Investigation of a novel host-directed therapy for malaria through ENU mutagenesis

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CONTENTS

SUMMARY

Treatments for malaria rapidly lose effectiveness due to parasite adaption. Using naturally occurring resistance to malaria as an example, it may be possible to circumvent this adaption by developing antimalarial therapies which render the host impervious to infection, rather than directly disabling the parasite. Known as host directed therapy, the success of this strategy depends on a detailed understanding of host factors which are critical to parasite survival.

An N-ethyl-N-nitrosourea (ENU) mutagenesis screen facilitated identification of two novel genes which influence malaria susceptibility in mice, *Tfrc* and *Sptb*. Mutations in these genes increased and decreased susceptibility to *Plasmodium chabaudi* infection respectively. In order to determine the mechanisms underpinning this susceptibility, a novel assay was developed based on flow cytometry of fluorescently labelled erythrocytes, and a dual staining approach towards the identification of parasitized erythrocytes. Using this assay it was determined that for *Tfrc*, which encodes the iron transport protein transferrin receptor 1, intraerythrocytic parasite survival was enhanced. For *Sptb*, which encodes erythrocytic beta spectrin, parasitized erythrocytes were found to be more susceptible to phagocytosis.

Overall, the study of these mutations and the mechanisms by which they influence malaria susceptibility has resulted in an increased understanding of host factors involved in this deadly disease. In future, it may be possible to manipulate these mechanisms to develop a host directed therapy, which would be less prone to parasite drug resistance.

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ETHICS STATEMENT

The work performed in this thesis was conducted under the agreement Ethics No ARA 2012/019 approved and obtained from the Animal Ethics Committee at Macquarie University.

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CHAPTER 1

LITERATURE REVIEW

MALARIA BURDEN

Malaria is a parasitic disease caused by the protozoan parasite *Plasmodium*, and has afflicted humans since prehistoric times[1]. Although global eradication efforts throughout the 20th century were successful in eliminating malaria in much of the developed world, including Europe, Canada, the USA and Australia, approximately half of the world's population remains at risk[2]. The majority of the at-risk population resides in developing countries, making malaria distribution and impact difficult to assess[2,3]. According to the World Health Organisation 473-789 thousand deaths were attributed to malaria in 2012, with a total of 135-287 million malaria cases that year[2]. Of these, 80% of cases and 90% of deaths occurred in sub-Saharan Africa, mostly in children under five years of age[2]. Indirect impacts from malaria, not conveyed in these estimations, include increased abortion rates and infant mortality due to placental malaria, impaired neurological and cognitive development of children both in utero and during early childhood, as well as increased poverty due to its significant economic burden[4-6].

A recent resurgence in funding and a global push to address the malaria problem has led to a substantial decline in malaria cases and mortality, with more than three million deaths averted since 2001 in Africa alone[2,7]. However, malaria is a wily and highly adaptive disease, and many significant obstacles must be overcome before the ultimate goal of global eradication is achieved. A combined strategy including vector control, drug and vaccine development, improved diagnostics, monitoring, evaluation, and surveillance is needed[7]. There is a general consensus that current drug treatments, including artemisinin-based combination treatments (ACTs), will not be sufficient to achieve eradication[8]. Indeed, parasite resistance to ACTs, the latest in a long line of malaria "wonder drugs", has already been observed in South-East Asia[9,10]. A major challenge in our quest to achieve eradication is the development of new drugs which are cheap, effective and, most importantly, less susceptible to the development of parasite drug resistance[8].

LIFECYCLE AND PATHOGENESIS

There are currently six species of *Plasmodium* known to infect humans; *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale curtisi*, *Plasmodium ovale wallikeri*, *Plasmodium malariae*, and *Plasmodium knowlesi*. As well as humans, *Plasmodium* afflicts many other vertebrate species, of which the rodent malarias are directly relevant to this thesis. The most common rodent malaria species used in the laboratory are *Plasmodium chabaudi*, *Plasmodium berghei*, *Plasmodium vinckei* and *Plasmodium yoelii*. All of these undergo a similar lifecycle with some important differences which can affect pathogenesis and transmission.

P. falciparum is the most deadly of the human malarias, accounting for the vast majority of malaria deaths[2]. All *Plasmodium* species require two hosts to complete their lifecycle; for *P. falciparum* these are the insect vector and a primate. The mammalian life cycle of *P. falciparum* begins when an infected female anopheles mosquito takes a blood meal, at which point the sporozoite form of the parasite moves from the salivary glands of the mosquito into the skin (Figure 1.1). Sporozoites migrate from the bite site to the liver where they transverse many cells before invading a suitable hepatocyte, at which point the asymptomatic liver-stage of infection begins[11]. Inside the hepatocyte the parasite matures through to schizogony, ultimately undergoing asexual reproduction to produce several thousand merozoites. Alternatively, in the case of *P. vivax* and *P. ovale*, the sporozoite can remain dormant in the liver in a hypnozoite stage for several years before maturing and replicating[12].

A single sporozoite can produce thousands of merozoites, which are released into the blood stream synchronously, thereby maximising the chance of establishing a blood stage infection. Once in the blood stream merozoites rapidly invade erythrocytes. *P. vivax*, *P. ovale*, and *P. malariae* preferentially invade only young erythrocytes, specifically reticulocytes. This generally restricts their parasitaemia to low levels[13]. On the other hand, *P. falciparum* and *P. knowlesi* are less selective and can occupy a significant proportion of the total circulating erythrocytes[13]. Amongst the rodent

malarias utilised in the laboratory, *P. berghei* and *P. yoelii* displays a preference for immature erythrocytes, whereas *P. chabaudi* and *P. vinckei* do not discriminate[14].

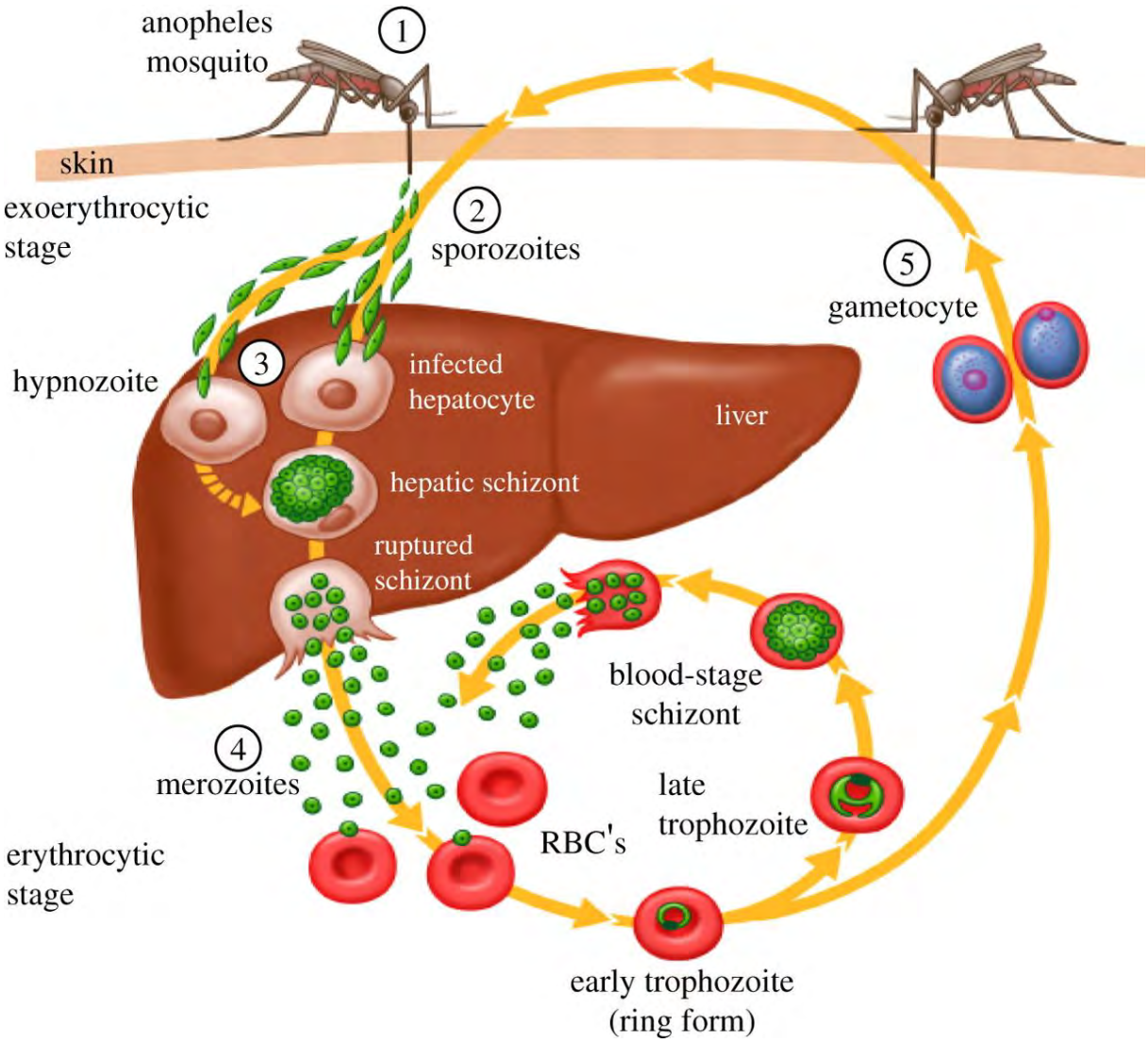


Figure 1.1. The mammalian life cycle of *Plasmodium*. Infection begins when an infected anopheles mosquito bites the host, releasing parasites from its salivary glands (1). Sporozoites migrate to the liver (2), where they invade hepatocytes, starting the liver stage of infection (3). Merozoites are released from hepatic schizonts into the blood stream, where they invade erythrocytes (RBCs) (4). Merozoites mature through the ring and trophozoite stage, replicate into schizonts, and multiple merozoites egress from the cell beginning the asexual blood cycle. Alternatively, merozoites mature into gametocytes which are taken up by uninfected mosquitoes, allowing disease transmission (5). Illustration obtained from Hill *et al.*[15] under an open access license.

Human and rodent Plasmodia incubate within the erythrocyte for 18-72 hours, depending on the species. During this period the parasite matures from ring stage to trophozoite stage and onto schizogony, at which point it reproduces asexually into several new merozoites. These merozoites then egress from the cell, restarting the blood cycle. In all human malarias, and in the rodent malarias *P. chabaudi* and *P. vinckei*, merozoites are released synchronously from all infected erythrocytes, creating a periodic cycle of asexual reproduction[14]. During this cycle a proportion of blood stage parasites develop into male and female gametocytes, the sexual form of the parasite. Gametocytes can be ingested by the mosquito vector, where they complete the sexual stage of the life cycle, eventually forming new sporozoites which facilitate the transfer of infection to a new mammalian host.

The asexual blood stage of infection is responsible for the symptoms of malaria. If left untreated the number of circulating parasites escalates rapidly, destroying erythrocytes and resulting in an array of potentially lethal side effects. During *P. falciparum* infection, with the exception of young forms (rings) and gametocytes, parasitised erythrocytes often adhere to the blood vessel walls, known as cytoadherence, or to uninfected erythrocytes in a process known as rosetting[16,17]. In this way the mature parasites are able to escape filtration by the spleen, an important part of the host's defence against infection[18]. Cytoadherence does not occur to the same extent in other forms of human malaria; in *P. falciparum* it is believed to be the main cause of mortality. The adherence and accumulation of parasites in capillaries within the brain eventually obstructs blood flow, ultimately leading to coma and death, a condition known as cerebral malaria. This condition is responsible for a large proportion of *P. falciparum* mortality and can result in long term neurological defects even after clearance of the parasite. A major cause of cerebral malaria is thought to be due to an excessive inflammatory response during malaria infection. This alters the expression of endothelial cell receptors which in turn enhances the cytoadherance of infected erythrocytes and promotes coagulation[19]. Cerebral malaria also occurs in the rodent malarias *P. berghei* and *P. yoelii*, although the relevance of these models to human cerebral malaria is debated[20]. While cerebral malaria does

not occur in *P. chabaudi*, cytoadherence, particularly of late stage parasites, is common[14]. Another major cause of *P. falciparum* mortality is severe anaemia. In this condition uninfected erythrocytes are cleared by the spleen even more rapidly than parasitised erythrocytes, greatly accelerating the loss of circulating erythrocytes[21]. This is exacerbated by an insufficient or abnormal erythropoietic response, which is suppressed during parasite infection[22]. Severe anaemia and ineffective erythropoiesis are often observed in mice infected with *P. chabaudi* and *P. vinckei*, making these relevant models for studying these effects. *P. falciparum* infection can also result in acidosis and hypoglycaemia, conditions produced when sequestered parasites impede blood flow to tissues, resulting in anaerobic glycolysis[23]. Biochemical variables related to these conditions are strongly predictive of disease mortality[24]. Hypoglycaemia also occurs in *P. chabaudi*, although the level of acidosis is unknown[14]. Additional symptoms of severe malaria include pulmonary oedema, acute kidney injury and jaundice, which along with the previously mentioned disease outcomes, usually correlate with the extent of microvascular obstruction due to parasite cytoadherence[25].

Despite the potential for serious outcomes, repeated exposure eventually results in partial immunity to malaria, and most infections remain asymptomatic. Factors such as parasite strain and species, host health status, age, immunity and genetics ultimately determine the host's susceptibility to malaria and the risk of developing severe disease.

MALARIA TREATMENT AND DRUG RESISTANCE

Current treatments for malaria are progressively becoming ineffective due to the development of parasite resistance, which now affects all classes of antimalarials[10,26]. The antimalarial chloroquine, which was discovered in 1934, disrupts the ability of the parasite to detoxify haematin, a by-product of haem digestion produced during parasite growth[27]. Resistance to chloroquine was first described in 1957, just 12 years after its large scale introduction in Thailand[28]. It was later determined that mutations in parasite genes encoding membrane transport proteins, namely *P. falciparum*

chloroquine resistance transporter (PfCRT)[29,30] and *P. falciparum* multidrug resistance protein (PfMDR1)[31], gave rise to this resistance. These mutations result in an increased rate of chloroquine transport out of the parasite, thereby decreasing intra-parasitic chloroquine concentration and its effectiveness[32,33]. Mutations in these transporters have also been shown to reduce the effectiveness of other antimalarials, highlighting a robust defence mechanism employed by the parasite against these drugs[33-37].

Another class of antimalarials that target the parasite's unique folate biosynthetic pathway are sulfadoxine and pyrimethamine. These two drugs target different enzymes in the folate pathway, namely dihydropteroate synthetase and dihydrofolate reductase (Dhfr), and are therefore often used in combination in an attempt to deter parasite resistance[38,39]. Nevertheless, resistance to this combination therapy has developed due to tandem mutations in the parasite target proteins[40-42]. Indeed, it seems the *Plasmodium* parasite has a remarkable ability to circumvent inhibitors targeted towards parasite proteins, a process facilitated by high genetic variation, intense selective pressure, and incomplete drug treatment regimens due to poor drug quality and supply in developing countries[43].

New antimalarials are desperately needed. However it is critical that any new drugs address the problems associated with drug resistance. To identify possible weak points in the parasites adaptability it is necessary to consider not only the parasite itself, but also its host environment. Indeed, malaria susceptibility is known to change dramatically depending on the host's genetic background[44,45]. Importantly, this intrinsic resistance to malaria has existed for thousands of years and yet continues to provide protection from this disease today, suggesting the parasite is unable to adapt and circumvent the protective mechanisms involved. Therefore, the study of genetic resistance to malaria, and the associated biological mechanisms, may prove crucial in the development of resistance proof treatment strategies.

GENETIC BASIS OF SUSCEPTIBILITY TO MALARIA

Host genetics significantly influences malaria susceptibility. It was suggested as early as 1933 that a form of "hereditary immunity" may exist in endemic malaria populations[46]. Later, it was observed that many genetic polymorphisms are geographically concordant with malaria incidence, which led to the finding that they can convey malaria resistance. Haldane proposed that the adverse effects of these polymorphisms, particularly in homozygotes, might be outweighed by their benefit during malaria infection; the alleles therefore convey an overall increase in fitness resulting in their genetic selection in populations in malaria afflicted areas[47]. Indeed, malaria is now considered to have been the most influential infectious disease in shaping the human genome[48]. Many polymorphisms have now been clearly associated with various degrees of malaria resistance in humans. Some of these polymorphisms will now be described, and, in the following section, the mechanisms by which they convey resistance to malaria will be discussed.

Haemoglobin polymorphisms

Haemoglobin comprises 96% of the dry weight of erythrocytes, and is responsible for their main function, the transport of oxygen throughout the body[49]. There are five types of polymorphisms involving haemoglobin found at high frequencies in both extant and historically malaria afflicted populations. Three of these involve single amino acid substitutions in the beta globin chain of haemoglobin; HbS (β6Glu to Val), HbC (β6Glu to Lys), and HbE (β26Glu to Lys). The remaining two types of polymorphisms, alpha and beta thalassemia, give rise to altered expression of the alpha and beta globin chains of haemoglobin respectively.

The HbS mutation is widespread in malaria endemic areas, reaching a prevalence of 15-20% in parts of sub-Saharan Africa[50]. Homozygotes for the HbS mutation develop sickle cell disease, a severe condition characterised by haemolytic anaemia, high morbidity and reduced life expectancy. Heterozygotes have sickle cell trait and are generally asymptomatic. The link between sickle cell trait and reduced malaria susceptibility was observed nearly 70 years ago[51]. Since then many studies have

confirmed this correlation, agreeing that sickle cell trait provides 30-50% protection from uncomplicated malaria and 70-90% protection from severe malaria[52-55]. HbC is most commonly observed in West Africa with homozygotes developing only mild haematological symptoms whereas heterozygotes exhibit intermediate symptoms[56,57]. HbC has been shown to be 90% and 30% protective against the occurrence of clinically diagnosed malaria episodes in homozygotes and heterozygotes respectively[58-60]. HbE is found almost exclusively in South-East Asia where it can be highly prevalent, reaching a frequency of 55% in parts of Thailand[61,62]. The heterozygous form of this condition is asymptomatic while homozygotes display only a mild haematological phenotype. Evidence for the protective of effect of HbE against malaria is complicated by the fact that many other polymorphisms are present in these populations creating hybrid phenotypes. Nevertheless, heterozygous HbE alone has been shown to be protective against severe malaria in a case control study in Thailand[63].

The thalassemias are the most common haemoglobin polymorphisms worldwide, with their distribution coinciding with historical and extant malaria endemicity[47,52,64]. Thalassemia is the result of one or both of the two α haemoglobin genes or the β haemoglobin gene being deleted or inactivated. α^0 thalassemia and β^0 thalassemia refer to the complete loss of gene expression. In the homozygous case, this condition is perinatal lethal for α^0 thalassemia and causes severe anaemia for β^0 thalassemia. A mild phenotype develops in heterozygous cases of this condition. α^+ thalassemia and β^+ thalassemia refer to a partial loss of expression. Mild anaemia results in homozygotes for both cases, while the condition if asymptomatic in heterozygotes. A combined heterozygosity of α^0 thalassemia and α^+ thalassemia is known as HbH disease and results in mild to moderate anaemia and jaundice. Clinical studies indicate heterozygous α^+ thalassemia, heterozygous α^0 thalassemia, and HbH provide protection from severe malaria, while convincing geographical correlations and *in vitro* studies also indicate a protective role for heterozygous β^0 thalassemia[65-68].

Erythrocyte enzyme polymorphisms

Glucose-6-phosphate dehydrogenase (G6PDH) is a key enzyme involved in controlling oxidative stress in the erythrocyte. Polymorphisms in the X-linked G6PDH gene, which result in reduced enzyme activity, have an increased prevalence in malaria afflicted populations[69]. Decreased risk of severe malaria has been reported in hemizygous males, heterozygous females, and homozygous females in Africa[70-72]. However, results are conflicting, with a more recent study in Mali reporting protection only in hemizygous males and homozygous females , while a study in Gambia and Kenya reported protection in heterozygous females as well[73,74].

Pyruvate kinase (PK) is a glycolytic enzyme involved in the production of ATP, and is critical in maintaining erythrocytic integrity[75]. It was first shown in mouse models that erythrocytic PK deficiency conveys resistance to the rodent malaria *P. chabaudi*[76]. This led to the finding that this condition provides protection against human malaria in both heterozygotes and homozygotes with a concomitant increase in prevalence in endemic malaria populations[77].

Erythrocyte membrane polymorphisms

Several polymorphisms in erythrocyte membrane proteins are well known to convey protection against malaria. The most striking example is Duffy negativity, a condition characterised by a point mutation which prevents transcription of the Duffy antigen gene in erythrocyte progenitors, and provides near complete protection from *P. vivax* malaria[78-81]. Duffy negativity can reach 100% prevalence in many parts of Africa[82]. Although common in other malaria afflicted regions, *P. vivax* is completely absent in regions of Africa with highly prevalent Duffy negativity, providing strong evidence for the role of this polymorphism in preventing malaria infection[83]. However, a recent study in Madagascar has identified Duffy negative individuals infected with *P. vivax*, indicating that this polymorphism is not 100% protective[84]. Outside of Africa, an alternate mutation that reduces Duffy antigen expression by half was found in a population in Papua New Guinea[85]. This polymorphism was

found to be 50% protective against clinical *P. vivax* infection and was associated with lower parasite densities[86].

ABO blood type has long been considered a factor in determining malaria susceptibility. Two recent, comprehensive, case-control studies have shown that O blood type is protective against severe *P. falciparum* infection, whilst A and AB types both increase susceptibility[87-89]. This is in keeping with the high (up to 80%) incidence of O blood type in parts of Africa[90].

Complement receptor 1 (CR1) is expressed on the surface of erythrocytes and plays a part in the complement immune system. Erythrocytic CR1 deficiency is extremely common in Papua New Guinea, with approximately 80% incidence[91]. Heterozygotes for point mutations associated with reduced CR1 expression display reduced risk of severe malaria, however, depending on geographic location, homozygotes are either unaffected or display increased risk of severe disease[92,93].

Null polymorphisms for the erythrocyte surface protein Glycophorin B (GYPB) are almost exclusively present in humans of African ancestry while Gerbich negativity (Glycophorin C (GYPC) polymorphism) is common in malaria endemic regions of Papua New Guinea[94,95]. *In vitro* studies suggest Gerbich negativity is protective against *P. falciparum* to some degree, however, studies in Papua New Guinea have found no association with malaria incidence or severe anaemia[96-98].

Erythrocyte cytoskeletal polymorphisms

The erythrocyte cytoskeleton is a network of interacting proteins positioned under the cell membrane. A polymorphism in band 3, a major protein of the cytoskeleton, causes a condition known as South-East Asian ovalocytosis (SAO)[99,100]. As the name implies, this condition reaches high frequencies (up to 30%) in parts of South-East Asia and Papua New Guinea [101]. SAO is embryonic lethal in homozygotes but asymptomatic in its heterozygous form, producing distinct, elliptical erythrocytes which nevertheless maintain functionality [102]. Strong evidence suggests heterozygous SAO protects against severe malaria, particularly cerebral malaria[103,104].

Hereditary elliptocytosis and hereditary spherocytosis are conditions caused by polymorphisms which disrupt the horizontal or vertical interactions between the cytoskeletal proteins, respectively. Interestingly, malaria endemic populations have an increased prevalence of these polymorphisms[105-107]. No association of these with malaria severity has been investigated in humans, however *in vitro* studies have indicated that they may protect against malaria[108-110]. In mouse models, several distinct mutations in the cytoskeletal proteins ankyrin and alpha spectrin have been shown to convey protection against *P. chabaudi* and *P. berghei*[111-113].

Endothelial receptor polymorphisms

The malaria parasite adheres to host endothelial receptors to escape immune detection and clearance by the spleen. This adherence is a major cause of severe malaria[114]. It therefore might be expected that polymorphisms which affect either the abundance or binding properties of these receptors would influence malaria susceptibility. Indeed, polymorphisms in intercellular adhesion molecule 1 (ICAM1), CD36, and CD31 (known parasite endothelial receptors) have been found in malaria endemic areas[115,116]. However, conflicting associations with severe malaria risk are reported, possibly as a result of parasite antigenic variability[117-121].

Immune polymorphisms

Human leukocyte antigen (HLA) molecules are ubiquitously expressed on the surface of nucleated cells, where they play a key role in the recognition of foreign antigens. Polymorphisms in HLA class I and II genes can coincide geographically with malaria incidence and have been associated with malaria susceptibility[122,123]. However, conflicting results have been found depending on sampling location (possibly due to parasite genetic variation), and the role of HLA in malaria infection has been questioned[124-126].

Cytokines play an important role in the pathogenesis of severe malaria. Polymorphisms in TNF-alpha[127], IFN-gamma[128,129], IFN-gamma regulatory factor 1[130,131], Interleukin 1[132], and Interleukin 4[133-135], have all been shown to influence malaria susceptibility. Additionally, polymorphisms in Fc gamma

receptors IIa and IIb, and CD40 ligand, which influence antibody regulation and B cell proliferation respectively, have been shown to play a role in malaria resistance[136-139].

Other polymorphisms

The haptoglobin protein binds free haemoglobin released during intravascular haemolysis, thereby facilitating iron recycling[140]. It has also been shown to play a role in regulating anti-inflammatory cytokines[141]. There is conflicting evidence regarding the role of haptoglobin polymorphisms in severe malaria[142-144]. However, later studies, which quantified levels of serum haptoglobin, agree that these polymorphisms reduce the risk of severe malaria[145,146].

Nitric oxide synthase 2 (NOS2) produces nitric oxide and is involved in the immune response to malaria. In some studies, a microsatellite polymorphism in *NOS2*, resulting in a shorter or longer CCTTT repeat length in the *NOS2* promoter region, are reported to influence malaria susceptibility, whilst others have reported no change[147-149]. Another type of polymorphism, resulting from a point mutation, has been associated with a reduced risk of severe malaria[150].

Summary

As evidence builds for the huge influence that naturally occurring polymorphisms can have on the outcome of malaria infection, the next question is how these polymorphisms convey this resistance so effectively. Indeed, polymorphisms such as Duffy negativity have resulted in the complete absence of *P. vivax* from Africa, a feat which likely could not be replicated through the use of modern day antimalarials. The study of the mechanisms by which these polymorphisms convey resistance is therefore of great interest to the malaria community.

MECHANISMS OF NATURALLY OCCURRING MALARIA RESISTANCE

Understanding how genetic polymorphisms protect against malaria may provide the key to eradication. If the biological processes underlying the resistance mechanisms of these polymorphisms can be identified, it may be possible to mimic the effect using pharmaceuticals. This could offer a novel means of disease treatment, which may be less susceptible to parasite resistance, as evidenced by the fact that polymorphisms, such as sickle cell trait, have provided resistance for thousands of years. This is the essence of host directed therapy.

Despite decades of research, the mechanisms underpinning malaria resistance in common polymorphisms such as sickle cell trait and thalassemia remain unknown. In many cases, multiple, often contradictory, mechanisms have been suggested for a single polymorphism. Although it is quite conceivable that these polymorphisms also affect liver stage parasite development, this review will focus only on effects described during distinct aspects of the parasites blood stage cycle (Table 1.1).

Table 1.1. Mechanisms of malaria resistance in common polymorphisms

Erythrocyte polymorphisms	Invasion	Growth	Cytoadherence/ rosetting	Susceptibility to phagocytosis	References
Sickle cell trait (HbAS)	↓ (low oxygen) or normal	↓ (low oxygen)	↓	↑	178, 229, 213, 234, 260, 68
Sickle cell disease (HbSS)	↓ (low oxygen) or ↑	↓ (low oxygen)	↓	Unknown	178, 229, 234, 260
HbAC	Normal	Normal	↓	Unknown	213, 261, 231
HbCC	Normal	↓	↓	Unknown	229, 261, 231
HbAE	↓	Normal	Unknown	↑	179, 300
HbEE	Normal	↓ or normal	Unknown	↑	226, 300
α^{+} thalassemia (-α/αα)	Normal	↓ (high oxygen)	↓	Normal	232, 262, 91
α^{0} thalassemia (--/αα)	↓ or normal	↓ or normal	↓	Unknown	179, 262
HbH thalassemia (--/-α)	↓ or normal	↓	↓	↑	179, 228, 262, 68
β^{0} thalassemia (-/β)	Normal	↓ (high oxygen)	Unknown	↑	232, 68
G6PD deficiency	Normal	↓ or ↓ (high oxygen)	Unknown	↑	232, 240, 242, 298
PK deficiency	↓	Normal	Unknown	↑	77
Duffy negativity	↓ (in *P. vivax*)	↑	Unknown	Unknown	159, 160,
ABO blood group	Normal	Normal	↓ (for O type)	↑ (For O type)	264, 265, 266, 299
CR1 deficiency	↓	Unknown	↓	Unknown	173, 269, 270
GYPA deficiency	↓	Unknown	Unknown	Unknown	167
GYPB deficiency	↓	Unknown	Unknown	Unknown	168, 169
GYPC deficiency	↓	Unknown	↓ (in *P. vivax*)	Unknown	170, 271
Basigin deficiency	↓	Unknown	Unknown	Unknown	174
Southeast Asian ovalocytosis (SAO)	↓	Normal	↑	Unknown	177, 263
Hereditary elliptocytosis (HE)	↓ or normal	↓	Unknown	Unknown	108, 109, 196
Hereditary spherocytosis (HS)	↓ or normal	↓	Unknown	Unknown	108, 113

↓ decreased, ↑increased. "Growth" refers to solely to the ability of the parasite to replicate and egress from the erythrocyte. Studies performed with *P. falciparum* unless otherwise indicated.

Erythrocyte Invasion

The brief interval between parasite egress from its host cell as a merozoite and subsequent reinvasion into a new erythrocyte has long been considered a potential weak point in the *Plasmodium* blood stage lifecycle. The invasion process has been extensively studied and is well understood (Figure 1.2). The merozoite attaches itself to the target erythrocyte in any orientation, but quickly realigns such that its apical end faces inward toward the erythrocyte membrane[151]. Organelles situated at the apical end then secrete various parasite proteins, which remodel the erythrocyte membrane preparing it for invasion[152,153]. A 'tight junction' is formed and an actin-myosin motor is initiated, leading to endocytosis of the merozoite within the erythrocyte membrane, afterwards known as the parasitophorous vacuole[154,155]. Finally, the erythrocyte membrane returns to its normal state. There is some evidence that this final stage is facilitated by the dephosphorylation of band 3 by the parasite phosphatase PfShelph2[156]. In *P. falciparum* this event is completed in less than 30 seconds, while the whole process, from parasite egress to reinvasion, takes around two minutes[157].

The speed of this process is critical as the extracellular merozoite is vulnerable to immune recognition due to direct contact with opsonins and immune cells, and is unable to replicate outside of the host cell[158]. Consequently, any disruption to the invasion pathway would be beneficial to the host. Selective pressure has therefore resulted in the evolution of several host polymorphisms associated with invasion inhibition in endemic malaria populations. These polymorphisms can be broadly split into two groups based on the mechanism underlying invasion inhibition: 1) those resulting in the loss or modification of a parasite ligand from the surface of the erythrocyte, thereby preventing parasite binding and entry; and 2) those which cause widespread remodelling or alteration of erythrocyte membrane properties.

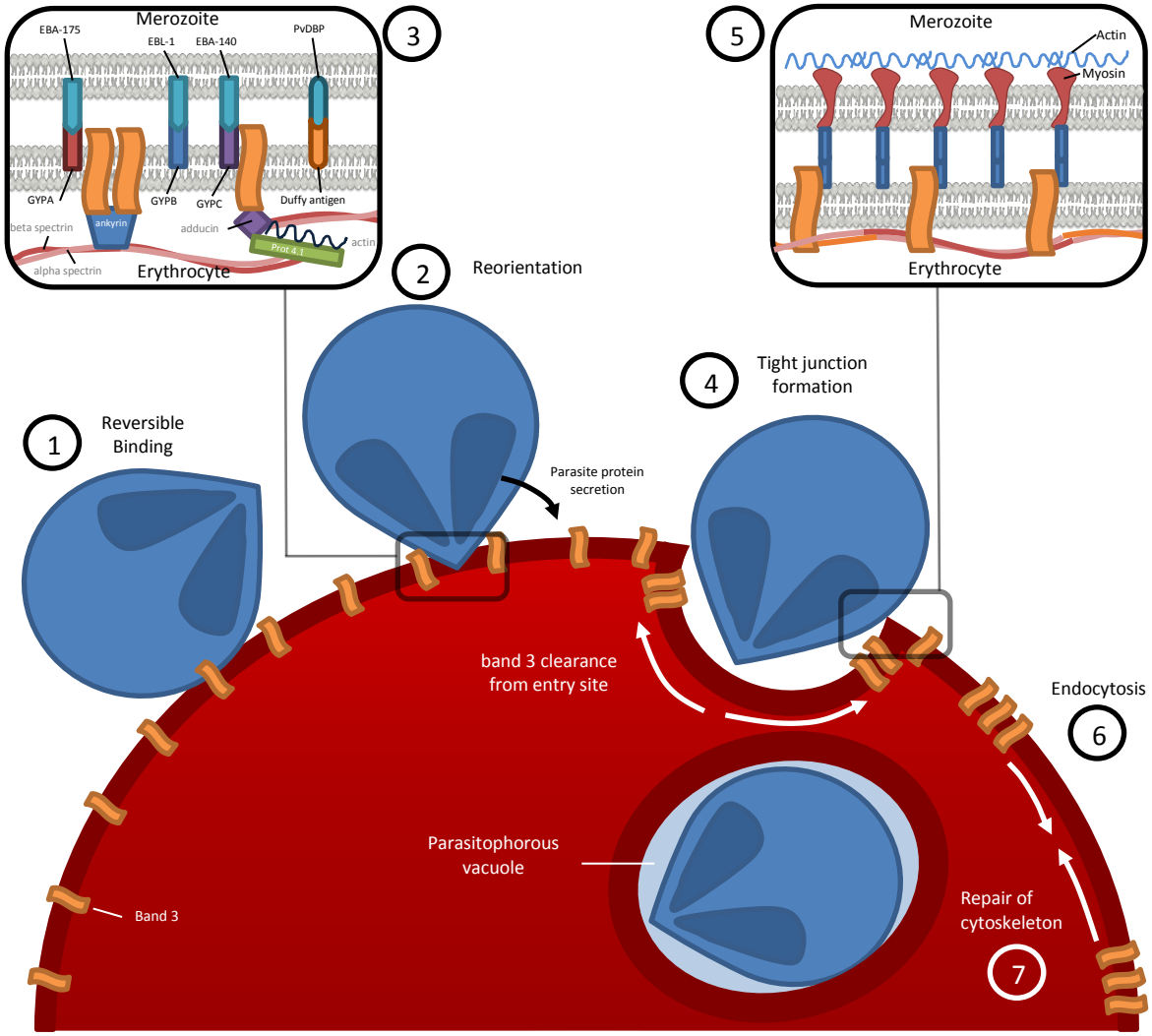


Figure 1.2. Invasion of erythrocytes by *Plasmodium*. The merozoite first adheres to the erythrocyte in a reversible manner (1). Reorientation follows, whereby the merozoite binds to various host ligands (2). These include Duffy antigen for *P. vivax*, and the glycophorins for *P. falciparum* (3). Proteins are secreted by the merozoite which result in remodelling of the erythrocyte surface including the removal of band 3 from the entry site (4). A tight junction is formed and an actin myosin motor is initiated (5). This allows the parasite to become engulfed and enter the erythrocyte by endocytosis (6). Finally, the erythrocyte cytoskeleton is returned to its normal state (7).

