value <0.01), and increased distribution width (RDW; p-value <0.01). The RBC count (RBC; p-value = 0.25) was found to be normal. Therefore, this suggests that the MRI47455 line exhibits a microcytic anemia with a haemoglobin defect. No differences in the heritability of the blood phenotype between males and females were observed. Together, this indicates that the blood phenotype was fully penetrant and inherited in an autosomal dominant mode. Additionally, based on the proportion of affected and

unaffected offspring generated from the G1 x SJL cross, G2 mutants can be assumed to be heterozygous for the causative mutation and therefore defined as MRI47455$^{+/-}$.

To determine whether mice homozygous for the mutation were viable and displayed a more profound phenotype, two G2 MRI47455$^{+/-}$ were intercrossed and their progeny (n= 43) analysed for full blood count (at 7 weeks of age). We found that 27 (65%) out of 43 analysed mice exhibited a low MCV, comparable to that of their parents (MCV <49.0 fl), whereas 16 animals (37%) displayed a normal MCV (MCV >51.0 fl). No mice with MCV values that were profoundly lower than their parents were observed. Since the calculated frequency of mutant littermates was less than an expected 75%, it is likely that the causative mutation(s) in a homozygous state is embryonic lethal.

Table 5 – Haematological parameters of G1 and G2 progenies.

	WBC	RBC	HGB	HCT	MCV	MCH	MCHC	RDW
G1 founder	9.8	8.6	118.0	0.40	47.6	13.8	291.0	17.1
MRI47455$^{+/+}$ (G2)	11.1 ± 0.6	9.8 ± 0.3	120.9 ± 9.2	0.52 ±.0.02	53.31 ± 0.28	13.1 ± 0.9	193.9 ± 2.5	14.6 ± 0.4
MRI47455$^{+/-}$ (G2)	11.4 ± 0.6	10.2 ± 0.1	105.1 ± 9.3	0.51 ± 0.01	**49.74 ± 0.44****	**8.9 ± 0.5***	**181.8 ± 3.2***	**17.3 ± 0.7***

Full blood analyses were conducted on the G1 founder mouse as well as on 26 MRI47455$^{+/+}$ and 32 G2 progenies between 7 and 8 weeks of age. Mutant mice were defined if their MCV was below 51.0 fl. All values are represented as mean ± SEM. Statistical differences to wild-type and performed by a student`s t-test were indicated as (*) p-value <0.05 and (**) p-value <0.005. WBC indicates white blood cell count (n x 10^9/L), RBC, red blood cell count (n x 10^{12}/L); HGB, haemoglobin (g/L); HCT, haematocrit (ratio); MCV, mean corpuscular volume (fl); MCH, mean corpuscular haemoglobin (pg); MCHC, mean corpuscular haemoglobin concentration (g/L); RDW, red cell distribution width (%);

MRI47455$^{+/-}$ mice display a thalassaemic phenotype

Adequate haemoglobin levels are essential for the development of functional RBC. Deficiency of the molecule is usually associated with several distinctive abnormalities in circulating RBCs. To further evaluate the microcytic anaemia phenotype in MRI47455$^{+/-}$, as indicated by the reduced MCV, MCH, MCHC and increased RDW, Giemsa stained peripheral blood smears were examined by light microscopy. The following abnormalities were observed: hypochromic erythrocytes that displayed a considerable variation in size (anisocytosis) and shape

(poikilocytosis), and elevated numbers of target cells and reticulocytes. Additionally, we observed a high frequency of RBCs containing small fragments of non-functional nucleus (Howell-Jolly bodies) which were not extruded during erythropoiesis (Figure 4.1A-B). Target cells are RBCs with a bull`s eye appearance and a darker perimeter ring and a result of an increased surface to volume ratio. As such they are often associated with decreased haemoglobin content (Singer, 1941, Cooper, 1970). The increased numbers of these cells were demonstrated by a manual count of cells on blood smears (p-value <0.005) (Figure 4.1G). Therefore, these findings are consistent with anaemia, where increased proportions of immature or inappropriately mature red cells are often observed.

Reduced haemoglobin content can either result from mutations in proteins that have a role during haemoglobin synthesis (thalassaemia) or due to a shortage of iron supply during RBC maturation. To determine if the RBC phenotype in our mutants is linked to iron deficiency, we measured the iron content in spleen tissue and RBCs of MRI47455$^{+/-}$ mice. Quantification analysis revealed a significant iron overload in MRI47455$^{+/-}$ spleens (4683.2 ± 271.4 AU MRI47455$^{+/-}$ versus 3211.0 ± 148.79 AU WT; p-value <0.05). However, no difference was observed in levels of labile iron in erythrocytes (2.68 x 10^6 ± 0.28 x 10^6 MFI MRI47455$^{+/-}$ versus 2.76 x 10^6 ± 0.08 x 10^6 MFI WT; p-value 0.78), suggesting that sufficient iron is available to the RBC for haemoglobin production (Figure 4.1H-I). Taken together we propose that the microcytic anaemia in MRI47455$^{+/-}$ is linked to a thalassaemic phenotype and is not a result of iron deficiency.

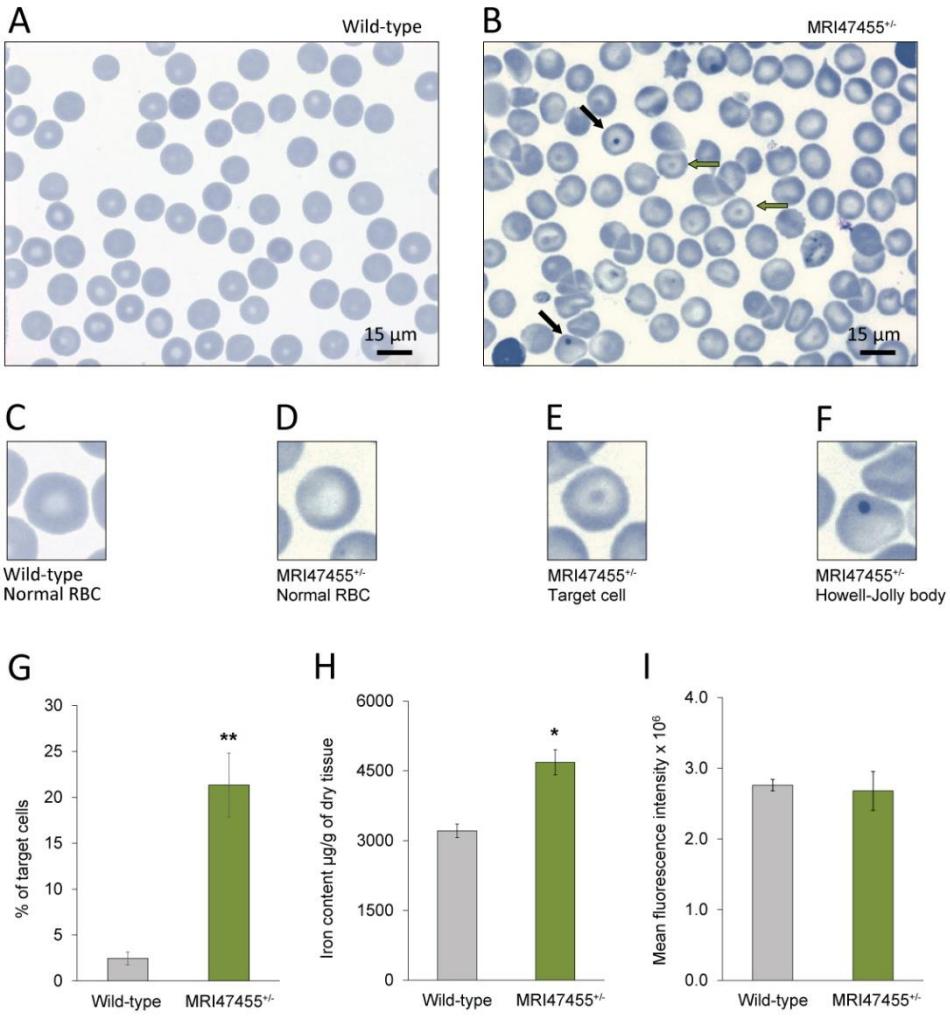

Figure 4.1 – MRI47455$^{+/-}$ mice display a thalassaemic phenotype. Giemsa stained blood smears from (A) wild-type and (B) MRI47455$^{+/-}$ mice, displaying hypochromic red blood cells (RBC) with anisocytosis and poikilocytosis. Increased number of Howell-Jolly bodies (black arrows), and target cells (green arrow) are other indices observed in MRI47455$^{+/-}$. (C-F) Zoomed view of normal WT and MRI47455+/- RBC, target cells and Howell-Jolly bodies. (G) Manual count of target cells (n=8 slides per group) with at least 200 counted cells per slide. (H) MRI4755+/- animals display iron overload in the spleen (WT n=7, MRI47455$^{+/-}$ n=6) but not in (I) RBCs (WT n=7, MRI47455$^{+/-}$ n=8). All values are represented as mean ± SEM and statistical differences to WT and performed by a student`s t-test are indicated with * (p-value <0.05) or ** (p-value 0.005).

Anaemia in MRI47455$^{+/-}$ is likely due to an up-regulated erythropoiesis

Reticulocytosis is a result of an up-regulated erythropoiesis and is either a response to compensate for increased RBC destruction/clearance or is caused by defects in the regulation of red cell production. Elevations in numbers of reticulocytes were observed by counting cells on thin blood smears (2.5 ± 0.4% in MRI47455$^{+/-}$ versus 5.7 ± 0.4%, p-value <0.05) and by flow cytometry analysis of CD71+ and TER119+ stained erythrocytes (6.3 ± 0.7% in MRI47455$^{+/-}$

versus 3.4 ± 0.6%, p-value <0.05) (Figure 4.2C-D & S4.1). To investigate if red cell destruction may drive this increase in cell production, the sensitivity of the mutant cells to haemolysis was tested by determining the cell osmotic fragility. Whole mouse blood was incubated in different salt concentrations and the proportion of cell lysis estimated by measuring the presence of cell free haemoglobin by absorbance (at 540nm). It was found that 50% of the red cells lysed at the following salt concentration: 4.37g/L NaCl for MRI47455$^{+/-}$; and 4.42g/L NaCl for WT (Figure 4.2A). This demonstrates that RBCs from the MRI47455$^{+/-}$ mice displayed a comparable osmotic fragility to WT. To further investigate if RBC destruction or clearance is the cause for the increase in erythropoiesis, we determined the half-life of circulating red cells. Blood cells were labelled *in vivo* with biotin (by IV injection) and the subsequent disappearance of labelled cells over time was assessed by flow cytometric analysis of blood samples taken from the tail. The half-life was 15.90 and 15.91 days for MRI47455+/- and WT respectively and was found to be comparable to previous studies in our laboratory (Figure 4.2B). Similarly, MRI47455$^{+/-}$ did not display a splenomegaly phenotype (0.57 ± 0.05% in MRI47455$^{+/-}$ versus 0.60 ± 0.03, p-value = 0.69), another indication that is associated with excessive red cell destruction (Figure 4.2E). Together, these sets of data suggest that the reticulocytosis in MRI47455$^{+/-}$ is unlikely to be a result of increased RBC destruction. Since we determined iron levels are normal in affected cells and therefore are unlikely to be a limiting factor in the rate of erythropoiesis, we hypothesised that the mutation in MRI47455$^{+/-}$ causes a defect during erythropoiesis.

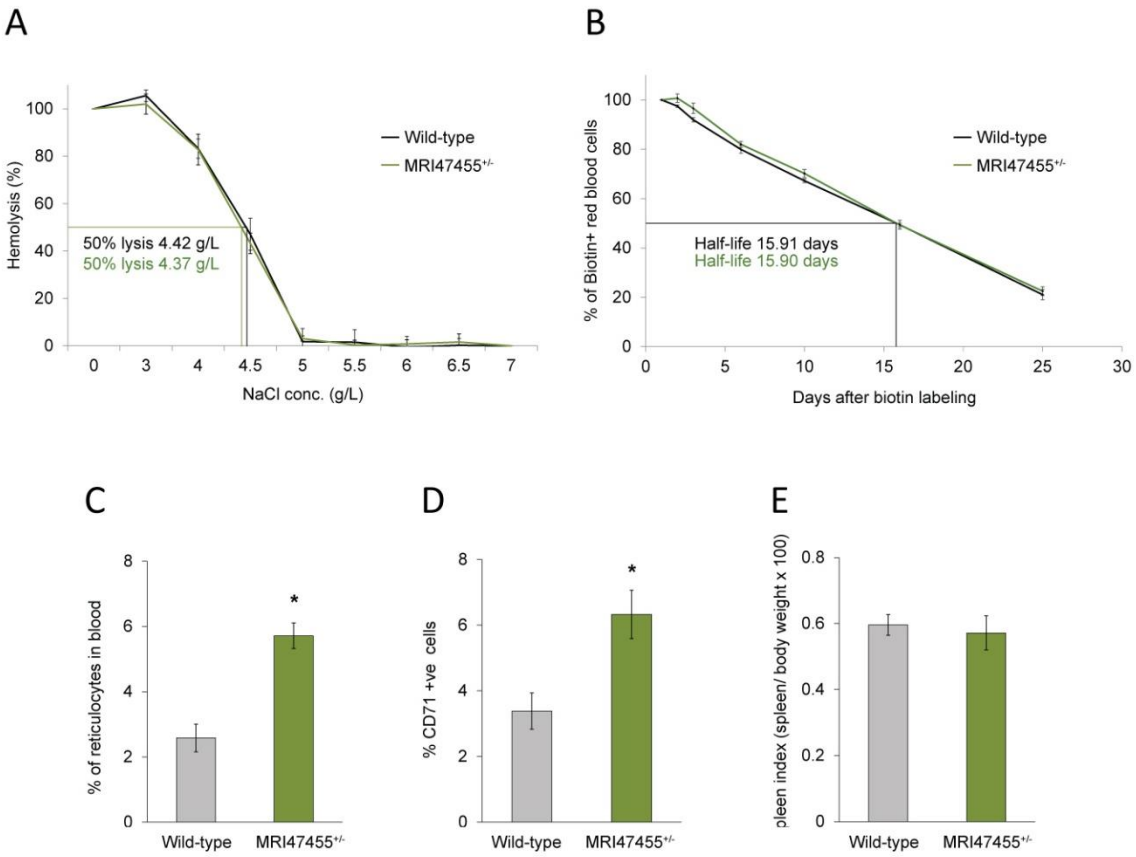

Figure 4.2 – The microcytic anemia MRI47455$^{+/-}$ is likely due to increased erythropoiesis and not excessive RBC destruction. (A) RBCs from MRI47455$^{+/-}$ display no increased osmotic fragility (n=8 for WT and 3 for MRI47455$^{+/-}$) or (B) reduced half-life (n=6 per group). However MRI47455$^{+/-}$ mice display increased reticulocytosis, quantified by a (C) manual count and by (D) flow cytometric analysis gating for CD71+/ TER119+ cells (n=8 per group). (E) No differences in spleen weights was found between WT (n=15) and MRI47455$^{+/-}$ (n=17). Error bars indicate ± SEM and * represents statistical significance to WT with a student`s t-test p-value <0.05.