In the first case, several parasite ligands are known to be essential for invasion. As previously mentioned, Duffy negativity is highly prevalent in Africa and largely prevents *P. vivax* infection. Work by Wertheimer *et al.*[159] revealed that the Duffy antigen is a ligand for *P. vivax* Duffy-binding protein (PvDBP), and that the interaction of these two proteins is essential for erythrocyte invasion[159,160]. The binding site of PvDBP to the Duffy antigen has been mapped to the PvDBPII domain of the *P. vivax* protein[161]. Using this information Grimberg *et al.*[162] found that antibodies raised against recombinant

PvDBPII can block this interaction and thereby inhibit invasion of Duffy positive erythrocytes *in vitro*. Furthermore, King *et al.*[163] have shown a strong negative correlation between the presence of naturally acquired antibodies that inhibit this interaction and the incidence of *P. vivax* in Papua New Guinea. Accordingly, PvDBPII is now under consideration as a vaccine for *P. vivax*, although there are concerns that variability in the PvDBPII domain among *P. vivax* strains may hinder its effectiveness[164,165].

For *P. falciparum* several redundant invasion pathways involving different host proteins are known. In 1977, Miller *et al.*[166] reported that erythrocytes from individuals with the rare En (a−) polymorphism were resistant to *P. falciparum* invasion. En (a−) erythrocytes lack Glycophorin A (GYPA), highlighting a possible role for this protein as a parasite ligand. Indeed, GYPA is now known to be a binding partner of erythrocyte binding antigen 175 (EBA175), and is important for *P. falciparum* invasion[167]. Likewise, erythrocyte surface proteins Glycophorin B and C (GYPB, GYPC) are polymorphic in endemic malaria populations, with later studies demonstrating their involvement in parasite invasion. The *P. falciparum* protein erythrocyte-binding ligand-1 (EBL1) binds to Glycophorin B[168,169], while Glycophorin C is a receptor for erythrocyte binding antigen 140 (EBA140)[170]. Antibodies against the binding domains of EBL1 and EBA140 inhibit parasite invasion in correlation with the extent to which the parasite is reliant on the respective invasion pathway[171,172]. CR1 has also been implicated in invasion, which may explain the protective effect of CR1 deficiency[173]. Most recently, the erythrocyte surface protein basigin has been identified as an obligatory parasite ligand for invasion across multiple different *P. falciparum* strains[174]. *In vitro* studies indicate polymorphisms in basigin (the Ok blood group antigen) prevent parasite invasion, although there is no evidence that polymorphisms in this antigen are increased in malaria endemic populations[174]. Overall, the identification of these ligand interactions, facilitated largely through the investigation of genetic polymorphisms in endemic malaria populations, has produced several possible vaccine candidates which are currently in development[171,175,176].

Aside from polymorphisms in erythrocyte surface antigens, invasion inhibition has also been reported in genetic conditions such as Southeast Asian ovalocytosis (SAO)[177], the haemoglobinopathies[178,179], and pyruvate kinase deficiency[77]. The reason for invasion inhibition in these cases is less clear and conflicting results are often reported. For SAO, the dominant effects of a heterozygous band 3 mutation are erythrocytes with alterations in band 3 quaternary structure and function[180-182], increased membrane rigidity[183-185], abnormal morphology, and decreased expression of surface antigens[186,187]. Early studies reporting a large decrease in invasion of SAO erythrocytes *in vitro*[177,183] were in direct contradiction with observations that SAO individuals often develop high parasitaemias[104]. Later studies report a more modest effect of band 3 mutations on parasite invasion which is strain specific, helping to explain previous discrepancies[185,188,189]. Together, these studies indicate a robust invasion inhibition in SAO, although the mechanism of inhibition remains unclear. Decreased membrane mobility of band 3 may interfere with invasion events, a notion supported by results showing decreased invasion into normal cells with artificially inhibited band 3 mobility[190-193]. Alternatively, the loss or reduction of surface receptors such as Glycophorin A (which forms a complex with band 3) may explain the inhibition[186,187]. Finally, increased membrane rigidity or direct interference with parasite/band 3 binding may play a role[183-185,194,195].

An early study on sickle cell trait reported reduced merozoite invasion of sickled cells under low oxygen tension[178], however later studies could not replicate this result[68]. Chotivanich *et al.*[179] reported reduced invasion into haemoglobin variant cells *in vitro* at high parasite densities. In this study HbAE, beta thalassemia/HbE, and alpha thalassemia (HbH and HbH Constant Spring), all inhibited invasion while HbEE did not. The results for HbH were contradicted by a later study which showed no difference in invasion or growth in these cells[68]. The homozygous form of pyruvate kinase deficiency has been shown to inhibit invasion, although the reason for this is unclear, while the heterozygous form does not [77]. A study by Schulman *et al.*[108] reported no difference in *in vitro* invasion of erythrocytes from patients with mutations causing hereditary elliptocytosis and hereditary spherocytosis. This study included erythrocytes partially deficient in alpha spectrin or with

spectrin conformational changes, as well as band 3 structural variants and erythrocytes devoid of protein 4.1. In contrast to this, a later study under similar conditions, on the same patient lacking protein 4.1 and another with a 25% reduction of protein 4.1, indicated a marked reduction of invasion into these cells[109]. The authors could not explain the discrepancy, although a contributing factor could be the strain of parasite used. Although increased membrane rigidity was suggested as the cause of decreased invasion, it is possible a secondary deficiency of GYPC (90% less in mutant cells) is the reason for this phenotype (the role of GYPC as a parasite ligand was unconfirmed at the time). Conflicting evidence is also reported in a study by Facer[196], in which mutations causing only a partial deficiency in protein 4.1 either increase invasion susceptibility or have no effect. Although, in the same study, two distinct mutations in alpha spectrin resulting in asymptomatic or symptomatic hereditary elliptocytosis were shown to inhibit parasite invasion, in agreement with previous studies. In mouse studies, Rank *et al.*[112] report that a heterozygous mutation in ankyrin had no effect on invasion by the murine malaria parasite *P. berghei in vitro*. A later study by Greth *et al.*[113] using the *P. chabaudi* parasite reported a possible invasion inhibition of erythrocytes from mice carrying an alternate ankyrin mutation using an *in vivo* assay.

Intraerythrocytic growth and egress

After gaining entry into the host erythrocyte the parasite must dramatically remodel its environment in order to grow, replicate, and eventually egress from the cell. The success of the parasite depends on several factors; digestion of haemoglobin within the cell, the ability to gain nutrients from inside and outside the cell, protein trafficking, scavenging of host proteins, and, finally, effective dissolution of the cell membrane during egress.

The digestion of haemoglobin is critical for the parasite as it must create space within the cell in order to grow. To achieve this the parasite employs the use of a digestive vacuole, an acidic organelle which degrades up to 80% of erythrocytic haemoglobin, converting the toxic haem by-product into inert haemozoin crystals[197-199]. While the parasite meets many of its nutrient needs through

haemoglobin degradation, it must also obtain extracellular nutrients not available within the erythrocytic cytosol[200-202]. Indeed, infected erythrocytes exhibit an increased membrane permeability compared to uninfected cells[203,204]. While it remains unclear precisely how the parasite develops these new permeability pathways, evidence suggests that both parasite encoded transporters, as well as the modulation of existing erythrocyte transporters, play a role[205,206]. Also critical for the uptake of extracellular nutrients is the formation of a tubovesicular network, without which the parasite is unable to import adenosine, glutamate and orotic acid[207]. Essential to the above processes is the ability of the parasite to distribute proteins appropriately throughout the cell. The parasite uses a conserved PEXEL motif to direct proteins to the erythrocyte membrane[208,209]. A sophisticated network of parasite trafficking proteins termed the *Plasmodium* translocon of exported proteins (PTEX) is responsible for the transport of proteins into the erythrocytic cytosol[210-212]. Trafficking is supported by unique organelles known as Maurer's clefts, which are attached to the erythrocyte membrane by host actin filaments[213]. As well as transporting its own proteins throughout the cell, the *Plasmodium* parasite makes use of several host proteins, some of which have been shown to be essential for parasite growth. Evidence suggests that during growth the parasite utilizes the host kinases PAK-1 and MEK-1[214], the antioxidant enzymes superoxide dismutase[215] and peroxiredoxin-2[216], and the haem biosynthetic enzymes aminolevulinate dehydratase and ferrochelatase[217-219]. At the end of its growth cycle the parasite undergoes schizogony, forming multiple merozoites which must escape from the erythrocyte. To facilitate merozoite egress, the parasite creates an osmotic pore which rapidly increases in size in a curling, buckling motion[220]. The parasite proteases falcipain-2 and plasmepsin-2 along with the host protease calpain-1 appear to be central to this process[221-223].

Given the complex interplay between host and parasite proteins, it is not surprising that many host genetic polymorphisms have been reported to impair parasite growth to some extent. Studies of parasite growth in erythrocytes with haemoglobin polymorphisms commenced shortly after the development of an *in vitro* culture system for *P. falciparum*[224]. Impaired growth in these cells (here defined specifically as intracellular parasite development and egress, independent of invasion or

factors which influence immune clearance) was frequently reported, however many studies were contradictory (for review see Taylor *et al.*[225]). Nagel *et al.*[226] reported that HbEE erythrocytes cause parasite growth impairment, however this was contradicted by a later study[227]. Nevertheless, both studies, as well as a later study, agree HbAE has no effect on parasite growth[226-228]. HbAC is reported to have no effect on growth while HbCC is consistently reported to impair growth[229-231]. Olson and Nagel[230] observed that HbCC were resistant to osmotic stress, and hypothesised that this may prevent efficient merozoite egress. However, a later study by Fairhurst *et al.*[231] found no evidence of schizont accumulation to support this, and instead suggested that the parasite undergoes spontaneous degradation in a subset of damaged HbCC erythrocytes with an increased haemoglobin concentration. Two studies by Friedman *et al.*[229,232] indicated a role for oxygen tension in parasite growth within HbAS, HbSS, alpha thalassemia trait, and beta thalassemia minor. Under low oxygen tension infected HbAS and HbSS cells underwent sickling and growth was attenuated. Conversely, in thalassemic cells growth impairment occurred only under high oxygen tension. In the more severe form of alpha thalassemia, HbH, studies agree that growth is impaired, however the mechanism remains unknown[228,233]. More recent studies have suggested two novel mechanisms of parasite growth impairment in these type of cells. A study by Cyrklaff *et al.*[213] describes a process whereby host actin filaments are utilized by the parasite in developing Maurer's clefts. In HbAS and HbAC cells the host actin filaments are in an abnormal state before infection, consequently, during remodelling by the parasite they fail to support the formation of normal Maurer's clefts. The authors suggest this may be due interference from oxidised forms of haemoglobin present in these cells, known as haemichromes. A later study by La Monte *et al.*[234] proposes an entirely different mechanism whereby specific host micro RNA's, which are more abundant in HbAS and HbSS cells, are translocated into the parasite where they fuse with parasite mRNA and inhibit normal translation of parasite proteins. It would be of great interest to see if these mechanisms occur in other types of polymorphic erythrocytes.

Recent evidence of host actin remodelling by the parasite[213], along with several studies detailing parasite protein interactions with the host cytoskeleton[235-238], suggest that cytoskeletal abnormalities in the erythrocyte may impact on parasite growth. Schulman *et al.*[108] reported that hereditary spherocytosis and hereditary elliptocytosis erythrocytes inhibited parasite growth in correlation with the extent of spectrin deficiency or spectrin dimer content respectively. This growth inhibition was not due to metabolic effects or haemolysis and seems to be caused solely by the abnormal cytoskeleton. These results are supported by a later study which also reports reduced parasite growth in hereditary elliptocytosis erythrocytes[109]. In a mouse model, Greth *et al.*[113] reported an increase in terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) of parasites inside ankyrin deficient erythrocytes. TUNEL staining indicates the presence of fragmented DNA and may indicate that parasite growth is impaired.

Early studies indicated parasite growth was normal within G6PD deficient erythrocytes, but impaired when exposed to oxidative stress[232,239]. This was not supported by later studies, which observed impaired growth even under normal conditions[240,241]. Furthermore, Usanga *et al.*[241] reported that the parasite was able to adapt to growth within the mutant cells by up-regulating its own copy of the G6PD gene. More recent studies have contradicted this result and indicate normal parasite G6PD expression and growth in G6PD deficient cells[242]. The authors suggest previous results could be due to long term storage sensitivity of the mutant cells. Despite these contradictory results, the inhibition of growth under oxidative stress in G6PD deficient cells is consistently observed[71,243].

Splenic filtration, cytoadherence and rosetting

The spleen is responsible for the clearance of defective, abnormal, or senescent erythrocytes[244]. The entire erythrocyte population is interrogated within the slow open microcirculation of the spleen once every 100-200 minutes[245]. Erythrocytes are forced through narrow inter-endothelial slits in what is the most challenging test to the cells deformability[244,246-248]. Erythrocytes which are not sufficiently deformable to pass through the slits, or those carrying markers of stress such as

phosphatidylserine exposure or auto-antibodies against aggregated band 3, are retained within the red pulp and exposed to resident macrophages for phagocytosis and recycling[249-251].

During malaria infection the parasite dramatically alters the properties of its host erythrocyte. The parasite progressively decreases erythrocyte deformability during its maturation[252-255]. Therefore, in order to avoid splenic clearance, the parasite alters the adhesive properties of its host cell as it develops. At later stages of growth this allows infected erythrocytes to bind to the endothelium, as well as to other erythrocytes via rosetting[252]. By removing themselves from circulation when they are at their most vulnerable stage, parasites may mature and egress from the cell before being cleared by the spleen. However, recent evidence suggests that even the mild decrease in deformability at the ring stage of growth results in significant parasite retention by the spleen[247,253]. Intriguingly, many erythrocyte polymorphisms, such as HbAS, result in a decreased erythrocyte deformability of uninfected cells[256]. This increased base erythrocyte rigidity has led to the hypothesis that this may predispose cells for retention by the spleen when infected, although this has not yet been tested[256].

A family of highly variant parasite proteins known as *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) are responsible for the cytoadhesion and rosetting of infected cells[257-259]. Interestingly, studies have found that infected HbAS[260], HbSS[260], HbAC[261], HbCC [261], and alpha thalassemic[262] erythrocytes do not bind as readily to human microvascular endothelial cells *in vitro* (compared with infected HbAA erythrocytes). It was found that infected HbAC and HbCC erythrocytes had reduced levels of surface PfEMP, despite displaying a normal amount of another membrane associated protein; knob-associated histidine-rich protein (KHARP)[231]. Interestingly, the reduced PfEMP expression in HbAC cells may be linked to the abnormal parasite growth in these cells described by Cyrklaff *et al.*[213]. It is hypothesised that the aberrant Maurer's clefts in these cells may inhibit parasite protein trafficking to the erythrocyte membrane[213]. In Southeast Asian ovalocytosis (SAO) it was found that infected erythrocytes had an increased affinity for the host receptor CD36 under flow conditions[263], which seems contradictory given the known protective effect of SAO against severe

malaria. However, the authors suggest that increased adherence to CD36 may remove parasites from circulation which would have otherwise caused clotting in the brain, where CD36 expression is low.

As well as influencing PfEMP display, polymorphisms in host receptors for PfEMP can influence the degree of cytoadhesion and rosetting during infection. Evidence indicates that the A and B blood groups enhance rosetting[264-266], possibly by acting as receptors for PfEMP[267,268]. It has also been shown that erythrocyte CR1 deficiency, which is associated with a reduced risk of severe malaria, reduces rosetting *in vitro*[269]. Furthermore, antibodies against CR1 and soluble CR1 both reduced rosetting in multiple *P. falciparum* strains, indicating CR1 may interact with PfEMP[269,270]. Impaired adhesion to PfEMP expressing COS-7 cells was observed in erythrocytes with a CR1 variant, Sl:-1,2, further supporting its possible role in PfEMP binding[269]. Interestingly, alpha thalassemic erythrocytes are also deficient in CR1, indicating that this mechanism may contribute to malaria protection conveyed by this polymorphism[91]. For *P. vivax*, Lee *et al.*[271] have shown that antibodies for GYPC, but not GYPA or CR1, inhibit rosetting. This study also reports reduced rosetting in GYPC knockdown erythrocytes derived from haematopoietic stem cells.

Barnwell *et al.*[272] determined that CD36 is an endothelial receptor for PfEMP. This indicates that protection from severe malaria reported in people with CD36 polymorphisms may be due to reduced cytoadhesion. Similarly, the host receptor's intracellular adhesion molecule 1 (ICAM-1) and CD31 are reported to bind to PfEMP and may influence severe malaria susceptibility in a similar way[118,273]. In support of this, infected erythrocyte affinity for the ICAM-1 receptor is associated with the development of cerebral malaria[274]. More recently, endothelial protein C receptor (EPCR) has been identified as a receptor for a specific subset of PfEMP proteins associated with severe malaria[275]. Furthermore, a polymorphic SNP in the EPCR gene has been associated with severe malaria, indicating that disruption of the PfEMP-EPCR interaction may be a protective mechanism[276].

Overall, significant evidence exists implicating reduced cytoadherence or rosetting as a mechanism of protection against severe malaria in several diverse host polymorphisms. When considering why this

would be the case, it is important to note that reduced cytoadherence would not only impair the parasite's ability to avoid splenic clearance, but would mitigate the severe pathologies associated with blood vessel obstruction, coagulation, and inflammation. Indeed these pathologies, which include cerebral malaria, are responsible for a large part of the deaths due to malaria infection. It is therefore unsurprising that polymorphisms which prevent these pathologies, even if they do not reduce parasite load significantly, would become fixed within malaria endemic populations.

Immune recognition of parasitised erythrocytes

The *Plasmodium* parasite distinguishes itself from most other pathogens in that its target cell, at least in mammals, is anucleated. Whilst most pathogens must subvert adaptive host cell defence mechanisms such as the interferon gamma response, erythrocytes do not have these capabilities. Therefore, there are only three known ways in which the immune system is able to specifically recognise and remove parasitised erythrocytes; 1) through the detection of parasite antigens which have been secreted onto the surface of the erythrocyte; 2) by the recognition of host protein damage or modification which occurs due to physiological stress in response to either parasitism or the direct action of parasite proteins; and 3) by the mechanical filtration of cells with decreased deformability by the spleen, as described above (Figure 1.3).

To avoid the immune recognition of parasite proteins on the erythrocyte surface, *Plasmodium* employs sophisticated antigenic variation. The best example of this is the var gene family, which encodes the *P. falciparum* protein PfEMP. Precise epigenetic control of this large family of genes allows parasites to express a vast array of PfEMP variants, which are monoallelically expressed in order to avoid immune adaption[277]. Indeed, although partial protective immunity to malaria occurs rapidly in high transmission areas, sterile immunity fails to develop[278]. In sickle cell trait, increased antibodies against PfEMP have been reported[279-281]. Although it is unclear why this is the case, this may contribute to the malaria resistance conveyed by this polymorphism.

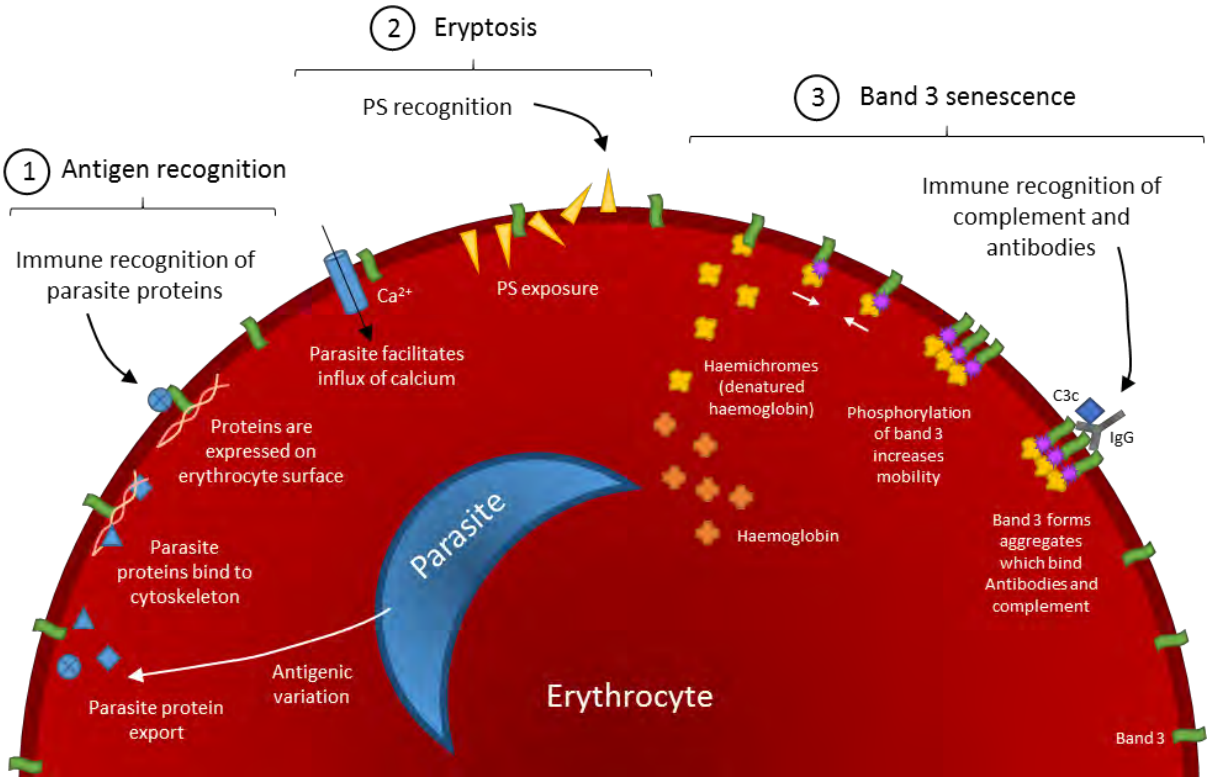


Figure 1.3. Immune recognition of parasitised erythrocytes. The immune system recognizes parasitised erythrocytes in three different ways. Parasite proteins on the surface of the erythrocyte can be recognised as "non-self", although sophisticated antigenic variation by the parasite works to prevent this (1). The parasite changes the permeability of the erythrocyte and actively imports calcium, calcium accumulation can induce an apoptotic like process termed "eryptosis", leading to PS exposure (2). PS exposing cells are recognised and cleared by the immune system. Parasitism induces oxidative stress and the formation of haemichromes. These bind to the cytoplasmic domain of band 3, inducing clustering. Band 3 aggregation is mediated by phosphorylation which reduces its affinity for cytoskeletal proteins and increases mobility. Aggregated band 3, which is a sign of erythrocyte senescence, binds naturally occurring antibodies and complement which are recognised by immune cells, leading to phagocytosis of the erythrocyte.

During their normal lifespan, erythrocytes undergo an array of physiological changes that eventually trigger their phagocytosis and recycling by macrophages. This senescence is characterised by cell shrinkage, increased density and rigidity, and changes to erythrocyte surface proteins. Two major models for erythrocyte senescence are commonly reported in the literature. In the first model, here referred to as "band 3 senescence", a build-up of haemichromes (haemoglobin denaturation products) results in crosslinking between band 3 cytoplasmic domains and band 3 aggregation[282,283]. This aggregation is facilitated by phosphorylation of band 3 which reduces its affinity for the cytoskeletal protein, ankyrin, and increases its mobility in the membrane[284]. Aggregated band 3 is

recognised by naturally occurring antibodies, which in turn promote complement protein C3 binding and activation[283]. This finally results in complement mediated phagocytosis of the senescent cell[285]. The second model is characterised by phosphatidylserine (PS) exposure, increased intracellular calcium, and activation of proteases in a process termed “eryptosis” which is considered an alternate form of apoptosis[286]. PS exposure leads to phagocytosis of the eryptotic cell[287].

Both eryptosis and band 3 senescence are elevated in malaria infected erythrocytes[250,288-290]. These processes increase as the parasite matures, and are thought to be caused by increased oxidative stress[288,291]. In situ, responses that include production of naturally occurring antibodies to band 3 are associated with improved malaria outcome, indicating that accelerated band 3 senescence is an important host defence mechanism against malaria[292].

Interestingly, band 3 senescence and eryptosis are also increased in polymorphic erythrocytes[293-297]. In the haemoglobin polymorphisms, aberrant haemoglobin is thought to contribute towards the build-up of haemichromes in erythrocytes, thereby accelerating band 3 senescence[298]. In G6PD deficiency and pyruvate kinase deficiency, increased oxidative stress in the erythrocyte is thought to be responsible for haemichrome formation, resulting in accelerated senescence[77,298]. Senescence is also accelerated in polymorphic erythrocytes infected with ring stage parasites, in comparison with ring stage infected control cells. This ultimately leads to increased phagocytosis of infected polymorphic cells. The increased susceptibility of ring stage polymorphic erythrocytes to band 3 senescence and eryptosis is thought to be an important and common mechanism of malaria resistance in these conditions[68,77,242]. It has also been recently reported that ring stage O-blood group erythrocytes display increased susceptibility to band 3 senescence and phagocytosis, indicating that this mechanism is responsible for the increased malaria resistance in O-blood group people[299]. Finally, infected HbAE and HbEE erythrocytes have shown an increased susceptibility to phagocytosis, however the mechanism behind this has not been determined[300].

Iron deficiency has been reported to reduce malaria susceptibility[301,302]. Studies of iron deficient mice infected with *P. berghei* indicate that this may be due to increased eryptosis of infected cells and associated phagocytosis[303,304]. Additionally, increased abundance of a high molecular weight aggregate in erythrocyte ghosts from iron deficient cells has been reported[305]. Although the nature of this finding was not explored, it may represent increased band 3 aggregation in these cells. Furthermore, many compounds have been shown to accelerate erythrocyte senescence, with several of these positively influencing the course of malaria infection. These include lead[306], chlorpromazine[307], the NO synthase inhibitor L-NAME[308], and the hormone analogue 16 alpha-bromoepiandrosterone[309]. In addition, indolone-N-oxides, which are being trialled as antimalarials, have also been shown to accelerate band 3 senescence, which may be their mechanism of action[310].

Summary

This section has described multiple ways in which naturally occurring genetic mutations in the host can lead to resistance to malaria. It is notable that a single mutation can lead to several distinct mechanisms which inhibit the parasites lifecycle, and conversely, multiple distinct mutations can impede the parasite through a single common mechanism. It should also be considered that the effect of these mutations may be cumulative and closely linked. A good example of this is HbAS. In this condition, parasite growth is inhibited by the action of micro RNAs[234] and aberrant actin remodelling[213]. This may explain the reduced surface expression of PfEMP-1 and associated reduction in cytoadherence seen in HbAS[260]. Simultaneously, elevated levels of oxidized haemoglobin lead to increased deposition of haemichromes onto the cytoplasmic region of band 3[68]. This leads to band 3 aggregation and binding of naturally occurring antibodies and complement to the erythrocyte surface. The combination of decreased cytoadherence and increased opsonisation results in increased phagocytosis of infected cells[68]. Increased uptake of parasitised erythrocytes may lead to improved presentation of surface antigens, resulting in the improved antibody response against PfEMP-1 observed in HbAS individuals[279-281]. Antibodies against PfEMP-1 may further inhibit

cytoadherence and facilitate phagocytosis in subsequent infections, which may explain why the protective effect of HbAS increases with age[311,312] (Figure 1.4).

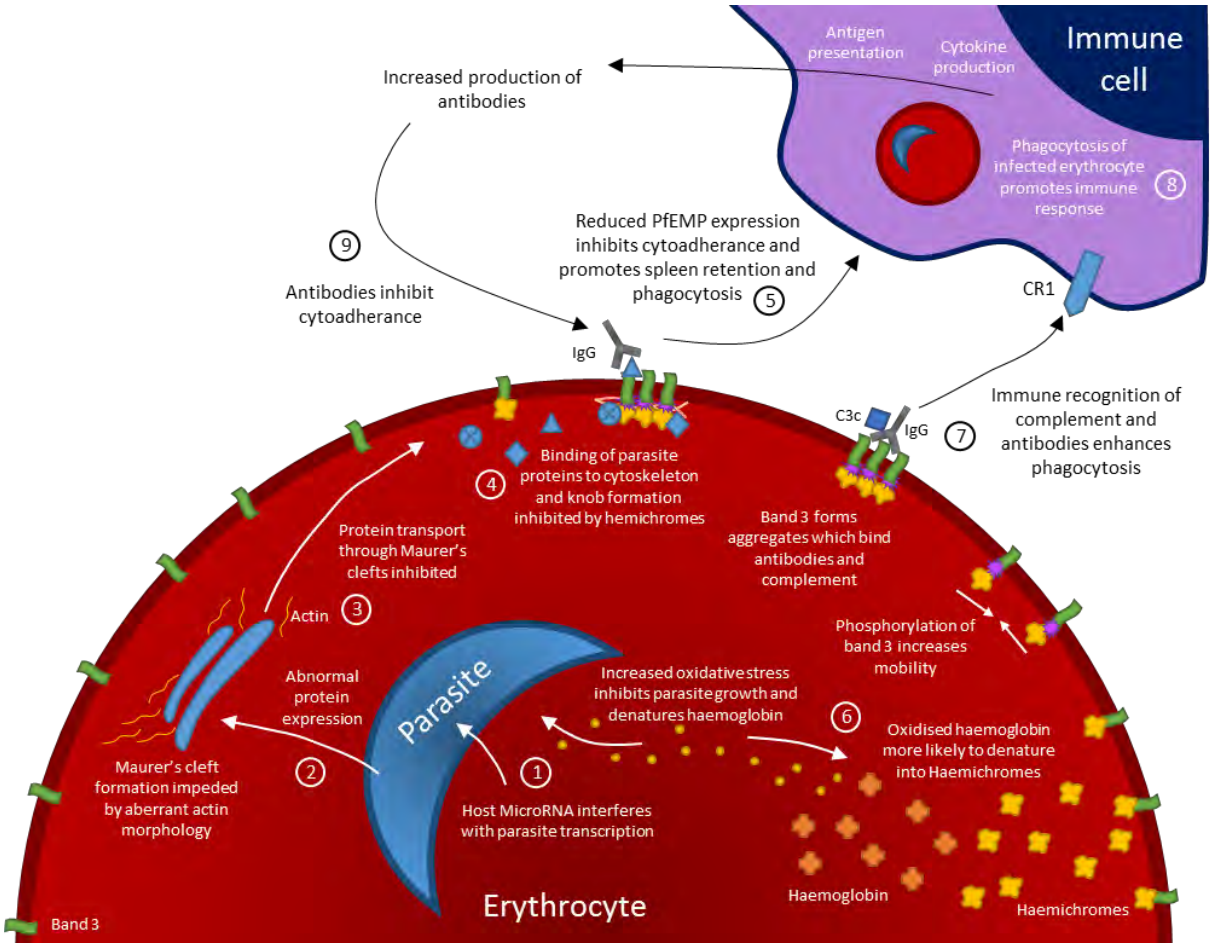


Figure 1.4. Multiple mechanisms of resistance in sickle cell trait (HbAS). Increased oxidative stress and host microRNAs in HbAS erythrocytes inhibit normal parasite development (1). This leads to inhibition of parasite transcription and protein expression (2). The formation of Maurer's clefts is impaired due to aberrant actin morphology, which prevent normal protein trafficking (3). Binding of parasite proteins which reach the erythrocyte membrane is inhibited by haemichromes, resulting in reduced surface expression of PfEMP (4). Reduced PfEMP expression results in less cytoadherence, which results in increased susceptibility to splenic clearance via phagocytosis (5). Oxidative stress also results in increased haemachrome formation (6), inducing band 3 mediated senescence and phagocytosis (7). Enhanced phagocytosis promotes an immune response (8). Increased production of antibodies against parasite proteins further inhibits cytoadherence and amplifies the effect (9).

IMPLICATIONS FOR THE DEVELOPMENT OF ANTIMALARIALS

The study of genetic polymorphisms and their associated mechanisms of malaria resistance have led to novel vaccine and drug targets. Drugs which accelerate erythrocyte senescence in similar ways to that seen in polymorphisms such as HbAS have already shown some promise[307-309]. Vaccines directed against the Duffy binding protein of *P. vivax* have also shown promise, however are hindered by parasite protein variability[164]. This problem may be avoided if there was a way to block the binding site on the host Duffy antigen, which is outside the parasite's genetic control, rather than the parasite Duffy binding protein. Indeed, the Duffy negativity polymorphisms have shown the power of this approach, whereby *P. vivax* is almost never seen in Duffy negative populations.

Host directed therapy (HDT)

Host directed therapy (HDT) is an approach which aims to solve the problem of drug resistance. Similar to a vaccine, HDT aims to give the host an advantage over a pathogen, rather than directly targeting the pathogen itself. Specifically, HDT is any therapy which targets a host protein in order to prevent disease. Drug resistance occurs when the pathogen mutates the drug target or changes itself in such a way so that the drug is no longer effective. Therefore, by using a therapy that doesn't directly target the pathogen, HDT deters the development of resistance. Many currently approved drugs which have known host targets can be re-purposed as HDTs. However, a disadvantage of HDTs is that they are more likely to have serious side effects as they are targeted at the host, rather than a unique pathogen process.

HDT has already proved to be an effective strategy against the vaccinia virus[313], Hepatitis C[314], Human immunodeficiency virus (HIV)[315], Pseudomonas aeruginsoa[316], listeria monocytogenes[317], and mycobacterium tuberculosis[318,319]. The development of effective HDTs for malaria will depend on the understanding of which host proteins are critical to the survival of the *Plasmodium* parasite. While the discovery of naturally occurring polymorphisms may provide a useful HDT target, non-

polymorphic targets may be missed, highlighting the potential utility of a forward genetics screen to identify novel malaria resistance genes.

APPROACHES FOR THE IDENTIFICATION OF NOVEL MALARIA RESISTANCE GENES

While a large array of genetic polymorphisms that convey malaria resistance have been discovered, it is likely that the vast majority of genetic based host resistance to malaria remains unknown. A study on Kenyan children indicated that, based on heritability, host genetics accounts for 25% of the risk of developing severe malaria[320]. Furthermore, only 8% of this amount could be accounted for, meaning that 92% of genes involved in malaria resistance are unknown. The discovery of these unknown genetic factors, along with other genetic factors which might not have occurred in nature, may provide valuable insights into the host-parasite interaction. One way to identify these genes is through genome wide association studies (GWAS). However, the success of this approach has been hampered by the large degree of heterogeneity between African populations[89], although work by Brisebarre *et al.* [321] has resulted in the identification of several new resistance loci which may lead to the identification of malaria resistance genes.

Another approach is to use mice. Different inbred strains of mice display a large range of susceptibility to malaria, highlighting an important role for host genetics in rodent malaria infection. A valuable approach for the discovery of genes influencing malaria susceptibility is the identification of quantitative trait loci (QTL). In this approach, an inbred mouse line which is susceptible to malaria infection is crossed with a second, more resistant inbred line. The malaria susceptibility of their offspring (from an F2 intercross for example) is then determined and QTLs associated with malaria resistance may be identified.

So far this approach has led to the identification of 11 resistance loci for *P. chabaudi* and 9 resistance loci for *P. Berghei*[322-325]. However, as this approach only provides a genomic region associated with resistance it is extremely difficult to identify a single gene responsible. Indeed, in many of these cases

malaria resistance may be due to the combined effect of multiple mutations within the genomic region. To date, only one of these QTLs has led to the identification of a gene involved in malaria resistance, *Pklr*[76]. Later studies found that mutations in this gene protect against malaria in human populations, highlighting the potential of this approach to identify genes involved in human malaria resistance[77].

N-ethyl-N-nitrosourea (ENU) mutagenesis

An alternate approach to identifying genes involved in malaria is ENU mutagenesis. In this approach, ENU is given to male mice in order to induce random point mutations in spermatogonial stem cells. ENU treated mice are then bred with wild type females to produce an array of mutagenised offspring. At this stage, mice can be infected with malaria to test for malaria phenotypes induced by dominant heterozygous mutations, or bred further to identify recessive phenotypes. This provides an unbiased approach for the identification of genes involved in malaria resistance, or susceptibility, across the whole genome.