MRI47455$^{+/-}$ mice are resistant to *P. chabaudi* infections

The primary aim of this project was to find novel mutations that result in a resistance to malarial infection. To test if the MRI47455 line was resistant, MRI47455$^{+/-}$ and WT mice were infected with a lethal dose of *P. chabaudi adami* DS and their parasitaemia levels monitored daily. Although MRI47455$^{+/-}$ displayed a slightly prolonged survival that was significant compared to WT (Mantel cox test p-value = 0.02) (Figure 4.3A), all mice succumbed to infection at the end of the experiment. This however may be explained by an increase in parasite virulence we have observed in our laboratory. The SJL mouse strain is normally highly susceptible to *P. chabaudi adami*. Monitoring of the development of blood parasitaemia throughout the course of infection revealed that MRI47455$^{+/-}$ mice exhibited a significant

decrease in proportions of infected red cells. Interestingly, this difference was already significant at a relatively low parasitaemia (5th day post-infection) and remained significant until the peak of parasitaemia was reached. This peak was delayed by one or two days in the MRI47455$^{+/-}$ mice (Figure 4.3B). Therefore, we concluded that the responsible mutation in MRI47455$^{+/-}$ does result in a resistance to malaria infection, and a reduced rate of parasite growth in the bloodstream.

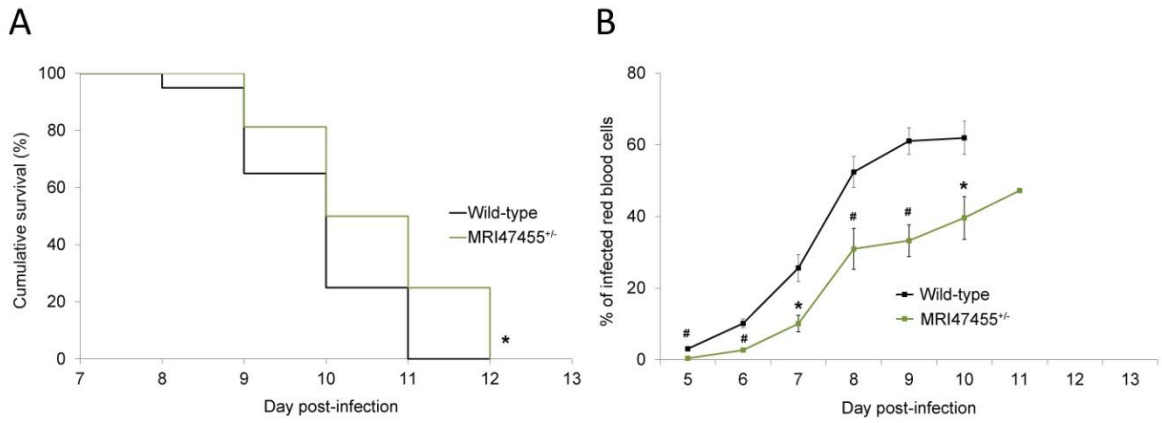

Figure 4.3 – MRI47455$^{+/-}$ mice are resistant to *P. chabaudi* infection. (A) Kaplan Meier survival curve. Infection dose was 4x10^4. (B) Parasitaemia curve of infected wild-type (n=18) and MRI47455$^{+/-}$ (n=14) female mice with the corresponding. Data is plotted as mean ± SEM and statistical differences from wild-type are indicated by student`s t-test p-values of * <0.05 and # <0.005.

Resistance to *P. chabaudi* in MRI47455$^{+/-}$ is due to an RBC autonomous effect

To determine if the malaria resistance phenotype in MRI47455$^{+/-}$ is due to a red cell autonomous effect, we employed an *in vivo* parasite growth and invasion assay. This assay was developed during the investigation of the line *Ank-1^{MRI2420}* and is described in detail in the preceding chapter (Greth et al., 2012). Briefly, isolated RBCs from uninfected MRI47455$^{+/-}$ and WT animals were incubated with separate fluorescent dyes and injected back into infected mice of both genotype groups at a late stage of parasite maturation (at approximately 23-24h post-invasion). After re-invasion of new red cells, as determined by examining Giemsa stained blood smears, blood was sampled at early ring and late trophozoite stages over two consecutive invasion cycles. This corresponded to sampling at the following respective time points: rings at 30 minutes and 30 hours; and trophozoites at 16 hours. The proportion of

infected RBCs for each labelled population was then quantified by flow cytometry. Results revealed that the proportion of infected MRI47455$^{+/-}$ RBCs was significantly reduced compared to WT. (Figure 4.4A-B). A reduction in the proportion of infected MRI47455$^{+/-}$ cells was already significant 30 minutes after injection of labelled RBCs into infected WT mice (42.8 ± 2.8% in MRI47455$^{+/-}$ versus 57.2 ± 3.7% in WT, p-value <0.05). This difference became more pronounced 4 hours later (43.5 ± 0.9% in MRI47455$^{+/-}$ versus 56.5 ± 0.8% in WT, p-value <0.0001) and remained highly significant for the consecutive cycle (39.5 ± 1.9% in MRI47455$^{+/-}$ versus 60.5 ± 2.3% in WT, p-value <0.0001). A similar trend was also observed in recipient animals on a mutant background, although statistical significance was only obtained for the 16 hour and 30 hour time points (30 min p-value 0.36, 16 h p-value <0.05, 30 h p-value <0.05). These differences were also apparent irrespective of the dye combinations or genotype of the recipient host. Because the decrease in the proportion of infected RBCs was independent of the background of the recipient animals, the clearance of RBCs from the circulation is unlikely to significantly contribute to resistance. Instead, the data indicate that the MRI47455$^{+/-}$ mutation confers resistance by directly affecting the ability of the erythrocyte to support either invasion, growth or egress of the parasite. An effect on invasion is most likely because we observed a significant difference as early as 30 minutes after injecting labelled cells.

To further verify that no other secondary effects could be contributing to resistance (such as altered splenic function or immune clearance), we also examined the contribution of RBC lysis and or clearance of RBCs during the infection. RBCs of uninfected MRI47455$^{+/-}$ and WT mice were equally labelled with separate fluorescent dyes and delivered into infected mice from both cohorts. Using flow cytometry, we daily quantified the rate of disappearance for each labelled subpopulation. Irrespective of the dye used and genetic background of the infected animals, the loss of MRI47455$^{+/-}$ and WT red cells did not differ (Figure 4.4C-D). This further confirms that neither red cell lysis, nor splenic or immune clearance were contributing to resistance in MRI47455$^{+/-}$.

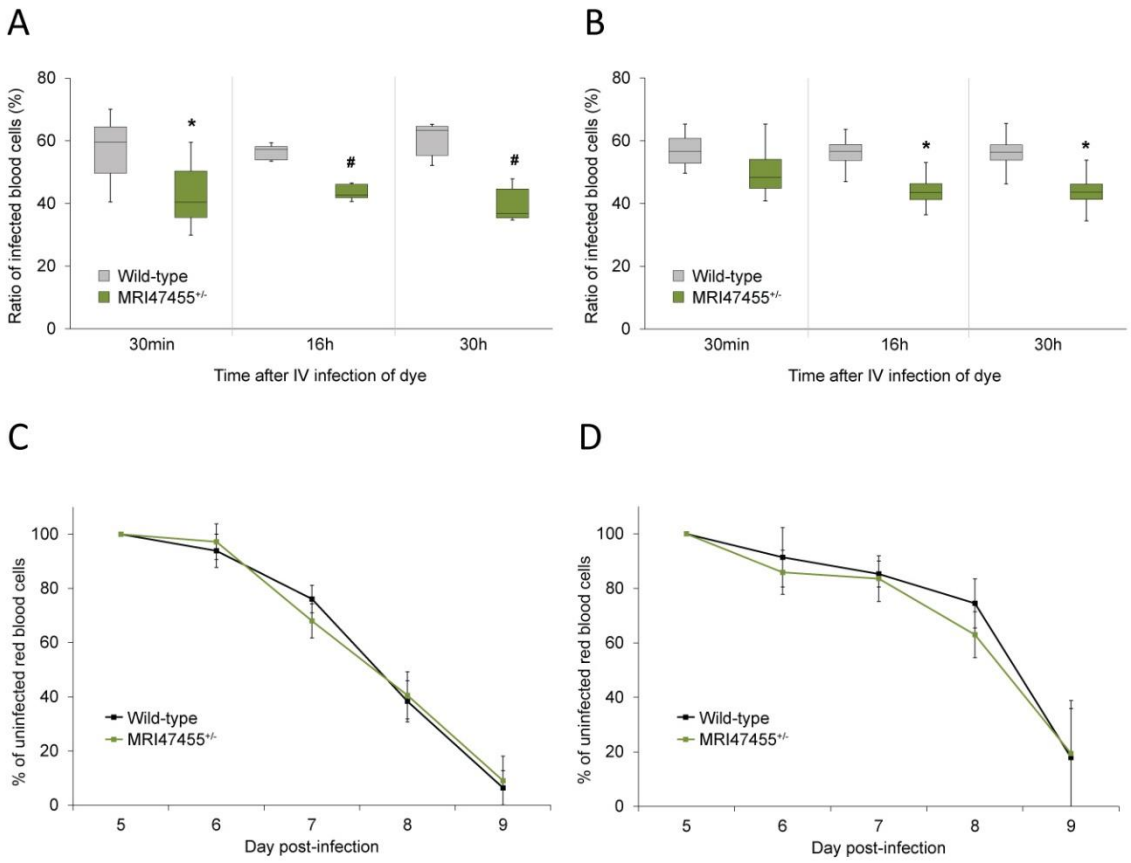

Figure 4.4 – Resistance of MRI47455$^{+/-}$ mice to *P. chabaudi* is due to a red blood cell autonomous effect. Uninfected red blood cells from wild-type and MRI47455$^{+/-}$ mice were individually labelled with different fluorescent dyes and injected back in a 1:1 ratio into infected animals of both cohorts (MRI47455$^{+/-}$ n=4, wild-type n=8). Blood samples were taken 30min, 16h and 30h later and the rate of infection was quantified by flow cytometry gating for RBCs +ve for either dye. Means of infected wild-type and MRI47455$^{+/-}$ RBCs were calculated by combining the separate means of normal and reversible labelled cells from (A) wild-type and (B) MRI47455$^{+/-}$ infected mice. Values are presented as a ratio, where the percentage of both groups of infected labelled RBCs together equals 100%. Based on the same methodology as for the *in vivo* invasion assay, ½ life of mutant and WT RBCs was assessed in infected WT (C) and MRI47455$^{+/-}$ mice (D) by tracking biotin labelled red cells throughout the infection. Data is graphed as mean ± SEM and statistical differences to wild-type, performed by a student`s t-test are indicated with either * for a p-value <0.05 or $^{\#}$ for a p-value <0.005 (MRI47455$^{+/-}$ n=4, WT=8).

The results from the *in vivo* invasion assay indicated that resistance is a result of an RBC autonomous effect, likely by impairing parasite invasion. However, at this point we cannot completely exclude the possibility that parasite growth is affected as well. To investigate if parasite growth may be impaired in the MRI47455$^{+/-}$ red cell, we conducted an analysis of intracellular parasite survival at early (6h post-invasion) and late stages (18h post-invasion) of parasite development. The presence of dead or dying parasites was investigated by using the terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labelling (TUNEL)

technique. The stain detects nuclei containing sheared or degraded DNA and is therefore indicative of dead or dying parasites, and has been used previously by our group (Greth et al., 2012, McMorran et al., 2009). Proportions of TUNEL+ parasites varied between 7-12% for both MRI47455$^{+/-}$ and WT at the 8am (p-value = 0.36) and 8pm (p-value = 0.13) time point without statistical differences (Figure 4.5). The frequency of dying or dead parasites was comparable to what has been observed previously in WT mice from the line MRI 23420 (Chapter 3). Therefore we considered that resistance in MRI47455$^{+/-}$ is unlikely to be a result of impaired parasite growth.

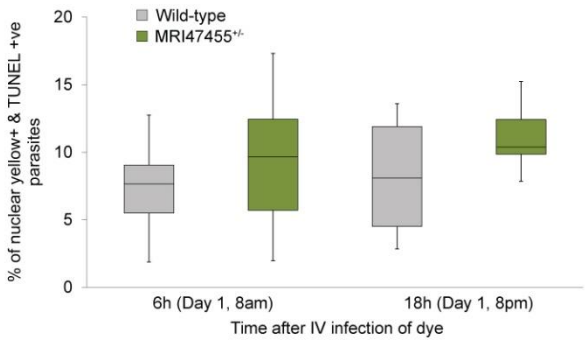

Figure 4.5 – Parasite maturation MRI47455$^{+/-}$ mice is normal. Separately labelled uninfected red blood cells (RBC) of WT and MRI47455$^{+/-}$ were injected at equal proportions into WT infected mice (MRI47455$^{+/-}$ n=4, WT=8) and sorted at early and late trophozoite stage, corresponding to the dye used. Sorted cells were then labelled with the DNA stain nuclear yellow and TUNEL, representing dying or dead parasites. Error bars indicate ± SEM (MRI47455$^{+/-}$ n=8, wild-type n=8).