When a phenotype of interest is identified, the causative ENU-induced mutation can be located by crossing with a different inbred strain and performing linkage analysis. Alternatively, whole genome sequencing can be used to identify all ENU-induced mutations in the line of interest. Due to the limited number of starting mutations, approximately one mutation per mega base depending on ENU dose, the identification of causative ENU mutations is relatively simple, making this a valuable approach to identifying malaria resistance genes.

THESIS AIMS

The current understanding of genes involved in malaria resistance is largely based on the investigation of easily diagnosable, common, genetic polymorphisms such as sickle cell trait, G6PD and the thalassemias. Previously it has been shown that genes involved in human malaria resistance also cause resistance in rodent malaria models. This study therefore sought to use rodent malaria models, in conjunction with ENU mutagenesis, to discover novel genes involved in malaria resistance. Thesis aims were as follows:

1. IDENTIFY NEW GENES WHICH INFLUENCE MALARIA SUSCEPTIBILITY

By screening ENU mutagenized mice for abnormal haematological properties and malaria susceptibility this study aimed to identify mouse lines of interest, and to then identify the causative mutations in these lines via linkage mapping and/or whole genome sequencing.

2. DETERMINE THE BIOLOGICAL MECHANISM RESPONSIBLE FOR ALTERING MALARIA SUSCEPTIBILITY

Once a genetic mutation has been associated with a change in malaria susceptibility, the biological mechanism responsible for this will be investigated. Firstly, the effect of the mutation in an uninfected animal will be determined. This will involve looking at the expression of the mutant protein, as well as haematological parameters and other relevant indices depending on the gene in question. Next, affected aspects of the parasite's lifecycle will be explored. The ultimate goal is to determine the mechanisms of resistance to malaria, which may lead to the identification of a suitable HDT target for development.

CHAPTER 2

EXPERIMENTAL PROCEDURES

MICE AND ETHICS

Mice were housed under controlled temperature (21°C) with a 12:12hr light-dark cycle. All procedures were conducted in accordance with the policies of Macquarie University and 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 ARA 2012/019 approved and obtained from the Animal Ethics Committee at Macquarie University or Ethics No A0104070 approved and obtained from the Animal Ethics Committee at University of Tasmania.

ENU MUTAGENESIS SCREEN

For N-Ethyl-N-Nitrosourea (ENU) mutagenesis two intra-peritoneal (IP) injections of 150 mg/kg ENU (Sigma-Aldrich, St Louis, MO) were given to male SJL/J mice one week apart. These mice were in-crossed with SJL/J female mice and their progeny (G1) were bled at seven weeks of age and screened for their peripheral blood parameters using an Advia 120 automated haematological analyser (Siemens, Berlin, Germany).

LINKAGE MAPPING

Causative mutations in all lines were mapped by linkage analysis on offspring from affected mice out crossed to C57BL/6 wild type mice. DNA was extracted from affected N2 mice, as determined by reduced MCV, using the Qiagen DNeasy Blood and Tissue kit according to the manufacturer's instructions (Qiagen, Limburg, Netherlands). A genome wide SNP analysis was performed on a MASSarray platform using the IPlex GOLD technology (Sequenom Inc, San Diego, CA) with over 300 polymorphic SNPs. This was performed by the Australian Genome Research Facility (AGRF). Candidate genes within the linkage interval were sequenced by amplifying each exon, including intron/exon boundaries, via PCR, using primers designed with Primer3 [326]. Single band PCR products were confirmed and purified using the SV Gel and PCR Clean-Up kit (Promega, Madison, WI). Sanger sequencing was performed by AGRF.

SCANNING ELECTRON MICROSCOPY

Erythrocytes were washed three times in MTRC (154mM NaCl, 5.6mM KCl, 1mM $MgCl_2$, 2.2mM $CaCl_2$, 20mM HEPES, 10mM glucose, 30U/mL heparin, 0.5% BSA, pH 7.4, 0.22μM filter sterilised) before being fixed in 2.5% gluteraldehyde for three days at 4°C. Fixed erythrocytes were dehydrated by incubation in increasing concentrations of ethanol followed by increasing concentrations of hexamethyldisilazane (HMDS). Samples were then gold coated with a Q150T sputter coater (Quorum, East Sussex, UK) before imaging on a JSM- 6480 LA scanning electron microscope (JEOL, Tokyo, Japan).

OSMOTIC FRAGILITY MEASUREMENT

In order to assess the membrane stability of mutant erythrocytes an osmotic fragility assay was performed. Ten microliters of blood from mutant and wild type mice was incubated for 30 minutes at room temperature in 1mL of 20mM phosphate buffer (pH 7.4) containing between 0 and 10g/L NaCl. Cells were then centrifuged at 500g for five minutes. The supernatant was removed and its absorbance measured at 540nm to determine the amount of haemoglobin in solution, and therefore the amount of haemolysis.

ERYTHROCYTE HALF-LIFE ASSAY

To determine the half-life of erythrocytes mice were injected with 1mg Sulfosuccinimidyl-6-(biotinamido) hexanoate (Sulfo-LC-NHS-Biotin) (Thermo Fisher Scientific, Waltham, MA) in MT-PBS via IV resulting in approximately 99% biotinylation of erythrocytes. Tail blood samples were taken on days indicated and prepared for flow cytometry as described. The percentage remaining biotinylated cells on each day was recorded.

ERYTHROCYTE DENSITY ASSAY

Erythrocyte density was determined based on separation of erythrocytes on a discontinuous Percoll gradient. A 90% v/v Percoll solution in MTRC was prepared and then diluted in MTRC to produce

eight solutions of decreasing density. These solutions were carefully layered in a 15ml flacon tube, from the most dense to the least dense, with 1.8ml of solution per layer. Freshly obtained blood was layered on top of the gradient before centrifugation at 800g for 30 minutes at 4C. Each layer was collected separately, washed, and erythrocytes were lysed in hypotonic solution. Samples were spun down, and the absorbance of the supernatant at 540nm was measured. The proportion of erythrocytes in each density layer was calculated as the sample absorbance divided by the total absorbance of all layers for that blood sample, multiplied by 100.

ERYTHROCYTE PURIFICATION

To isolate erythrocytes from whole blood for analysis the following procedure was used. Whole blood was collected by cardiac puncture of anaesthetized mice. Blood was layered onto ice cold 60% v/v Percoll (Sigma-Aldrich, St Louis, MO) in MTRC and centrifuged at 800g for 30 minutes. This facilitated the separation of platelets and leukocytes (at the interface) from erythrocytes and reticulocytes (in the cell pellet). The erythrocyte fraction was then incubated with 1μg/mL anti-CD71 FITC (clone R17217), 1μg/mL anti-CD45 FITC (clone 30-F11), and 1μg/mL anti-CD41 FITC (clone MWReg30) (eBioscience San Diego, CA) for 1hr at 4C. Cells were washed and labelled with anti-FITC magnetic beads and run through a LD magnetic separation column according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Erythrocytes were analysed by flow cytometry to determine purity, which was maintained above 99%.

PREPARATION OF ERYTHROCYTE GHOSTS, SDS-PAGE, AND WESTERN BLOTTING

Purified erythrocytes were washed three times in MTRC. Cells were then incubated in hypotonic lysis buffer (5mM PBS, 1mM EDTA) with Roche complete protease inhibitor cocktail (Roche, Basel, Switzerland). Ghosts were pelleted at 20,000g for 20 minutes and washed in hypotonic lysis buffer until the supernatant became clear. The volume of packed ghosts was determined and an equal volume was used for all SDS-PAGE and western blot experiments. Packed ghosts were diluted 5x in laemmli buffer with 5% v/v beta mercaptoethanol and heated at 70°C for 10 minutes before being

separated by SDS-PAGE on precast 4-15% Tris/glycine gels (Biorad, Hercules, CA). For the measurement of band 3 aggregation beta mercaptoethanol was excluded from the sample buffer. Gels were stained overnight in coomasie before washing and imaging. For western blotting gels were transferred to nitrocellulose membranes using the iBlot system (Life Technologies, Carlsbad, CA). Anti-beta spectrin (clone N-19), anti-ankyrin (clone 8C3) (Santa Cruz Biotechnology, Dallas, TX), anti-alpha spectrin (clone 17C7), anti-actin (clone C4) (Abcam, Cambridge, UK), anti-C3 (clone GTX72994) (Gentex, Irvine, CA), anti-band 3 and anti-protein 4.1 (a kind gift from Connie Birkenmeier) were used to probe blots. Secondary antibodies conjugated to HRP (Abcam, Cambridge, UK) were used in conjunction with Immobilon Western Chemiluminescent HRP Substrate (Merck, Darmstadt, Germany) to image blots.

IDENTIFICATION OF SPLENIC ERYTHROBLASTS AND RETICULOCYTES BY FLOW CYTOMETRY

For the characterisation of erythropoietic cells, whole spleens were disassociated through a 70μM cell strainer and diluted in MTRC. Approximately $1x10^7$ cells were incubated in 50μL of 1μg/mL anti-CD16/CD32 (eBioscience, San Diego, CA) in MTRC for 15mins at 37°C to prevent non-specific antibody binding. Cells were then incubated for 30mins at 37°C in the dark with 1μg/mL anti-TER119 PE-Cy7, 1μg/mL anti-CD71 (Tfr1) PerCP eFluor 710 (eBioscience, San Diego, CA), and 4μM Hoechst 33342 (Sigma-Aldrich, St Louis, MO). Cells were analysed by flow cytometry and cell populations were identified as described.

MEASUREMENT OF CELL SURFACE TFR1

As Tfr1 molecules undergo a constant endocytosis cycle in the presence of iron, spleen cells were first washed in MTRC to remove excess iron. Cells were then incubated at 37°C for 15mins to allow Tfr1 molecules to return to the surface of the cell before proceeding with antibody staining as described above. The amount of cell surface Tfr1 was determined by the mean fluorescence intensity (MFI) of staining with the CD71 (Tfr1) antibody.

IRON MEASUREMENTS

Total non-heme iron was measured as described by Patel *et al.*[327]. Briefly, tissue samples (~50-100mg) were dried at 45°C for 48hrs before being digested in 300µL of 10% trichloroacetic acid/10% HCl at 65°C for 48hrs. Samples were centrifuged at 16,000g for five minutes and then 200µL was added to 1mL chromogen solution (0.01% bathophenanthroline-disulfonic acid, 0.1% thioglycolic acid, 2.5M sodium acetate). Samples were incubated for 15mins and absorbance at 535nm was measured. Plasma iron was measured in a similar way using 50µL plasma digested in 70µL 10% trichloroacetic acid/10% HCl, of which 100µL was added to 100µL of chromogen solution. A set of standard iron solutions between 10 and 500µg/mL were made using a standard 1M iron solution (Sigma-Aldrich, St Louis, MO). These were used to construct a standard curve and samples were diluted to fit within the linear range of the curve.

Serum and erythrocytic ferritin were measured using a mouse ferritin ELISA kit according to manufacturer's instructions (Kamiya biomedical, Seattle, WA). For erythrocytic ferritin, cells were lysed in hypotonic buffer and the total protein concentration was measured using the Pierce BCA protein assay kit according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA). Labile iron pool (LIP) was measured as described by Prus and Fibach[328]. Briefly, spleen cells stained as described above were incubated with 2µM calcein-aceto-methyl ester (calcein-AM) (Sigma-Aldrich, St Louis, MO) for 15mins at 37°C. Cells were washed twice in MTRC and then divided between two tubes. 100µM deferiprone (L1, Sigma-Aldrich) was added to one tube while the other was left as is. Cells were incubated for one hour at 37°C before being analysed by flow cytometry as described. The LIP was calculated by subtracting the Calcein-AM mean fluorescence intensity (MFI) of L1 treated cells from untreated cells.

RNA EXTRACTION AND QUANTITATIVE PCR

For the measurement of hepcidin expression, RNA was extracted from livers using Trizol (Life Technologies, Carlsbad, CA). Reverse transcription was performed using anchored-oligo $(dT)_{18}$

primers with a Transcriptor High Fidelity cDNA Synthesis Kit according to the manufacturer's instructions (Roche, Basel, Switzerland). Quantitative PCR was performed with the GoTaq qPCR Master Mix (Biorad, Hercules, CA) using primers for *Hamp1* (F 5′-TTGCGATACCAATGCAGAAGA-3′, R 5′-GATGTGGCTCTAGGCTATGTT-3′) and *Actb* (F 5′-GGCTGTATTCCCCTCCATCG-3′, R 5′-CCAGTTGGTAACAATGCCATGT-3′) which were designed so that either the forward or reverse primer spanned an exon/exon boundary to avoid amplification of genomic DNA. Results were calculated using the $\Delta\Delta C_t$ method as the fold change in cDNA of mutant compared to wild type based on *Hamp1* expression compared to *Actb.*

MALARIA PARASITES AND INFECTION

For experimental malaria infection stock blood infected with *P. chabaudi adami* DS or *P. berghei* ANKA was stored at -80°C. 250µL of thawed parasitized blood was injected into the intraperitoneal cavity of C57BL/6 donor mice. Once C57BL/6 donors reached 5-15% parasitaemia they were anesthetized with isofluorane and bled by cardiac puncture before being sacrificed. Parasitized blood was diluted in Krebs's buffered saline containing 0.2% glucose according to Jarra and Brown[329], and the required dose of parasitized erythrocytes were injected into the intraperitoneal cavity of mice to be infected. All experiments were performed on SJL/J mice unless otherwise stated. Mice were monitored daily by tail bleed using microscopy or flow cytometry as described.

MICROSCOPY

Microscopy was used to determine parasitaemia of thin blood smears or to analyse cells sorted by flow cytometry. Sorted cells were spun down and concentrated before being allowed to settle onto glass slides coated with 0.1% polyethylenimine (PEI) (Sigma-Aldrich, St Louis, MO). Cells were fixed in methanol for one minute before being stained in a 10% Giemsa solution (Sigma-Aldrich, St Louis, MO) at pH 7.4 for 10mins. Parasitaemia was calculated by counting at least 500 parasitized cells by light microscopy at 100 × magnification.

ENZYMATIC TREATMENT OF ERYTHROCYTES

In order to validate the ability of the labelled erythrocyte assay to detect the inhibition of parasite invasion, erythrocytes were treated with enzymes reported to inhibit *P. falciparum* invasion *in vitro* as previously described[330]. Briefly, erythrocytes were incubated with 20mU/mL neuraminidase, 0.5mg/mL trypsin, and 1mg/mL chymotrypsin in MTRC for 30mins at 37°C.

LABELLED ERYTHROCYTE ASSAYS

Labelled erythrocyte assays were used to determine erythrocyte autonomous changes to malaria susceptibility *in vivo.* Donor blood from wild type and mutant mice was labelled and transfused into infected mice using the following protocol. Blood was collected by cardiac puncture of anaesthetized mice, and suspended at 20% haematocrit in MTR (154mM NaCl, 5.6mM KCl, 1mM $MgCl_2$, 2.2mM $CaCl_2$, 20mM HEPES, 10mM glucose, 30U/mL heparin, pH 7.4, 0.22μM filter sterilised). Erythrocytes were enzymatically treated as described or left untreated before labelling. Depending on the experiment erythrocytes were incubated in 6μg/mL or 20μg/ml hydroxysulfosuccinimide Atto 633 (Atto 633), 20μg/ml hydroxysulfosuccinimide Atto 488 (Atto 488) (Sigma-Aldrich, St Louis, MO), or 125μg/mL sulfosuccinimidyl-6-(biotinamido) hexanoate (Biotin) (Thermo Fisher Scientific, Waltham, MA) at 4°C for 1hr with constant slow mixing. Stock solutions of 2mg/mL Atto 633 and 25mg/mL Biotin were prepared in dimethylformamide (DMF) and stored at -20°C. Labelled erythrocytes were washed three times and mutant erythrocytes were combined with wild type erythrocytes in approximately equal proportions in different label combinations to correct for any dye effects. Alternatively, enzyme treated erythrocytes were combined with untreated erythrocytes in a similar fashion. Approximately 1-2 x 10^9 labelled erythrocytes were transfused into host mice by I.V. Depending on the assay, host mice were infected with *P. chabaudi* for 7 days before transfusion, and were injected during the time of schizogony (approximately 6hrs into the dark cycle); otherwise host mice were uninfected at the time of transfusion. In one assay host mice underwent splenectomy before infection with *P. chabaudi* and subsequent transfusion of labelled cells. In another assay, host

mice were intraperitoneally injected with 0.2ug/g Escherichia coli lipopolysaccharide (0111:B4) (Sigma-Aldrich, St Louis, MO). Blood samples were taken from host mice at time points indicated. The parasitaemia of each labelled population was calculated as the number of labelled erythrocytes containing a parasite divided by the total number of labelled erythrocytes in that population. This value varies considerably depending on factors such as host parasitaemia and number of circulating merozoites. For this reason parasitaemia ratio is reported, which is calculated as the parasitaemia of the mutant labelled population divided by the parasitaemia of the wild type labelled population in each individual mouse. The percentage labelled erythrocytes was calculated as the number of labelled cells divided by the total number of erythrocytes. The remaining labelled erythrocytes is reported as the percentage labelled erythrocytes at each time point divided by percentage labelled erythrocytes at the first time point of the assay, this value also varies greatly depending on host parasitaemia. Notably, this can increase above 100% if the unlabelled, host, erythrocyte population is reduced at greater rate than the labelled population. The remaining labelled erythrocyte ratio is reported as the remaining labelled mutant erythrocytes divided by the remaining labelled wild type erythrocytes in each individual mouse.

STAINING OF BLOOD SAMPLES FOR FLOW CYTOMETRY

For the measurement of parasitaemia, reticulocytosis, erythrocyte count, and for labelled erythrocyte assays, samples were prepared for flow cytometry as follows. Three microliters of tail blood was collected directly into 50μL staining solution which contained 12μM JC-1 (Life Technologies, Carlsbad, CA), 10ul Sphero blank calibration beads (for assays measuring erythrocyte count) (BD Biosciences, Franklin Lakes, NJ), 1μg/mL Streptavidin PE-Cy7 (for assays with biotinylated erythrocytes), 1μg/mL anti-CD45 APC eFluor 780 (clone 30-F11), and 1μg/mL anti-CD71 (Tfr1) PerCP eFluor 710 (clone R17217) (eBioscience, San Diego, CA) in MTRC pre-warmed to 37°C. Samples were incubated at 37°C for 20 minutes before adding 500ul of 4μM Hoechst 33342 or 2μM Hoechst 34580 (Sigma-Aldrich, St Louis, MO) in MTRC. Samples were incubated at room temperature for 20 minutes before being washed and re-suspended in 700μL MTRC followed by analysis by flow cytometry. For

the evaluation of SYTO-16, Dihydroethidium, and Thiazole orange in detecting parasitized erythrocytes, cells were incubated with 1µg/mL anti-CD45 APC eFluor 780 and 1µg/mL anti-CD71 PerCP eFluor 710, along with either 2.5µM SYTO-16, 5µg/mL Dihydroethidium, or 100ng/mL Thiazole Orange for 20mins at room temperature before being washed and analysed. For the measurement of eryptosis samples were incubated in either 5µM Fluo-3-AM or Annexin V Alexa Fluor 488 (Life Technologies, Carlsbad, CA) according to the manufactures instructions and analysed by flow cytometry.

DETERMINING THE SENSITIVITY OF HOECHST 33342 AND JC-1 DETECTION OF PARASITISED ERYTHROCYTES

To assess the sensitivity of the Hoechst/JC-1 staining method at a different parasitaemia, blood from an infected mouse at approximately 1% parasitaemia was serially diluted with uninfected blood. Mice were anesthetized with isofluorane and bled by cardiac puncture before being sacrificed. Infected blood was divided into three aliquots and each aliquot was serially diluted threefold with uninfected blood a total of five times. The expected parasitaemia was calculated as the initial parasitaemia (determined by counting of parasites on a Giemsa stained slide) divided by the dilution factor. For each dilution the parasitaemia was measured using the Hoechst/JC-1 flow cytometry method described above as well as by using Hoechst 33342 or JC-1 fluorescence alone.

FLOW CYTOMETRY AND CELL SORTING

Samples were run and 1,000,000-10,000,000 events were collected using either BD FACS Diva or BD FACS Sortware with a BD Aria II, BD LSRFortessa, or BD Influx flow cytometer (BD Biosciences, Franklin Lakes, NJ). The BD Aria II was equipped with a 50mW 405nm laser, 20mW 488nm laser, and 18mW 633nm laser, The BD LSRFortessa with a 20mW 355nm laser, 50mW 488nm laser, and 50mW 640nm laser, while the BD Influx was equipped with a 100mW 355nm laser, 200mW 488nm laser, and 120mW 640nm laser. Hoechst 33342 was excited using the 355nm laser and detected through a 460/50 filter. Hoechst 34580 was excited using the 405nm laser and detected through a 460/50 filter.

For the 488nm laser, events were detected through a 530/40, 580/30, 692/40, or 750LP filter as required, while a 670/30 or 750LP filter was used to detect events from the 633nm or 640nm laser. Cell debris and noise were removed from analysis based on FSC/SSC properties and single cells were gated based on either trigger pulse width or by using the FSC peak area to height ratio. Beads were selected based on FSC/SSC and relative erythrocyte count was determined based on the bead count of infected mice versus three uninfected mice included in each analysis. Cell sorting was performed on the BD Influx into collection tubes and slides prepared as described earlier. Compensation and further analysis was performed using FlowJo v10.0.6 (Tree Star, Ashland, Oregon, USA).

PHAGOCYTOSIS ASSAY

Blood was collected from infected mice and incubated with 20μg/ml hydroxysulfosuccinimide atto 633 (NHS-Atto 633) (Sigma-Aldrich, St Louis, MO) and 4μM Hoechst 33342 (Sigma-Aldrich, St Louis, MO) in MTR for 30 minutes at room temperature. Blood was washed three times in MTRC. Intraperitoneal cells were harvested by lavage with 1.5mL RPMI 1640 from mice previously injected with 200ul sterile filtered 2% BSA 48hrs before. Intraperitoneal cells were combined with an equal number of mutant or wild type labelled cells before being allowed to settle onto slides at 37C for 30 minutes. Non-adherent cells were removed by washing slides three times with ice cold MTRC, while bound but non-phagocytized erythrocytes were lysed by washing slides in ice cold water for 30 seconds. Samples were examined using a fluorescence microscope (Zeiss, Jena, Germany).

STATISTICS

For malaria survival, statistical significance was determined by the Mantel-Cox test. Linkage analysis scores were calculated based on a chi-squared goodness-of-fit test. For ratios obtained in the labelled erythrocyte assays significance was determined using the one sample *t*-test with 1 as the hypothetical mean. For other results a two-tailed Students *t*-test was used to determine statistical significance.

CHAPTER 3

A FLOW CYTOMETRIC ASSAY TO QUANTIFY INVASION OF RED BLOOD CELLS BY RODENT *PLASMODIUM* PARASITES *IN VIVO*

CHAPTER INTRODUCTION

The study of the host/parasite interaction *in vivo* is hampered by an excess of experimental variables. Whilst it may be tempting to move to a culture system, whereby conditions can be tightly controlled and variables reduced, this will result in an incomplete picture of the real world situation. For example, in malaria infection, parasitised red blood cells (RBCs)* are constantly moving through the circulatory system, undergoing interrogation by immune cells, squeezing through narrow capillaries and splenic sinusoidal slits, and adhering to vessel walls. The parasite changes its behaviour dramatically according to its host environment, even employing alternate invasion pathways in order to avoid immune detection whilst maintaining its ability to enter RBCs.

In order to fully understand the mechanisms by which genetic mutations can influence malaria susceptibility, the *in vivo* environment needed to be maintained, while the number of experimental variables needed to be reduced. To achieve this, a novel assay was developed which allowed a comparison between different types of RBCs within an identical host environment. This allowed a reduction in variables associated with parasite load and synchronicity, immune response, and animal condition. Furthermore, by using this assay, interactions between the mutant RBC and parasite could be delineated from any systematic effects brought on by the mutation, i.e. changes in iron homeostasis.

This chapter has previously been published and is a direct excerpt from: Patrick M Lelliott, Shelley Lampkin, Brendan J McMorran, Simon J Foote and Gaetan Burgio. A flow cytometric assay to quantify invasion of red blood cells by rodent *Plasmodium* parasites *in vivo*. *Malar J*. **13**:100 (2014).

PML wrote the manuscript, helped conceive the study, and carried out all experiments with the exception of maintaining the parasite lines and performing mouse malaria infections; these were carried out by SL and GB. BJM, SJF, and GB helped conceive the study, contributed toward experimental design and analysis, and assisted in drafting the manuscript.

* In this chapter erythrocytes are referred to as RBCs to maintain consistency with the published study

BACKGROUND

Malaria is one of the most deadly infectious diseases, resulting in nearly one million deaths annually[331]. The symptomatic stage of infection occurs when the merozoite form of the *Plasmodium* parasite invades circulating RBCs, and undergoes development and replication. Interfering with merozoite invasion is regarded a potentially useful and novel anti-malarial approach, and understanding of the process is relatively advanced. The merozoite first binds to the RBC at an indiscriminate orientation before aligning itself so its apical end faces the RBC surface whereby its binding becomes irreversible[151]. Parasite proteins are then secreted resulting in remodelling of the RBC surface, tight junction formation, and endocytosis of the merozoite[157,332-334]. Genetic variations in the parasite[335-339] and the host[79,170] have been reported to alter parasite invasion efficiency, and several key protein interactions have been identified in this process[156,174,333,340]. However, all of these studies have been conducted using *in vitro* cultured parasites[330,334,341-343], and there is a need for methods to test and translate these findings *in vivo*. This is of particular importance when considering the interaction between the host's immune system and invading merozoite. Indeed, during infection the production of antibodies against merozoite antigens, which inhibit invasion, is thought to be an important mechanism in malaria immunity[344-346]. In concordance with this, it has been shown that the parasite possesses several alternate invasion pathways, and that it may switch between these pathways in response to immune action[168,334,335,347,348]. The development of vaccines against merozoite antigens, or other invasion blocking therapies, may, therefore, benefit from an *in vivo* invasion assessment, which would account for the role of the immune system in this interaction.

Rodent malaria parasites have long been utilized as a model for human malaria and several rodent *Plasmodium* species are now in widespread use, including *Plasmodium berghei*, *Plasmodium chabaudi*, *Plasmodium yoelii*, and *Plasmodium vinckei*. These species display substantial genotypic and phenotypic similarities to the human malarias *Plasmodium falciparum* and *Plasmodium vivax*[349] and, therefore, offer the potential to explore invasion phenotypes *in vivo* in mice. However, to use these models two issues need to be addressed.

Firstly, it is often challenging to accurately determine parasitaemia in *in vivo* samples. This is particularly pertinent at low parasitaemia levels when microscopic examination of blood smears is impractical. Automated methods such as flow cytometry are preferred, but accuracy can be hindered by the presence of additional cell types, especially RBC progenitors and leukocytes. Recently, several studies have reported the use of novel dyes combined with autofluorescence or fluorescently conjugated antibodies to accurately determine parasitaemia *in vivo*[350-352] and these are explored in this study. Another option is to use transgenic green fluorescent protein (GFP) expressing rodent malarial parasites as described by Franke-Fayard *et al.*[353]. However, the use of these parasites is so far restricted to two species of rodent malaria, *P. berghei* strain ANKA[353] and *P. yoelii*[354], with other parasite strains less suitable for transgenesis due to difficulties in maintaining the parasite *in vitro*. Another limitation of these parasites is that they must be maintained under constant drug selection to preserve a pure GFP expressing line. The second issue preventing accurate determination of invasion efficiency *in vivo* is the variation in synchronicity and parasitaemia between individual animals, which is not as pertinent a problem when using *in vitro* cultures. This variation is due to factors inherent in *in vivo* studies such as imperfect injection of the starting dose of parasites, small differences in the age or weight of individual animals, parasite variability and natural variation in the immune response to infection. Furthermore, during an *in vivo* infection, invasion can occur over a period of six hours or more making it difficult to distinguish between invasion and early stage growth phenotypes. To overcome this drawback it is necessary to discriminate between newly invaded parasitized RBCs (pRBCs) and those pRBCs already in circulation. This can be achieved by fluorescently labelling RBCs before exposing them to the parasite as previously described for *in vitro* assays[330,342]. Additionally, in order to avoid inaccuracies due to inter-individual variation it is also necessary to include a second population of labelled cells to act as a control. In this way the treated RBCs, or RBCs of interest, can be compared to control RBCs within one animal, thereby negating variations in parasite synchronicity and environmental conditions.

The study presented here describes a novel flow cytometric *in vivo* invasion assay, which addresses these issues. The assay was developed and optimized using mice infected with *P. chabaudi adami* strain DS*,* and its ability to analyse treatments known to block invasion in *in vitro* studies was verified. The assay allowed accurate determination of *in vivo* parasitaemia in mice, and distinguishes leukocytes, RBC progenitors, and RBCs containing Howell Jolly bodies. The ability of this assay to analyse the precise time of parasite invasion and correct for inter-individual variability through the use of two distinct RBC labels was also demonstrated.

RESULTS

A novel flow cytometric method to detect parasitized RBCs in in vivo *samples*

In preliminary studies using uninfected mice, a flow cytometric cell staining and analysis protocol was tested that took advantage of the DNA-specific dye, Hoechst 33342, which distinguishes DNA-containing blood cells[355], in conjunction with fluorescently-labelled antibodies raised against the leukocyte common antigen (CD45) and a red cell progenitor marker (CD71) to exclude leukocytes and non-parasitized nucleated blood cells. It was found that even after excluding Hoechst-CD45 and Hoechst–CD71 dual-positive events, there remained a Hoechst-stained population accounting for 0.3-0.9% of blood cells (Figure 3.1 A-D). Furthermore, the Hoechst fluorescence intensity of these cells was equal to that of pRBCs (Figure 3.1 E). To determine their identity, the cells were sorted onto glass slides, fixed, stained with Giemsa and examined under light microscopy. Most of the cells resembled red blood cells, with the addition of intracellular spherical basophilic-stained particles consistent with the appearance of Howell-Jolly (HJ) bodies (Figure 3.1 F). HJ-RBCs were observed at similar frequencies in both SJL/J and C57BL/6 strains of mice, and were also detected when other nucleic acid-specific dyes, including SYTO-16, dihydroethidium, and thiazole Orange were used (Supplementary figure 3.1).

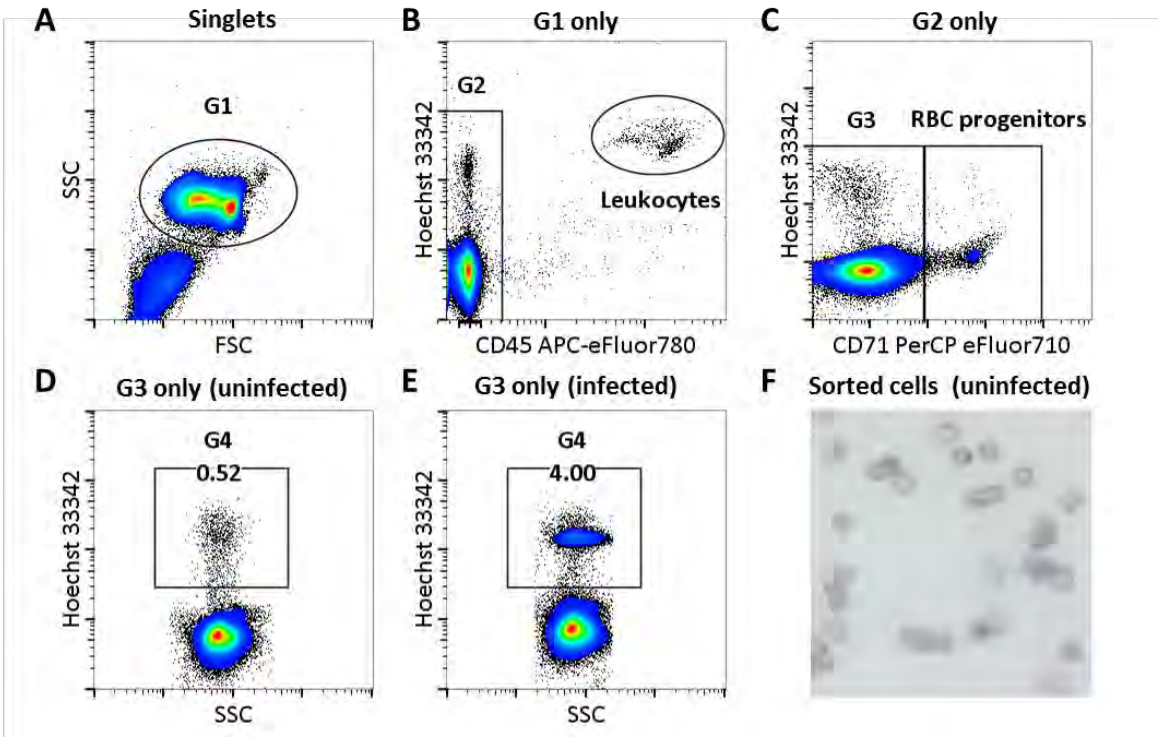


Figure 3.1. Gating strategy of uninfected whole blood from SJL/J mice. Single cells were gated based on trigger pulse width and from these cells, debris, noise and platelets were gated out based on forward scatter/side scatter (FSC/SSC) as G1 (A). Leukocytes were then gated out by selecting anti-CD45 APC eFluor780 negative cells (G2) (B) and RBC progenitor cells were gated out by selecting anti-CD71 PerCP eFluor710 negative cells (G3) (C). Finally, the remaining cells were gated based on positive DNA staining by Hoechst 33342 (G4). Representative results are shown from uninfected (D) and *P. chabaudi adami* DS infected (E) mice. At each stage only the cells in the previous gate were analysed as indicated. A representative image of cells from gate G4 in uninfected mice, which were sorted and Giemsa stained is shown, these are characteristic Howell-Jolly bodies (F).

To distinguish HJ-RBCs from pRBCs in blood from infected mice, the inclusion of a mitochondrial membrane potential dye, JC-1, which has been used previously in *P. falciparum* studies to determine parasite viability, was investigated[356]. In uninfected samples, fewer than 0.005% of cells were Hoechst 33342 and JC-1 positive when leukocytes and RBC progenitors were excluded from analyses (Figure 3.2 A). That is, the vast majority of Hoechst positive red cells identified above as containing HJ-bodies, did not stain with JC-1. To determine the optimal staining conditions of JC-1 blood samples from *P. chabaudi adami* DS infected mice at 2-10% parasitaemia were prepared. Concentrations ranging from 0.75µM to 24µM were tested and it was found that optimal staining conditions were incubation with 12µM of JC-1 for 20 mins at 37°C (Supplementary figure 3.2). Under

these staining conditions infected samples contained a population of Hoechst 33342 positive and JC-1 positive cells, which corresponded to sample parasitaemia (Figure 3.2 B). Samples from *P. berghei* ANKA infected mice exhibited a similar staining pattern indicating this method is consistent across different parasite species (Figure 3.2 C, D). In order to make this technique more broadly accessible, another DNA dye, Hoechst 34580, was tested, which can be used with a standard 405 nm violet laser. Results indicate that although Hoechst 34580 results in less separation between uninfected and infected RBC populations, it can be used effectively when combined with JC-1 and provides similar sensitivity to Hoechst 33342 (Supplementary figure 3.3). To test the sensitivity of this staining protocol in distinguishing parasite-infected cells, infected blood was serially diluted with uninfected blood, and the percentage of infected cells determined (% parasitaemia). The limit of detection was approximately 0.007% when using JC-1 and Hoechst 33342 together compared to 0.64% when using Hoechst 33342 alone (Figure 3.2 E). Finally, this protocol was assessed in the absence of anti-CD71 and anti-CD45 antibodies. Although sensitivity was reduced due to an inability to distinguish leukocytes from infected cells, this method reduced costs and simplified sample analysis (Supplementary figure 3.4).