P. chabaudi parasites display impaired invasion into MRI47455$^{+/-}$ RBCs

Based on the data obtained in the *in vivo* invasion/growth and TUNEL assay we hypothesised that parasite invasion is likely to be a major mechanism for the malaria resistance phenotype in the MRI47455 line. We assessed the invasion capacity of *P. chabaudi* within a controlled environment by adopting an *ex vivo* invasion assay that was previously developed in our laboratory and described in detail in the 'Methods' section. Briefly, the assay involves labelling uninfected RBCs of both WT and MRI47455$^{+/-}$ mice with different dyes, and then mixing them together with infected WT RBCs under *in vitro* tissue culture conditions. The proportions of

infected cells derived from each red cell type are then determined at different time points using flow cytometry. Because *P. chabaudi* cannot be maintained under culture conditions long term, it was only possible to examine one cycle of parasite invasion and growth. We observed that the parasitaemia in MRI47455$^{+/-}$ RBCs was significantly lower 3 hours post-invasion, when compared to WT (44.03 ± 1.52% MRI47455$^{+/-}$ versus 55.97 ± 2.90% WT; p-value <0.05) (Figure 4.6). This was similar to our data from the *in vivo* assay where we observed a significant reduction in infected mutant RBCs 30 minutes after invasion. Therefore, this provides further evidence that the RBC autonomous effect is due to impaired invasion of *P. chabaudi* into MRI47455$^{+/-}$ RBCs.

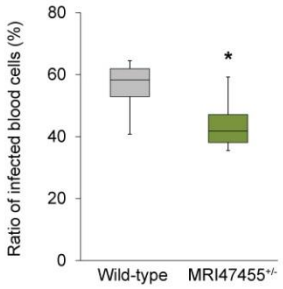

Figure 4.6 – Invasion of *P. chabaudi* is impaired in MRI47455$^{+/-}$ red blood cells. Red blood cell (RBC) invasion efficacy was determined under *ex vivo* conditions by adding infected WT RBCs to uninfected individually labelled MRI47455$^{+/-}$ and WT cells. Rate of infection was determined by flow cytometry. Error bars indicate ± SEM and statistical significance with a student`s t-test p-value of <0.05 to WT is represented by * (MRI47455$^{+/-}$ n=8, WT=8).

Identification of two ENU mutations in the genes *Ahctf1* and *Tubb4a*

We hypothesised that the underlying phenotype in MRI47455$^{+/-}$ mice is due to a genetic mutation by the action of ENU. Therefore, to identify the mutation we sought to sequence all exons, intron-exon boundaries and promoters in affected animals using next generation sequencing technologies. Four microcytic mutants (G5-G7) from distantly related and progeny tested parents in the pedigree were selected. DNA was extracted and sent for next generation sequencing to Oto Genetics (Atlanta, USA) on an Illumina platform (HiSeq 2000).

From the identified variants we filtered out all common single nucleotide polymorphisms (SNPs), and selected only for novel non-synonymous intronic and exonic mutations that were shared between all four mutants (Table S1). We found 17 variants that fulfilled these criteria. Four were located in introns. Since they were located >30bp away from the intron-exon border, we considered that they were unlikely to affect splicing of the transcript. Therefore, we restricted the investigation to the exonic variants (13 variants). The first step in the analysis was to determine the segregation pattern for each SNP with the microcytic phenotype in a cohort of MRI47455$^{+/-}$ animals. This was done by genotyping a minimum of four progeny tested breeders from different parents by Sanger sequencing. The results showed that only two out of the 13 gene mutations were found in all sequenced affected individuals. The two mutations were found in two genes located on different chromosomes, namely *Ahctf1* (AT hook containing transcription factor; chromosome 1) and *Tubb4a (*Tubulin Beta 4A Class IVa; chromosome 17).

The *Ahctf1* mutation is a single point transversion (T to A) at nucleotide position 3936, located in exon 29. It results in a missense change in the coding sequence, replacing an aspartic acid for glutamic acid at the amino acid position 1312 (Figure 4.7). The affected residue corresponds to a highly conserved region between mouse and human (Kimura et al., 2002b). Further, the site of the mutation was found in an inhibitory domain (second one from the N-terminal), which has been implicated to regulate the expression level of *Ahctf1*. In addition, the *Ahctf1* transcript (termed ELYS) is ubiquitously expressed in haematopoietic tissue and plays a functional role during cytokinesis (Kimura et al., 2002b, Rasala et al., 2006). We hypothesise that the ENU mutation may therefore impact on the expression levels of ELYS and thereby induce the underlying blood phenotype in MRI47455$^{+/-}$.

The *Tubb4a* mutation is an A to G substitution located at nucleotide position 482 in exon 4. This results in the replacement of aspartic acid for glycine at amino acid position 161 in the β-tubulin 4a protein (Figure 4.8). Protein alignment analysis (www.ensembl.org) revealed that the position of the variant is highly conserved across mammalian species. Furthermore, the mutation is found within a large region of the protein that has been proposed to possess GTPase activity and to be necessary for microtubule assembly (Wang et al., 2007). It was found

that the gene is abundantly expressed in haematological tissues by consulting the open-source databases: protein atlas (http://www.proteinatlas.org), biogps (http://biogps.org), and mouse genome informatics (www.informatics.jax.org). Since microtubules form an important part of the cytoskeleton in most cells, including erythroid precursors (but not mature RBCs), we concluded that the *Tubb4a* variant is another candidate that could potentially impact developmental processes of maturing red cells.

Interestingly, we observed viable mice that are homozygous for the mutation in either *Ahtcf1* or *Tubb4a*. However, full blood count analysis on separate mice for each genotype did not reveal any significant differences between heterozygotes and homozygotes. No mice that carry a homozygous mutation in both genes have been detected so far. Therefore, we conclude that Ahctf1$^{-/-}$ and Tubb4a$^{-/-}$ are viable. Although, whether the double homozygotes are embryonic lethal cannot be confirmed at this point due to the limited number of mice genotyped.

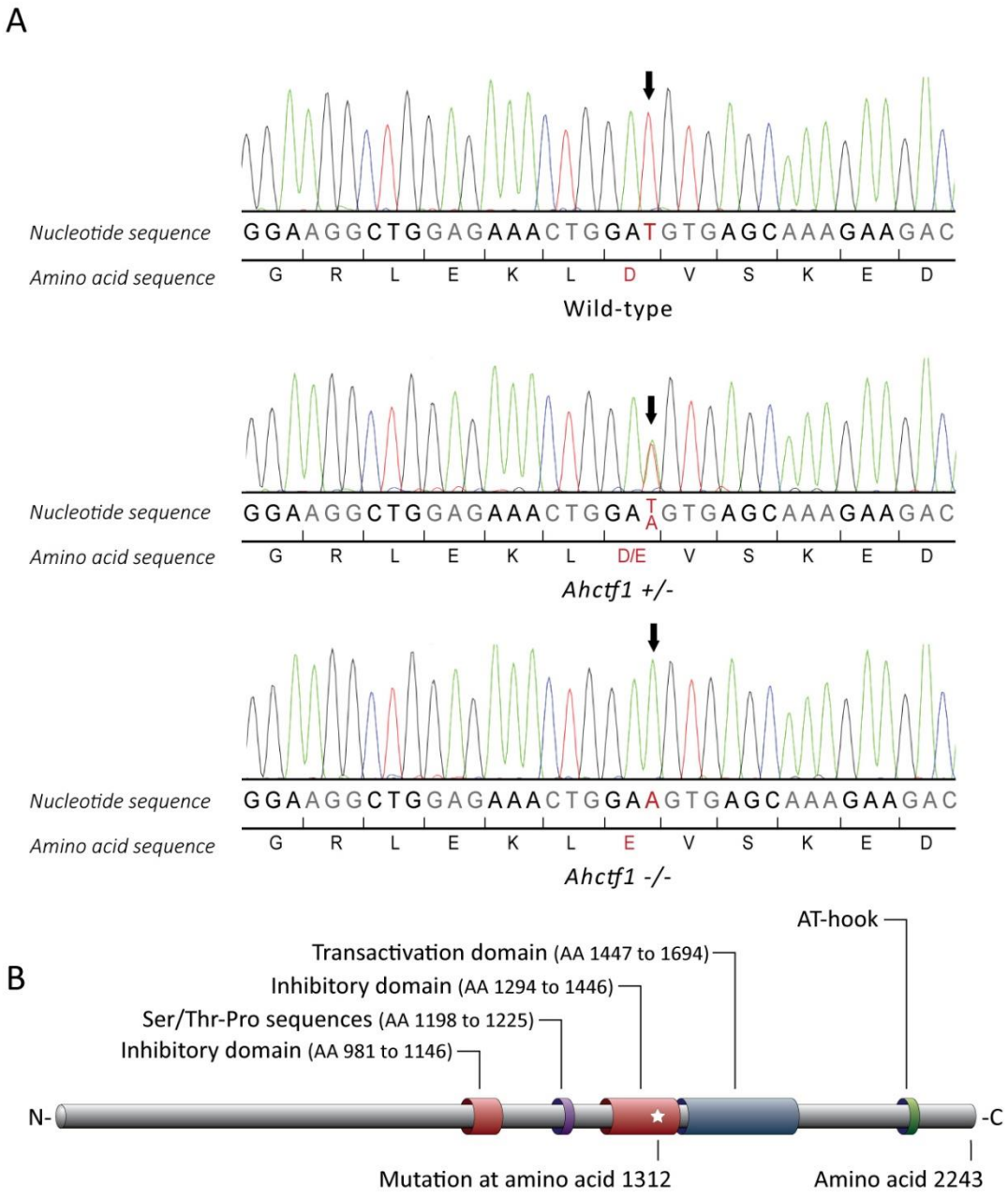

Figure 4.7 - Identification of the *Ahctf1* allele. (A) DNA sequence electropherograms showing the T to A transversion in exon 29 for WT, *Ahctf1*$^{MRI47455/+}$ and *Ahctf1*$^{MRI47455/MRI47455}$ mice including the resulting amino acid change. (C) Schematic visualisation for the location of the mutation within the protein including important domain structures.

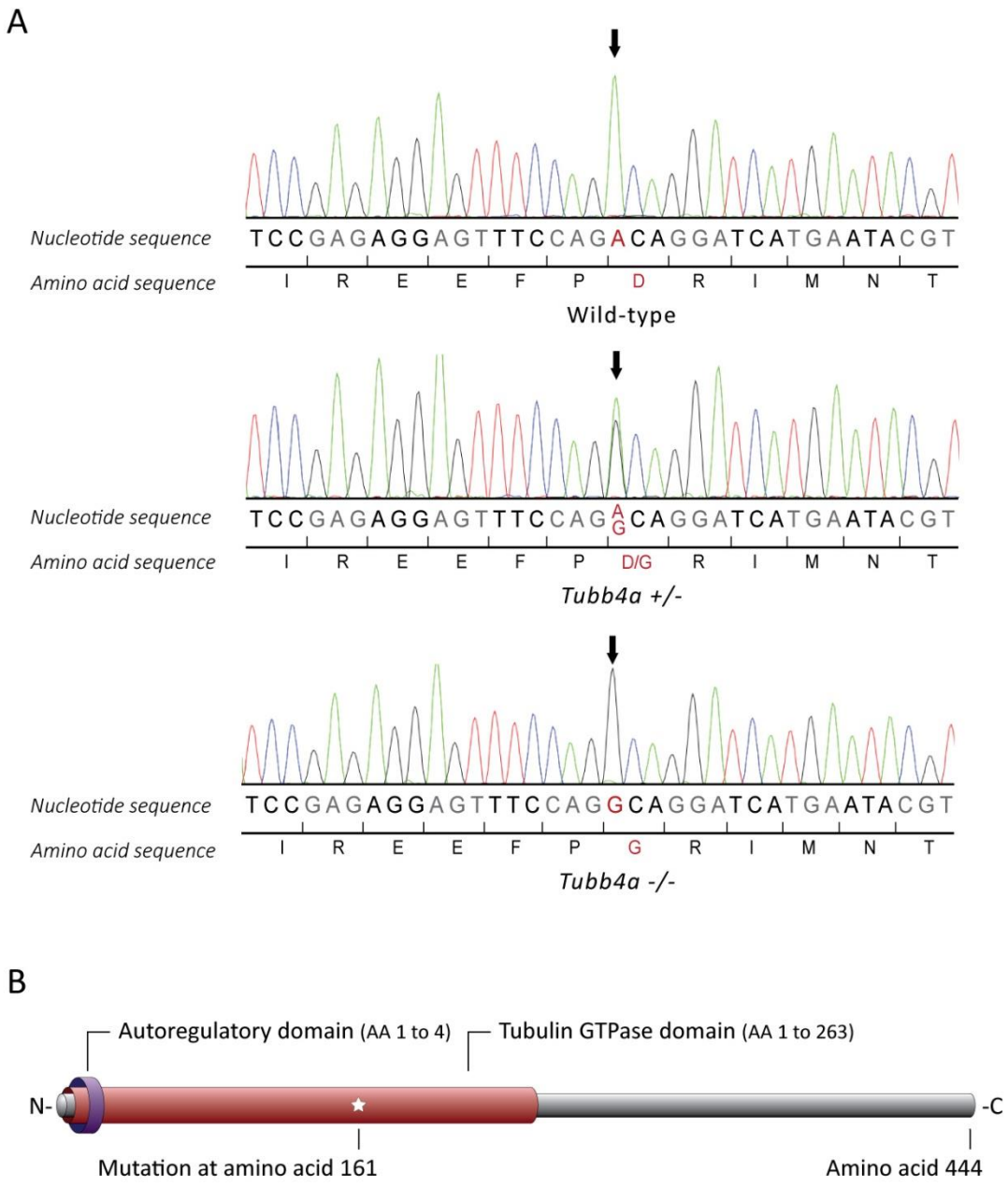

Figure 4.8 – Identification of the *Tubb4a* allele. (A) DNA sequence electropherograms representing the A to G transversion in exon 4 for WT, *Tubb4a*$^{MRI47455/+}$ and *Tubb4a*$^{MRI47455/MRI47455}$ mice. Designated below is the corresponding amino acid. (B) Visual representation of the encoded protein and the position of the mutation.