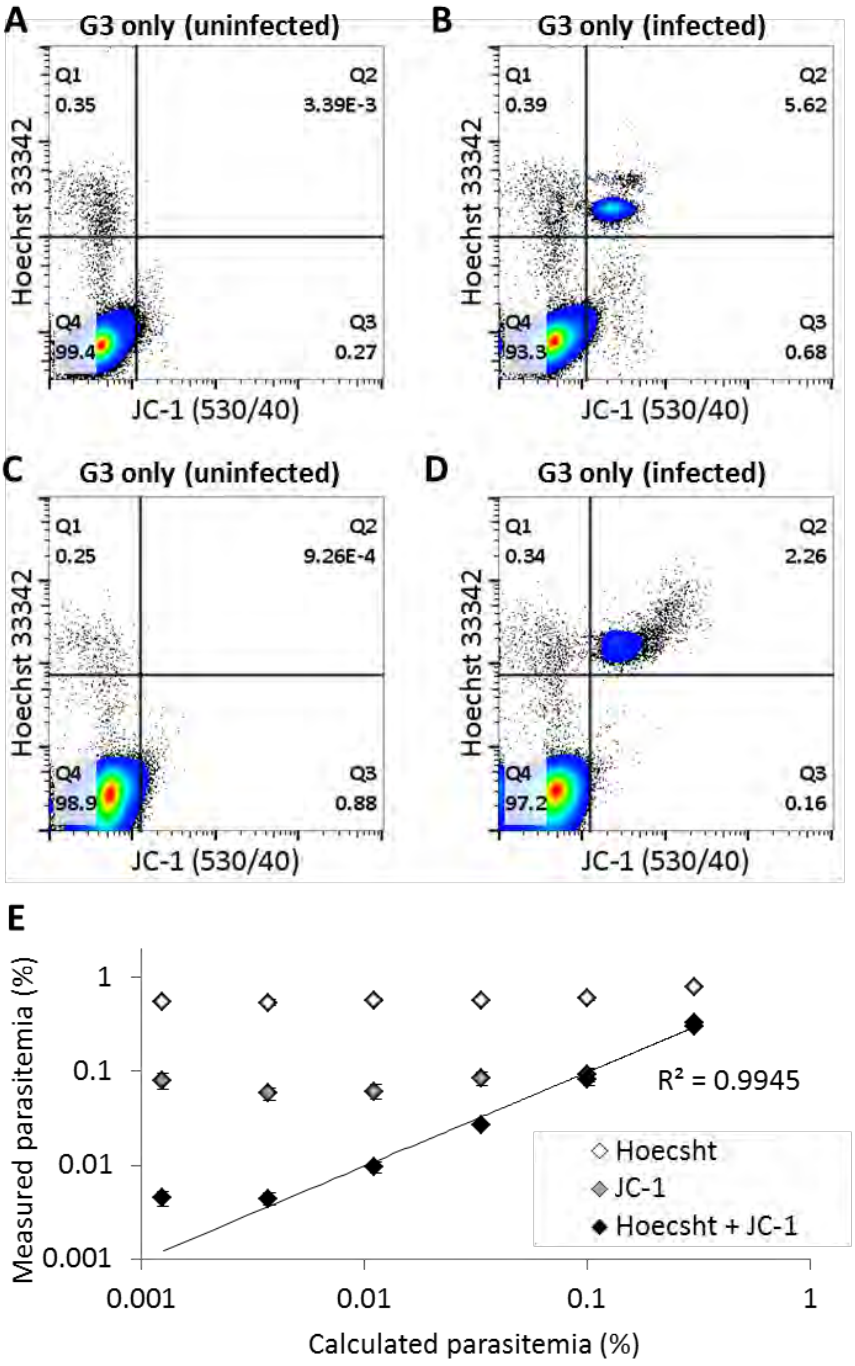


Figure 3.2. Hoechst and JC-1 staining of uninfected and infected blood. In one experiment blood samples were collected from uninfected (A) and *P. chabaudi adami* DS infected (B) mice. In another experiment blood was collected from uninfected (C) and *P. berghei* ANKA infected (D) mice. Blood samples were stained with JC-1, anti-CD45 APC eFluor780, anti-CD71 PerCP eFluor710, and Hoechst 33342. Note that plots are a typical representation of results and instrument voltages were slightly different between experiments. Samples were gated up to G3 as in Figure 3.1. A blood sample from a *P. chabaudi adami* DS infected mouse at approximately 1% parasitaemia was split into three aliquots and serially diluted with uninfected blood to create a dilution curve (E). Calculated parasitaemia was estimated based on the parasitaemia of the undiluted sample, determined by light microscopy, and the dilution factor. Measured parasitaemia for Hoechst was calculated as Q1 + Q2, for JC-1 it was calculated as Q2 + Q3 and for Hoechst + JC-1 it was calculated as Q2 alone (D). Error bars represent SEM for the three replicates.

In vivo parasite invasion assay

In addition to a method for quantifying parasitaemia, a flow cytometric method for quantifying parasite invasion *in vivo* was developed. A major hurdle in this type of analysis is distinguishing parasite invasion from parasite growth or clearance. To do this it is necessary to distinguish newly infected RBCs from those already in circulation. This was achieved by labelling two populations of RBCs and transfusing them into infected mice. Two criteria had to be met in selecting suitable RBC labels for this assay. Firstly, labels had to be compatible with the Hoechst/JC-1 method of pRBC detection and secondly, allow parasite invasion to occur as normal. Hydroxysulfosuccinimide Atto 633 (NHS-Atto 633) and sulfosuccinimidyl-6-(biotinamido) hexanoate (Sulfo-LC-NHS-Biotin) were selected as suitable labels. The NHS conjugate binds to primary amines on the surface of RBCs. Both labels were optimized for the minimum concentration required to distinguish labelled cells from unlabelled, and were clearly distinguishable from each other without need for compensation (Figure 3.3 A).

To determine that the RBC labels did not affect the ability of parasites to invade RBCs, blood conjugated with the two different labels was combined and injected into infected SJL/J mice during schizogony at 2-10% parasitaemia. The time of day was critical in this assay in order to maximize the number of invasion events. It was determined that schizogony peaked approximately half way through the dark cycle, under the conditions used here, this corresponded to between 10pm and 2am. Blood was sampled 30mins and 3hrs after injection, and stained and analysed as described in Methods. The results showed that the parasitaemia of the two labelled populations for each of the infected mice were virtually identical (Figure 3.3 D, F and Supplementary figure 3.5), indicating that these labels do not differentially affect parasite invasion. It was however observed that the parasitaemia of the fluorescently labelled populations varied between mice and did not correlate with the endogenous parasitaemia of the host mouse (Supplementary figure 3.5). This was presumably due to variations in the number of parasites undergoing schizogony in each mouse during the course of the assay.

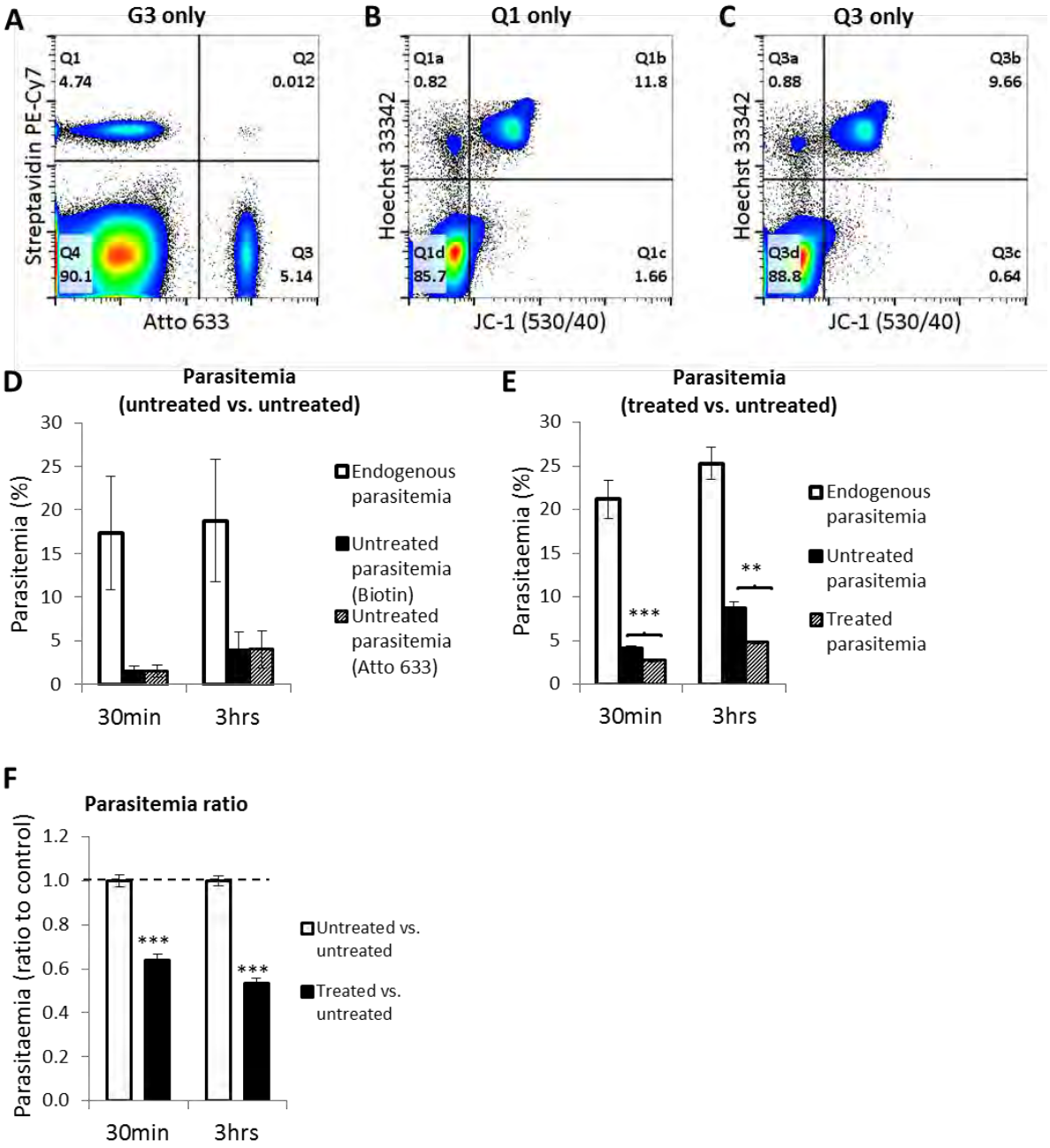


Figure 3.3. *In vivo* parasite invasion assay. Blood samples were taken from *P. chabaudi adami* DS infected SJL/J mice 30mins and 3hrs after injection with labelled RBCs and stained with JC-1, Hoechst 33342, anti-CD45 APC eFluor780, anti-CD71 PerCP eFluor710 and streptavidin PE-Cy7. Cells were gated to G3 as described in Figure 3.1 and then gated based on their respective label (A). Cells from Q1 (Biotin labelled) were gated based on JC-1 and Hoechst fluorescence and the parasitaemia was determined as Q1b divided by Q1 (B). Similarly, parasitaemia of cells from Q3 (Atto 633 labelled) was determined as Q3b divided by Q3 (C), and endogenous parasitaemia was determined based on the cells from Q4 (unlabelled). As a control experiment the two labelled RBC populations were untreated (D). To quantify the effect of protease treatment on invasion one labelled RBC population was treated with trypsin, chymotrypsin and neuraminidase, while the other was left untreated (E). Parasitaemia ratio calculated as the parasitaemia of the labelled RBCs of interest divided by the parasitaemia of the labelled control RBCs for each individual sample (F). Results are from five or six infected mice with RBC labels switched between treated and untreated to account for any invasion difference due to the RBC label. Error bars represent SEM, **p-value < 0.01, ***p-value < 0.001.

It was next determined if this method could be used to compare rates of invasion between normal cells, and cells treated ex vivo with a combination of proteases (neuraminidase, trypsin, and chymotrypsin) that are known to remove host cell molecules necessary for merozoite interaction and entry into red cells[334,336]. Blood collected from a donor mouse was divided in two. One aliquot was subjected to protease treatment, and the other was left untreated. Aliquots were labelled, combined and injected into infected mice during peak schizogony (see Supplementary figure 3.6 for schematic). Blood samples were collected at 30mins and 3hrs after injection and parasitaemia of the two populations determined (Figure 3.3 B, C, and E). Similar to the above-mentioned assay results, parasitaemia values varied between individual mice (Supplementary table 3.1). However, at each time point the parasitaemia of protease-treated cells was 30-60% less than in untreated cells in the same mouse, corresponding to parasitaemia ratios ranging between 0.4 and 0.7. These differences were observed at each time point, were irrespective of the dye combination, and were highly statistically significant (Figure 3.3 F and Supplementary figure 3.5).

DISCUSSION

In this report, a novel flow cytometry based assay is presented which allows the quantification of erythrocytic parasite invasion *in vivo*. To develop this assay the specificity of current fluorescent dyes used for the detection of pRBCs were evaluated in an *in vivo* model of malarial infection. To do this, several DNA specific dyes alone or in combination previously reported for parasitaemia measurement were assessed: Hoechst 33342[355,357], SYTO 16[350], Dihydroethidium[352,358], and Thiazole Orange[355,359]. Surprisingly, it was found that samples from uninfected mice stained with these dyes resulted in a positively stained population of 0.3-0.9% which was considerably larger than previously reported values for *in vivo* analysis[350-352]. To explain this discrepancy, this cell population was isolated and examined. This resulted in the detection of basophilic intra-erythrocytic staining indicating the presence of Howell-Jolly (HJ) bodies. HJ-RBCs are usually quite rare in humans, and are associated

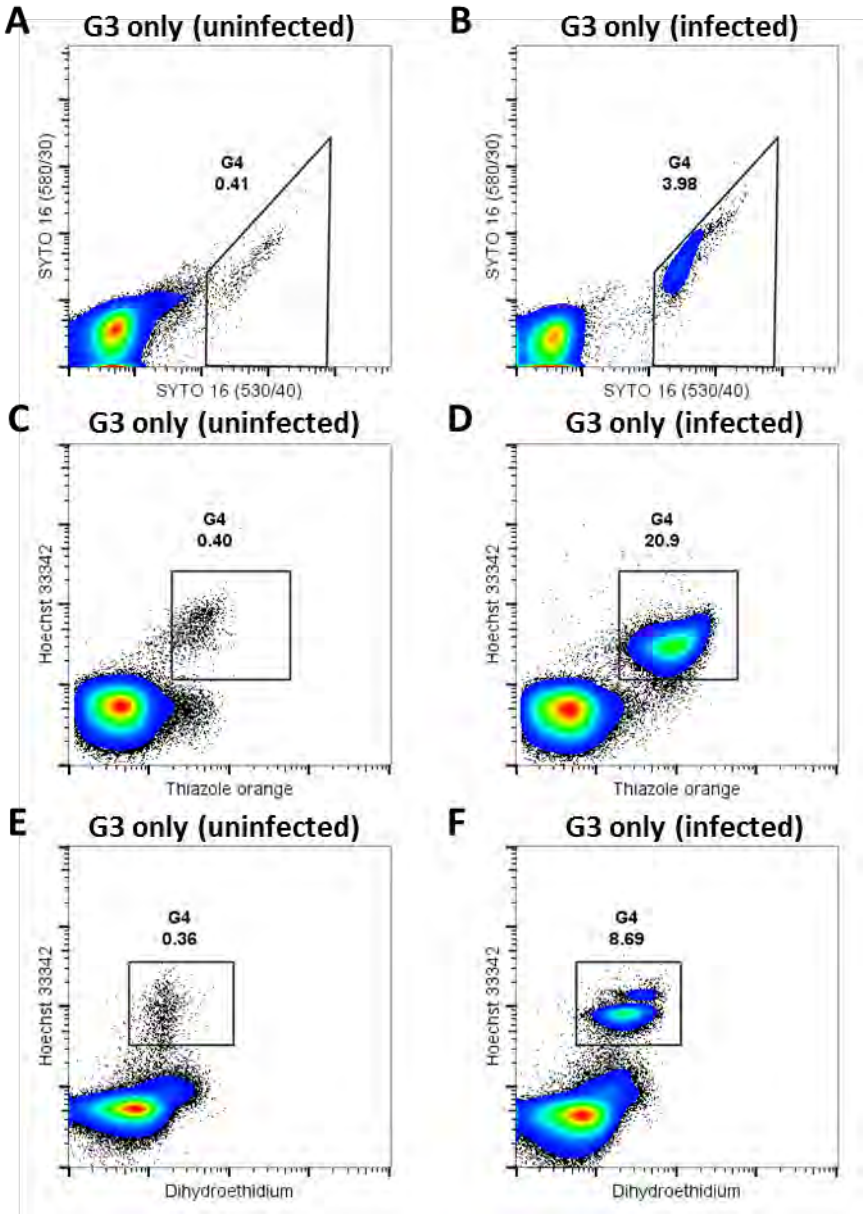
with abnormal splenic function[360]. In mice, some studies report HJ-RBC frequencies in control animals comparable to the data obtained in this study[361,362] while others report lower levels[363], it is not clear why this is the case. HJ-RBCs occur when remnants of DNA remain in mature RBCs due to incomplete expulsion of the nucleus during erythropoiesis. As pRBCs and HJ-RBCs could not be distinguished from each other based on DNA staining alone, JC-1, a mitochondrial membrane dye, was investigated to determine if it would allow for increased specificity in pRBC staining. It was found that the combination of mitochondrial (JC-1) and nucleic acid stain (Hoechst 33342) provided an increase in the LOD of pRBCs from 0.64% to 0.007% compared with Hoechst 33342 alone. Similar levels of sensitivity were observed in mice infected with *P. chabaudi adami* DS or *P. berghei* ANKA. Although mitochondrial membrane potential dyes have previously been employed to assess parasite viability[356,364] and to determine parasitaemia[365,366] to the best of this author's knowledge the combination of these with DNA specific dyes has not been used to quantify parasitaemia *in vivo*. In addition to JC-1 and Hoechst, selective, fluorescently labelled antibodies were employed to detect and exclude RBC progenitors and leukocytes, further improving the sensitivity of the assay. As well as using the Hoechst 33342 dye, which must be excited with a 355 nm (UV) laser, the use of an alternative Hoechst 34580 dye, which is excited by the more commonly available 405 nm laser, was demonstrated, offering a broader applicability for this assay. The later dye has been used previously to measure parasitaemia[113]. In a practical setting this assay allows accurate quantification of parasitaemia down to approximately 0.013% utilizing a 355/488/633 nm three-laser instrument, with the detection of pRBCs as low as 0.007%. However, in order to accurately measure low parasitaemia a sufficient number of events must be analysed to overcome the incidence of noise related to sample or machine impurities, in some cases this will require the analysis of > 5,000,000 events.

In addition to parasitaemia measurement a method was established to directly compare rates of parasite invasion in different RBC donor cells within a single recipient animal. RBC labels were evaluated that could be detected in conjunction with the Hoechst and JC-1 dyes, and the NHS-Atto 633 and Sulfo-LC-NHS-Biotin (combined with streptavidin PE-Cy7) were selected. To ensure the

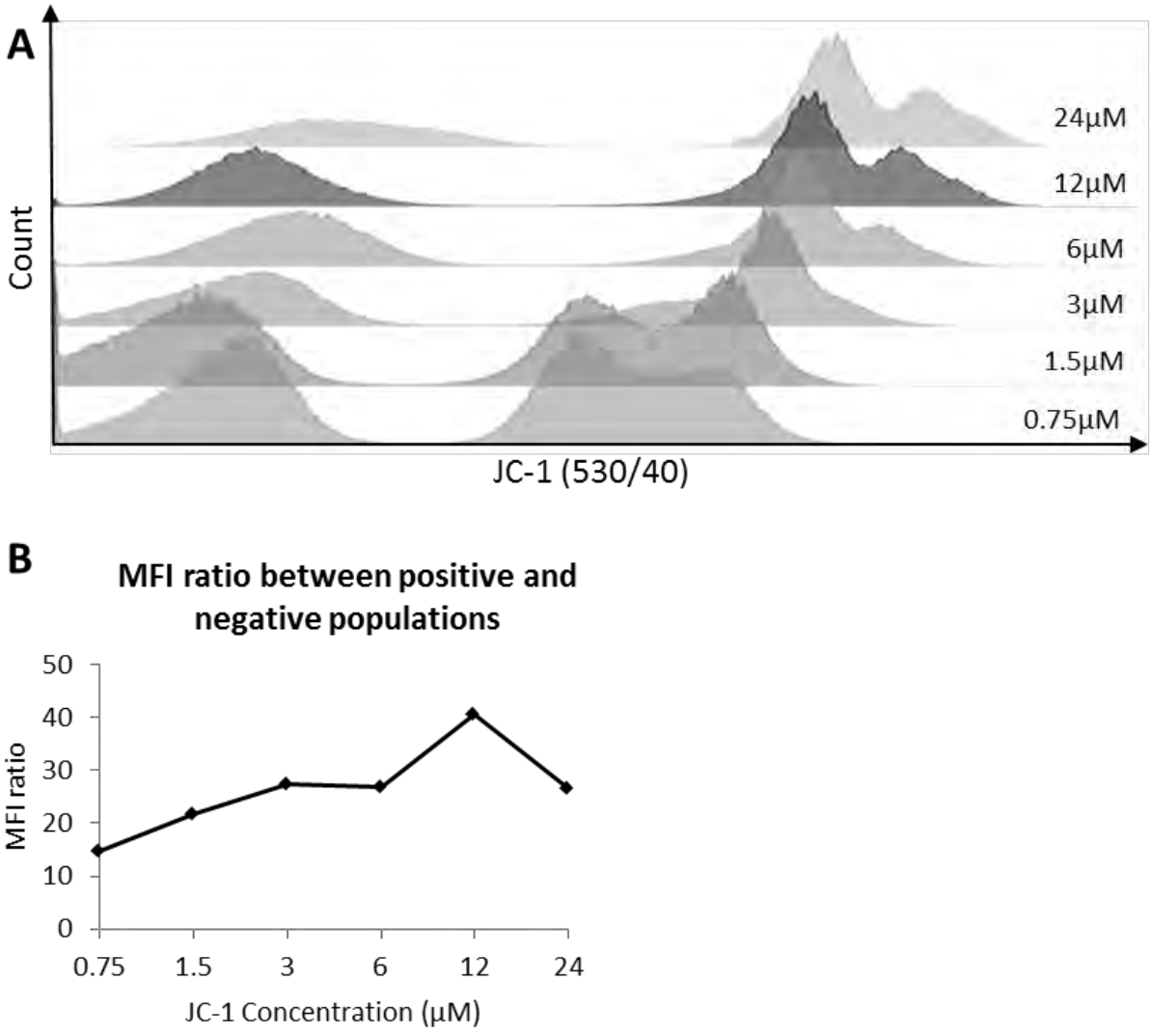
accuracy of this assay the effect these labels might have on parasite invasion was addressed. Theron *et al.*[330] suggested that surface labels, such as FITC, may inhibit invasion, while Pattanapanyasat e*t al.*[367] report that using biotin as a surface label has no effect on invasion. Under the conditions used here, labelling red cells with these molecules did not affect parasite invasion *in vivo*. The high quantum yield of the molecules allowed concentrations of the labels to be minimized. By using two populations of labelled cells, rather than one population as used in *in vitro* assays[330,342,367], the assay was able to be performed in mice with variable parasite loads and parasite stage synchronicity with little effect on results. In addition, by optimizing the starting time of this assay to coincide with peak schizogony significant numbers of newly invaded RBCs were detected after just 30 minutes; this timeframe was also sufficient to detect differential invasion rates between protease-treated and untreated cells. The limited time frame is likely to specifically reflect an invasion phenotype, rather than parasite growth as reported in previous assays[113]. However, results suggest that by continuing this assay over longer time periods other aspects of the parasite life cycle such as growth, splenic clearance, and sequestration can also be investigated.

Once this assay was established it was determined if invasion inhibition produced by treatment of RBCs with trypsin, chymotrypsin, and neuraminidase could be detected. It was found that protease treatment reduced invasion by 35%. This effect on invasion was not as great as what may be expected, although treatments such as this have been shown to have variable effect between different strains of *P. falciparum* parasites[342]. Importantly, the magnitude of the invasion inhibition was highly consistent between mice despite differences in parasitaemia and synchronization, and was not affected by label combination. The ability to accurately study the interaction between the parasite and its host cell is of utmost importance in determining factors which are essential for parasite survival. To date, techniques for assessing parasite invasion have been exclusively carried out *in vitro*. The assay presented here allows the accurate measurement of both parasitaemia and erythrocytic invasion *in vivo*. The validity of this assay to detect invasion inhibition was demonstrated.

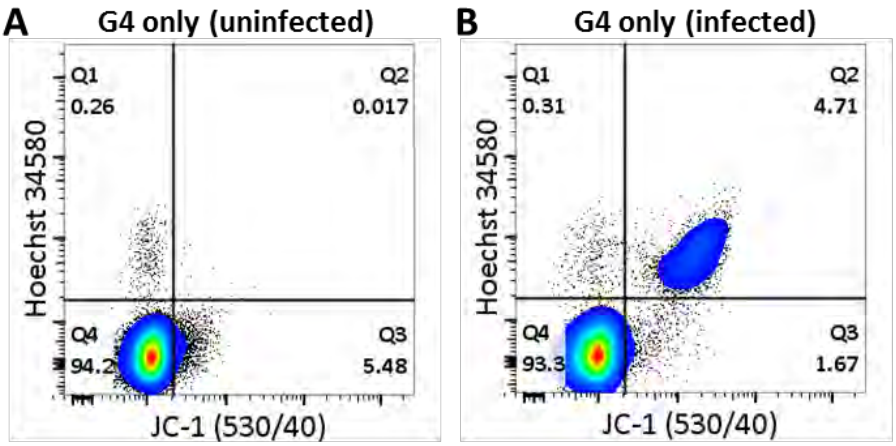
SUPPLEMENTARY FIGURES



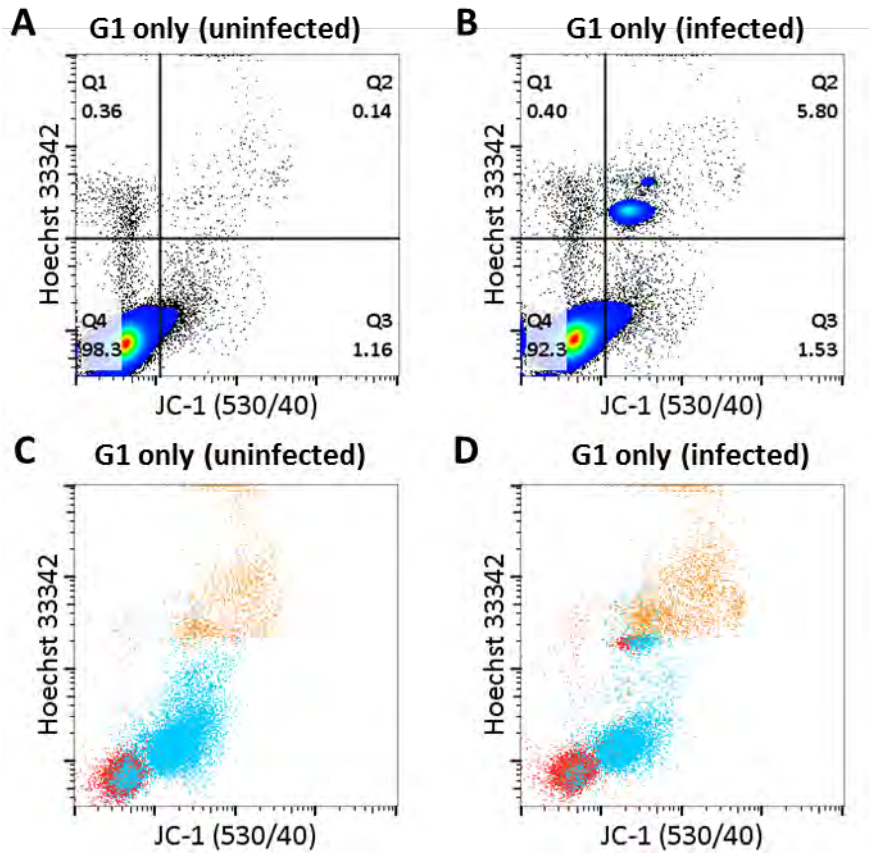
Supplementary figure 3.1. Background staining using nucleic acid-specific dyes. Background staining of HJ-RBCs in uninfected mice was observed when using the nucleic acid-specific dyes SYTO 16 (A), thiazole orange (C) and dihydroethidium (E). These populations were indistinguishable from parasitized cells in equivalent samples from infected mice (B, D, and F).



Supplementary figure 3.2. Optimization of JC-1 staining. A blood sample was taken from a *P. chabaudi adami* DS infected mouse and incubated with different concentrations of JC-1. Cells were analysed by flow cytometry (A), and the optimal staining concentration was 12µM as determined by the ratio of the mean fluorescence intensity (MFI) of the JC-1 positive population compared to the negative population (B).



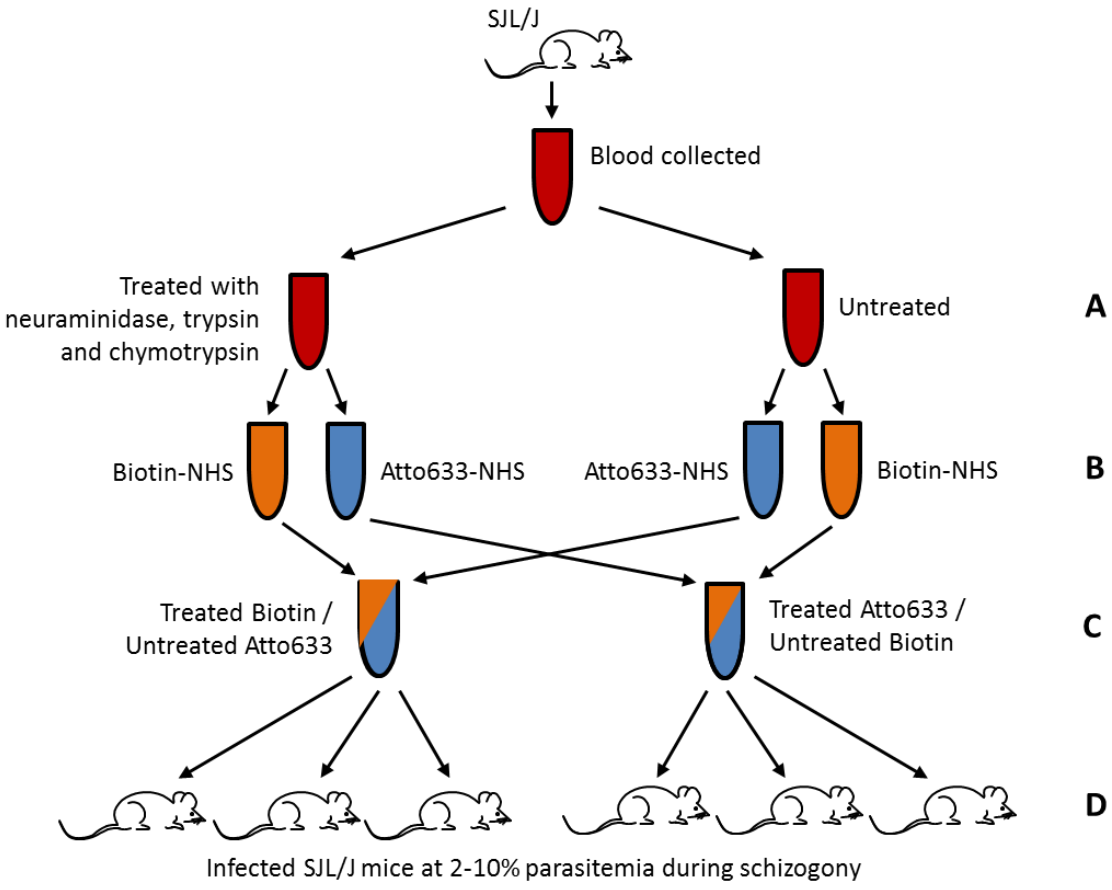
Supplementary figure 3.3. Hoechst 34580 and JC-1 staining of uninfected and infected blood. Blood samples were collected from uninfected (A) and *P. chabaudi adami* DS infected (B) mice and stained with JC-1, anti-CD45 APC eFluor780, anti-CD71 PerCP eFluor710, and Hoechst 34580. Samples were gated up to G4 as in Figure 3.1 except that the forward scatter peak height to area ratio was used to distinguish single cells rather than trigger pulse width.



Supplementary figure 3.4. Hoechst and JC-1 staining of uninfected and infected blood without using antibodies. Blood samples were collected from uninfected (A) and *P. chabaudi adami* DS infected (B) mice and stained as in Figure 3.2. Samples were gated based on trigger pulse width and FSC/SSC up to G1 as in Figure 3.1 without using antibody staining to remove leukocytes and reticulocytes from the analysis. The overlap between mature red blood cells (red), leukocytes (orange) and reticulocytes (blue) is shown (C, D).

Mouse	Biotin label	Atto633 label	30 minutes after transfusion				3 hours after transfusion			
			Endogenous parasitemia*	Biotin parasitemia	Atto633 parasitemia	Parasitemia ratio#	Endogenous parasitemia*	Biotin parasitemia	Atto633 parasitemia	Parasitemia ratio#
Untreated vs. untreated										
1	Untreated	Untreated	8.20%	0.50%	0.50%	1.02	8.60%	0.80%	0.80%	1
2	Untreated	Untreated	25.10%	1.30%	1.30%	0.99	27.00%	3.80%	3.80%	1
3	Untreated	Untreated	42.40%	4.30%	4.40%	0.98	45.80%	12.90%	13.30%	0.97
4	Untreated	Untreated	2.80%	0.40%	0.40%	1	3.10%	0.50%	0.50%	0.94
5	Untreated	Untreated	8.30%	0.80%	0.90%	1.1	9.50%	1.70%	1.70%	1.03
Treated vs. untreated										
1	Treated	Untreated	30.00%	2.20%	4.10%	0.54	32.00%	4.50%	8.30%	0.54
2	Treated	Untreated	15.40%	3.10%	4.50%	0.68	20.00%	5.60%	10.00%	0.56
3	Treated	Untreated	15.80%	3.20%	4.90%	0.64	19.90%	4.80%	11.40%	0.42
4	Untreated	Treated	18.70%	4.30%	2.90%	0.68	23.40%	8.80%	5.20%	0.59
5	Untreated	Treated	21.50%	4.00%	2.70%	0.66	27.20%	8.20%	4.60%	0.56
6	Untreated	Treated	25.70%	3.20%	2.40%	0.76	29.20%	6.20%	4.10%	0.66

Supplementary figure 3.5. Results from individual infected mice included in the *in vivo* parasite invasion assay. Complete data set for each mouse from the *in vivo* parasite invasion assays. *Endogenous parasitemia is the parasitemia of unlabelled RBCs in each mouse. # Parasitemia ratio is treated parasitemia dived by untreated parasitemia



Supplementary figure 3.6. Schematic representation of the *in vivo* parasite invasion assay. Blood was collected from uninfected SJL/J mice and divided into two tubes. One tube is treated with neuraminidase, trypsin, and chymotrypsin, which are known to inhibit parasite invasion while the other sample is left untreated (A). These tubes are again divided into two tubes and one is labelled with Biotin-NHS and the other with Atto 633-NHS (B). Samples were then combined in two combinations; Biotin labelled treated RBCs with Atto 633 labelled untreated RBCs and Atto 633 labelled treated RBCs with Biotin labelled untreated RBCs (C). These two combinations were injected separately into two lots of infected mice during schizogony at 2-10% parasitaemia (D).

CHAPTER 4

TFRC MUTANT MOUSE LINE MRI24910

CHAPTER INTRODUCTION

This chapter describes results from the study of the mouse line MRI24910. This line was identified from the dominant ENU mutagenesis screen based on a significantly reduced mean corpuscular volume (MCV), which was predicted to reduce malaria susceptibility in these mice. Although mice instead turned out to be more susceptible to malaria, they remained a line of interest for two reasons. Firstly, The ENU-induced mutation responsible for reduced MCV was found in the *Tfrc* gene, which is involved in iron transport and homeostasis. The role of iron in malaria infection is hotly debated and has major implications regarding iron supplementation in malaria endemic areas. This study therefore sought to assess the role of iron in the increased malaria susceptibility of these mice. Secondly, these mice provided a unique opportunity to study physiological changes in the erythrocyte and mouse which increased, rather than decreased, susceptibility to *Plasmodium*. This assisted in narrowing down which factors inhibit *Plasmodium* infection of abnormal erythrocytes, by the examination of common traits between these mice, and other conditions which decrease malaria susceptibility.

BACKGROUND

Iron is essential for cellular growth in almost all organisms through its involvement in DNA synthesis, oxygen metabolism, as an enzyme cofactor and as part of the haem molecule. Microbial pathogens such as protozoa, fungi and bacteria are no exception, and are reliant on iron for growth and survival[368]. The potential to exploit this dependence has important therapeutic implications. The iron dependence of *Plasmodium* is well documented, and has been demonstrated by the inhibitory effects of iron chelators on parasite growth *in vitro*[368,369]. However, translating this outcome into chelation antimalarials *in vivo* has been controversial, with several clinical trials producing inconclusive results[370-375]. In fact, due to widespread iron deficiency, iron supplementation is commonplace in malaria endemic areas, and there are several reports that this may exacerbate the risk of malaria infection[376-378]. Notably a large iron supplementation trial in Pemba was terminated

due to an increased risk of malaria in people receiving iron[379]. A clearer understanding of parasite and host iron metabolism is needed to inform decisions regarding iron supplementation and the use of iron chelators, both of which have widespread implications in treatment practices for malaria.

Iron homeostasis in the host during malaria infection is complex and incompletely understood[380]. During infection, expression of the iron regulatory hormone, hepcidin, increases significantly, leading to decreased iron availability, both through reduced dietary absorption and increased storage[381,382]. It has been speculated that this upregulation of hepcidin may exacerbate malaria anaemia due to a suppression of erythropoiesis[381-384]. Additionally, absorption of supplemental iron, often given during malaria to offset anaemia, is reduced, possibly due to the effects of hepcidin upregulation[384,385]. However, it has also been suggested that the restriction of iron bioavailability may be a host defence mechanism designed to inhibit parasite access to iron[386,387]. Additionally, it has been found that this host iron sequestration prevents the development of *Plasmodium* sporozoites in the liver[381]. The inhibition of liver stage malaria, in people already carrying the disease, partially protects against compounding infections by multiple parasite strains and species[381]. Expression of the iron regulatory protein lipocalin 2 (Lcn2) is also increased during malaria infection[388], and studies of knockout mice indicate a key role for Lcn2 in modulating the innate and adaptive immune response to infection through its influence on iron recycling[389]. It is speculated that Lcn2 redistributes iron to control erythropoiesis and immune cell development during infection, although its exact role remains uncertain.

While iron homeostasis can have a direct impact on the outcome of malaria infection in terms of systemic iron availability and immune modulation, it also impacts erythrocyte physiology, the target cell of blood stage malaria infection. Iron forms part of the haem molecule which, as a complex with haemoglobin, comprises 96% of the dry weight of erythrocytes[49]. Iron deficiency impedes normal erythrocyte development, leading to reduced cell volume and haemoglobin density. Interestingly, parasite invasion and replication are inhibited in iron deficient erythrocytes compared to normal erythrocytes *in vitro*, although the reason for this has not been determined[390]. In addition,

parasitized iron deficient erythrocytes are more likely to display phosphatidylserine, which results in enhanced phagocytosis of these cells relative to equivalent parasitized control erythrocytes{Koka, 2007 #911}[304]. The reasons for this increased exposure are also undetermined, although it seems that iron deficient RBCs are more susceptible to eryptosis caused by calcium influx{Matsuzaki-Moriya, 2011 #912}. Overall, evidence suggests that alterations in both iron homeostasis and erythrocyte physiology significantly impact on the course of malaria infection, however, the biological mechanisms underlying these changes remain unclear.

Transferrin receptor 1 (Tfr1) is a major protein involved in transporting iron into cells via the iron carrier transferrin and is essential for erythropoiesis[391] (Figure 4.1). It is involved in iron homeostasis through its interaction with the haemochromatosis protein (Hfe) by the following mechanism: iron loaded transferrin competes with Hfe in binding to Tfr1, such that increased/decreased binding sites on Tfr1 lead to decreased/increased Hfe in circulation[395-397]. Mice with mutations induced in Tfr1 which promote/prevent binding of Hfe display decreased/increased hepcidin expression respectively[393]. Finally, hepcidin influences dietary iron absorption and iron recycling by inactivating the iron export protein ferroporitin[392-394]. The expression of Tfr1 also impacts erythrocyte physiology, with Tfr1 deficiency resulting in reduced erythrocyte volume in a similar manner to systematic iron deficiency[391].

This study investigated the role of Tfr1 in iron homeostasis and erythrocyte physiology, in the context of rodent malaria infection, through the characterization of an ENU mutagenised mouse line. It was hypothesized that physiological changes in the erythrocyte, brought on by insufficient iron availability during erythropoiesis due to the lack of Tfr1, would impair parasite invasion and replication within these cells. Furthermore, changes in iron homeostasis in these mice were predicted to alter the course of infection by influencing iron availability and modulating the erythropoietic response.

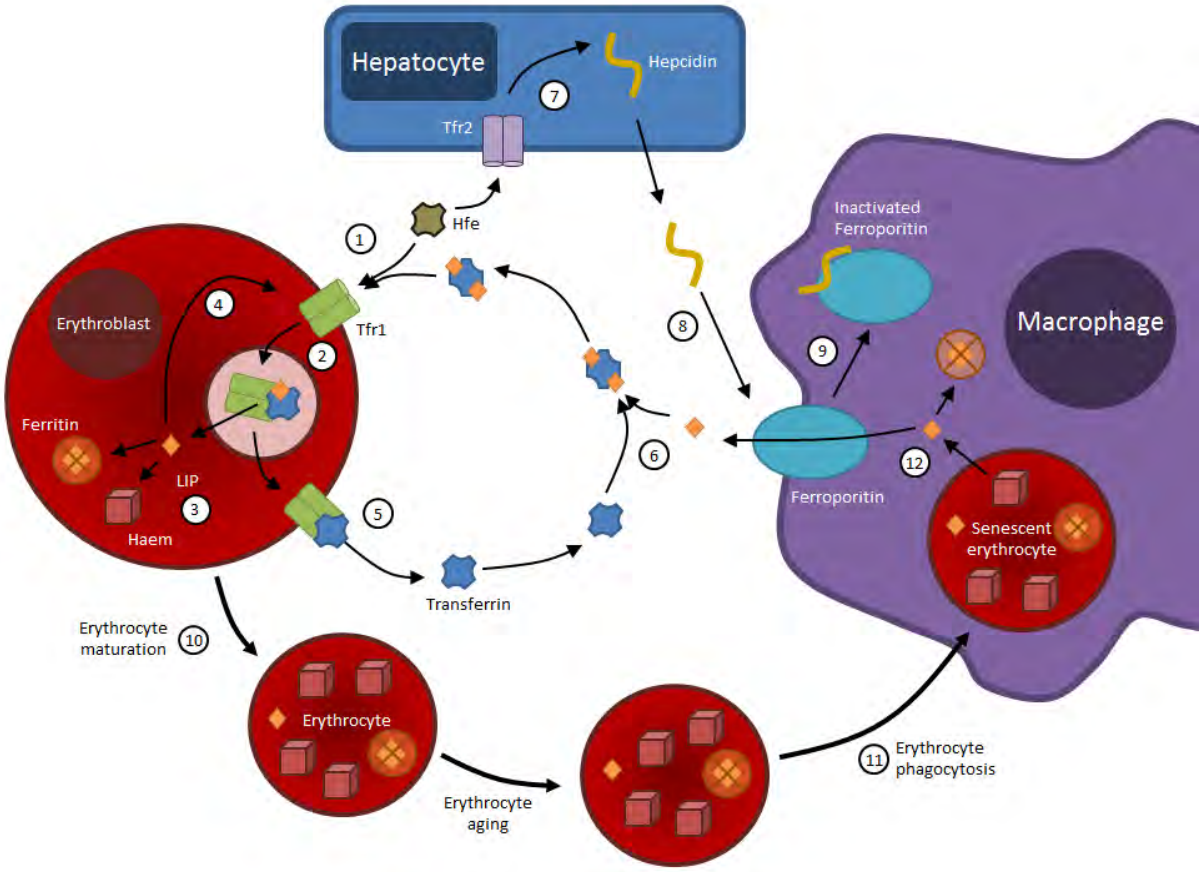


Figure 4.1. Tfr1 iron transport and influence on iron homeostasis. Iron loaded transferrin (Tf) competes with Hfe in binding to Tfr1 (1). The Tfr1-Tf complex is internalized and iron exported into the cytosol (2). The labile iron pool (LIP) of the cytosol is either stored in ferritin complexes or converted to haem (3). The LIP also influences expression of ferritin andTfr1 in a feedback loop (4). After release of iron, the Tfr1-Tf complex is returned to the cell surface where Tf is disassociated (5). Free serum iron binds to Tf, restarting the cycle (6). Meanwhile, unbound Hfe associates with transferrin receptor 2 (Tfr2), which induces expression of hepcidin by hepatocytes (7). Hepcidin binds to the iron export protein ferroportin (8), triggering its internalization and inactivation, and preventing iron release from macrophages and enterocytes (9). Erythrocyte recycling is the main source of iron. As erythroblasts mature, erythrocytic iron is contained mainly within haem, but also within ferritin and in the LIP (10). Senescent erythrocytes are phagocytized by splenic macrophages (11), which break down haem into free iron for storage in ferritin or export by ferroportin (12).

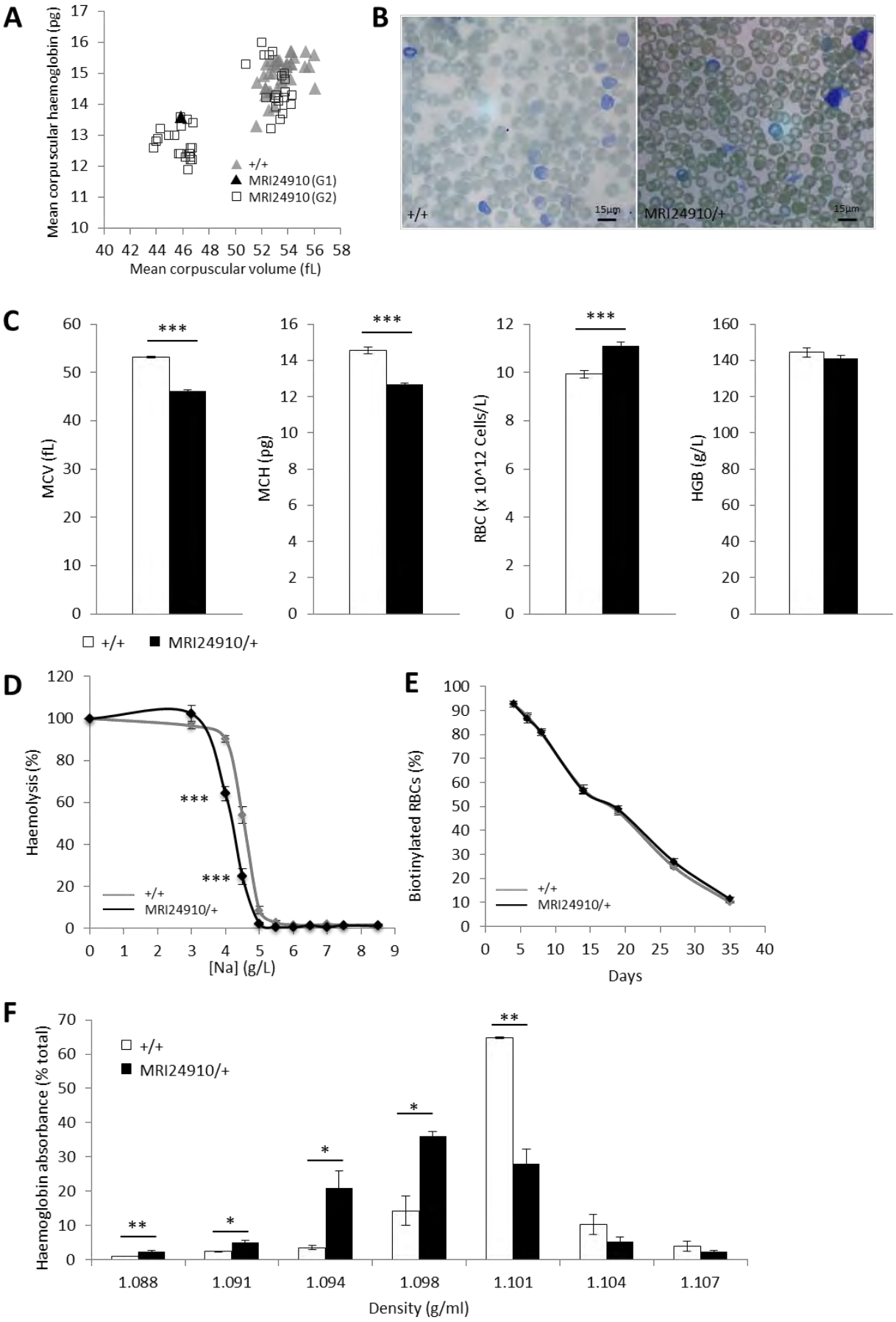
RESULTS

Identification of a novel ENU-induced mutation in Tfrc *in a mouse strain with a dominant microcytosis phenotype*

An automated full blood count was performed on seven week old G1 offspring from ENU mutagenized mice to identify mutations causing erythrocyte abnormalities. The G1 mouse MRI24910 was selected from the ENU dominant mutagenesis screen based on a decreased mean cell volume

(MCV) of 45.9 fL, which was more than three standard deviations below the mean (53.1 ± 1.3 fL) (Figure 4.2 1A). G2 mice displayed this phenotype in a one-to-one ratio confirming a fully penetrant, dominant mutation in the MRI24910 line causing microcytosis (46.8 ± 0.6 fL) (Figure 4.2 A, C). To determine if microcytosis was associated with any morphological defects in erythrocytes, blood smears were obtained from mutant and wild type mice. Erythrocytes from mutant mice (MRI24910/+) were indistinguishable from wild type (+/+), and displayed a normal, discoid shape, with no identifiable abnormalities (Figure 4.2 B). Additional parameters from the automated full blood count indicated mutant mice had reduced erythrocyte haemoglobin, which was compensated for by an increased erythrocyte count, and resulted in a normal level of haemoglobin by blood volume (Figure 4.2 C). The response of mutant erythrocytes to osmotic stress was assessed by measuring the extent of haemolysis of erythrocytes in different concentrations of salt solution. Mutant erythrocytes had significantly decreased osmotic fragility, which indicates the erythrocyte membrane of these cells holds together at least as well as wild type under stress (Figure 4.2 D). To investigate the *in vivo* half-life of mutant erythrocytes mutant and wild type mice were injected with biotin and the proportion of biotinylated erythrocytes was monitored over time. Mutant biotinylated erythrocytes were cleared from circulation at equal rate to wild type, indicating erythrocytes are not more susceptible to senescence or splenic clearance (Figure 4.2 E).

Figure 4.2. **Haematological properties of mutant line MRI24910.** ENU mutagenized offspring including the G1 mouse MRI24910, and G2 offspring. Two populations exist in the G2 population at a Mendelian ratio of 1:1, mice were assumed to be carrying the mutation if their MCV was less than 48 fL, and are referred to as MRI24910/+, while mice with MCV greater than 48fL are referred to as wild type (+/+) (A). Giemsa stained blood smear of wild type, and mutant mice (B). Haematological properties from an automated blood count. MCV – mean corpuscular volume, MCH – mean corpuscular haemoglobin, RBC – erythrocyte count, HGB – total haemoglobin by blood volume (C). Osmotic fragility of erythrocytes based on haemolysis in salt solution (D). Erythrocyte half-life based on the percentage biotinylated cells remaining in circulation after an initial biotin injection which labelled >99% of erythrocytes (E). Quantification of the relative number of erythrocytes, based on haemoglobin absorbance, in each density layer separated on a discontinuous Percoll gradient (F). Error bars indicate SEM, * $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$. Data is from 20 mice per group for the automated blood count, 4-9 per group for the osmotic fragility test, erythrocyte half-life, and density.


A
Mean corpuscular haemoglobin (pg)
Mean corpuscular volume (fL)
+/+
MRI24910 (G1)
MRI24910 (G2)
B
+/+
15µm
MRI24910/+
15µm
C
MCV (fL)
MCH (pg)
RBC (x 10^12 Cells/L)
HGB (g/L)
***
+/+
MRI24910/+
D
Haemolysis (%)
[Na] (g/L)
***
***
+/+
MRI24910/+
E
Biotinylated RBCs (%)
Days
+/+
MRI24910/+
F
Haemoglobin absorbance (% total)
Density (g/ml)
+/+
MRI24910/+
**
*
*
*
**
1.088
1.091
1.094
1.098
1.101
1.104
1.107

To determine if the mutation resulted in a change in erythrocyte density erythrocytes were separated on a discontinuous Percoll gradient. The proportion of erythrocytes in each density layer was then determined by lysing cells and measuring the amount of haemoglobin absorbance produced. A significantly higher proportion of mutant erythrocytes were found in lower density layers, indicating the mutation may reduce the density of erythrocytes (Figure 4.2 F). Overall, these results indicate a dominant mutation, which has resulted in a regenerative microcytic anaemia, without the loss of erythrocyte membrane cohesion under osmotic stress.

To determine if homozygosity of this mutation is compatible with survival, mice with the abnormal haematological phenotype were mated. Offspring from these pairs only exhibited two phenotypes, the parent's phenotype, and a wild type phenotype. The lack of a third phenotype indicates the homozygous mutation may result in perinatal lethality. In support of this, timed matings of microcytic mice bred together revealed evidence of complete embryo re-absorption at E-13.5, further indicating that homozygosity for this mutation is not compatible with life.

The ENU-induced mutation responsible for this phenotype was mapped by backcrossing the original G1 mouse, MRI24910, to a C57BL/6 mouse. Affected mice were identified based on microcytosis (MCV <48 fL) and linkage analysis was performed as described in Chapter 2. This analysis identified a significant linkage peak on chromosome 16, between 7Mbp and 67Mbp (Supplementary figure 4.1 A, B). The *Tfrc* gene at 32.6Mbp was an ideal candidate gene as a deficiency in this gene has been reported to cause a similar dominant microcytosis phenotype to that observed here[391]. Sanger sequencing of all exons of the *Tfrc* gene revealed a T to C transition in exon 5 leading to a serine to proline substitution (S161P) (Supplementary figure 4.1 C). This mutation is in the protease-like domain of the transferrin receptor 1 protein (Tfr1), which in the equivalent region of the human protein is involved in the binding of transferrin[398] (Figure 4.3 A). The S161 amino acid is highly conserved indicating it plays a key role in protein function (Figure 4.3 B). Due to the wide spread mutagenesis caused by ENU treatment, It is possible that the mutation in *Tfrc* is not responsible for the observed phenotype. However, this is unlikely for several reasons: the causative mutation must

be within the interval defined by linkage mapping, therefore discounting ENU-induced mutations in other regions of the genome, the haematological phenotype closely matches that of previous *Tfrc* mutant mice and it is unlikely a mutation in a different gene would present the same phenotype, finally the mutation is in a highly conserved amino acid which suggests this mutation would produce a phenotype consistent with that observed. It was therefore hypothesized that this mutant line, designated $Tfrc^{MRI24910}$, results in perturbations in iron homeostasis which may explain the abnormal haematological phenotype of these mice.

The MRI24910 mutation in Tfrc *results in reduced protein expression and altered splenic iron homeostasis*

To investigate the effect of this mutation on Tfr1 expression the cell surface levels of protein on splenic erythroblasts, which express particularly high levels of Tfr1 in order to obtain iron for haem synthesis, were measured. Tfr1 transports iron into the cell through endocytosis, therefore, only a proportion of the protein is present on the cell surface at any one time. To account for this, cells were incubated in iron free media to stimulate and maximize the externalization of the protein before analysis. Cells were then stained with anti-CD71 (Tfr1) antibody, and also the DNA dye Hoechst and anti-TER119 antibody to facilitate the identification of erythroblasts and reticulocytes by flow cytometry (Figure 4.3 C, D). TER119 is a cell surface marker only present on late erythroblasts, reticulocytes, and mature erythrocytes. The mean fluorescence intensity (MFI) of staining with the CD71 (Tfr1) antibody was used to quantify levels of surface Tfr1. Splenic erythroblasts and reticulocytes from $Tfrc^{24910/+}$ mice had significantly less surface Tfr1 compared to wild type per cell based on mean fluorescence intensity (38% and 42% less, respectively) (Figure 4.3 E). This indicates that the mutant gene copy is either not expressed, resulting in haploinsufficiency of Tfr1 in this cell type, or that the mutant protein is expressed but does not undergo normal endocytic recycling and reach the surface of cell. In either case, this indicates that microcytosis in these mice is likely caused by insufficient iron transport into erythroblasts during erythropoiesis.

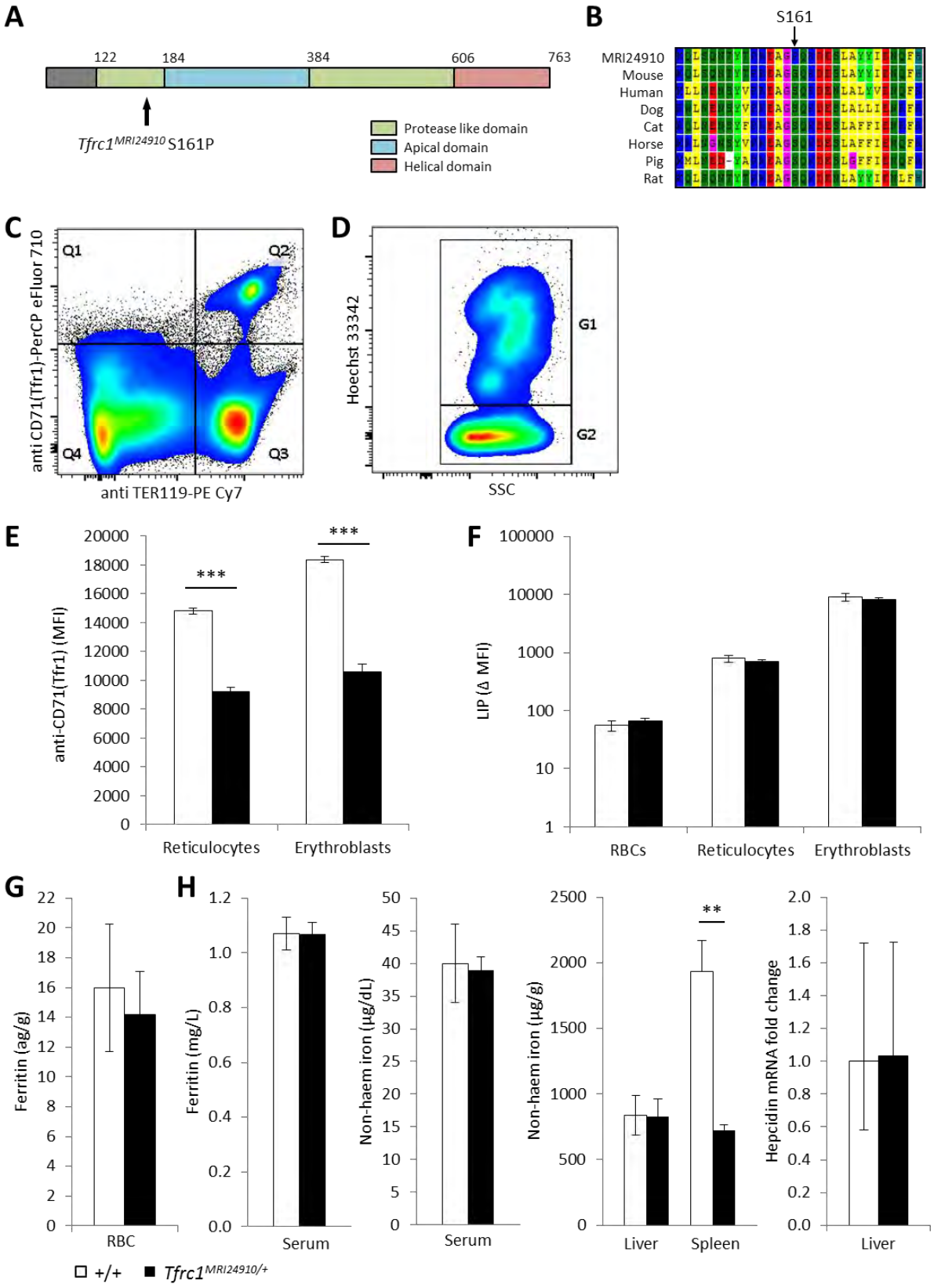

A
122
184
384
606
763
Tfrc1MRI24910 S161P
Protease like domain
Apical domain
Helical domain
B
S161
MRI24910
Mouse
Human
Dog
Cat
Horse
Pig
Rat
C
Q1
Q2
Q3
Q4
anti CD71(Tfr1)-PerCP eFluor 710
anti TER119-PE Cy7
D
G1
G2
Hoechst 33342
SSC
E
anti-CD71(Tfr1) (MFI)
***
***
Reticulocytes
Erythroblasts
F
LIP (Δ MFI)
RBCs
Reticulocytes
Erythroblasts
G
Ferritin (ag/g)
RBC
H
Ferritin (mg/L)
Serum
Non-haem iron (µg/dL)
Serum
Non-haem iron (µg/g)
**
Liver
Spleen
Hepcidin mRNA fold change
Liver
+/+
Tfrc1MRI24910/+

Figure 4.3. **Identification and characterisation of the MRI24910 mutation in *Tfrc*.** A S161P point mutation was located in the protease like domain of *Tfrc* (A). The S161 residue is highly conserved, indicating it has a critical function in the protein (B). Splenic erythroblasts and reticulocytes where identified based on positive staining with the markers TER119 and CD71 (Tfr1) (Q2), and erythrocytes based on negative CD71 and positive TER119 staining (Q3) (C). From the Q2 gate, erythroblasts (G1) were distinguished from reticulocytes (G2) based on Hoechst staining (D). Mean fluorescent intensity (MFI) of staining with anti-CD71 (Tfr1) was determined in each cell population as a measure of Tfr1 surface expression (E). The labile iron pool (LIP) of each cell population determined based on the change in calcein-AM MFI with/without the iron chelator L1 according to Prus and Fibach[328] (F). Ferritin content as measured by ELISA in erythrocytes (G) and serum (H). Non-haem iron content measured colourmetrically in serum, liver and spleen, and hepcidin mRNA expression in liver relative to wild type using beta actin as a housekeeper gene (H). Error bars indicate SEM, ** $p < 0.01$, *** $p < 0.001$. Data is representative of 1 of 3 experiments with 4 mice per group for Tfr1 MFI. For other experiments, data is from 4 mice per group for LIP, ferritin, serum iron, and hepcidin, and 7-8 mice per group for liver and spleen iron.

To investigate if the decreased surface expression of Tfr1 alters cellular iron management during erythropoiesis, the labile iron pool (LIP) of erythrocytes, reticulocytes, and erythroblasts, as well as intraerythrocytic ferritin content, was measured. Cellular iron homeostasis and Tfr1 expression within erythroblasts is tightly regulated by the iron regulatory proteins (Irp1 and Irp2), which in turn sense the cellular LIP status[399-401]. An excess LIP results in up-regulation of ferritin expression, while low LIP level leads to increased expression of Tfr1. Analysis of the LIP in erythroid progenitors (erythroblasts and reticulocytes) as well as erythrocytes found no difference between mutant and wild type cells (Figure 4.3 F). This indicates that while Tfr1 expression is reduced, cellular iron homeostasis is still maintained within mutant cells. In support of this, intraerythrocytic ferritin levels were also found to be normal in mutant mice (Figure 4.3 G). There was also no difference in the proportion of splenic erythroblasts and reticulocytes between mutants and wild type mice, and no difference in the proportion of peripheral blood reticulocytes (Supplementary figure 4.2 A, B). Overall, these results indicate that the *Tfrc* mutation causes reduced surface expression of Tfr1, but does not result in any major perturbations to cellular iron homeostasis. Erythroblasts are able to mature into apparently normal functioning erythrocytes, albeit of a reduced size and reduced haemoglobin content.

It was next investigated if the reduction of Tfr1 surface expression on erythroblasts affected systematic iron homeostasis. The iron regulatory protein Hfe binds to Tfr1, therefore, a reduction in

Tfr1 could plausibly lead to an increase in the amount of circulating Hfe. An increase in circulating Hfe would in turn influence systematic iron homeostasis through its role in regulating hepcidin expression[393]. Analysis of mutant and wild-type mice did not detect any differences in serum ferritin or non-haem bound iron to indicate a change in systematic iron homeostasis (Figure 4.3 H). Additionally, no difference was found in the level of non-haem bound iron, and no difference in hepcidin expression in the liver (Figure 4.3 H). In contrast, non-haem bound iron in the spleen was significantly reduced in mutant mice (Figure 4.3 H). The spleen is directly involved in erythropoiesis and erythrocyte iron recycling. Since cellular iron homeostasis and abundance of splenic erythropoietic cells was normal (Figure 4.3 F, G; Supplementary figure 4.2), this result could instead indicate an abnormality in the recycling of erythrocytes. Perhaps the phagocytosis of senescent erythrocytes by splenic macrophages is impeded, or alternatively iron export may be accelerated in these cells to compensate for the reduced uptake by erythroid progenitors. Together these results suggest that systemic iron availability is normal in mutant mice. Instead the microcytic phenotype may be the result of impaired iron uptake in the erythropoietic system.

Tfrc$^{MRI24910/+}$ *mice display increased susceptibility to Plasmodium* chabaudi adami *DS infection*

Iron deficiency anaemia has been reported to reduce susceptibility to malaria infection in humans and mice[302,304]. Therefore, it was next investigated if the altered Tfr1 expression of erythroid cells and erythrocyte physiological abnormalities in *Tfrc1*$^{MRI24910/+}$ mice would influence the course of malaria infection. Mice were challenged with the rodent malaria parasite *Plasmodium chabaudi adami* DS and parasitaemia and survival were monitored daily. Parasitaemia of mutant mice increased more rapidly than their wild type littermates, resulting in a significantly higher parasitaemia during the acute stage infection, before peak parasitaemia (Figure 4.4 A). After the peak of infection parasitaemia decreased at an equal rate in both mutant and wild type mice. SJL/J mice are naturally susceptible to *P. chabaudi* infection and rarely survive when challenged with this strain. Nevertheless, mutant mice succumbed to infection earlier than wild type, although the difference in survival was not significant (p = 0.067) (Figure 4.4 B).

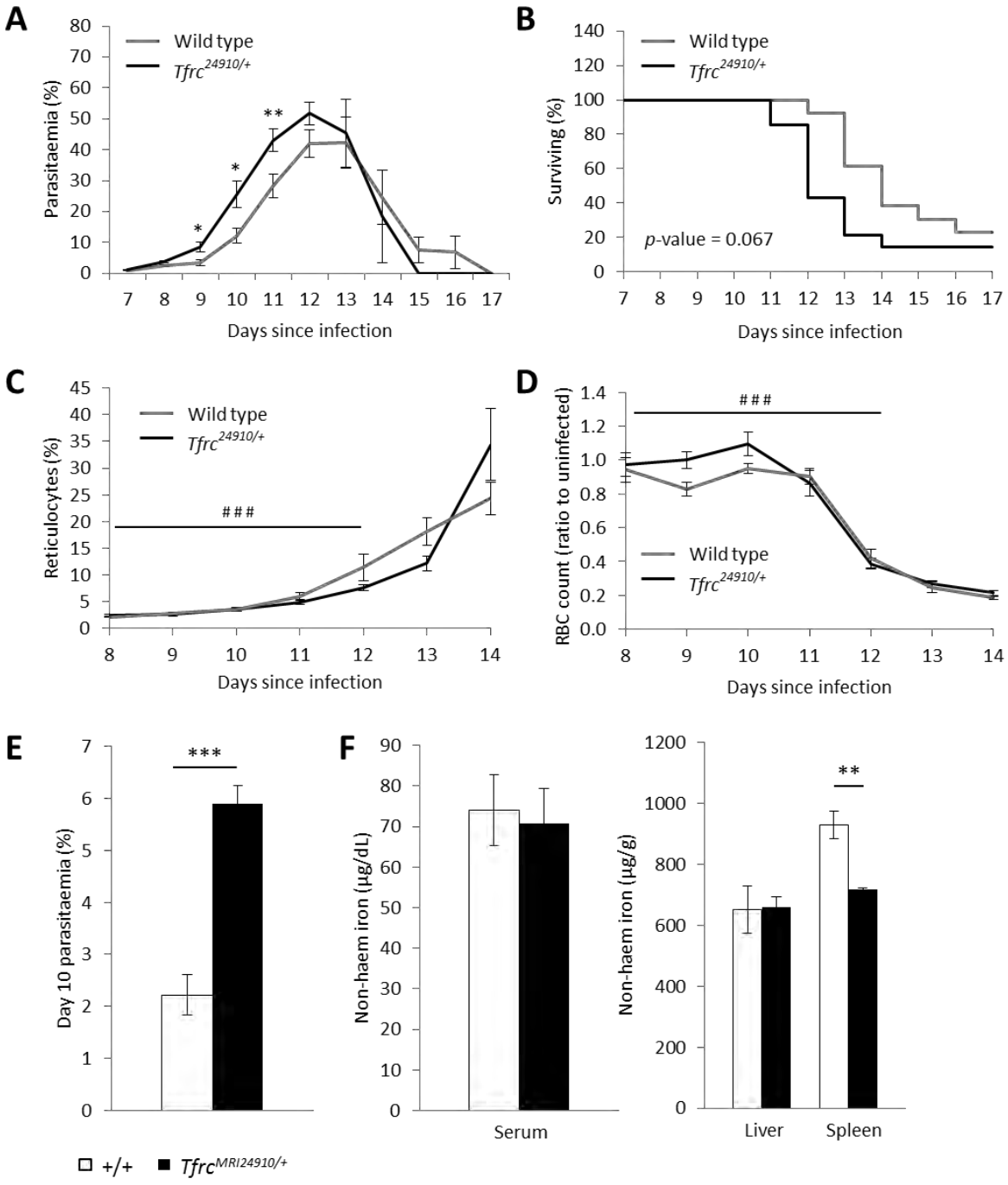


Figure 4.4. **Susceptibility of *Tfrc*$^{MRI24910/+}$ to *P. chabaudi adami* DS infection.** Parasitaemia (A), and survival curve (B) for female mice combined from three separate experiments. Parasitaemia was determined by either counting of Giemsa stained slides or by flow cytometry using the DNA dye Hoechst 34580. Mice were infected with 1x10^4 parasitised erythrocytes. In one experiment reticulocyte (C) and erythrocyte (D) count was determined by flow cytometry. In another experiment mice were sacrificed on day 10 of infection. Parasitaemia (E), and non-haem iron in serum, liver, and spleen (F) was determined. Error bars indicate SEM, the log-rank (Mantel-Cox) test was used to calculate *p*-value for survival. * $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$ between wild type and mutant. ### $p < 0.001$ between wild type mice on day 8 and wild type mice on day 12 of infection. Data is combined from 3 experiments with 4-12 female mice per group for parasitaemia and survival. Data is from 1 experiment with 4-6 mice per group for reticulocyte and erythrocyte count, and 1 experiment with 4-5 mice per group for day 10 parasitaemia and iron measurement.