To further assess if any of the two candidate genes are primarily responsible for the underlying phenotype in mutant mice, we genotyped an additional 63 mice derived from different generations and parents (Table 6). We considered that the causative ENU mutation should be present in all mice that exhibit the microcytic phenotype. Results showed that out of 36 mice that carried a mutation (heterozygous or homozygous) in both genes, 27 animals displayed a low MCV, while 9 mice did not express the phenotype (LOD = 3.22). From 6 mice that had a mutation only in *Ahctf1* (LOD = 0.57), 3 were microcytic and 3 not. A similar LOD score was calculated for the 9 mice with a mutation exclusively in *Tubb4a* (5 microcytic, 4 normal; LOD = 0.49). Additionally, we observed 12 mice (out of the 63 genotyped animals) that are W.T for both genes from which 5 exhibited a low MCV phenotype. This suggests that both mutations are probably needed to induce the microcytic phenotype in MRI47455$^{+/-}$, or that they have associated with the MCV phenotype by chance and that another unidentified mutation is instead causing the phenotype. The notable lack of complete segregation to these mutations was also in contrast to the apparent fully penetrant phenotype observed in the G2 progenies. Nevertheless, given that both mutations segregated significantly with microcytosis over several generations, we hypothesise that the mutations in *Ahctf1* and *Tubb4a* are linked to the MRI47455$^{+/-}$ phenotype. Furthermore, to investigate the contribution of each mutation in regard to the underlying phenotype, we are currently in the process of crossing animals to segregate the two mutations into separate lines for further assessing the contribution of each variant for the haematological and malaria susceptibility phenotype.

Table 6 – Segregation of the mutations in *Ahct1* and *Tubb4* with the microcytic phenotype.

	Number of animals			
Mutant strain	**Total**	**MCV >51.0 fl**	**MCV <49.0 fl**	**LOD**
Ahctf1^{MRI47455}*Tubb4a*^{MRI474555}	36	9 *(9 het/het)*	27 *(21 het/het, 4 het/hom, 2 hom/het)*	3.22
Ahctf1^{MRI47455}	6	3 *(3 het)*	3 *(2 het, 1 hom)*	0.57
Tubb4a^{MRI474555}	9	4 *(4 het)*	5 *(3 het, 2 hom)*	0.49
Wild-type	12	7	5	

Table shows the number of mice that carry a mutation in one or both genes and display a low MCV (<49.0 fl) or normal MCV (MCV >51.0 fl). Linkage is presented with the LOD score.

Discussion

This chapter investigated the mouse line MRI47455, which was generated through ENU mutagenesis and dominant screening for mice that display an abnormal blood phenotype. We demonstrated that MRI47455$^{+/-}$ exhibits a microcytic anaemia and several other clinical symptoms that are associated with thalassaemia. Furthermore, it was assessed if affected animals were less susceptible to malaria infection. Evidence was provided that MRI47455$^{+/-}$ animals were significantly more resistant to experimental infection by impairing RBC invasion by *P. chabaudi* parasites. Furthermore, through whole exome sequencing of affected animals we identified two separate mutations in the genes *Ahctf1* (AT-hook containing transcription factor) and *Tubb4a* (Tubulin, Beta 4A Class IVa) that may cause the microcytic phenotype in MRI47455$^{+/-}$. However this last finding requires additional work to confirm if mutations in these genes are indeed causing the phenotype.

MRI47455$^{+/-}$ display a thalassaemic-like phenotype

This chapter identified that MRI47455$^{+/-}$ are characterised by displaying a microcytic anaemia. Further, it was demonstrated that affected mice exhibit a reduced cellular haemoglobin concentration that is unlikely to be a result of impaired iron uptake and incorporation into maturing RBCs. Therefore, we hypothesised that the MRI47455$^{+/-}$ phenotype is more consistent with what is seen in thalassaemia. In support, affected mice share several clinical features with what is observed in thalassaemia, a group of inherited blood disorders that affect the body`s ability to produce haemoglobin due to gene mutations or defective genes (Roberts and Williams, 2003, Weatherall and Clegg, 2001). These include low mean corpuscular haemoglobin concentrations, hypochromic erythrocytes, abundant target cells and reticulocytes, as well as iron overload in the spleen. Additionally, our data suggest that homozygous mutants are embryonic lethal, which is consistent with what has been reported in the mouse models *RBC13* and *RBC14*, which reproduce a human β-thalassaemia on the genomic level (Brown et al., 2013). However, despite these similarities, MRI47455$^{+/-}$ exhibited an overall less severe anaemia and absence of splenomegaly when compared to either *RBC13* or *RBC14*. Additionally we proposed that the reticulocytosis in our mutants is due to an increased erythropoiesis that is not caused by increased RBC destruction. In comparison, there is ample evidence that the thalassaemic anaemia is a result of the weakening and destruction

of affected RBCs (Giani and Filosa, 2001, Muanprasat et al., 2013, Origa and Galanello, 2011). Therefore, it seems likely that the underlying phenotype in MRI47455$^{+/-}$ is instead a result of an as yet unknown defect during the production of erythrocytes that affects haemoglobin incorporation and not its synthesis.

MRI47455$^{+/-}$ mice are resistant to *P. chabaudi* infection by impairing parasite invasion

Another aim of this study was to investigate the host response of MRI47455$^{+/-}$ during malarial infection. Microcytic mice displayed a significant increased survival rate compared to littermates with a normal MCV. Likewise, infected ENU mutants exhibited a significant reduction in parasite levels at low and high parasitaemia resulting in a delayed peak infection. Moreover, based on our results we showed that resistance is primarily due to an impaired parasite invasion mechanism. By delivering fluorescently labelled mutant and WT uninfected RBCs into infected MRI47455$^{+/-}$ and WT mice we found a significant reduction in the infection rate of mutant cells. A very similar difference was observed when the invasion assay was performed under *ex vivo* conditions. These experiments excluded any likely role for resistance mechanisms outside the red cell (eg immune cell and spleen-mediated clearance), and instead clearly demonstrate the resistance mechanism is due to differences specifically within the MRI47455$^{+/-}$ red cell. Additionally, we did not detect an increase of dying or dead intracellular parasites in MRI47455$^{+/-}$ when compared to their control group. Therefore, preferential clearance of infected erythrocytes or impaired parasite growth as a contributing mechanism to resistance was excluded.

Many of the genetic disorders that are associated with microcytosis, such as the thalassaemias and a number of RBC structural variants, have been shown to offer protection against malaria in humans and mice alike (Mohandas et al., 1984, Schofield et al., 1992). For example, a case-control study in Papua New Guinea with children who are homozygous for α^+ thalassaemia, found a strong correlation between subjects displaying microcytic RBCs with reduced haemoglobin content, and reduced risk to contracting severe malaria (Fowkes et al., 2008). Additionally it has been shown that mouse models with β-thalassaemia (Roth et al., 1988) or carrying mutations in the structural proteins ankyrin-1 (Greth et al., 2012), spectrin (Shear et al., 1991) and band 3 (Allen et al., 1999) exhibit increased resistance against *P. chabaudi*

infection. While the protective mechanisms owing to these RBC variants differ, a number of common strategies affecting parasite burden have been observed. For example, impaired parasite growth has been associated with deficiencies in the structural proteins ankyrin-1 (Greth et al., 2012) and spectrin (Shear et al., 1991), as well as in individuals that are homozygous for the haemoglobin E (HbEE) disorder (Nagel et al., 1981). On the other hand, malaria resistance in α-, β-thalassaemia (Pantaleo et al., 2012, Smith et al., 2002, Williams et al., 2002) and Southeast Asian ovalocytosis (Genton et al., 1995) has been postulated to be due to increased phagocytosis, clearance or destruction of infected RBCs as a result of an abnormal cell membrane. Interestingly, so far a primary invasion mechanism has not been attributed to any of the examples discussed above. In contrast, an invasion mechanism has been primarily attributed to mutations in RBC receptors. This includes the Duffy antigen negative mutation, which has been shown to prevent the invasion of *P. vivax* parasites (Miller et al., 1976), whereas deficiencies of glycophorin A (*GYPA*) and B (*GYPB*) reduce the RBC entry of *P. falciparum* (Facer, 1983). These may suggest that MRI47455$^{+/-}$ RBCs possess an unidentified surface or membrane abnormality which renders them resistant to malaria infection. To our knowledge, this invasion phenotype is therefore unique in regard to other identified genetic mutations that are associated with a microcytic anaemia phenotype.

Identification of two gene candidates that associate with the underlying phenotype

The overall aim of this study was to discover novel host gene candidates that offer protection against malaria. By exome sequencing four affected mice we found two separate mutations in the unlinked genes *Ahtctf1* and *Tubb4a* that significantly associated with the microcytic phenotype in MRI47455$^{+/-}$ mice. We therefore hypothesised that the mutations in these candidate genes cause a defect during erythropoiesis, thereby inducing the underlying blood phenotype in affected mice. *Ahctf1* has been identified to be part of a multi-protein complex termed Nup107-160, which is important for spindle assembly and subsequent division of the cell (Orjalo et al., 2006). Alongside this, *Tubb4a* encodes the protein β-tubulin 4a, which dimerizes with α-tubulins to form the main building blocks of the globular protein tubulin, a major constituent of microtubules (Kirschner and Mitchison, 1986). While both genes have been shown to be expressed in erythroid precursors, to date there are no studies in the

literature that have addressed a specific role for either *Ahctf1* or *Tubb4a* during erythropoiesis.

However, it has been shown that microtubules, as well as correct spindle assembly, play important functions during the late stage of RBC maturation (McGrath et al., 2008, Yoshida et al., 2005, Konstantinidis et al., 2012). For example, microtubules are an important determinant in the enucleation process of orthochromatic erythroblasts during their differentiation into reticulocytes (McGrath et al., 2008, Yoshida et al., 2005, Konstantinidis et al., 2012). Evidence of a role for correct spindle assembly during terminal differentiation of erythroblasts is given from a survivin knockout study in mice, a protein that functions in the maintenance of an active spindle assembly checkpoint. Heterozygous survivin-deficient mice display anaemia with increased reticulocytosis and a number of RBC abnormalities, including red cell fragmentation, hypochromia and anisocytosis (Leung et al., 2007). Therefore, it is possible that either a defect in *Ahctf1* or *Tubb4a* may affect processes during differentiation of erythroid precursor cells, thereby disturbing erythropoiesis.

Moreover, given the MRI47455$^{+/-}$ phenotype most strongly associates when both mutations are present, it is possible that the two genes either interact with each other or affect similar processes during erythropoiesis. During the mitotic phase of the cell cycle in dividing eukaryotic cells, microtubules are used to create the basic machinery of the mitotic spindle. Moreover, microtubules stabilise spindles during prometaphase and metaphase and enable spindle pole separation during the later stages of mitosis (Scholey et al., 2003). Therefore, it is plausible that defects in microtubules likely affect correct spindle assembly and vice versa. In support, another mouse study reported that survivin null mutants exhibit disrupted microtubule formation and therefore suggested that the protein is essential for coordinating the organisation of microtubules (Uren et al., 2000). Disruption of both spindle assembly and microtubule formation could potentially result in a more severe phenotype and therefore explain why we observed only a genotype-phenotype association when both mutations were present. Furthermore, our results suggested that mice that are homozygous for the phenotype are embryonic lethal. While this is in contrast to the observation of viable *Ahctf1*$^{-/-}$ and *Tubb4a*$^{-/-}$ animals, it could provide an explanation for why we did not detect *Ahctf1*$^{-/-}$

/*Tubb4a*$^{-/-}$. However, this remains to be confirmed. Another possibility is that these mutations have been inherited by chance and there exists another mutation that is responsible for the microcytic anaemia. Similarly, the lack of complete association with the microcytic phenotype could be due to a combined effect of *Ahctf1*, *Tubb4a* and other not yet identified mutation(s). At this point of time, we cannot conclude if the *Ahctf1* and or *Tubb4a* candidate genes are the main contributors or have only partial or no effect in regard to the underlying phenotype in MRI47455$^{+/-}$. Future work on this mutant line will therefore be necessary to correctly identify and verify the responsible mutation.

Future perspectives

The work in this chapter is limited by our results in aiming to identify the causative ENU mutation. While we discovered two mutations in separate genes that segregated with microcytosis in MRI47455$^{+/-}$ over several generations, no complete genotype-phenotype association was found. Additionally, neither of the two identified genes have been assigned a direct role with a haematological disorder, further limiting our conclusion on the contribution of the *Ahctf1* and *Tubb4a* mutations. Therefore, future work will address how these gene variants relate to the microcytic phenotype. This will include investigating if the ENU mutations have an effect on the transcription or protein levels by immunoblotting and quantitative real-time PCR experiments. Other experiments will determine if the function of the proteins is modulated in MRI47455$^{+/-}$. For example, immunofluorescence studies with an anti-tubulin antibody can be performed on erythroid precursors to assess if spindle and microtubule formation is impaired during erythropoiesis. Moreover, cell cycle assays and quantification of each erythroid precursor stage will allow us to assess if the mutations affect a particular stage during terminal erythropoiesis and if this is a result of impaired cell division. Additionally, to address if the MRI47455$^{+/-}$ phenotype is due to an additive effect between the two gene variants, work will be carried out as well on animals that only harbour a mutation in either *Ahctf1* or *Tubb4a*. If the hypothesis that both variants are needed is true, it can be expected that the results from the above experiments will differ between mice that either carry one or both mutations.

Finally, our data pointed to the possibility that the line MRI47455 may carry other gene mutations that have not been identified yet and contribute to the phenotype. Such variants could have been missed during the bioinformatics pipeline in order to reduce the number of possible candidates. Alternatively, additional mutation(s) may reside in intron sequences that are not captured by whole exome sequencing. While this is highly possible, more genotyping is necessary for the correct identification of the responsible variant.

We demonstrated that resistance in MRI47455$^{+/-}$ is primarily due to impaired invasion and not growth of the intracellular parasite, which is in contrast to most of the classical RBC disorders. Given the unique resistant phenotype of this line, continuing work should also be aimed to further dissect the molecular mechanism behind impaired invasion. As such, proteomics on membrane fractions of affected RBC will reveal if there are important changes in protein concentrations, and may assist in the discovery of novel RBC receptors and proteins required by the parasite for successful cell entry.