Iron homeostasis and the erythropoietic response to infection is normal in Tfrc$^{MRI24910/+}$ *mice*

In order to explain the increased parasitaemia in *Tfrc1*$^{MRI24910/+}$ mice it was considered if the increased susceptibility phenotype was due to a systematic effect, i.e. mutant mice have an impaired erythropoietic response during infection, or if this was because of physiological changes to erythrocyte itself, i.e. the parasite is able to survive more easily within the mutant erythrocyte.

To address the first hypothesis, erythropoietic response and iron homoeostasis were investigated during the course of infection. To assess the erythropoietic response, the total remaining cells (erythrocytes and reticulocytes), as well as the proportion of reticulocytes, were measured during infection. Reticulocyte abundance increased significantly, in concordance with decreasing total remaining cells (Figure 4.4 C, D). However, there were no differences between mutant and wild type mice, indicating that the erythropoietic response to infection is unchanged in *Tfrc1*$^{MRI24910/+}$, and is not the reason for the increased parasitaemia observed. During malaria infection the parasite must obtain iron from its host in order to survive and replicate. It has been suggested that the host sequesters iron during infection in order to reduce parasite growth[387]. It was therefore investigated if a change in iron homeostasis during infection could explain the increased parasitaemia of mutant mice. Serum and tissue iron abundance were measured in a cohort of mice on day 10 of infection. Although mutant mice had a significantly higher parasitaemia there was no difference in serum iron or liver iron to indicate abnormal iron homeostasis during infection (Figure 4.4 E, F), while spleen iron remained reduced in mutant mice as observed when uninfected (Figure 4.4 F). Together these results suggest that the increased parasitaemia of mutant mice is not due to a change in these systematic responses to infection.

Parasites have a survival advantage within Tfrc$^{MRI24910/+}$ *erythrocytes*

To investigate if the increased parasitaemia was due to an erythrocyte autonomous effect, two parameters were assessed that influence parasite abundance during infection, 1) the ability of the parasite to invade erythrocytes, 2) the survival of the parasite within the erythrocyte until its

replication and egress. To measure these parameters a labelled erythrocyte assay was performed as previously described[402] (see chapter 3). In these experiments the parasitaemia of two labelled erythrocyte populations are compared within one mouse, allowing for direct comparison of the parasite's interaction with different erythrocyte types (Figure 4.5 A, B). In this assay it was found that three hours after the injection of labelled mutant and wild type erythrocytes into an infected wild type mouse, equal numbers of parasites were found in each erythrocyte population (Figure 4.5 C, E). This indicates that the *Tfrc* mutation has no effect on the parasites ability to invade erythrocytes and therefore differential parasite invasion between *Tfrc1*$^{MRI24910/+}$ and wild type mice cannot explain the increased parasitaemia of mutant mice. In contrast, at 7, 15, and 23 hours after injection, by which stage parasites will have progressively matured from ring stage, to trophozoite stage, and onto schizogony, parasitaemia of the mutant erythrocyte population was significantly higher than wild type (Figure 4.5 C, E). Furthermore, a similar result was obtained when labelled erythrocytes were injected into an infected mutant mouse (Figure 4.5 D, F). It was therefore hypothesised that parasites growing within *Tfrc* mutant erythrocytes have a survival advantage compared to those within wild type erythrocytes. To explain this, it was further hypothesised that parasitised wild type erythrocytes are removed from circulation more rapidly than parasitised mutant erythrocytes. To investigate the later hypothesis the proportion of total remaining labelled erythrocytes were measured during this assay. However, labelled erythrocytes were cleared from circulation at an equal rate (Figure 4.5 G, H), indicating that reduced clearance of *Tfrc* mutant erythrocytes cannot explain the observed increase in parasitaemia in these cells. Overall, these results suggest that although parasites invade mutant and wild type erythrocytes equally, they have an erythrocyte autonomous survival advantage in mutant erythrocytes. Although this survival increase was modest (9-15%), the cumulative effect of this survival advantage over multiple parasite replication cycles could plausibly explain the increased parasitaemia of *Tfrc1*$^{MRI24910/+}$ mice during infection.

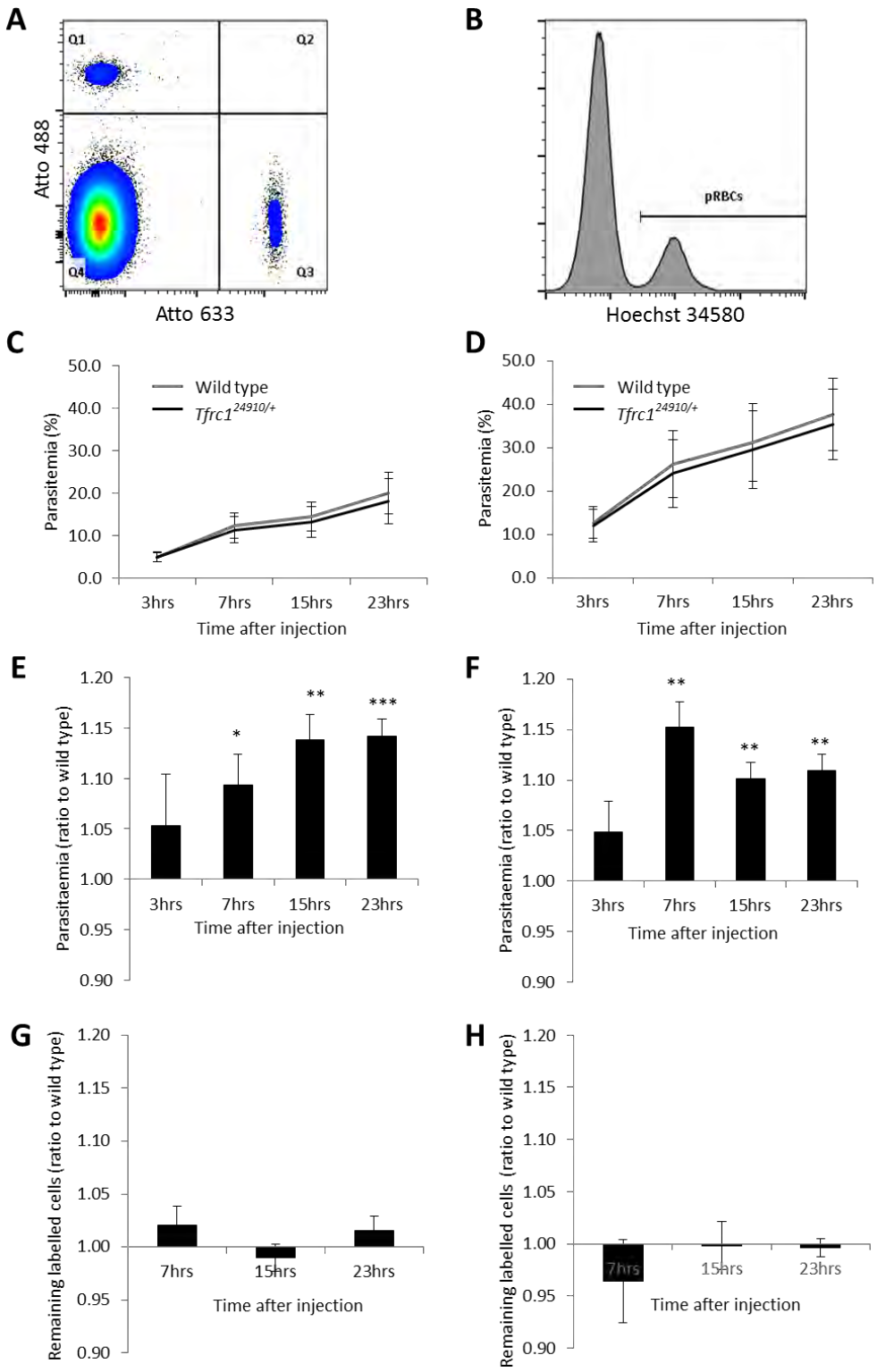


Figure 4.5. Increased *P. chabaudi adami* DS survival within *Tfrc*$^{MRI24910/+}$ erythrocytes. Transfused erythrocytes from mutant and wild type mice were identified according to their fluorescent label, Atto 488 (Q1), and Atto 633 (Q3) (A). In each labelled population parasitaemia was determined according to Hoechst 34580 fluorescence (B). Parasitaemia of labelled populations transfused into infected wild type (C), and mutant (D) mice. Ratio of parasitaemia of labelled mutant erythrocytes compared to labelled wild type erythrocytes in wild type (E), and mutant (F) mice. Ratio of total labelled mutant erythrocytes compared to labelled wild type erythrocytes in wild type (G), and mutant (H) mice. Two combinations of dye label were used to account for any dye effects. Error bars indicate SEM, * $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$. Data is from 7-8 mice in each experiment.

DISCUSSION

Iron homeostasis during malaria infection is complex and remains poorly understood[380]. Clinical studies have shown a 30-51% reduction in the risk of malaria in iron deficient, compared to iron replete, children in malaria endemic areas[302,403,404]. While research into the mechanisms responsible for this are lacking, evidence suggests that erythrocytes from patients with iron deficiency anaemia (IDA) inhibit parasite invasion and growth[303,390]. Furthermore, mouse models of IDA display a reduced susceptibility to rodent malaria infection[303,304]. Here, the response to malaria was assessed in a mouse line carrying a novel ENU-induced missense mutation in the *Tfrc* gene. Given the critical role of Tfr1 in facilitating iron uptake by erythroblasts, and its influence on systematic iron homeostasis, it was hypothesised that this mutation would reduce malaria susceptibility in a similar manner to IDA. In fact, the opposite was found to be true, with *Tfrc* mutant mice displaying an increased susceptibility to malaria.

There are several key differences between the mouse line described here and IDA. During iron deficiency, a systematic lack of iron leads to inadequate iron supply during erythropoiesis, resulting in microcytosis and hypochromia. As iron deficiency becomes more severe, IDA occurs, which is characterised by a reduced overall haemoglobin level, haematocrit, and blood count. For $Tfrc^{+/MRI24910}$ mice, erythroblasts have lower levels of surface Tfr1, which likely results in reduced iron transport into cells, and concomitant microcytosis. This outcome is similar to other mutant mouse lines which exhibit microcytosis and reduced surface levels of Tfr1 on erythroblasts, including the heterozygous knockout mouse line $Tfrc^{+/-}$[391,405,406]. In contrast to IDA, the $Tfrc^{+/MRI24910}$ microcytosis was accompanied by an increased blood count, resulting in a normal level of total haemoglobin (Figure 1), and a normal mean cell haemoglobin concentration (MCHC) (data not shown). It therefore seems that $Tfrc^{+/MRI24910}$ mice are able to compensate for the heterozygous *Tfrc* mutation by producing smaller erythrocytes, but in greater number. Likewise, systematic iron availability in $Tfrc^{+/MRI24910}$ mice was normal, as indicated by serum iron parameters, hepcidin expression, and liver iron stores, indicating mice are able to compensate for any perturbations in iron homeostasis due to this

mutation. This study therefore provides a unique insight into the influence of a compensatory reaction to iron restricted erythropoiesis on malaria infection, as distinct from the influence of systematic iron deficiency seen in IDA.

It has been previously suggested that parasite growth is inhibited in IDA due to the reduced bioavailability of iron[407]. While it remains unclear where and how the parasite obtains iron necessary for growth, evidence suggests that the parasite meets some of its need by importing iron from serum[408-410]. Reduced serum iron in IDA may therefore reduce the parasite's access to iron, thereby inhibiting growth. Systematic iron availability in the *Tfrc*$^{+/MRI24910}$ line is normal when uninfected, however, malaria infection results in a redistribution of host iron in order to limit iron availability to the invading parasite[381,389]. Iron redistribution was therefore investigated during infection in *Tfrc*$^{+/MRI24910}$ mice. However, iron homeostasis in the *Tfrc*$^{+/MRI24910}$ line was found to proceed in a similar manner to wild type mice during infection, indicating that the observed increase in malaria susceptibility is not attributable to differences in iron homeostasis.

It was next considered whether an altered erythrocyte physiology could account for the observed increase in malaria susceptibility. IDA erythrocytes have several similarities to *Tfrc*$^{+/MRI24910}$ erythrocytes; both are microcytic, display reduced osmotic fragility and reduced erythrocyte density[305]. However there are several key differences: while IDA erythrocytes have a reduced erythrocyte half-life, mean corpuscular haemoglobin concentration (MCHC), and intraerythrocytic ferritin content, in *Tfrc*$^{+/MRI24910}$ erythrocytes these parameters are normal. In terms of malaria susceptibility, the merozoite invasion rate of *Tfrc*$^{+/MRI24910}$ erythrocytes was unchanged compared to wild type. This is in contrast to IDA erythrocytes, which have been reported to be less susceptible to invasion[390]. In addition, parasites had an increased survival rate in *Tfrc*$^{+/MRI24910}$ erythrocytes compared to wild type. Parasite survival within the erythrocyte depends on its ability to grow, maintain erythrocyte integrity, avoid immune detection and phagocytosis, and pass through the small capillaries and filtering beds in the spleen. The increased survival of parasites within *Tfrc*$^{+/MRI24910}$ erythrocytes suggests that one or more of these aspects is altered in the mutant

parasitised erythrocytes. This finding is at odds with IDA erythrocytes, in which the parasite is more susceptible to clearance and therefore has a reduced rate of survival[303,304]. This enhanced clearance in IDA is thought to be caused by increased phosphatidylserine exposure, which mediates increased phagocytosis, although the reasons for this increased exposure are unknown[304]. These results indicate that physiological characteristics including microcytosis, density, and osmotic fragility of erythrocytes do not necessarily influence erythrocyte susceptibility to invasion or its ability to support parasite growth, at least in the *P. chabaudi* model. This finding is supported by several studies, both *in vivo* and *in vitro*, which indicate that microcytosis *per se* does not inhibit parasite growth, or reduce the risk of malaria[411,412]. It therefore seems that parameters such as haemoglobin concentration, intraerythrocytic ferritin levels, or other parameters not measured here, may have a greater impact on the parasite than factors such as microcytosis. This raises the question of whether iron deficiency without anaemia (IDWA), in which microcytosis is present without a reduction in haemoglobin concentration, influences malaria risk differently than IDA? Current studies have not addressed this possibility, with study groups defined either including both IDA and IDWA in one group[302,403,404], or IDWA being excluded from the study[390]. It would be of interest in future studies to determine if the differences in physiological characteristics in these conditions have an impact on the parasite. It also remains possible that other systematic factors not tested in this study, such as inflammation or the immune response, may contribute towards the increased susceptibility of *Tfrc*$^{+/MRI24910}$ mice. Overall, it can be concluded that the survival advantage of parasites within *Tfrc*$^{+/MRI24910}$ erythrocytes is a major factor in the increased susceptibility to malaria observed in this mouse line.

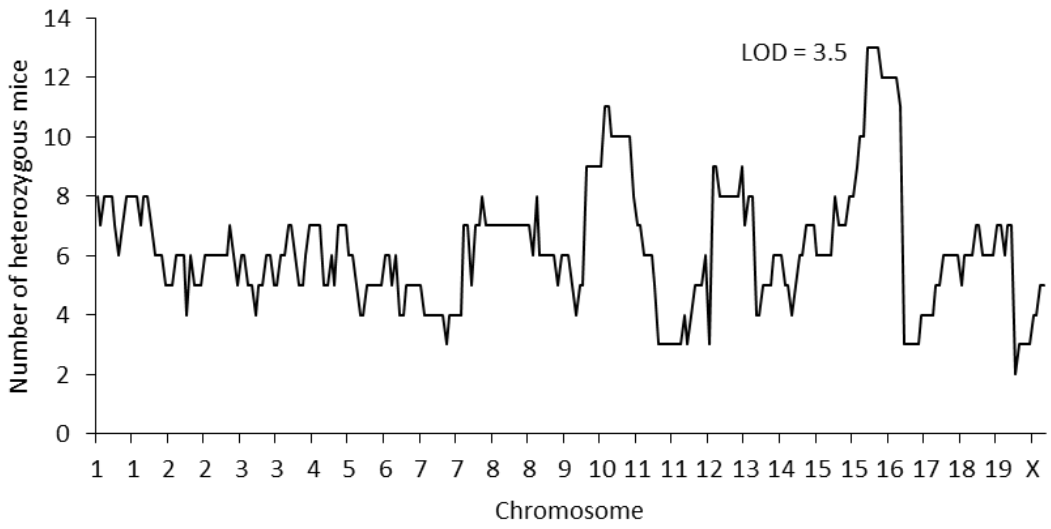
To the best of this author's knowledge, an increased susceptibility to malaria in microcytosis has not been reported. However, increased malaria susceptibility has been observed in people receiving iron supplements in several clinical studies (reviewed by Clark *et al.*[413]). One study reported that iron supplementation in people with IDA increased the risk of malaria by 41%[414]. While increased iron availability to the parasite may play a role in increasing malaria susceptibility, evidence also suggests

that erythrocytes from people with IDA receiving iron supplementation are better able to support parasite growth *in vitro*, outside of the influence of serum iron or other factors[390]. One hypothesis suggested to explain this, is that increased erythropoiesis, brought on by iron supplementation to compensate for anaemia, results in an increased proportion of reticulocytes and young erythrocytes in circulation[390]. Young erythrocytes have been shown to be more susceptible to malaria infection, including by *P. falciparum*, and therefore may account for the general increase in malaria susceptibility[390,415,416]. However, in this study, both erythropoiesis and erythrocyte half-life is normal, indicating this is unlikely to be the reason for increased susceptibility in this case.

In conclusion, this study reports a novel mutation in *Tfrc,* which reduces the surface expression of the protein on erythroblasts. Phenotypically, the mutation causes mild iron restricted erythropoiesis, without anaemia, in heterozygous mice. Surprisingly, mice displayed elevated susceptibility to malaria infection and exhibited an increased parasitaemia during the acute phase of infection. Furthermore, increased parasitaemia levels were likely due to an erythrocyte autonomous increase in parasite survival. These findings indicate that while severe iron deficiency associated with anaemia may be protective against malaria, iron restricted erythropoiesis resulting from Tfr1 deficiency, without anaemia, enhances malaria susceptibility.

SUPPLEMENTARY FIGURES

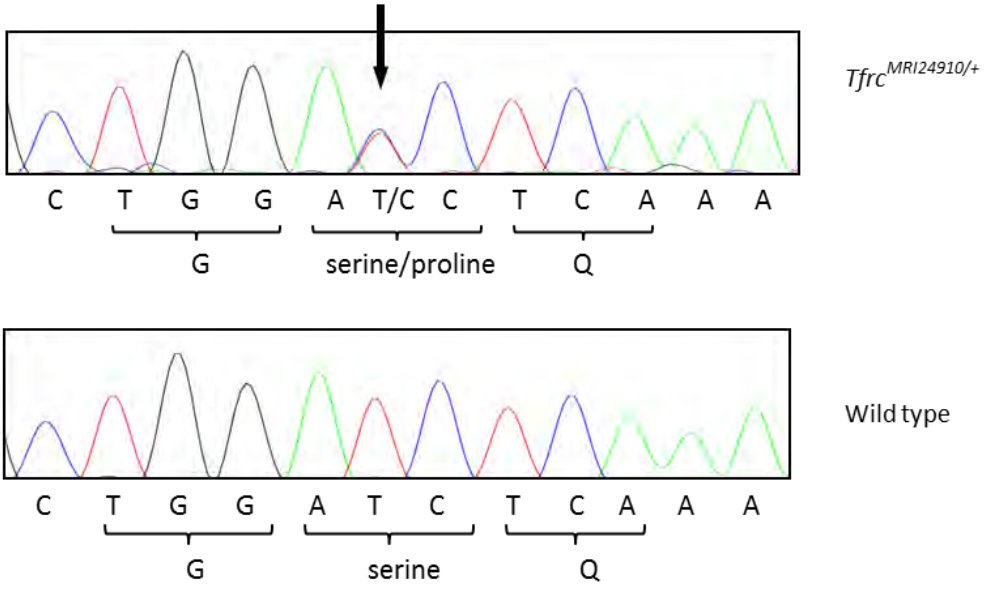
A



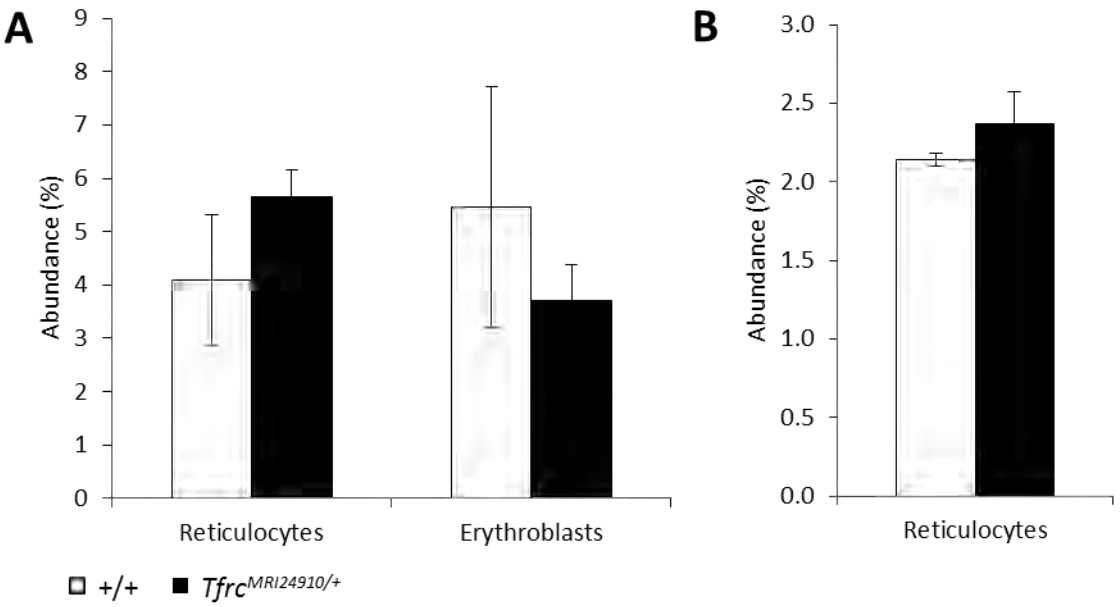
B

Microsatellite	Chromosome	Position	Number of heterozygotes	Number of homozygotes	LOD score
D16Mit12	16	39Mbp	39	4	7.0

C



Supplementary figure 4.1. Mapping of the MRI24910 mutation causing microcytosis. Heterozygosity of 13 microcytic N2 mice (MCV < 48 fL) indicates a linkage peak on chromosome 16 between 7Mbp and 67Mbp (A). The interval was confirmed in 43 additional microcytic N2 mice using the microsatellite marker D16Mit12 (B). *Tfrc* was selected as a candidate gene within this interval as it is known to cause a phenotype similar to that reported here. Sanger sequencing of all *Tfrc* exons revealed a T to C heterozygous mutation in exon 5, indicated with an arrow, resulting in a serine to proline amino acid substitution (C). LOD score was calculated using a chi squared test based on the expected number of heterozygotes.



Supplementary figure 4.2. Erythroblast and reticulocyte levels in *Tfrc*$^{MRI24910/+}$. Reticulocytes and erythroblasts in the spleen, identified as in Figure 2 (A). Reticulocytes in the peripheral blood as determined by positive staining with anti-CD71 (Tfr1) using flow cytometry (B). Error bars indicate SEM, data is from 4 mice per group for spleen reticulocyte and erythroblast abundance, and 7-8 mice per group for peripheral blood reticulocyte abundance.

CHAPTER 5

SPTB MUTANT MOUSE LINES MRI26194 AND MRI53426

CHAPTER INTRODUCTION

This chapter examines the ENU-induced mutant lines MRI26194 and MRI53426. Like MRI24910, these lines were originally identified based on reduced MCV. Interestingly, additional parameters measured by the automated haematological analyser were very similar between these lines and the MRI24910 line. However, in this case, mice displayed a significant reduction in malaria susceptibility, and a more detailed haematological analysis, including erythrocyte osmotic fragility and erythrocyte lifespan, revealed significant differences between these lines.

The ENU-induced mutations responsible for reduced MCV in these lines were identified in *Sptb*, which encodes the erythrocyte cytoskeletal protein beta spectrin. The host cytoskeleton is known to interact with the parasite during invasion, growth, and egress from the erythrocyte, however, genetic abnormalities in cytoskeletal genes are poorly studied, and mutations in *Sptb* have not been examined in the context of malaria infection. Therefore, the investigation of these mutant lines provided an opportunity to determine the role of beta spectrin in malaria infection, and relate these interactions and associated malaria resistance mechanisms to other erythrocyte mutations.

BACKGROUND

Malaria has exerted the strongest known selective pressure on the human genome in recent history[48]. One of the outcomes of this is an increased prevalence of erythrocytic polymorphisms, which convey protection against malaria infection in endemic regions[417,418]. Studies of the biological mechanisms underlying this protection have led to an increased understanding of the interactions between host and parasite. For example, genetic polymorphisms such as Duffy negativity and glycophorin C null mutations, which alter erythrocyte surface ligands and impair parasite invasion, have facilitated the identification of parasite proteins involved in the invasion process[159,338,419]. In other erythrocytic polymorphisms, the elucidation of a diverse array of protective mechanisms has led to valuable insights regarding the parasite's intraerythrocytic growth. These mechanisms include impaired cytoadherence of infected cells[260,262], reduced erythrocyte rosetting[87], enhanced

phagocytosis of infected cells[68,242], interference with parasite protein trafficking[213], and modulation of parasite protein translation due to changes in erythrocytic microRNA composition[234]. Indeed, mechanisms of resistance in these conditions are multifactorial and diverse, and remain incompletely understood.

An important aspect of the *Plasmodium* parasite's life cycle is its interaction with the erythrocytic cytoskeleton, which it manipulates in order to enter, develop within, and egress from the erythrocyte[222,238]. The cytoskeleton is a complex protein lattice, which provides structure and deformability to the circulating erythrocyte. This protein lattice is largely composed of alpha and beta spectrin, which are interconnected at nodes providing linkage points to the band 3 protein, located in the cell membrane. Interestingly, populations in endemic malaria areas have an increased prevalence of mutations in cytoskeletal proteins causing hereditary spherocytosis (HS) or hereditary elliptocytosis (HE)[105-107]. However, with the exception of South-East Asian ovalocytosis (SAO), a form of HE caused by a mutation in band 3, no association has been investigated between these types of mutations and malaria resistance[103]. Nevertheless, *in vitro* studies have demonstrated impaired invasion and/or growth in erythrocytes from individuals with SAO, HE and HS, indicating a role for these mutations in malaria resistance[108,110,177,420]. Additionally, several distinct mutations in *Ank1* and *Spta1*, associated with HS, have been shown to convey resistance to *P. chabaudi* and *P. berghei* infection in mice[111-113].

In SAO, impaired merozoite invasion of erythrocytes has been suggested as a protective mechanism conveyed by this condition. It has been suggested that the loss of merozoite binding partners[189], increased membrane rigidity[183], or reduced mobility of band 3 in the erythrocyte membrane[191], hinder invasion in these cells. In one form of HE, mutations in the *Spta1* self-association site are reported to inhibit *P. falciparum* invasion of these cells in correlation with increasing spectrin dimerization, although evidence is conflicting[108,196]. In support of a role for spectrin dimerization in invasion, studies have shown that artificial cross-linking of spectrin decreases erythrocyte deformability and reduces invasion by *P. falciparum*[421-423]. In both HE and HS, impairment of parasite

growth has been demonstrated[108,420]. One study indicates this impairment correlates with the degree of spectrin dimerization in HE, and the degree of spectrin deficiency in HS, indicating a role for spectrin in facilitating normal parasite development[108]. This finding is supported by studies indicating that parasite proteins, such as ring-infected erythrocyte surface antigen (RESA), bind to spectrin during parasite development[238]. Finally, parasite growth is also reportedly inhibited in a mutant mouse line carrying a mutation in *Ank1*, as evidenced by increased terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) of parasites[113]. TUNEL stains DNA which has begun to break-down and has been shown to be a reliable measure of parasite death. Overall, while several studies indicate a role for erythrocyte cytoskeleton mutations in inhibiting parasite invasion and growth, the biological mechanisms underlying these defects are incompletely understood.

This study describes two novel ENU-induced mutations in the cytoskeletal gene *Sptb* in mice, and investigated the response of the mutant mice to malaria infection. Mutations in *Sptb* are associated with both HE and HS, depending on how the protein is affected by the mutation. It was therefore hypothesised these mutations would alter the erythrocyte cytoskeleton, and consequently impede normal parasite invasion and growth in these cells. In fact, this was found not to be the case, and this study instead demonstrates that these mutations result in an enhanced phagocytosis of mutant parasitized erythrocytes.

RESULTS

Identification of two novel ENU-induced mutations in Sptb *in mouse strains with a dominant microcytosis phenotype*

G1 offspring from SJL/J ENU mutagenized mice were screened for erythrocyte physiological parameters outside of the normal range, determined using a haematological analyser. The G1 mice MRI26194 and MRI53426 displayed microcytosis, with a mean corpuscular volume (MCV) more than three standard deviations below the normal distribution (47.0 fL and 48.7 fL respectively versus 53.1 ± 1.3 fL in wild type). Each of these G1 mice were crossed with SJL/J mice and in both cases 50% of

G2 offspring were microcytic, indicating a fully penetrant, dominant, ENU-induced mutation responsible for the abnormal haematological phenotype. Aside from reduced size, inspection of erythrocytes by both light microscopy and scanning electron microscopy revealed no obvious morphological differences compared to wild type (Figure 5.1 A, B). Complete blood counts of mice from both mutant lines revealed a decrease in mean corpuscular haemoglobin (MCH), which is a measure of the total haemoglobin content per cell, in concordance with reduced cell volume (Figure 5.1 C). Mice also displayed an increased erythrocyte count (RBC count). The increased number of erythrocytes compensated for the fact that each cell contained less haemoglobin, thereby resulting in a normal level of total haemoglobin by blood volume (HGB) (Figure 5.1 C). This indicates that while mice exhibit abnormal erythrocyte physiology, this does not result in anaemia. The percentage of reticulocytes, an immature form of erythrocyte, was unchanged from wild type. This indicates that erythropoiesis in the mutant lines is normal. It was hypothesized that the alterations to erythrocyte physiology observed may influence the cell's susceptibility to osmotic lysis. Indeed, mutant erythrocytes were found to display a significant increase in osmotic fragility (Figure 5.1 D). To determine if these abnormalities altered the *in vivo* half-life of mutant erythrocytes, cells were labelled with biotin and their rate of clearance monitored. The half-life of mutant erythrocytes was found to be reduced by approximately 25% in both lines, indicating cells are more susceptible to clearance (Figure 5.1 E).

To determine if homozygosity of either of these mutations is compatible with life, microcytic mice were in-crossed and their offspring monitored. Rarely for MRI26194, a pup was born displaying jaundice, rapid breathing and a maximum lifespan of 48hrs (Supplementary figure 5.1). Blood smears from these jaundiced pups revealed severe erythrocyte fragmentation, spherocytic erythrocytes, and an increased abundance of reticulocytes (Supplementary figure 5.1). These mice were predicted to be carrying the homozygous mutation and were utilised in experiments described below. For MRI53426, offspring segregated into only two populations with either a wild type or heterozygous microcytosis phenotype.

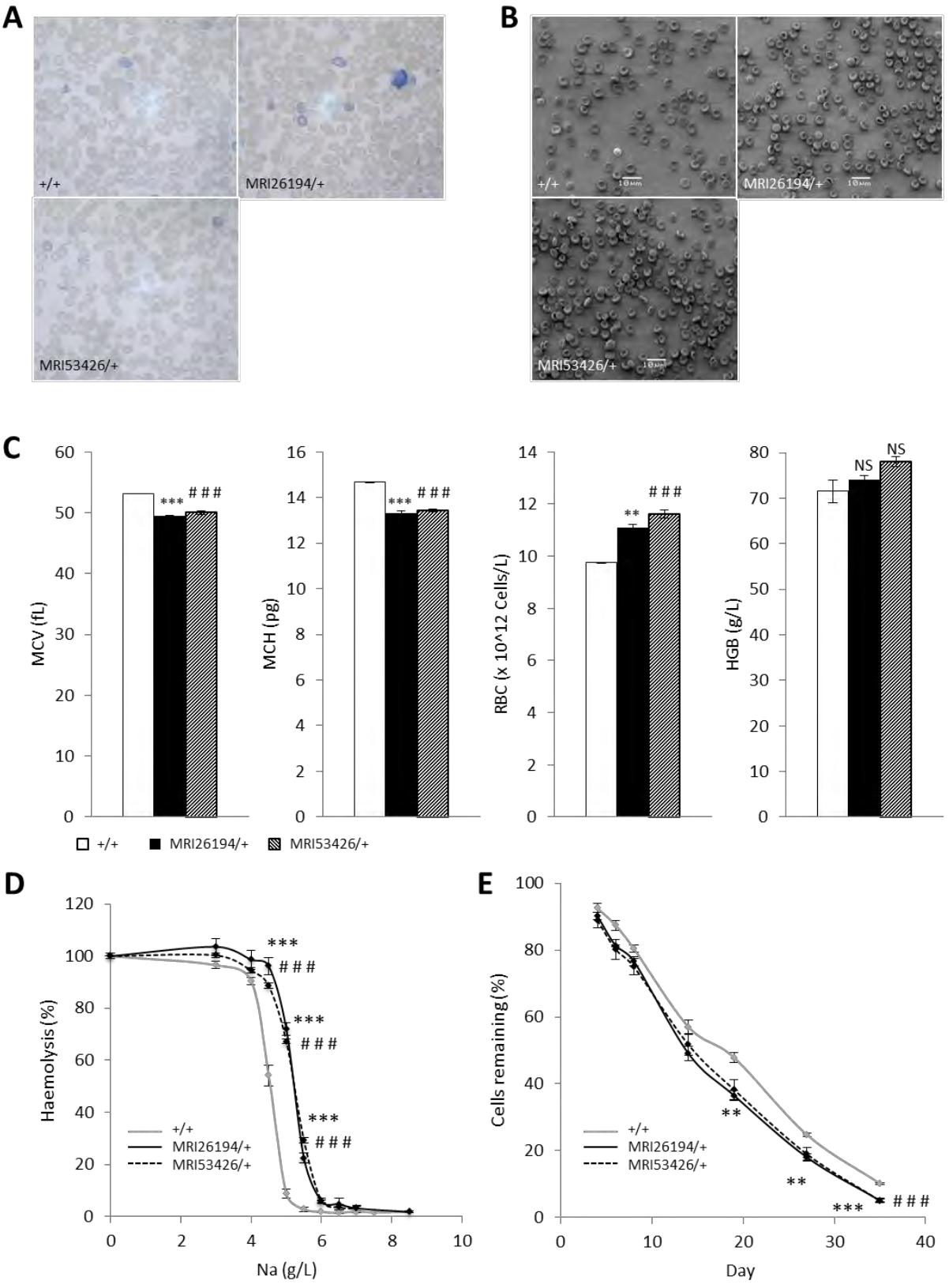

A
+/+
MRI26194/+
MRI53426/+
B
+/+
MRI26194/+
MRI53426/+
C
MCV (fL)
MCH (pg)
RBC (x 10^12 Cells/L)
HGB (g/L)
NS
+/+
MRI26194/+
MRI53426/+
D
Haemolysis (%)
Na (g/L)
E
Cells remaining (%)
Day

Figure 5.1. **Haematological properties of mutant lines MRI26194 and MRI53426.** Representative Giemsa stained smears, and SEM images, of erythrocytes from wild type (+/+) and both mutant lines (A, B). Haematological properties from an automated blood count. MCV – mean corpuscular volume, MCH – mean corpuscular haemoglobin, RBC – erythrocyte count, HGB – total haemoglobin by blood volume (C). Osmotic fragility of erythrocytes based on haemolysis in salt solution (D). Erythrocyte half-life based on the percentage biotinylated cells remaining in circulation after an initial biotin injection which labelled >99% of erythrocytes (E). Half-life was approximately 14 days for both mutant lines and 18 days for wild type. Error bars indicate SEM. ** $p < 0.01$, *** $p < 0.001$ between MRI26194 and wild type. $^{\#\#\#}$ $p < 0.001$ between MRI53426 and wild type. NS – $p > 0.05$. Data is from 14-34 mice per group for automated blood analysis, 8-35 per group for osmotic fragility, and 3 per group for erythrocyte half-life.

To investigate possible embryonic re-absorption of homozygotes, timed mates were performed and embryos collected on E9.5, E11.5, and E13.5. Embryo re-absorption was evident as early as E9.5, suggesting homozygosity for MRI53426 is lethal before this stage of development and therefore responsible for re-absorption. Together, these results indicate that these mutations disrupt the normal function of a gene which is critical to life. To identify the ENU-induced mutations responsible for these phenotypes, microcytic G2 mice (MCV < 50 fL) from each line were outcrossed C57BL/6 mice. Microcytic F1 mice were backcrossed again with C57BL/6 mice to produce N2 mice, and linkage analysis was performed on microcytic N2 mice. Genome wide SNP analysis indicated a significant linkage peak on chromosome 12, between 69Mbp and 87Mbp for MRI26194, and between 23Mbp and 87Mbp for MRI53426 (Supplementary figure 5.2 A, B). The *Sptb* gene encoding the major erythrocyte cytoskeletal protein beta spectrin (Sptb1) is positioned within these intervals. The *ja* mutation in *Sptb*, a premature stop codon introduced approximately mid-way through the protein, has previously been reported to result in a phenotype closely matching that reported here, with microcytosis, reduced cell haemoglobin content, reduced erythrocyte half-life, as well as early homozygous lethality[424]. It was therefore hypothesized that the phenotype observed in these lines was caused by mutations in the *Sptb* gene which likewise disrupt protein function. All exons and intron/exon boundaries of *Sptb* were sequenced in affected mice from both mutant lines. The MRI26194 line was found to contain a A to T transversion which introduces a premature stop codon in place of a splice site at the exon/intron boundary at the end of exon 6 (IVS6+2T>A)

(Supplementary figure 5.2 C). The mutation in this line, hereafter designated *Sptb*^{MRI26194}, likely disrupts splicing and leads to a transcript encoding a truncated protein of 188 amino acid residues (Figure 5.2 A). In the MRI53426 mutant line, designated *Sptb*^{MRI53426}, a T to A transversion was located in exon four resulting in a valine to glutamic acid missense mutation (V116E) in the calponin homology domain (Figure 5.2 A, Supplementary figure 5.2 D). The calponin homology domain, also known as the actin binding domain, is at the N-terminus of the protein and is involved in binding to beta actin. Analysis of this mutation using SIFT indicates that the V116 residue is conserved across 46 species, suggesting a critical function of this amino acid[425]. The predicted severity of these mutations, as well as the match in phenotype to the *ja* mutant line, strongly suggests these ENU-induced mutations are responsible for the abnormal haematological phenotype in these mice.

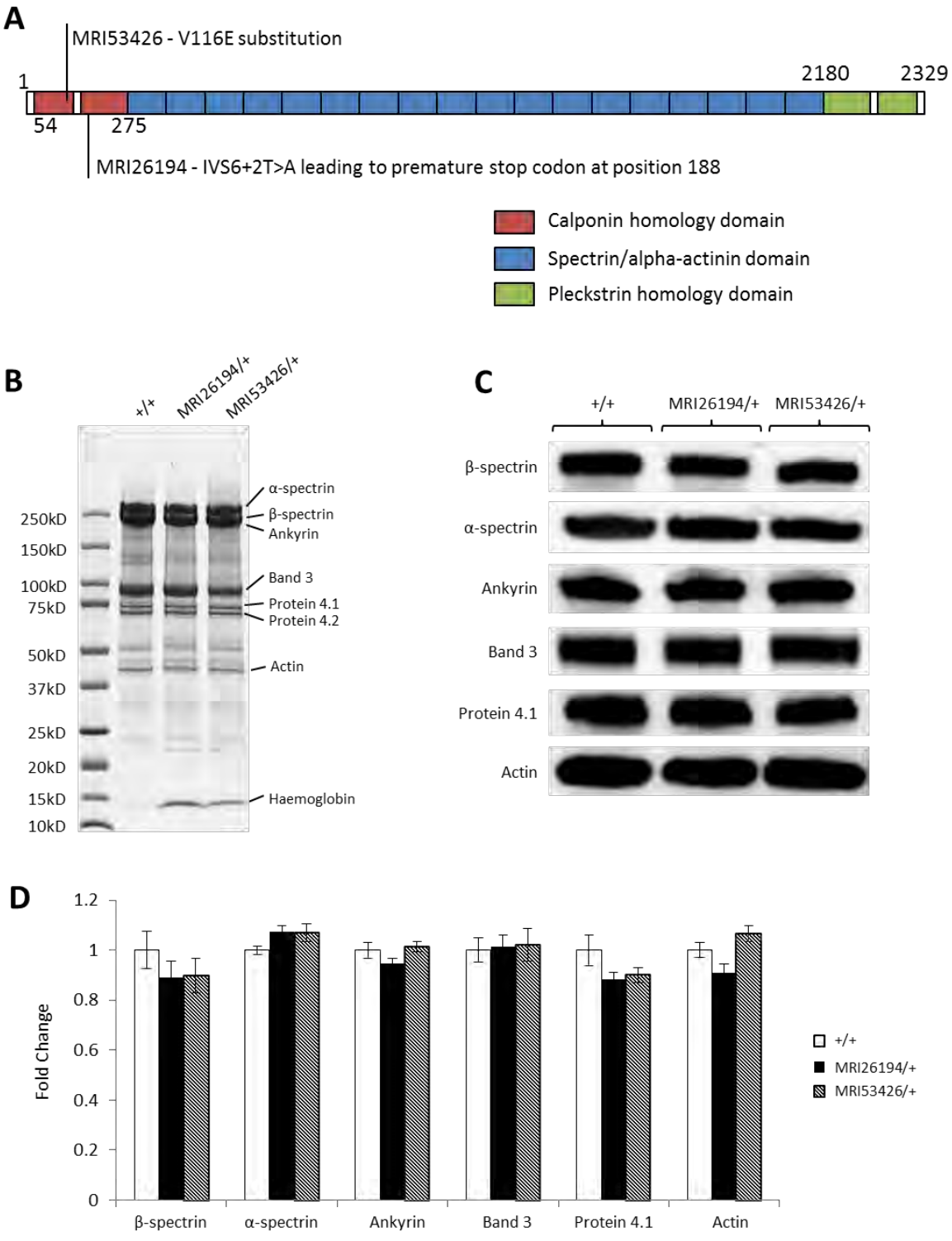


Figure 5.2. **Identification and characterisation of the MRI26194 and MRI53426 mutations in *Sptb*.** For MRI26194 a splicing site mutation introducing a premature stop codon at amino acid residue 188 was identified, while for MRI53426 a missense mutation causing a valine to glutamic acid substitution was identified (A). Coomasie stained erythrocyte membrane ghosts from mutant and wild type mice separated by SDS-PAGE on a 4-20% gel (B). Western blots of major cytoskeletal proteins in erythrocyte membrane ghosts from mutant and wild type mice (C). Quantification of indicated protein bands detected by western blot in samples from 3 mice per group (D). Western blots images were taken below signal saturation. Data representative from 1 of 3 experiments. Error bars indicate SEM.

The MRI26194 and MRI53426 mutations in beta spectrin result in normal cytoskeletal abundance in heterozygotes despite homozygous lethality

To determine the effect of the two ENU-induced *Sptb* mutations on protein expression, beta spectrin abundance was first investigated in homozygous mice. For *Sptb* $^{MRI26194/MRI26194}$, western blotting with an antibody raised against the N-terminus of beta spectrin failed to detect either full length protein or the expected truncated form (Supplementary figure 5.1). Tissues could not be obtained for *Sptb* $^{MRI53426/MRI53426}$ due to the early stage of embryo absorption. Next, heterozygote protein expression was investigated. To quantify protein abundance in mature erythrocytes, platelets, leukocytes and reticulocytes were removed from whole blood samples, as confirmed by flow cytometry (Supplementary figure 5.3). Coomasie staining of erythrocyte ghosts separated using 1D SDS-PAGE revealed no major differences in the most abundant membrane associated proteins, including beta spectrin (Figure 5.2 B). A noticeably darker band in the mutant samples at approximately 15kDa corresponds with the size of alpha and beta haemoglobin, and indicates increased membrane bound haemoglobin in mutant erythrocytes (Figure 5.2 B). This phenomenon has been described in HbAS due to increased oxidative damage to haemoglobin, however in this case the reason for increased binding is unclear. Western blotting of erythrocyte ghosts confirmed equal abundance of beta spectrin, alpha spectrin, ankyrin, band 3, protein 4.1, and actin, as normalised to packed erythrocyte ghost volume (Figure 5.2 C, D). Overall, these results indicate that the MRI26194 mutation prevents Sptb1 protein expression in homozygotes, while for heterozygotes of both mutant lines, the mutated allele is partially compensated for, producing erythrocytes of normal cytoskeletal protein abundance, although of smaller size.

Sptb$^{+/MRI26194}$ *and* Sptb$^{+/MRI53426}$ *mice are less susceptible to infection with Plasmodium* chabaudi adami *DS*

Mutations in genes encoding the erythrocyte cytoskeletal proteins band 3[101], ankyrin[113], protein 4.1[108], and alpha spectrin[108] are reported to reduce susceptibility to malaria infection; therefore, the

malaria susceptibility of the *Sptb* mutant mouse lines was assessed. Mice were infected with the rodent malaria strain *P. chabaudi adami* DS, which is normally lethal in the SJL/J mouse line utilized in this study. Mice were inoculated with a high ($1x10^7$), and low ($1x10^4$), dose of parasitized erythrocytes to determine their susceptibility to early stages of infection (days 1-5), and later stages (days 6 onwards). When inoculated with a high dose of parasitized erythrocytes, both mutant and wild type mice displayed a similar response to infection. Parasitaemia increased exponentially over five 24 hour growth cycles equally in all lines, while the erythropoietic response was minimal, based on reticulocyte count (Figure 5.3 A). This indicates that the parasite is able to invade, develop, and multiply normally in the *Sptb* mutant lines under these conditions.

In contrast, low dose inoculation resulted in a significantly altered course of infection in *Sptb* mutant lines. Parasitaemia was significantly reduced from day eight and day nine of infection in the $Sptb^{+/MRI26194}$ and $Sptb^{+/MRI53426}$ lines respectively, ultimately resulting in a 42% percent drop in peak parasitaemia of both lines (Figure 5.3 B). The erythropoietic response was also enhanced, with a significant increase in the proportion of reticulocytes from day nine onwards in both lines, along with an increased blood count from day 12 onwards (Figure 5.3 B). This cumulated in a significant increase in survival of $Sptb^{+/MRI26194}$ and $Sptb^{+/MRI53426}$ mice compared to wild type (91% and 100% respectively, compared to 8% of wild type) (Figure 5.3 B).

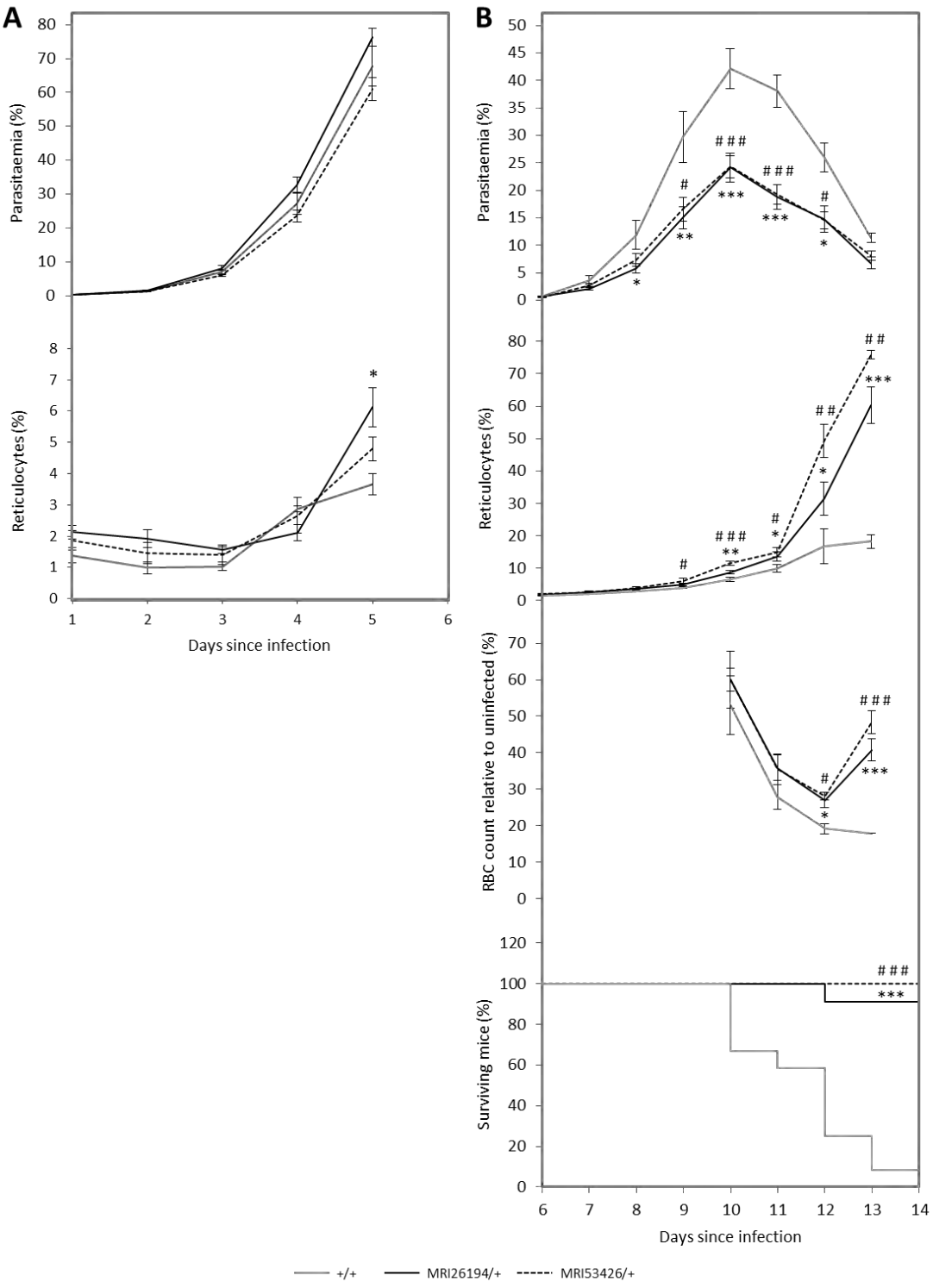


Figure 5.3. Increased resistance to *P. chabaudi adami* DS infection in *Sptb*$^{+/MRI26194}$ and *Sptb*$^{+/MRI53426}$ mutant mice. Mice were infected with a high ($1x10^7$) (A) or low ($1x10^4$) (B) dose of parasitised erythrocytes. Parasitaemia and reticulocyte count was determined daily by flow cytometry. In the low dose infection erythrocyte count is reported as a percentage compared to three uninfected mice included in each day of analysis. In the high dose challenge mice were sacrificed on day 5 of infection while for the low dose infection survival was monitored daily. Error bars indicate SEM. The log-rank (Mantel-Cox) test was used to calculate *p*-value for survival while the student's *t*-test was used otherwise. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ between *Sptb*$^{+/MRI26194}$ and wild type. $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$ between *Sptb*$^{+/MRI53426}$ and wild type. Data is from 4 mice per group for high dose infection, and 6-12 per group for low dose infection.

Reduced ring-stage parasite survival and enhanced clearance of Sptb *mutant erythrocytes*

The parasite growth rate results indicate that while there are no inherent barriers to parasite proliferation within the mutant lines, the mice appear to have an increased ability to control infection and recover, directing attention to the immune response. The immune response to infection with *P. chabaudi* is primarily cell mediated, and involves the production of Th1 cytokines and activation of phagocytic cells[426,427]. The timing of this response depends on parasite dose, strain, and mouse background[428]. It is therefore plausible that this response is altered in the *Sptb* mutant lines, and may explain their reduced susceptibility during later stages of infection. To investigate this possibility two hypotheses were considered. Firstly, mutant mice may have an enhanced immune response, resulting in an earlier, more potent, activation of immune cells and accelerated removal of parasites; or secondly, parasites may be disadvantaged in some way within mutant erythrocytes rendering them more susceptible to clearance or destruction by immune cells. As a potential role of *Sptb* in the immune system was not immediately apparent, the second hypothesis was focused on.

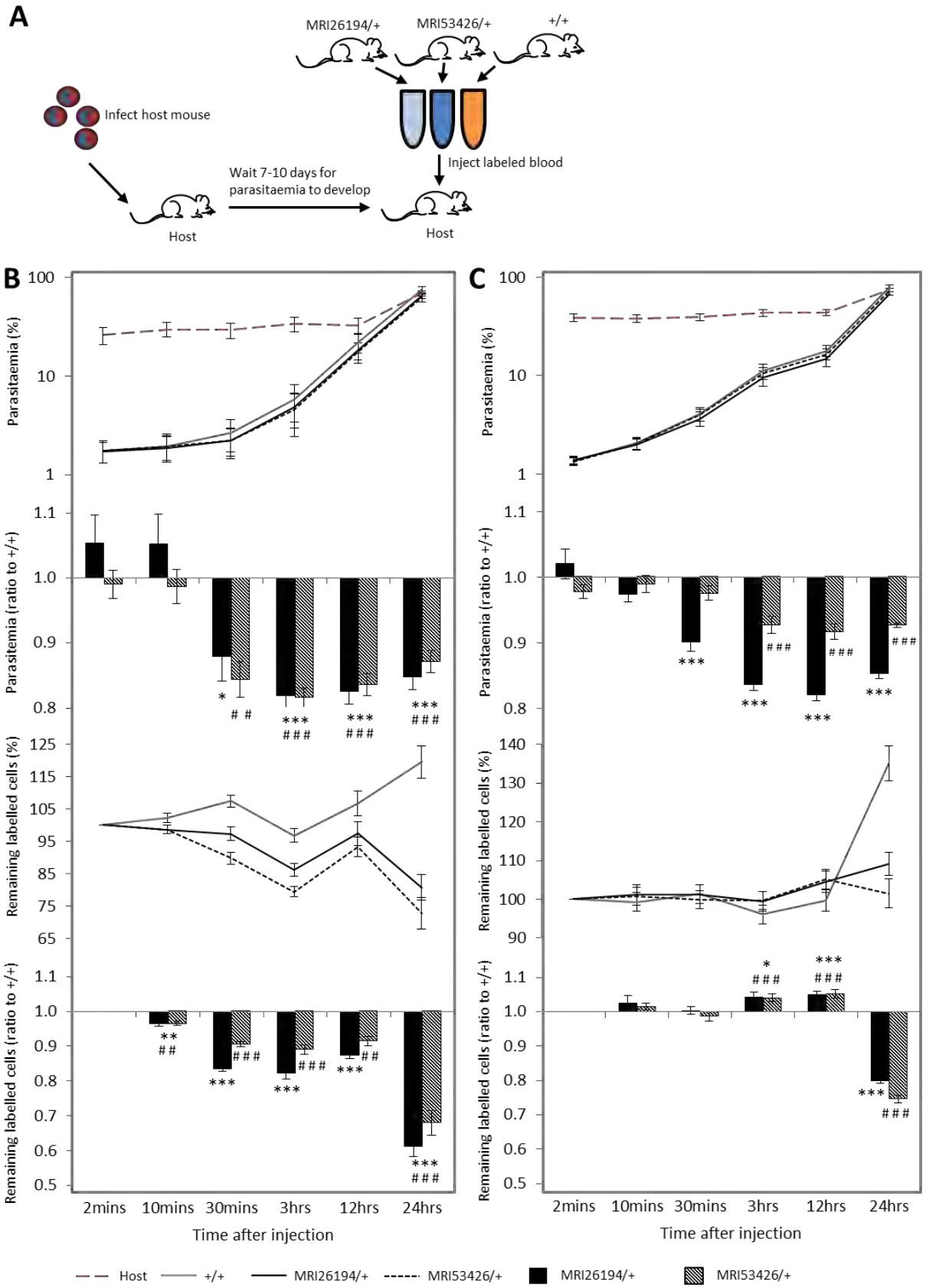
To determine if parasites were disadvantaged when established within *Sptb* mutant erythrocytes, an *in vivo* erythrocyte labelling experiment was performed as previously described[402] (see chapter 3). In this assay, erythrocytes from both mutant and wild type mice were fluorescently labelled, combined, and transfused into a cohort of infected mice (Figure 5.4 A). This facilitated a direct comparison of parasite invasion and survival, defined as the likelihood of the parasitised erythrocyte to remain in circulation until schizogony, within each respective erythrocyte population.

Wild type mice, inoculated with a low dose of parasites, were monitored until reaching 20-30% parasitaemia, on day nine of infection. Labelled blood was transfused into infected mice, and the proportion of each labelled erythrocyte population, as well as its parasitaemia, was determined as described in experimental procedures. Two minutes after transfusion, labelled wild type, $Sptb^{+/MRI26194}$, and $Sptb^{+/MRI53426}$, erythrocytes had equal parasitaemia (Figure 5.4 B). This supports earlier results, and indicates that merozoite invasion of *Sptb* mutant erythrocytes is unimpeded. A

similar result was observed ten minutes after transfusion, further strengthening this conclusion. In contrast, 30 minutes after injection, both *Sptb* mutant labelled erythrocyte populations displayed significantly reduced parasitaemia relative to the labelled wild type population (12% and 16% reduction in $Sptb^{+/MRI26194}$, and $Sptb^{+/MRI53426}$ respectively). This indicates that *Sptb* mutant ring-stage parasitised erythrocytes (RPEs) have a reduced likelihood of survival compared to wild type RPEs. This result was maintained for the remainder of the assay, while there was no significant difference in parasitaemia between the two mutant lines.

It was hypothesised that the decreased survival of *Sptb* mutant RPEs may be associated with a general increase in the clearance of mutant erythrocytes. To determine if this was so, the total number of remaining labelled erythrocytes in each population was monitored during the assay. This value varied considerably in response to the rate of haemolysis and clearance of unlabelled erythrocytes within each transfused mouse. Nevertheless, the proportion of remaining *Sptb* mutant erythrocytes was consistently less than remaining wild type erythrocytes, starting from 10 minutes are transfusion (Figure 5.4 B). The difference in these populations increased during the assay, and by 24 hours after transfusion there were 39%, and 32%, fewer labelled $Sptb^{+/MRI26194}$, and $Sptb^{+/MRI53426}$ erythrocytes remaining respectively. Importantly, the rate of this clearance outpaced that expected if only parasitised erythrocytes were being cleared, therefore, this indicates an enhanced non-specific clearance of both uninfected and parasitised mutant erythrocytes. Notably, this result was not observed when labelled erythrocytes were transfused into uninfected mice, in which there was no difference in the rate of clearance of *Sptb* mutant erythrocytes (Supplementary figure 5.4).

Figure 5.4. Decreased survival of *P. chabaudi adami* DS in erythrocytes from $Sptb^{+/MRI26194}$ and $Sptb^{+/MRI53426}$ mutant mice. Wild type mice, used as hosts, were infected with $1x10^4$ parasitised erythrocytes. At day 7-10 of infection, at the peak of schizogony (mid-way through the dark cycle), labelled blood from both mutants, and wild type (+/+) mice was transfused into host mice (A). In one experiment host mice were intact (B), while in another they underwent splenectomy before beginning the assay (C). Labelled erythrocytes and parasitaemia were determined at the indicated time points by flow cytometry. Fluorescent labels were switched to account for any dye effects. Similar results were obtained using mutant mice as hosts. Error bars indicate SEM. The one sample *t*-test was used to calculate *p*-value for ratios. * $p < 0.05$, *** $p < 0.001$ for $Sptb^{+/MRI26194}$ ratio to wild type. $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$ for $Sptb^{+/MRI53426}$ ratio to wild type. Data is from 6 intact mice and 9 splenectomised. Labelled blood is from 3 mice per group.


A
MRI26194/+
MRI53426/+
+/+
Infect host mouse
Wait 7-10 days for parasitaemia to develop
Inject labeled blood
Host
Host
B
C
Parasitaemia (%)
Parasitaemia (ratio to +/+)
Remaining labelled cells (%)
Remaining labelled cells (ratio to +/+)
2mins
10mins
30mins
3hrs
12hrs
24hrs
Time after injection
Host
+/+
MRI26194/+
MRI53426/+
MRI26194/+
MRI53426/+

Overall, these results indicate that, while the parasite is able to invade *Sptb* mutant erythrocytes normally, at an early stage of development, less than 30 minutes after invasion, survival of parasites within mutant erythrocytes is reduced, which may be related to an enhanced non-specific clearance of mutant erythrocytes.

The enhanced clearance of Sptb *mutant erythrocytes is partially due to the action of the spleen but cannot explain reduced survival of ring-stage parasites*

The spleen plays a central role in malaria immunity, particularly during the early stages of infection[247], it was therefore hypothesised that the spleen may play a role in the increased clearance of *Sptb* mutant erythrocytes, and the reduced parasitaemia of these cells. To investigate this, the *in vivo* erythrocyte labelling experiment was repeated as described, but blood was transfused into infected mice which had previously been splenectomised. Surprisingly, splenectomy had little effect on results in terms of parasitaemia. *Sptb* mutant labelled erythrocytes again displayed reduced parasitaemia compared to wild type, beginning 30 minutes after transfusion (Figure 5.4 C). Although, in the case of $Sptb^{+/MRI53426}$ the reduction in parasitaemia was more modest in transfused splenectomised mice compared to intact mice (2-8% reduction in splenectomised, compared to 13-18% in intact mice). The clearance of labelled erythrocytes, however, was dramatically different in splenectomised mice. In contrast to clearance in intact mice, labelled *Sptb* mutant erythrocytes were cleared at an equal, or even lesser rate, compared to labelled wild type erythrocytes up to 12 hours after transfusion (Figure 5.4 C). However, 24 hours after transfusion, labelled *Sptb* mutant erythrocytes were again less abundant than wild type erythrocytes, indicating that perhaps at high parasitaemia, mutant erythrocytes are more susceptible to clearance regardless of splenic activity.

To confirm this result, splenectomised wild type and $Sptb^{+/MRI26194}$ mice were inoculated with 1×10^4 parasitized erythrocytes and daily parasitaemia monitored. Splenectomised mutant mice maintained their malaria resistance phenotype and displayed significantly reduced parasitaemia compared to splenectomised wild type mice (Supplementary figure 5.5). Notably, all mutant and wild type mice

succumbed to the lose dose infection, indicating a critical role for the spleen in controlling malaria infection. Presumably due to the heightened susceptibility to malaria in both groups, no difference in survival was observed in mutant mice. Overall, these results suggest that the spleen contributes toward the increased clearance of *Sptb* mutant erythrocytes, however, only plays a limited role in the reduced survival of parasites within these cells.

Parasite survival within Sptb *mutant erythrocytes is influenced by lipopolysaccharide treatment*

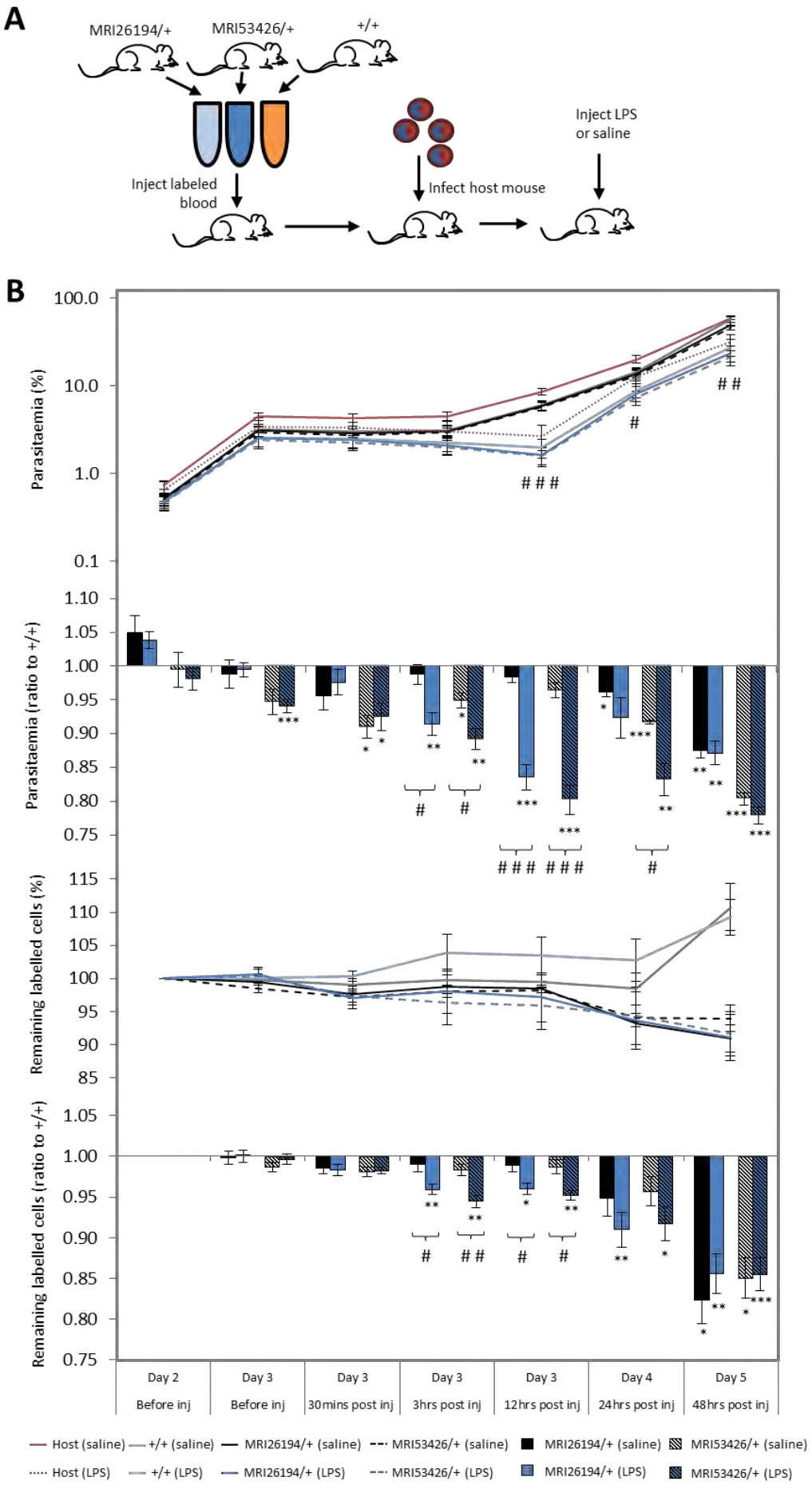
To address the hypothesis that reduced parasite survival within mutant erythrocytes is due to an enhanced susceptibility to immune clearance, parasite survival was assessed in mice treated with *Escherichia coli* lipopolysaccharide (LPS) (0.2 µg/g). *E. coli* LPS is a well known activator of cell mediated immunity, and induces the production of Th1 cytokines and activation of phagocytes[429,430]. Erythrocytes from each of the lines were fluorescently labelled, and transfused into uninfected mice. Mice containing the labelled erythrocytes were then inoculated with a high dose of parasitized erythrocytes ($1x10^7$), and the proportion of labelled erythrocytes and parasitaemia were monitored daily (Figure 5.5 A). On day three of infection, one cohort of infected mice was injected with LPS and one with saline. LPS injection resulted in a significant drop in total parasitaemia of mice compared to those that received saline injection, indicating that LPS enhances the clearance of all infected red cells in these mice (Figure 5.5 B). However, the parasitaemia of labelled *Sptb* mutant erythrocytes within the LPS cohort was also significantly reduced compared to the parasitaemia of labelled wild type erythrocytes in these mice. This difference was most dramatic 12 hours after injection, at which point parasitaemia was 16-20% less in the mutant labelled populations compared to wild type populations. No difference was observed between these populations in saline injected control mice (Figure 5.5 B). LPS injection also increased the clearance rate of labelled (uninfected) erythrocytes. The total remaining mutant erythrocytes was significantly reduced compared to wild type in the LPS cohort, 3 and 12 hours after injection, while clearance of these cells remained equal in saline injected mice. Additionally, no difference was found in the parasitaemia or rate of clearance of labelled

erythrocytes among the three populations on day two of infection (Figure 5.5 B). This result supports our earlier finding that at this stage of infection there are no barriers to parasite invasion and growth within *Sptb* mutant erythrocytes. Overall, these results indicate that an LPS induced immune response results in an enhanced clearance of parasites within *Sptb* mutant erythrocytes compared to those within wild type erythrocytes. Furthermore, this response also results in the preferential clearance of mutant erythrocytes relative to wild type.

Sptb *mutant ring-stage parasitized erythrocytes are more susceptible to phagocytosis*

The phagocytosis of parasitised erythrocytes is a major mechanism of cell mediated immunity to *P. chabaudi* infection[431]. Furthermore, LPS is known to induce the activation of phagocytic cells via the production of Th1 cytokines[429,430]. As an LPS induced immune response was found to reduce survival of parasites within *Sptb* mutant erythrocytes, the possibility that mutant parasitized erythrocytes may have an increased susceptibility to phagocytosis was addressed. Additionally, the reduced survival of parasites in mutant erythrocytes was found to occur as early as 30 minutes after invasion, therefore, this hypothesis was tested in ring-stage parasitised erythrocytes (RPEs).

Figure 5.5. Survival of *P. chabaudi adami* DS in erythrocytes from *Sptb*$^{+/MRI26194}$ and *Sptb*$^{+/MRI53426}$ mutant mice changes during the course of infection and in response to LPS injection. Wild type host mice were transfused with labelled blood before being infected with $1x10^7$ parasitised erythrocytes. On day 3 of infection, mice were injected with LPS or saline (A). Labelled erythrocytes and parasitaemia were determined at the indicated time points by flow cytometry (B). Fluorescent labels were switched to account for any dye effects. Error bars indicate SEM. The one sample *t*-test was used to calculate *p*-value for ratios, while the student's *t*-test was used otherwise. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ for *Sptb*$^{+/MRI26194}$ and *Sptb*$^{+/MRI53426}$ ratios to wild type. $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$ between LPS and saline injected mice. Data is from 4 saline, and 6 LPS injected mice. Labelled blood is from 3 mice per group.