Overall summary

In this study we presented the mutant line MRI47455$^{+/-}$, which is characterised as a classical thalassaemic-like phenotype, but also includes a number of clinical features that are not related with this type of RBC disorder. We therefore proposed that the affected animals possess a defect in the development of maturing RBCs. Furthermore, it was shown that affected mice are significantly more resistant to *P. chabaudi* infection than their non-affected littermates. We demonstrated that the mechanism of resistance is the result of a red cell autonomous effect by inhibiting parasite invasion, which contrasts with most other classical inherited RBC disorders that have been associated with this type of phenotype. Finally, we identified two separate mutations in *Ahctf1* and *Tubb4a* that associated with the microcytosis in MRI47455$^{+/-}$. Because both genes play important functions during mitosis it is possible that the two mutations disrupt cellular processes during erythropoiesis and thereby contribute to the observed RBC defect. However, this remains to be further verified. Nevertheless, MRI47455$^{+/-}$ represents a very interesting model to further dissect the host response during malarial infections.

Supplementary data

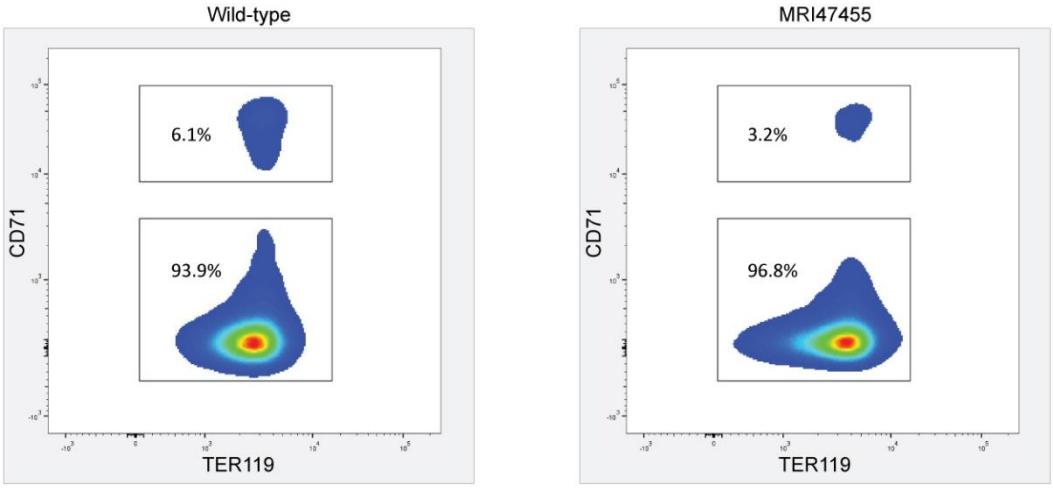

Figure S4.1 – Example of flow cytometry plots corresponding to CD71+ cells quantification. Example of increased proportion of CD71+/TER119+ cell in MRI47455$^{+/-}$ using flow cytometric analysis. Analysed with FlowJo V10.0.5 (http://flowjo.com) software.

Table S1 – List of non-synonymous gene candidates shared between all four mice sent for genome-wide next generation gene sequencing.

Gene	Chromosome number	Chromosomal location	Exon	Nucleotide change	Amino acid change	Number of animals		LOD
						Alternative	Wild-type	
Mr1	1	155137734	2	T96A	D32E	3	5	0.54
Ahctf1	1	179762771	29	T3936A	D1312E	8	0	2.33
Nes	3	87971375	1	A173G	D58G	0	4	0.00
Tmem79	3	88333605	2	T35A	M12K	0	4	0.00
Usp1	4	98932899	8	T1493C	V498A	0	4	0.00
Vmn2r52	7	10158815	6	A2396G	Y799C	0	4	0.00
Otog	7	46289740	37	T6193A	C2065S	0	4	0.00
Olfr714	7	107074239	1	T410A	M137K	0	4	0.00
Sez6l2	7	126968063	intron	A/G	>30bp away from intron/exon borders			
Sesn3	9	14310348	4	T443C	V148A	0	4	0.00
Lrrc9	12	72469295	intron	T/A	>30bp away from intron/exon borders			
Csmd3	15	47888732	25	T4141A	C1381S	2	6	0.32
C4b	17	34740365	11	G1234A	D412N	0	8	0.00
Gm11131	17	35380204	intron	C/T	>30bp away from intron/exon borders			
H2-Q4	17	35380204	intron	C/T	>30bp away from intron/exon borders			
Tubb4a	17	57081543	4	A482G	D161G	8	0	2.33
Kdm2az	19	4356922	6	T312G	S39R	3	4	0.59

Between 4 and 8 mice that were selected for a low MCV (MCV <49 fl) were genotyped for all exonic variants. Number of mice that carried a mutation (affected) in any of the gene candidates are presented and strength of linkage is shown by the LOD score.

Chapter 5 – ENU MUTANT MOUSE LINE MRI47495

Introduction

This chapter investigates another mutant mouse line, which was generated through N-ethyl-N-nitrosourea (ENU) mutagenesis. Similar to *Ank-1*^{MRI23420} and MRI47455, the reported mutant line MRI47495 was discovered by a dominant screen of 1st generation G1 mice for haematological phenotypes. Affected mice were primarily characterised by red blood cells (RBC) that display an increased mean corpuscular volume (MCV). This chapter is therefore directed to identify the ENU mutation responsible for the underlying abnormal blood phenotype. Additional investigations are aimed to further examine how the mutation affects the biology of MRI47495 and if this line exhibits a selective advantage during malarial infection.

Results

Identification of MRI47495 mice

Line MRI47495 was derived from a G1 mouse that displayed an increased mean corpuscular volume (MCV) that was three standard deviations higher than the normal population. Progeny testing, conducted by crossing the G1 founder (female) to wild-type SJL mice, revealed that half of the G2 littermates displayed a comparable high MCV (p-value <0.0001), while the other half displayed an MCV similar to wild-type mice (Table 7). Otherwise, affected G2 mice exhibited a comparable haematocrit (HCT, p-value = 0.82), RBC count (RBC, p-value = 0.41), mean cellular haemoglobin (MCH, p-value = 0.61), mean cellular haemoglobin concentration (MCHC, p-value = 0.51) and red cell distribution width (RDW, p-value = 0.78) to WT. The same heritability pattern of crosses between SJL and macrocytic mice was repeatedly observed over several subsequent generations. Furthermore, inheritance of the phenotype was consistent between males and females alike. Together, this suggests that the phenotype is inherited in a Mendelian autosomal dominant mode and is fully penetrant. Furthermore, we considered from the frequency of affected and unaffected mice resulting from G1 x SJL crosses that the G2 mutants are heterozygous (MRI47495$^{+/-}$) for the causative mutation.

To test if homozygous mice may be viable and exhibit a more profound phenotype, we intercrossed MRI47495$^{+/-}$ animals and conducted a full blood count on their progenies. This revealed, in addition to the two MCV classes observed in the G2 animals, a third group that exhibited a more dramatic MCV (64.11 ± 0.68 fl: different to WT, p-value <0.0001 and to *Rbl1*$^{MRI47495+/-}$, p-value <0.005) than MRI47495$^{+/-}$. In particular, out of the 25 mice analysed, 5 animals displayed an MCV greater than 61.0 fl, whereas 6 exhibited a normal MCV (MCV <54.0 fl) and 14 mice an increased MCV that was comparable to their parents (MCV between 56.0 fl and 59.0 fl). This equates to an approximate 1:2:1 ratio and therefore indicates that the third group of mice is homozygous (MRI47495$^{-/-}$) for the underlying mutation. Similar to their heterozygous littermates, MRI47495$^{-/-}$ displayed no other significant deviations in other blood parameters (HCT, p-value = 0.75; RBC, p-value = 0.16; MCH, p-value = 0.88; MCHC, p-value = 0.37; RDW, p-value = 0.64) when compared to WT. Furthermore, breeding from a cross between two homozygous animals produced a litter size of 7 healthy looking animals, all with an MCV >61.0 fl, which is comparable with what was observed in progeny from the G2 intercross. Taken together, since the heterozygous phenotype is intermediate to the homozygous phenotype we can assume that the trait is inherited in an incomplete dominance mode, and breeding capacity of MRI47495$^{-/-}$ is normal.

Table 7 – Automated full blood count parameters on MRI47495 and wild-type mice.

	WBC	RBC	HGB	HCT	MCV	MCH	MCHC	RDW
Founder (G1)	4.8	4.91	79	0.28	57.7	16.1	278	15.7
MRI47495$^{+/+}$ (G2-5)	4.6 ± 0.6	4.8 ± 0.5	70.8 ± 2.6	0.26 ± 0.03	53.05 ± 0.73	16.9 ± 0.7	266.1 ± 3.1	14.9 ± .0.6
MRI47495$^{+/-}$ (G2)	4.3 ± 0.4	5.1 ± 0.5	69.9 ± 2.0	0.26 ± 0.03	**58.02 ± 0.43***	16.6 ± 0.3	265.8 ± 1.8	14.7 ± 0.2
MRI47495$^{-/-}$ (G3-5)	4.3 ± 0.5	4.6 ± 0.3	71.2 ± 2.4	0.24 ± 0.02	**64.11 ± 0.68*#**	16.5 ± 0.1	264.7 ± 1.6	15.7 ± 0.4

Full blood analyses were conducted on the G1 founder mouse as well as on 18 MRI47495$^{+/+}$, 43 MRI47495$^{+/-}$, and 21 MRI47495$^{-/-}$ mice at 7 weeks of age. Generations of analysed mice are indicated in brackets and all values are represented as mean ± SEM. Statistical differences to WT and performed by a student`s t-test are indicated with * (p-value <0.0001), whereas differences to MRI47495$^{+/-}$ are shown by # (p-value <0.005). WBC represents white blood cell count; RBC, red blood cell count; HBG, haemoglobin; HCT, haematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; and RDW, red cell distribution width.

To further verify and evaluate the aetiology of the macrocytosis, Giemsa stained thin blood smears from MRI47495$^{+/-}$ and MRI47495$^{-/-}$ mice were examined by light microscopy. This revealed a noticeable increase in cell size in all red cells for MRI47495$^{-/-}$ (Figure 5.1A-C). Possible causes of the observed increase in RBC size were next investigated. The origins of macrocytosis can be broadly categorised as megaloblastic or non-megaloblastic. The spectrum of megaloblastic disorders generally includes nutritional deficiencies (eg B12 vitamin or folic acid) and primary complications in the bone marrow (eg myelodysplasia and leukaemia) (Aslinia et al., 2006). For example, macrocytosis due to vitamin B12 or folate deficiency is a result of defective DNA synthesis and is characterised by the presence of macro-ovalocytes, hyper-segmented neutrophils, and red cells with a notable variation in shape (poikilocytosis) and size (anisocytosis). These megaloblastic-like features have also been also implicated with primary bone marrow disorders. Examination of blood smears from MRI47495$^{+/-}$ and MRI47495$^{-/-}$ did not indicate any RBC progenitor abnormalities. However, a thorough examination of the bone marrow was not conducted to confirm this. Non-megaloblastic processes that cause macrocytosis can develop from multiple mechanisms. One possible cause for this type of macrocytosis is the presence of defects in the RBC membrane. Another possible cause is an increase in RBC production secondary to peripheral red cell destruction or loss. Thin blood smears from mutants were not indicative of elevated numbers of target cells suggestive of cell membrane abnormalities. Observations also did not reveal any signs of haemolysis: no fragmented RBCs (shizocytes) or an increase in the number of reticulocytes were detected. The absence of reticulocytosis was further verified by cell counting of the smears and calculating the proportion of reticulocytes from the total number of counted cells (different to WT: p-value = 0.61 for MRI47495$^{+/-}$, p-value = 0.56 for MRI47495$^{-/-}$) (Figure 5.1D). Furthermore red cell destruction secondary to increased erythropoiesis is usually accompanied with an increase in spleen size. Interestingly, weights of excised spleens (15 homozygotes and 9 WT) adjusted for body weights (spleen index) were found to be approximately 30% smaller in *Rbl1*$^{MRI47495/MRI47495}$ animals (4.82 ± 0.10g in *Rbl1*$^{MRI47495/MRI47495}$ versus 6.59 ± 0.20g in WT, p-value <0.0001). Taken together, we therefore suspect the cause of the macrocytosis is non-megaloblastic and without anaemia, but the exact aetiology remains unknown.

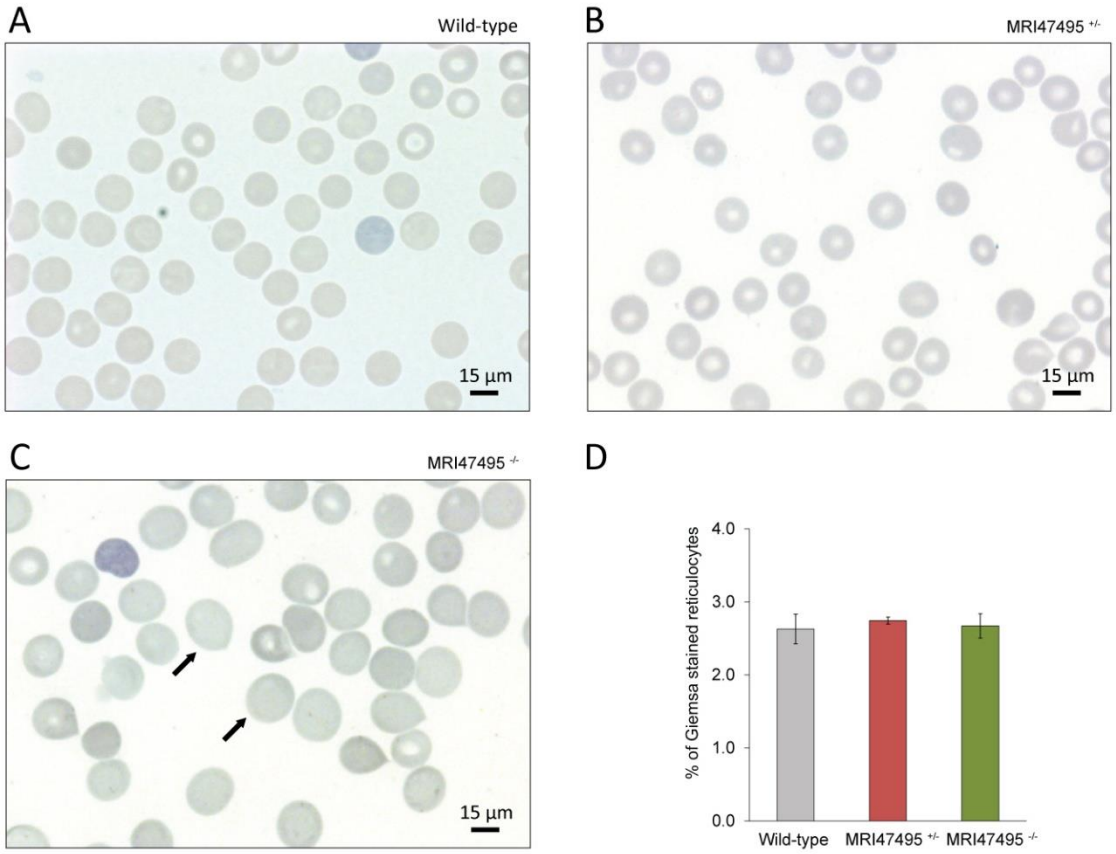

Figure 5.1 – *Rbl1*^{MRI47495} mice display macrocytic red blood cells. Giemsa stained blood smears of wild-type (A), MRI47495$^{+/-}$ (B), and MRI47495$^{-/-}$ (C) mice. Black arrows indicate macrocytic red blood cells. (D) Manual count of reticulocytes on blood smears (n=6 per group). Error bars indicate ± SEM.

Identification of a novel ENU mutation in the retinoblastoma-like 1 gene.

To identify the causative mutation for the elevated MCV phenotype, we selected two progeny tested mutants (G2) displaying a high MCV for exome sequencing. Extracted DNA was sequenced on an Illumina platform (HiSeq 2000) by Oto genetics (Atlanta, USA). We considered that to cause the observed phenotype: a causative mutation must be heterozygous as a result of crossing affected G1 to SJL/J. Further, we assumed that the mutation would be unique in the mutant mice selected for sequencing and not present in other ENU-induced mutant mice (in an SJL/J background) that we had previously sequenced. Therefore, we first annotated unique variants that were in common between both affected mice. We then retained only heterozygous variants that were found in both samples and that had not been described previously. This allowed us to reduce the total number of exonic and intronic variants to 26 (Table S2). Four of the mutations were found outside of putative splicing

sites (>30bp away from intron/exon borders) and were therefore excluded as they were considered unlikely to have a significant effect on the encoding protein. Since the mutation results in an abnormal RBC phenotype, we made the additional assumption that the causative gene should be expressed in haematological tissue or RBC progenitors. Therefore, we investigated the gene and protein expression profiles of the genes in which the remaining 22 mutations were located using the following open-source databases: protein atlas (http://www.proteinatlas.org), biogps (http://biogps.org), and mouse genome informatics (www.informatics.jax.org). Only five of the 22 genes were reported to be significantly expressed in haematological tissue and these were selected for further investigation. To test if any of the 5 selected variants associated with the macrocytic phenotype, 5 mice (2 WT, 1 heterozygous and 2 homozygous for the phenotype) were genotyped using Sanger sequencing. We found that a mutation in the retinoblastoma-like protein 1 (*Rbl1*) gene located on chromosome 2 was present only in mice with a high MCV and not in WT mice. Genotyping of the other four loci did not detect any such associations (Table 8). Additional genotyping for the *Rbl1* mutation in 15 distantly related MRI47495 mice (10 heterozygous and 5 homozygous for the phenotype) revealed that all genotyped MRI47495$^{+/-}$ mice carried one copy of the *Rbl1* mutation, and all of the MRI47495$^{-/-}$ animals possessed the mutation in both alleles. This demonstrated full segregation of the *Rbl1* mutation with the macrocytic phenotype. We therefore defined this line of mouse as *Rbl1*^{MRI47495}.

Table 8 – Linkage analysis of selected gene candidates with the macrocytic phenotype in *Rbl1*^{MRI47495}.

	Number of animals						
Variant	**MCV <54.0**	**MCV 57.0-61.0**	**MCV >63.0**	**WT**	**Heterozygous**	**Homozygous**	**LOD**
Rbl1	2	11	7	2	11	7	5.11
Psma7	2	1	2	5	0	0	0.00
Skint11	2	1	2	5	0	0	0.00
Man2a2	2	1	2	5	0	0	0.00
Cpd	2	1	2	5	0	0	0.00

Linkage is presented with the LOD score.

The ENU-induced mutation in *Rbl1* is a T -> C transversion in *Rbl1* in exon 22 (Figure 5.2). It is a missense mutation, replacing a leucine at position 1049 to serine. The mutation is located at the C-terminal end of the protein, a region that is highly conserved between human and mouse (Huppi et al., 1996). The encoded protein of *Rbl1* is known as p107 and belongs to the cell cycle inhibitor family comprising the retinoblastoma protein 1 (Rb) and retinoblastoma-related protein 2 (p130) (Cobrinik, 2005, Classon and Dyson, 2001). Together, these proteins make up a critical component of the cell cycle machinery, and share multiple overlapping cellular functions such as entry and exit from the cell cycle, differentiation and cell death (reviewed in (Wirt and Sage, 2010)). Additionally, they share several structural features including an A and B pocket, separated by a spacer region, as well as a C-terminal binding site for E2F transcription factors (Ewen et al., 1991, Li et al., 1997, Zhu et al., 1995). The MRI47495 mutation is located in the latter domain. Knockout of *Rb* (Sankaran et al., 2008), as well as *E2F4* (a member of E2Fs) (Kinross et al., 2006), results in ineffective erythropoiesis, as well as impaired erythroid maturation. Therefore, we hypothesise that the identified mutation impairs the function of p107 and affects the proliferation and/or differentiation of erythroid precursors.

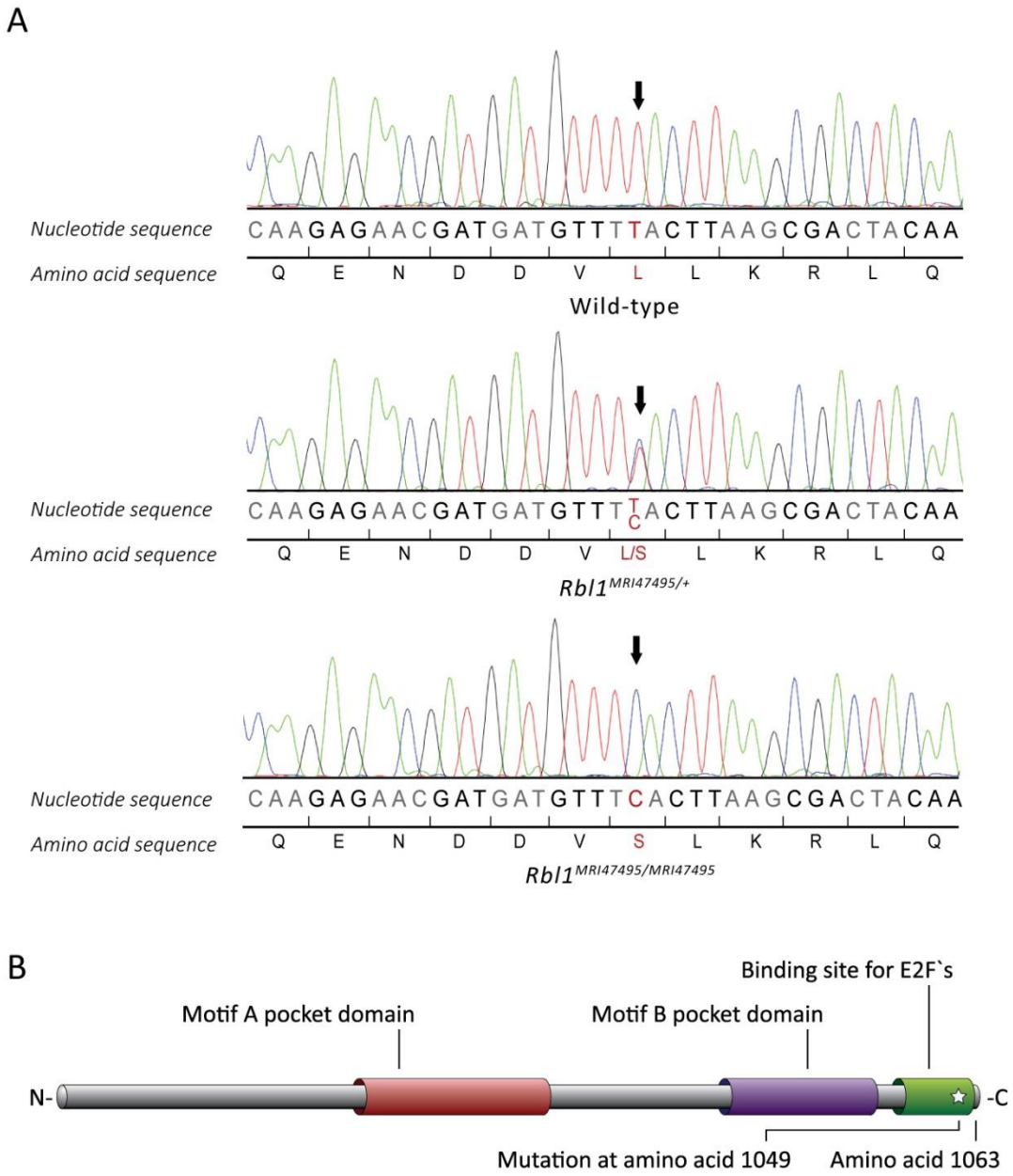

Figure 5.2 – Identification of the Rbl1 allele. (A) Electropherograms of the DNA sequence showing the T to C transversion in exon 22 for WT, *Rbl1*$^{MRI47495/+}$ and *Rbl1*$^{MRI47495/MRI47495}$ mice including the resulting amino acid change. (C) Schematic visualisation for the location of the mutation within the protein including previously identified domain structures.

Rbl$^{MRI47495/MRI47495}$ exhibit an impaired cell cycle in spleen cells

All members of the retinoblastoma family have been shown to negatively regulate cell cycle functions in proliferating cells (LeCouter et al., 1998, Vanderluit et al., 2004). As such, control of cell division by p107, as well as Rb and p130, has been primarily shown to occur during the preparative stage of cell division, known as interphase. Interphase precedes mitosis (M phase) and cytokinesis. Interphase is initiated by the G1 growth phase, which prepares the cell and ensures that the cell is ready for the replication of DNA that occurs in S phase. This is followed by another growth phase, known as G2, during which the cell readies itself for M phase. As well as being expressed in erythroid progenitors, it is also expressed in other organs that display high rates of cell division. This includes the spleen (Garriga et al., 1998). Therefore, we hypothesised that if the *Rbl1* mutation affects the function of the protein then *Rbl*^{MRI47495} mice may exhibit an abnormality in any of the three cell cycle phases of proliferating spleen cells. As such, the average cell cycling pattern of all cells from dissected spleen tissue of *Rbl*$^{MRI47495/MRI47495}$ mice was determined by flow cytometric analysis using the DNA stain Propidium Iodide (PI). The premise of this stain is that it binds in a stoichiometric proportion to the amount of DNA present in the cell. Since cells will approximately double the amount of DNA during the S phase, the relative amount of cells in each of the three phases can be determined. The mean fluorescence intensity (MFI) of cells in the G2/M phase will be about twice as high as that of cells in the G1 phase. In support, flow cytometry plots of analysed spleen cells (Figure 5.3A) display two major peaks, which relates to the MFI of cells at G1 and G2/M. The area between therefore presents cells in the S phase. Quantification of the MFI from the total MFI for each gated population revealed no differences between homozygotes and WT in the G1 (p-value = 0.90) and S (p-value = 0.47) phases (Figure 5.3B). However, *Rbl1*$^{MRI47495/MRI47495}$ exhibited an approximate 33% decrease in MFI at G2/M (p-value <0.05) and 50% increase at the sub-G1 phase (p-value <0.05) when compared to WT. These results therefore provide support for our hypothesis that the function of p107 in cell cycle regulation is affected by the identified mutation in *Rbl1*^{MRI47495} mice. However it remains to identify if all spleen cells or only a sub-population is affected. Further, at the time that this thesis was submitted, there was no information available about the effect of the mutation on expression of transcript or protein levels, or indeed the specific function of the mutant protein. Therefore,

if the arrest at the G2/M phase enhances the activity of mutant p107 as a cell cycle inhibitor and thereby decreases proliferation of spleen cells is currently being investigated.

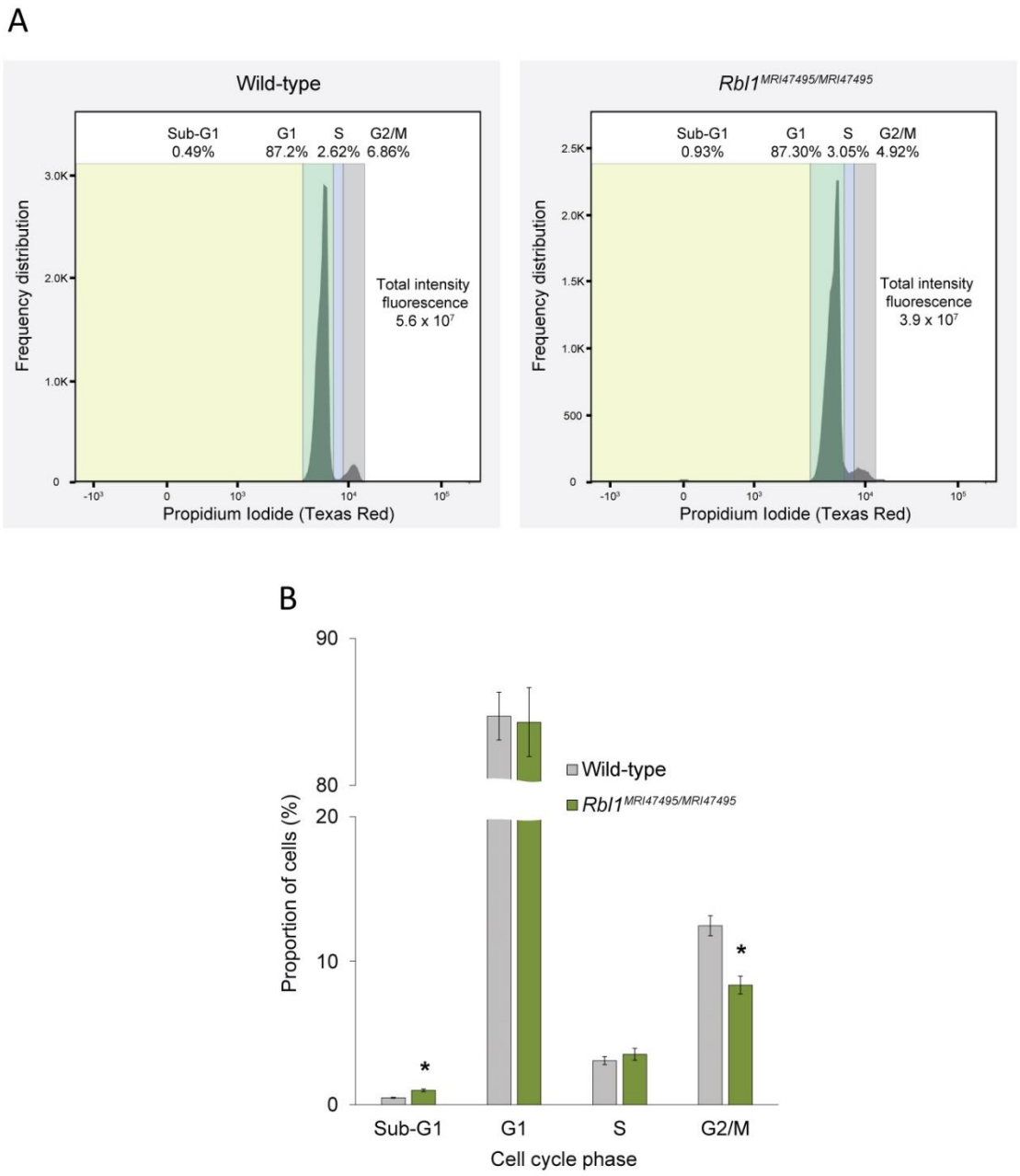

Figure 5.3 - *Rbl1*$^{MRI47455/MRI47495}$ mice exhibit cell cycle defect. (A) Cell cycle assay using flow cytometry analysis in spleen cells of *Rbl1*$^{MRI47495/MRI47495}$ and WT. (A) Flow plot examples of stained cells with the DNA stain Propidium Iodine. The frequency distribution was determined by gating each cell cycle stage using FlowJo V10.0.5 (http://flowjo.com) software. (B) Quantification of the proportion of spleen cells for each gate in *Rbl1*$^{MRI47495/MRI47495}$ (n=4) and WT (n=4). Data demonstrated a cell cycle arrest at G2/M phase in homozygous animals. Error bars represents ± SEM and statistical significance to wild-type, performed by a student`s t-test is indicated with * (p-value <0.05).

The *Rbl*^{MRI47495} mice are not resistant to *P. chabaudi* infection

The major aim of this thesis was to identify novel gene variants that offer protection against malaria infection. Therefore, we tested whether *Rbl1*^{MRI47495} mice showed any level of resistance to *P. chabaudi* infection. Mice hetero- and homozygous for the *Rbl1*^{MRI47495} mutation, as well as wild-type littermates, were infected with a lethal dose of *P. chabaudi adami* DS and monitored daily for parasitaemia and survival. The rate of survival in *Rbl*$^{MRI47495+/-}$ (p-value = 0.75) and *Rbl*$^{MRI47495/MRI47495}$ (p-value = 0.92) was comparable to WT (Figure 5.4). Likewise, the development of blood parasitaemia was virtually identical in all three cohorts throughout the infection. Therefore, we concluded that macrocytic *Rbl*^{MRI47495} mutants display no increased or decreased resistance to malarial infection.

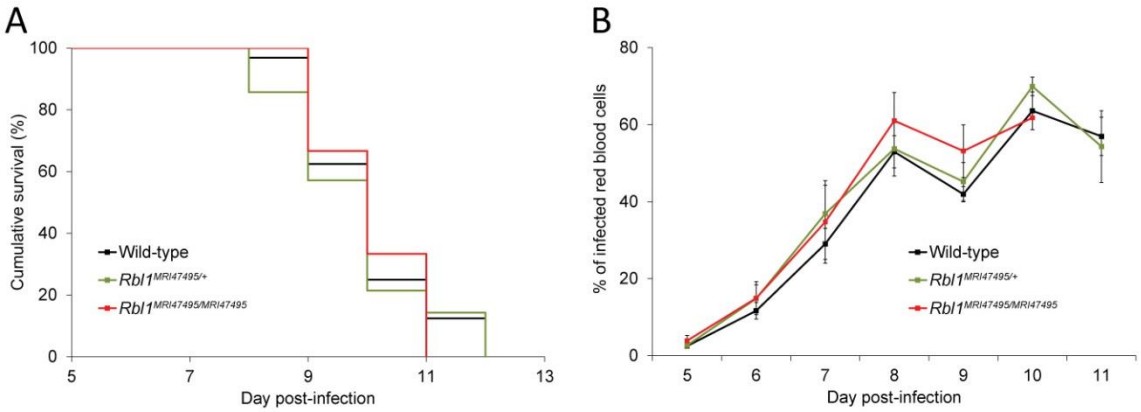

Figure 5.4 – *Rbl1*^{MRI47495} is not resistant to *P. chabaudi* parasites. (A) Number of infected RBCs and (B) Kaplan Meier curve for female wild-type (n=32), *Rbl1*$^{MRI47495/+}$ (n=17), and *Rbl1*$^{MRI47495/MRI47495}$ (n=4) mice. Animals were infected with *P.chabaudi* at a dose of 1 x 10^4 iRBC. Error bars are presented as ± SEM.

Discussion

We report here a novel ENU mutant line, MRI47495, which was identified during dominant screening of mice that display an abnormal blood phenotype. We showed that MRI47495 mice were characterised by a macrocytic phenotype. Further, we discovered a novel mutation in the cell cycle inhibitor gene *Rbl1* that completely associated with the underlying phenotype in *Rbl1*^{MRI47495}. Additionally, we demonstrated that spleen cells in homozygous mutants are arrested at the G2/M phase during the cell cycle. However, the *Rbl1*^{MRI47495} mice were not resistant to *P. chabaudi* malarial infection.

Rbl1^{MRI47495} mice display an abnormal blood phenotype with macrocytosis

Rbl$^{MRI47495/MRI47495}$ mice were identified by displaying a macrocytic phenotype. Observation of thin blood smears revealed that macrocytosis is a result of an increased cell volume of RBCs and not secondary to elevated numbers of larger reticulocytes. There is evidence that cell size of the mature red cell is controlled during its development in the bone marrow (Dolznig et al., 2004). Therefore, we hypothesised that the abnormal blood phenotype is due to an impairment during this process. The most common form of macrocytosis is a megaloblastic anemia and often associated with either vitamin B12 or folate deficiencies (Aslinia et al., 2006, Koury et al., 1997). In this type, red cells are larger as they are unable to adequately synthesise DNA in order to divide at the right time, and are therefore often associated with macro-ovalocytosis (oval shaped erythrocytes) and nuclear remnants in immature RBCs in the periphery (Morris et al., 2007). Since we did not observe any of these characteristics in *Rbl1*^{MRI47495} mice, we suspect that the cause of the increased cell size is due to another non-megaloblastic defect during the development of erythrocytes.

Genotyping and association with the MCV phenotype provided strong evidence that the causative mutation in MRI47495 is in the *Rbl1* gene. Further, we showed that the mutation is located within a site that is important for binding E2F transcription factors. However, our results could not provide further details on how the mutation relates to the macrocytosis in affected mice. Nevertheless, this study is the first to describe a mutation in *Rbl1* that is linked to an abnormal blood phenotype. Although it has been shown that knockout of p107 in mice causes hypercellularity in the marrow of the sternum, the study did not report if

erythropoiesis, as well as the functionality of mature RBCs, is affected or not (LeCouter et al., 1998). Whether the authors did not observe or simply not test for an abnormal blood phenotype is not known. Nonetheless, it is important to note that we do not know if the mutation in *Rbl1*^{MRI47495} mice affects p107 levels or its molecular function. As such, one explanation could be that the novel blood phenotype is due to an impairment in the interaction between p107 and its binding partners. However, to further investigate how the *Rbl1* mutation causes the underlying phenotype, it is important to increase our understanding of the functional role of p107 during the maturation process of RBCs.

In non-haematological tissue both p107 and Rb have been shown to control proliferation and differentiation of cells. There is also some evidence for a similar role for p107 in erythropoiesis. Knockout of the closest homolog of p107, the Rb protein, causes deregulated erythropoiesis as a result of arresting differentiation of early to late erythroblasts (Sankaran et al., 2008). The authors concluded that Rb mediates its activity by interacting with E2F, thereby coupling the process of mitochondrial biogenesis with cell cycle exit during erythropoiesis. Other work showed that, similar to Rb, p107 is also highly expressed in differentiating erythroid precursors, and declines rapidly once the cells exit the cell cycle (Kastner et al., 1998, Ikeda et al., 1996, Condorelli et al., 1995). Furthermore, there is evidence that p107 complexes with E2F4, a member of the E2F family, in cycling erythroid precursors (Kinross et al., 2006). Therefore, it is plausible that p107 may control processes during erythropoiesis by interacting with E2F4, and that the *Rbl1*^{MRI47495} mutation causes an impairment in the interaction between p107 and E2F4. Specifically, the mutation could increase in binding affinity between p107 and E2F4 and thereby lower the activity of the transcription factor.

A closer look at what is known about the functions of E2F4 provides further support for this hypothesis. A knockout study showed that adult *E2F4*$^{-/-}$ mice exhibit a defect in differentiating and proliferating erythroid precursors, resulting in a marked macrocytic anaemia with megaloblastic features (Humbert et al., 2000). In addition, work by Hurford et al. (1997) demonstrated that loss of p107 activity has no obvious effect on expression of E2F4. Therefore, if p107 primarily mediates its activity through E2F4 in erythroid precursors then this provides a possible explanation to why an abnormal blood phenotype has not been

observed in p107 knockout mice. However, so far we cannot predict from our results if the underlying RBC phenotype in *Rbl1*^{MRI47495} is caused by an impaired interaction between p107 and E2Fs. Such a hypothesis therefore remains to be investigated in future studies. Moreover, it remains possible that other mechanisms with not yet identified binding partners contribute to the *Rbl1*^{MRI47495} phenotype.

Homozygous *Rbl1*^{MRI47495} mice exhibit an arrest in the cell cycle

Studies have shown that p107 is an important cell cycle regulator. We considered that if *Rbl1* is the causative gene, it can be expected that *Rbl1*^{MRI47495} mice display an impaired cell cycle. By quantifying the DNA content of spleen cells in homozygotes, we demonstrated that about one third of cells exhibited an inhibitory defect at the G2/M phase. In addition, we observed that the spleen size in these mice was reduced by a similar degree, although whether the reduction in spleen tissue is due to a reduction in cell proliferation caused by the G2/M defect is not known. However, the effect of the *Rbl1*^{MRI47495} mutation appears to contrast with what has been observed in a p107 knockout mouse model. As well as an increase of cell proliferation in the bone marrow, *p107*$^{-/-}$ mice also display hypercellularity in the spleen, liver and isolated fibroblasts (LeCouter et al., 1998). It is therefore possible that the mutation in *Rbl1*^{MRI47495} enhances the inhibitory role of p107.

Both homologs of p107, Rb and p130, have been associated with inhibitory functions during G1, S and G2 phase (Stark and Taylor, 2006, Sun et al., 2007). However, p107 has only been implicated in an inhibitory role during the G1 and S phase of the cell cycle, which was demonstrated by transiently transfecting osteosarcoma SAOS-2 cells with physiologic levels of p107 (Jiang et al., 2000). It may therefore be possible that the mutation in *Rbl1*^{MRI47495} disrupts a regulatory function of p107 that has not been identified yet and perhaps occurs only during G2. So far, it has been shown that p107 is predominantly inactivated by hyper-phosphorylation between the S and G2/M phases. Although there is evidence that a small pool remains under-phosphorylated throughout the cell cycle (Beijersbergen et al., 1995, Voorhoeve et al., 1999, Balciunaite et al., 2005). It has been shown that this population interacts with members of the Smad family and E2F4/5, mediating transcriptional repression of other downstream cell cycle genes (Chen et al., 2002). However, the same authors have

also shown that these interactions are not exclusive to the G2/M phase. Therefore, it remains to be investigated how the mutation in p107 causes the cell cycle defect in *Rbl1*^{MRI47495} spleen cells. Moreover, it is currently not known if erythroid precursors display a similar inhibition during the G2/M phase or not.

Rbl1^{MRI47495} mice are not resistant to *P. chabaudi* infection

In addition, we also aimed to find novel gene variants that offer protection against malarial infections. We therefore infected *Rbl1*$^{MRI47495/+}$ and *Rbl1*$^{MRI47495/MRI47495}$ mice with the murine malaria parasite *P. chabaudi*. However, both heterozygous and homozygous mutants did not deviate from WT in respect to survival and levels of infected RBCs. While several RBC disorders have been identified that offer protection against malaria (Bunyaratvej et al., 1992b, Ferreira et al., 2011, Greth et al., 2012, Shear et al., 1991), none of these have been associated with a macrocytic phenotype. Similarly, to our knowledge there are no known mutations in cell cycle regulators that alter susceptibility to malaria. Therefore, our results suggest that affected RBCs of *Rbl1*^{MRI47495} offer hospitable conditions for the parasite to develop and progress its life cycle, despite their abnormal phenotype.

Future directions

While this chapter identified a significant association between *Rbl1*^{MRI47495} mice and the abnormal blood phenotype, no formal explanation for how the mutation in *Rbl1* could cause the observed macrocytosis was offered. Further investigation will therefore focus on how the ENU mutation affects the maturation process of RBCs. In the first instance, immunoblotting and real-time PCR can be performed to assess whether the mutation directly affects protein and transcript levels. Secondly, quantification of erythroid precursors for specific sub-populations will reveal if the defect contributes to a particular stage during terminal differentiation. Furthermore, assessment of the phosphorylation status of p107 during the cell cycle, as well as binding assays with other associated partners (in particular E2F transcription factors), will help to reveal if p107 activity is modulated. In addition, protein complex immunoprecipitation can be used to identify if the cell cycle defect is a result of impaired interactions between p107 and other not yet identified binding partners. All of these

experiments will help demonstrate how the *Rbl1*^{MRI47495} mutation causes the blood phenotype, as well as if the mutation causes a loss or a gain in function.

Overall summary

Taken together, in this chapter we presented the mutant line *Rbl1*^{MRI47495} which exhibits a haematological phenotype that is mostly consistent with a non-anaemic, non-megalocytic macrocytosis. We further identified the first ever ENU mutation in the cell cycle inhibitor *Rbl1* that associated with an RBC phenotype, although no mechanistic details could be resolved with respect to how the mutation causes the macrocytic phenotype. However, we demonstrated that *Rbl1*^{MRI47495} mice display a cell cycle defect in cycling spleen cells, providing support that the *Rbl1* mutation affects the function of the protein. Finally, it was shown that the gene variant has no effect on the host response of affected mice during malaria infection. Because of the novel phenotypic features, *Rbl1*^{MRI47495} represents an ideal model to further investigate the functional role of *Rbl1* during the development of maturing RBCs.

Supplementary figures

Table S2 – Identified gene candidates from Next Generation gene sequencing.

Gene	Chromosome number	Chromosomal location	Exon	Nucleotide change	Amino acid change	Expressed in haematological tissue
Pgap1	1	54511913	17	A1607G	L536P	No
Tns1	1	73953360	19	A158G	S720P	No
Rbl1	2	157147552	22	T3146C	L1049S	Yes
Psma7	2	180039430	2	G146A	S49L	Yes
Mup2	4	60661805	2	T158A	D53V	No
Skint11	4	114244753	4	T389C	L130S	Yes
5031414D1	6	34905440	intronic	C/A	>30bp away from intron/exon border	
Gm9268	7	43024207	4	A709T	S247C	No
Klk1b9	7	43979598	intronic	T/A	>30bp away from intron/exon border	
Mrgbr65	7	48168681	1	G305A	T102I	No
Mrgbr65	7	48168700	1	T286C	R96G	No
Pcsk6	7	66033890	15	A1808T	H603L	No
Man2a2	7	80364058	10	C1244T	R415Q	Yes
Trim12c	7	104348211	2	G137A	T461I	No
Trim12c	7	104348227	2	C121A	V41L	No
Defa23	8	21191644	1	A31G	I11V	No
Csgalna	8	68362046	intronic	T/C	>30bp away from intron/exon border	
Gtpbp3	8	71491093	5	T619A	W207R	No
Itgb1	8	128720417	11	T1412C	I471T	No
493044	10	22067994	1	A86C	V29G	No
Sfi1	11	3156809	9	G896A	T299I	No
Cpd	11	76782377	21	C394A	C1314F	Yes
Serpin6b	13	32977639	6	A694T	M232L	No
Prss51	14	64095094	intronic	A/G	>30bp away from intron/exon border	
Tdh	14	63493927	8	A797T	M266K	No
Ctdp1	18	80469347	1	C200G	R67P	No

List contains all nonsynonymous gene variants that were shared between the two macrocytic mutants selected for sequencing. Genes in red were selected for further association studies.

Chapter 6 – FINAL REMARKS

The achievability of using ENU mutagenesis as a tool to discover gene mutations responsible for a particular phenotype is governed by the approach of how to select mutants of interest for sequencing and further experimentation. In order to find novel host factors that can potentially be targeted by a host-directed therapy (HDT), our laboratory employed a two screen strategy. In screen one, 1st generation (G1) mice are initially tested for an abnormal RBC phenotype by conducting a full blood count. An animal that displays a deviation in one or more blood parameters is then selected and mated with normal SJL to confirm the heritability of the observed abnormality. Thereafter, the resulting offspring from that cross are screened again to assess if the RBC defect also causes resistance to malaria infections. In contrast, screen two consists of additional screening for increased malaria survival of G1 mice that do not express an abnormal blood phenotype in the first place. Similarly, these mice are then selected and progeny tested. Therefore, identification of the causative gene is either based on an abnormal RBC, or survival phenotype, depending on which screen mutants are selected from. This research project investigated out of over 80 (generated in our laboratory), six mutant lines. Three were identified from screen two; these demonstrated a significant increase in survival and reduced parasitaemia but no other phenotype. The other three lines were generated from screen one and are discussed in this thesis.

Overall, we found that blood mutants, in particular those with a distinct RBC phenotype, are easier to identify than survival mutants. This is exemplified by the identification of the causative gene in two out of the three investigated lines with an RBC defect, namely *Ank-1*^{MRI23420} and *Rbl1*^{MRI47495}. While we could not confirm the identity of the variant in MRI47455, two possible gene candidates were found that associated with the observed RBC abnormalities in this line. In contrast, no responsible mutation could be found in the other investigated survival mutants. A similar trend was repeatedly observed in other studies performed by other members in our laboratory. For example, out of 40 generated mutant lines with an RBC defect, a causative mutation was found in 23, whereas causative mutations in only 4 out of 40 survival lines have been identified so far. Several factors contribute to this observation. RBC defects are usually a more consistent and reliable phenotype compared

survival and parasitaemia levels. This aided in the efficient discrimination of true from false positive mutants. In comparison the malaria screen involves a second live organism (*P. chabaudi),* which carries a number of inherent variable factors that are difficult to control for. Our laboratory has observed for example a distinctive shift in the virulence of the parasite over the course of several years of infections (G. Burgio, personal comment). Additionally, the observed RBC defect allowed assumptions to be drawn as to what biological functions are affected by the gene mutations. The use of this knowledge assisted in prioritising possible gene candidates. Furthermore, obtaining a small volume of blood instead of infecting mice with malaria, aided in the phenotyping of animals in a relatively short period of time without risking their lives and preventing them from being used in further experimentation. Nevertheless, it is important to consider that screening for a phenotype, additional to malaria resistance, is more likely to result in the identification of genes that are otherwise involved in important biological functions of the mouse. Disruption or inhibition of such host factors can result in unwanted side effects and may decrease the suitability of them as possible targets for an HDT. Nevertheless, our results show prove of concept that ENU mutagenesis in conjunction with an abnormal blood and resistant phenotype screen is a valid approach to discover new host targets that alter the susceptibility to malarial infection.

It was shown that the mutant lines presented here, exhibited a defect in RBCs that results in a decreased or increased red cell size. Both *Ank-1*^{MRI23420} and MRI47455 are characterised by a microcytic anaemic phenotype. However, the aetiology of the microcytosis between these mutants was found to be quite contrasting. We demonstrated that *Ank-1*^{MRI23420} mice display a classical hereditary spherocytosis (HS) phenotype, including splenomegaly, increased osmotic fragility of RBCs, and spherocytes on blood smears. Additionally, we identified a mutation in the *Ank-1* gene, which encodes a cytoskeletal/membrane protein important for the structural integrity of RBCs. Defects in the ankyrin-1 protein have been associated as the primary cause for HS (Gallagher, 2005, Eber et al., 1996a), and we provided convincing evidence that the identified ENU mutation is responsible for the underlying clinical symptoms in *Ank-1*^{MRI23420}. In contrast, MRI47455 mice displayed a thalassaemic-like phenotype, with low cellular haemoglobin content, but normal iron levels in RBCs. The results suggest that microcytosis is caused by an as yet unknown mechanism that affects haemoglobin

incorporation rather than its synthesis. The third mutant line, *Rbl1*^{MRI47495}, differed from the other two in that affected mice displayed an increase, rather than decrease, in RBC size without anaemia. Support for the hypothesis that the identified novel mutation lies in the cell cycle inhibitor gene *Rbl1*, was given by demonstrating that affected mice exhibit a cell cycle arrest in spleen cells. However, how the mutation is responsible for the blood phenotype remains unanswered.

Furthermore, we found that two out of the three investigated lines are resistant to *P. chabaudi* infection. We demonstrated that survival of *Ank-1*^{MRI23420} and MRI47455 was significantly increased, which correlated with a profound reduction in parasitaemia. We further provided novel insights into the mechanism of resistance. It was shown that ankyrin-1 deficiency in *Ank-1*^{MRI23420} results in an impairment of intracellular parasite growth. In contrast, we found that resistance of MRI47455 mice is primarily caused by the inability of parasites to invade RBCs. Overall the work presented in this thesis has opened new avenues for future investigations into a host-directed antimalarial therapy which may avoid the common problem of parasite resistance.

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APPENDICES

Appendix A – Ethics approval letter University of Tasmania

Letter of ethic clearance to conduct the live animal research as presented in this thesis:

UTAS

University of Tasmania
Animal Ethics Committee
ETHICS APPROVAL PERMIT

University of Tasmania
Research Services
Phone: (03) 62267283
Facsimile: (03) 62267148
Email: Animal.Ethics@utas.edu.au

To:	Dr Gaetan Burgio
From:	Marilyn Pugsley, Acting Exec Officer, Animal Ethics Committee
Date	21 September 2011
Project:	A12086 – Discovery of novel anti-malarial compounds
Approved on:	16 September 2011
Approval expires:	16 September 2014
Annual Report 1 due:	16 September 2012

PLEASE READ THIS PERMIT CAREFULLY AS APPROVAL MAY BE WITHDRAWN FOR PROJECTS THAT DO NOT COMPLY WITH THE CONDITIONS OUTLINED BELOW.

On 16 September 2011, the Animal Ethics Committee approved this project. The duration of the approval is until 16 September 2014 and is subject to the review and approval of an annual report. The annual report is due on the anniversary date of approval each year. You will receive an email reminder 4 weeks before the report is due.

If the project is to continue past 16 September 2014 a new initial application will need to be submitted as a project can only be approved for a maximum of 3 years.

As Chief Investigator on the project, you are responsible for ensuring:

(a) that all aspects of the work conform to the requirements of the current edition of the *Australian code of practice for the care and use of animals for scientific purposes*

(b) that a full record is maintained of all animals used in this project; and

(c) that any animals under an experimental regime are clearly identified. On this point, the Committee requests that you use an identification card on the cage or enclosure as illustrated on the following page. This identification is not required for animals being held as stock but only for those under the experimental regime.

(d) that you contact the Animal Welfare Officer (Dr Barrie Wells) to advise him of when and where your experiments will be conducted. Sufficient notice needs

to be given so that if the AWO wishes to make an inspection, this can be easily arranged. Dr Wells contact is 0427680782 or barrie.wells@utas.edu.au

The Animal Ethics Committee is to be promptly notified of any unexpected adverse events which occur during the period of the approved project and which may impact on the welfare of the animals. (2.2.28 *Australian Code of Practice*).

Autopsy by a qualified veterinarian should be performed when animals die unexpectedly. Any foreseeable departure from this requirement must have been outlined and approved in the initial application.

Detailed experimental records must be kept and must be available to the Committee on request.

Conditions which apply to some projects only:

(i) If the investigation necessitates a Parks & Wildlife permit you are required to send a copy of this permit to the AEC Secretary before commencing work.

(ii) If you intend to obtain laboratory animals directly from a source other than the University Central Animal House, the Animal Welfare Officer must be notified when the order is placed and when the animals are received.

Special Conditions of approval (if applicable): There are no special conditions of approval applicable to this project.

Marilyn Pugsley
Acting Executive Officer Animal Ethics

Appendix B – Ethics approval letter MacquarieUniversity

Letter of ethic clearance to conduct the live animal research as presented in this thesis:

MACQUARIE UNIVERSITY

ANIMAL RESEARCH AUTHORITY (ARA)

AEC Reference No.: 2012/017 **Date of Expiry:** 19 April 2013

Full Approval Duration: *20 April 2012 to 19 April 2015 (36 Months)*

Principal Investigator:		**Other people participating**	
Prof Simon Foote		Shelley Lampkin	0439 617 344
ASAM		Andreas Greth	0405 415 984
simon.foote@mq.edu.au		Patrick Lelliott	0450 314 761
0407 855 438		Elinor Hortle	0400 067 447
Associate Investigators:		Hong Ming Huang	0411 299 659
Gaetan Burgio	9812 3517		
Brendan McMorran	0400 391 529		

In case of emergency, please contact:

the Principal Investigator / Associate Investigator named above

or Manager, CAHF: 9850 7780 / 0428 861 163 and **Animal Welfare Officer**: 9850 7758 / 0439 497 383

The above-named are authorised by MACQUARIE UNIVERSITY ANIMAL ETHICS COMMITTEE to conduct the following research:

Title of the project: **Study of host response to malarial infections**

Purpose: **4- Research : Human or animal health and welfare**

Aims:

1. **To identify host response mechanisms to malarial infection**
2. **To identify loci contributing to the outcome of disease and breed congenic lines to further characterise the genes contributing to resistance / susceptibility to malarial infection**
3. **To characterise the role platelets play in malarial infection**
4. **To characterise the biology of malarial infection in mice with genetic changes**

Surgical Procedures category: **7 (Major Physiological Challenge), 8 (Death as an endpoint)**

All procedures must be performed as per the AEC-approved protocol, unless stated otherwise by the AEC and/or AWO.

Maximum numbers approved (for the Full Approval Duration):

Species	Strain	Age/Sex/Weight	Total	Supplier/Source
01: Mice	Mus mus domesticus/musculus	m/f/18-30g	3000	WEHI/Jax/CAHF or other
		Total	**3000**	

Location of research:

Location	*Full street address*
Australian School of Advanced Medicine (ASAM)	Level 1, Clinic Building, 2 Technology Place, Macquarie University NSW 2109
Central Animal House Facility (CAHF)	Building F9A, Research Park Drive, Macquarie University NSW 2109

Amendments approved by the AEC since initial approval: N/A

Conditions of Approval: N/A

Being animal research carried out in accordance with the Code of Practice for a recognised research purpose and in connection with animals (other than exempt animals) that have been obtained from the holder of an animal suppliers licence.*This ARA remains in force until the Date of Expiry (unless suspended, cancelled or surrendered)* **and will only be renewed upon receipt of a satisfactory Progress Report before expiry (see Approval email for submission details).**

Prof Michael Gillings (Chair, Animal Ethics Committee) **Approval Date: 19 April 2012**