A
MRI26194/+
MRI53426/+
+/+
Inject labeled blood
Infect host mouse
Inject LPS or saline
B
Parasitaemia (%)
Parasitaemia (ratio to +/+)
Remaining labelled cells (%)
Remaining labelled cells (ratio to +/+)
Day 2 Before inj
Day 3 Before inj
Day 3 30mins post inj
Day 3 3hrs post inj
Day 3 12hrs post inj
Day 4 24hrs post inj
Day 5 48hrs post inj
Host (saline)
+/+ (saline)
MRI26194/+ (saline)
MRI53426/+ (saline)
MRI26194/+ (saline)
MRI53426/+ (saline)
Host (LPS)
+/+ (LPS)
MRI26194/+ (LPS)
MRI53426/+ (LPS)
MRI26194/+ (LPS)
MRI53426/+ (LPS)

The phagocytosis of uninfected mutant and wild type erythrocytes by intraperitoneal macrophages was first compared. These cells were rarely phagocytised, irrespective of genetic background (Figure 5.6 A, B). The phagocytosis of RPEs was next compared. These were obtained from mice on day two of infection, one hour into the light cycle, at which time over 95% of parasites were at ring-stage. RPEs were phagocytised significantly more often than uninfected erythrocytes, while *Sptb* mutant RPEs were phagocytised approximately twice as often as wild type RPEs (Figure 5.6 B). This indicates that *Sptb* mutant RPEs have an inherent susceptibility to phagocytosis, which likely explains the increased resistance to malaria in these mice.

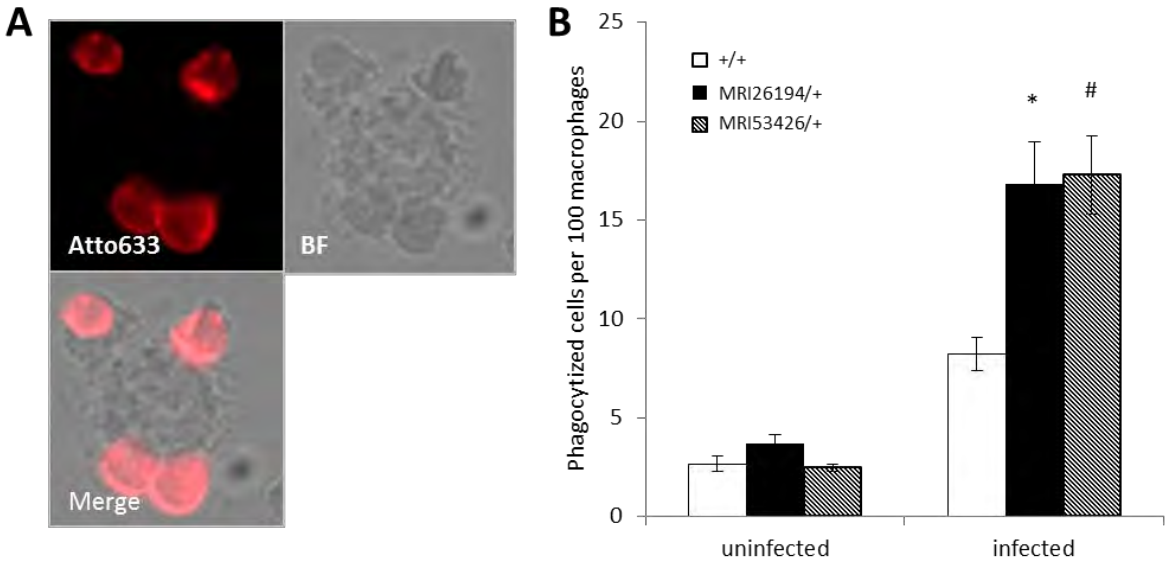


Figure 5.6. Enhanced phagocytosis of ring stage parasitised *Sptb*$^{+/MRI26194}$ and *Sptb*$^{+/MRI53426}$ erythrocytes. Phagocytised erythrocytes labelled with Atto633, and macrophages, were identified by fluorescence microscopy (A). The total number of phagocytised erythrocytes per 100 macrophages was determined when incubated with uninfected erythrocytes (UI), and ring stage parasitised erythrocytes (RPE) using samples from four mice in each group (B). Error bars indicate SEM. * $p < 0.05$ between *Sptb*$^{+/MRI26194\ and}$ wild type. $^{\#}$ $p < 0.05$ between *Sptb*$^{+/MRI53426}$ and wild type. Data is from 4 mice per group.

Eryptosis and erythrocyte senescence cannot explain the enhanced phagocytosis of Sptb *mutant ring stage parasitised erythrocytes*

Phagocytosis of parasitised erythrocytes is reported to occur via three distinct mechanisms; eryptosis[287], accelerated senescence[285], and by the recognition of parasite antigens[432]. It was therefore investigated if one or more of these mechanisms is enhanced in *Sptb* mutant RPEs. Eryptosis is characterised by an influx of calcium, resulting in the activation of proteases, and surface exposure of phosphatidylserine[286]. Phosphatidylserine exposure signals immune cells to phagocytise the cell[287]. In agreement with previous studies a significant increase in eryptosis was found, as indicated by Fluo-3-AM fluorescence and Annexin V binding, in RPEs compared to uninfected erythrocytes (Supplementary figure 5.6 A, B). However, no differences in these indices were observed between mutant and wild type RPEs, indicating this is not the reason for the increased phagocytosis of these cells.

The senescence of erythrocytes occurs through a sequential series of events beginning with the aggregation of band 3 protein in the cell membrane[282]. Naturally occurring antibodies attach to aggregated band 3 and promote binding of complement component C3[283]. Antibodies and complement are recognised by immune cells, leading to phagocytosis[250]. Band 3 aggregation, and membrane bound IgG and C3, were all increased significantly in RPEs compared to uninfected erythrocytes (Supplementary figure 5.6 C-E). However, again no difference was found between mutant and wild type RPEs, indicating erythrocyte senescence is not involved in the enhanced phagocytosis of mutant RPEs. Together these results suggest that enhanced phagocytosis of mutant RPEs does not occur via increased eryptosis or senescence and instead may occur via enhanced recognition of parasite antigens.

DISCUSSION

The malaria parasite dramatically remodels the host erythrocytic cytoskeleton during merozoite invasion and intraerythrocytic development[252,433]. Defects in cytoskeletal proteins brought on by genetic mutations in *ANK1*[111-113], *SPTA1*[108,111,196], *PROT4.1*[108,420], *GYPC*[420] and *SLC4A1*[177,183,191], have been demonstrated to obstruct these processes *in vitro*, and to impede malaria infection *in vivo*, although the biological mechanisms underpinning this inhibition are uncertain. This study shows for the first time that mutations in the cytoskeletal gene, *Sptb*, are able to deter rodent malaria infection and provide a survival advantage against this disease. Furthermore, it was demonstrated that rather than inhibiting invasion or growth of the parasite, these mutations result in enhanced phagocytosis of parasitised erythrocytes.

The mutations described here result in a phenotype which closely matches that of the *Sptb*^{ja} mutant line, which contains a premature stop codon at amino acid position 1160 of beta spectrin[434]. The *ja* mutation results in dramatically reduced levels of mRNA in erythroid progenitors leading to a lack of detectable beta spectrin in the erythrocyte membrane[434]. It is possible that *MRI26194 Sptb* mutation, which also results in the incorporation of a premature stop codon, has a similar effect on mRNA stability. In support of this, the phenotype of these mice is closely matched, both in homozygotes, which are jaundiced, lack a truncated erythrocytic beta spectrin form, and display severe anaemia, reticulocytosis, and spherocytosis; and heterozygotes, which are microcytic and have a reduced erythrocyte half-life[424,434]. The effect of the *MRI53426* missense mutation is more difficult to predict, and unfortunately homozygous tissues for these mice could not be obtained. However, if the *MRI53426* mutant protein was expressed and incorporated into the erythrocyte membrane, an altered phenotype of *Sptb*$^{+/MRI53426}$ compared to *Sptb*$^{+/MRI26194}$ would be expected, which was not the case. It can therefore be speculated that the *MRI53426* mutation also results in the loss of mRNA or protein stability, which is supported by the fact that the *MRI53426* mutated amino acid residue is highly conserved. The microcytic phenotype of the *Sptb*$^{+/MRI26194}$ and *Sptb*$^{+/MRI53426}$ lines indicates that the mutant allele is partially, but not fully, compensated for, while the lack of spherocytosis and

normal cytoskeletal protein abundance suggests a relatively normal cytoskeletal arrangement. Notably, a similar microcytosis with normal cytoskeletal protein abundance has been reported in mice carrying a heterozygous null mutation in the major cytoskeletal protein, Ank-1, indicating a robust cytoskeletal regulatory system in erythroid progenitors[112]. Importantly, here we have only assessed the relative abundance of the major cytoskeletal proteins. It is plausible that the *Sptb* mutations described have altered the phosphorylation state of these proteins, or perhaps have led to changes in the abundance of other proteins present in the cytoskeleton. Indeed, the increased osmotic fragility of mutant erythrocytes indicates cytoskeleton stability has been affected. While the exact changes to the erythrocyte skeleton could not be elucidated here, further studies of protein modifications such as phosphorylation, as well as the quantification of less abundant proteins may help explain the phenotypes observed.

When subjected to lose dose malaria infection, $Sptb^{+/MRI26194}$ and $Sptb^{+/MRI53426}$ mice displayed a marked resistance phenotype. Survival was 100% and 91% in the mutant lines respectively, compared to only 8% in wild type. This resistance phenotype is similar to that reported for the $Ank\text{-}1^{+/1674}$ and $Ank\text{-}1^{+/MRI23420}$ mutant lines, which display 92% and 94% survival compared to 25% and 10% in wild type respectively, and indicates a critical role for cytoskeletal mutations in malaria resistance[112,113]. In a high dose infection, no difference in response was seen in the *Sptb* mutant lines, indicating parasite invasion and growth is unaffected by these mutations. This is in contrast to previous *in vitro* studies of cytoskeletal mutations, which have indicated parasite invasion and growth are reduced in these conditions[108,196]. It is plausible that during the high dose infection, the immune system does not have sufficient time to respond before mice succumb to infection. If this was the case it would indicate a role for the immune system in the resistance phenotype of the *Sptb* mutant lines.

In considering why parasite invasion and growth was inhibited in previous studies of cytoskeletal mutations, but was unaffected in this study, it is critical to examine the nature of the genetic mutations under question. Mutations in cytoskeletal proteins result in a diverse array of disorders,

which have varying effects on erythrocyte morphology, protein abundance, and biomechanical properties. *In vitro* studies have only reported invasion inhibition in mutations resulting in severe HE, such as the band 3 mutation resulting in SAO, and those resulting in the loss of protein 4.1[177,196,420]. In these same studies, it was demonstrated that other mutations, including those resulting in milder forms of HE, do not influence parasite invasion. In a separate study, mutations causing HS also had no effect on invasion[108]. The mutations described in this study do not result in identifiable HE or HS in heterozygotes, as observed on thin blood smears and by SEM, nor do they result in spectrin deficiency. Therefore, the unrestricted invasion in the *Sptb*$^{+/MRI26194}$ and *Sptb*$^{+/MRI53426}$ lines examined in this thesis are consistent with previous studies. Likewise, *in vitro* studies have indicated that while parasite growth is reduced in erythrocytes with severe HE or HS, it is not effected in those conditions which result in a normal abundance of spectrin, or those without morphological abnormalities[108,420]. *In vivo* studies have described reduced parasitaemia and increased survival to rodent malaria strains due to heterozygous mutations in *Ank1* and *Spta1*[111-113]. These mutations resulted in a milder HS phenotype, without major morphological abnormalities or changes in protein abundance, similar to that reported here. However, the nature of these protective effects could not be unequivocally assigned to a reduction in parasite invasion or growth. Furthermore, one study indicated that invasion of *P. berghei* was unimpeded in erythrocytes with a heterozygous *Ank1* mutation[112]. Given that parasite invasion and growth in the *Sptb*$^{+/MRI26194}$ and *Sptb*$^{+/MRI53426}$ lines was unaffected, an alternate explanation for the malaria resistance was sought.

In vitro, parasite abundance, and therefore growth depends on the ability to invade the erythrocyte, replicate, and egress from the cell. *In vivo*, the situation is quite different; the parasite must invade erythrocytes and multiply, but it must also maintain the integrity of its cell as it passes through the small capillaries and the spleen, as well as avoid detection by the host immune system. It was therefore considered if these aspects of parasite survival were influenced in the *Sptb* mutant lines.

The role of splenic clearance in malarial resistant cytoskeletal mutations has not previously been investigated. However, a study in a mouse model of sickle cell trait reported that splenectomy

abolished the malaria resistance associated with this condition[435]. Here, splenic clearance had only a minor effect on the ability of the *P. chabaudi* parasite to survive within *Sptb*$^{MRI26194/+}$ and *Sptb*$^{MRI53426/+}$ mutant erythrocytes, and therefore was unlikely to be a major reason for the reduced malaria susceptibility in these lines. Interestingly, the spleen did play a significant part in the clearance of uninfected mutant erythrocytes, but this only occurred during infection or under stimulus by LPS. This result highlights a novel role for the spleen in the clearance of abnormal erythrocytes under immune stimulus, and may have implications in the management of erythrocytic diseases such as sickle cell anaemia.

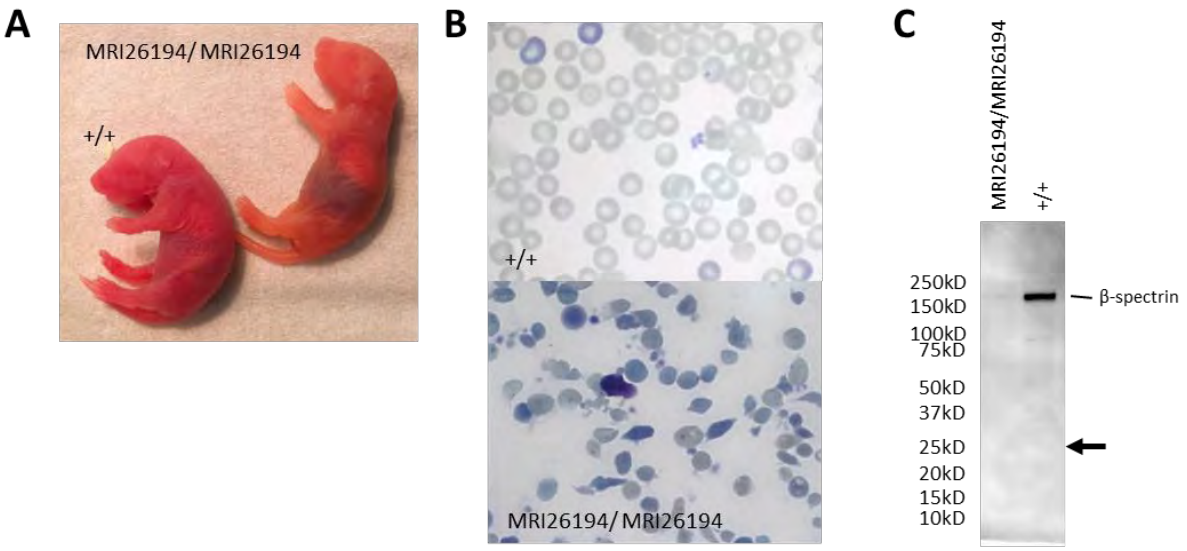
Immune clearance is also previously unexplored as a mechanism of malaria resistance in conditions resulting from cytoskeletal mutations. This study demonstrated that reduced survival of parasites within mutant erythrocytes was dependent on immune activation, and furthermore, that phagocytosis of mutant parasitised erythrocytes was enhanced. Increased susceptibility to phagocytosis has previously been reported in several common erythrocyte polymorphisms associated with malaria resistance including: sickle cell trait[68], G6PD deficiency[242], beta thalassemia[68], HbH[68], pyruvate kinase deficiency[77], and in O-blood type[299]. In contrast to these polymorphisms, which have all been shown to exhibit accelerated senescence and complement opsonisation associated with phagocytosis, no change was found in the senescence of *Sptb* mutant erythrocytes, either parasitised or uninfected. Additionally, no change was found in eryptosis in these cells to account for the increase in phagocytosis. In support of this result, previous studies have reported that deficiency in either the alternate or classical complement pathways does not have a major effect on the response to *P. chabaudi*, indicating that complement mediated phagocytosis plays only a minor role in the host defence against this infection[436]. These results are intriguing because they indicate a potentially novel mechanism of enhanced phagocytosis induced by these mutations. One possibility is that non-opsonic phagocytosis, a major mechanism of immune clearance in *P. chabaudi* infection[432], is enhanced. In both *P. falciparum* and *P. chabaudi*, non-opsonic phagocytosis has been shown to largely occur via macrophage receptor CD36 binding of parasitized erythrocytes, which in *P.*

falciparum likely occurs via binding to *P. falciparum* erythrocyte membrane protein (PfEMP-1)[437]. PfEMP-1 expression is altered in several erythrocyte polymorphisms, which evidence suggests may be due to abnormal protein trafficking in these cells[213,261]. It is possible a similar mechanism may be occurring in *Sptb* mutant erythrocytes, which may lead to increased surface expression of parasite antigens. A systematic approach towards blocking potential antigen-receptor interactions may help elucidate the reasons for the enhanced phagocytosis observed, although this was beyond the scope of this thesis. Furthermore, some benefit may be gained from an assessment of parasite proteins expressed on the surface of infected mutant erythrocytes compared to infected control erythrocytes, although again, this study could not be completed in the timeframe of this project. Overall, this study has demonstrated that parasites are more susceptible to phagocytosis within *Sptb* mutant erythrocytes, and this likely explains the reduced susceptibility of these mice to malaria infection.

Although enhanced phagocytosis has been demonstrated in other erythrocyte defects, it has not been reported in conditions brought on by cytoskeletal mutations. This study further strengthens the hypothesis that cytoskeletal mutations convey malaria resistance in human populations. Very few studies have investigated the prevalence of these types of mutations in endemic malaria populations, and these have focussed on conditions characterised by the presence of erythrocytes with abnormal morphology[102,106,107]. Notably, the conditions described here do not result in morphological abnormalities, aside from microcytosis, and therefore the prevalence of these types of mutations in endemic malaria populations has not been examined. Interestingly, studies have reported up to an 8% prevalence of microcytosis, unrelated to common erythrocyte polymorphisms, in malaria endemic populations[412]. The cause of microcytosis in these reports is undetermined, and indeed, difficult to determine, although results from this study raise the possibility that microcytosis may be due to cytoskeletal mutations which convey malaria resistance. Although, as described in chapter 4 of this thesis, microcytosis may also cause increased susceptibility to malaria, making it imperative to determine the underlying defect causing microcytosis in these studies.

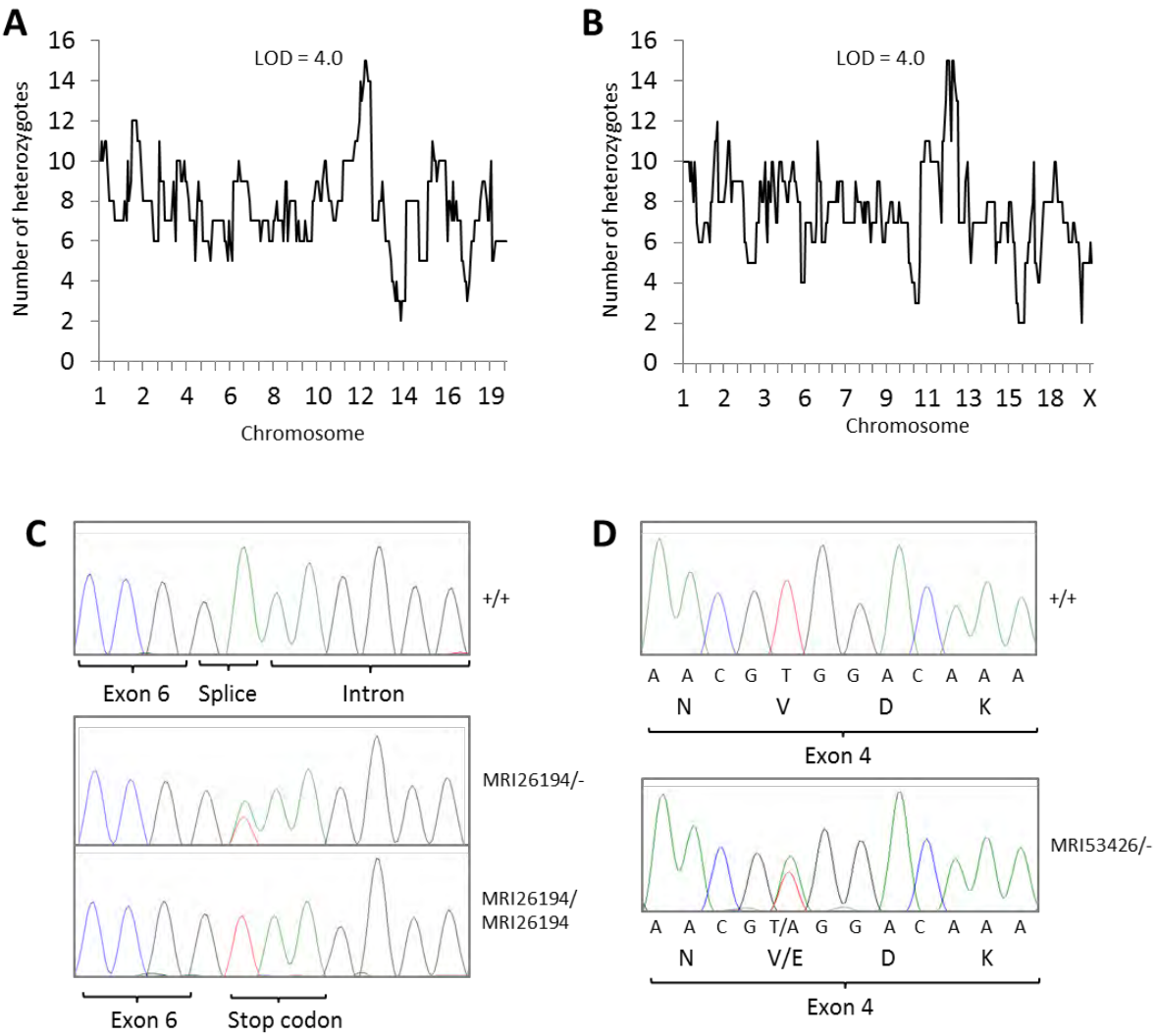
In conclusion, an association between novel mutations in the erythrocyte cytoskeletal gene *Sptb* and malaria resistance in mice is reported. In contrast to previous studies of cytoskeletal mutations, parasites were able to invade and grow within mutant erythrocytes normally. However, mutant parasitized erythrocytes were demonstrated to be more susceptible to immune clearance via phagocytosis.

SUPPLEMENTARY FIGURES

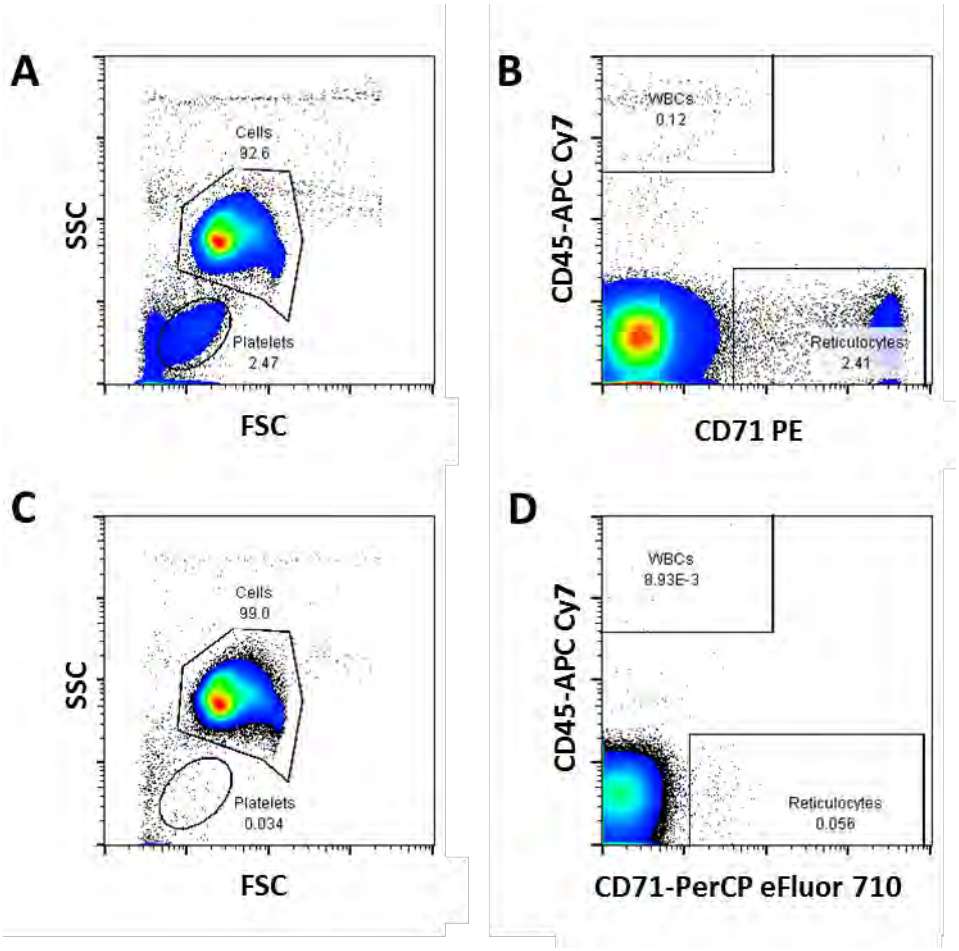


Supplementary figure 5.1. *Sptb*$^{MRI26194/MRI26194}$ mice are jaundiced and lack detectable β-spectrin.

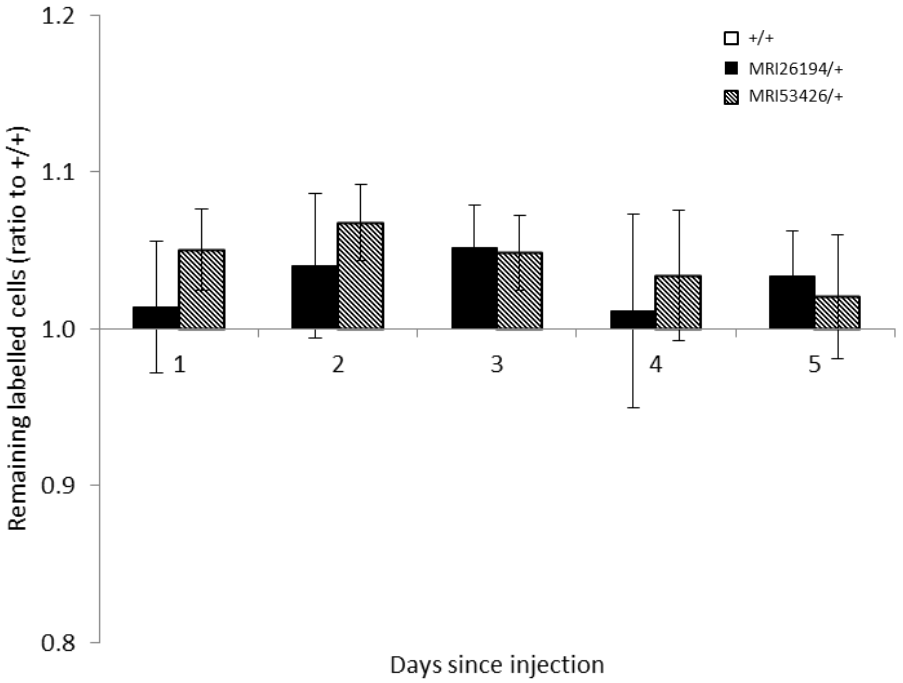
Sptb$^{+/MRI26194}$ by *Sptb*$^{+/MRI26194}$ mating's occasionally produced homozygous pups displaying jaundice (A). Giemsa stained blood smears from wild type, and homozygous pups (B). Western blot of whole erythrocyte lysates from mutant and wild type mice separated by SDS-PAGE on a 4-20% gel and probed using an antibody against the N-terminal of β-spectrin (C). The expected size of the truncated β-spectrin (24kD) is indicated with an arrow.



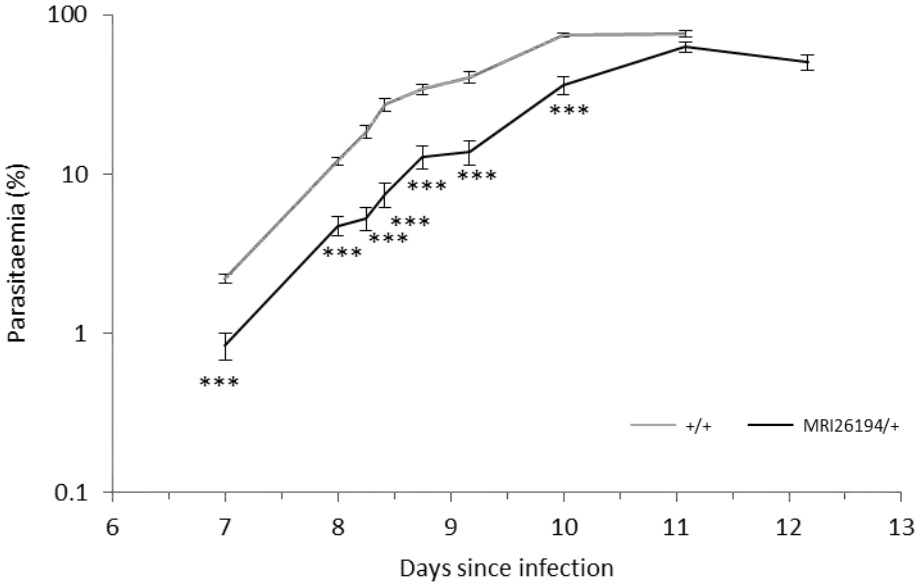
Supplementary figure 5.2. Mapping of the MRI26194 and MRI53426 mutations. Heterozygosity frequency of 15 microcytic N2 mice (MCV < 50 fL) indicate linkage peaks on chromosome 12, between 69Mbp and 87Mbp for MRI26194 (A), and between 23Mbp and 87Mbp for MRI53426 (B). The *Sptb* gene is within this interval and was selected as a candidate gene due to the similar phenotype of *Sptb* mutant mice previously described. *A*ll *Sptb* exons were Sanger sequenced in both mutant lines. For MRI26194, a T to A transversion in a splice site at the exon/intron boundary at the end of exon 6 was identified which introduces a premature stop codon. For MRI53426 a T to A transversion was located in exon four resulting in a valine to glutamic acid substitution. LOD score was calculated using a chi squared test based on the expected number of heterozygotes.



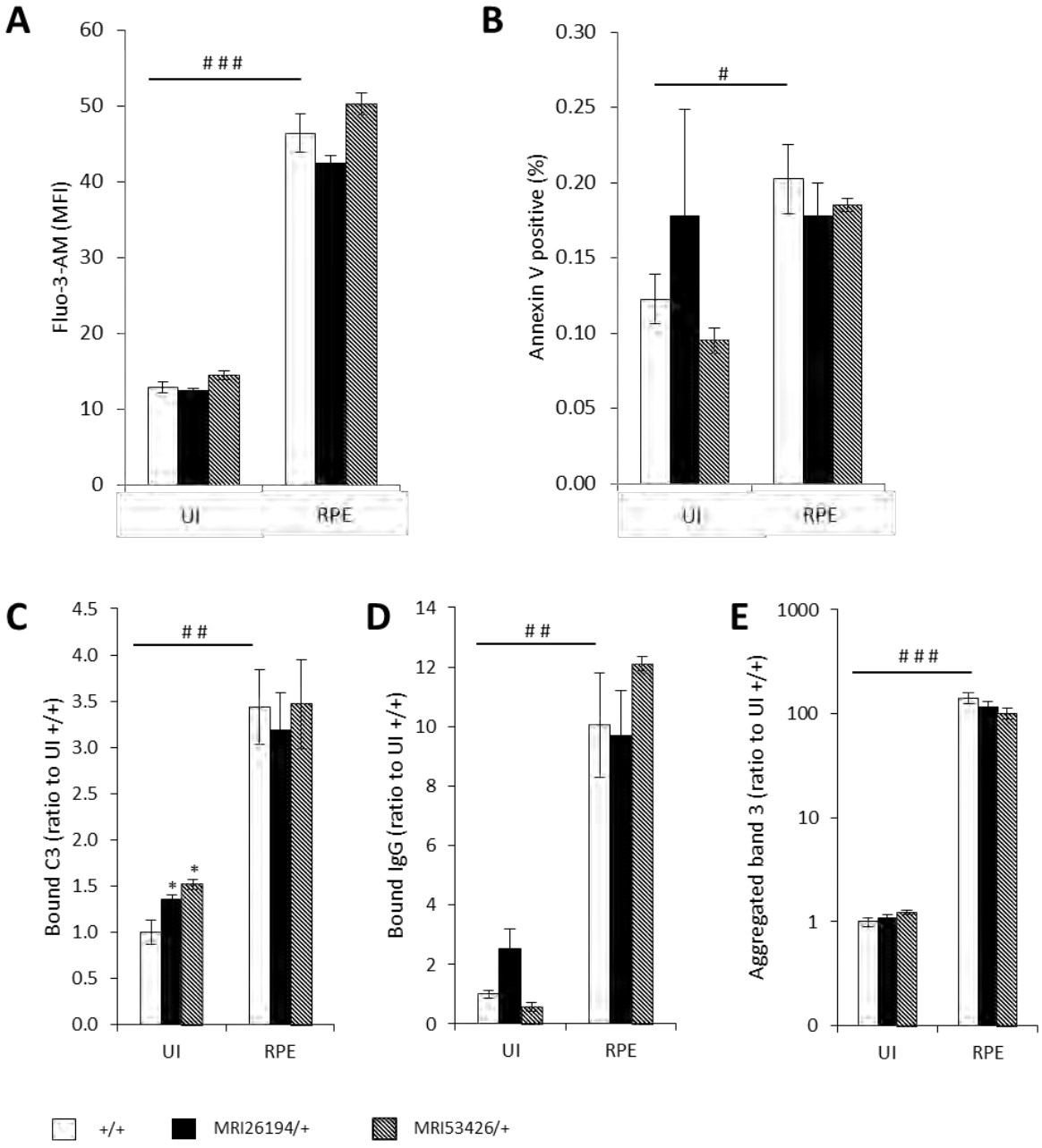
Supplementary figure 5.3. Assessment of erythrocyte purity. Representative plots of a blood sample before purification (A, B) and after purification (C, D). Platelet, WBC, and reticulocyte abundance is indicated based on FSC/SSC and staining with antibodies for CD45 and CD71.



Supplementary figure 5.4. Remaining labelled cells in uninfected mice. Labelled blood from both mutant lines, and wild type (+/+) mice was transfused into uninfected host mice. The remaining labelled cells was determined at the indicated time points by flow cytometry. Fluorescent labels were switched to account for any dye effects. Error bars indicate SEM. Data is from 3 mice and labelled blood from 3 mice per group.



Supplementary figure 5.5. Increased resistance to *P. chabaudi adami* DS infection in *Sptb*$^{+/MRI26194}$ regardless of splenectomy. Splenectomised wild type and *Sptb*$^{+/MRI26194}$ mice were infected with 1x10^4 parasitised erythrocytes. Parasitaemia was determined daily by flow cytometry. Error bars indicate SEM. *** $p < 0.001$. Data is from 6-8 mice per group.



Supplementary figure 5.6. Eryptosis and erythrocyte senescence in *Sptb*$^{+/MRI26194}$ and *Sptb*$^{+/MRI53426}$ ring stage parasitised erythrocytes. Blood samples from infected mice were analysed by flow cytometry. Uninfected erythrocytes (UI) and ring stage parasitised erythrocytes (RPE) were identified based on Hoechst 33342 staining. Fluo-3-AM mean fluorescence intensity (MFI) (A), and percentage of cells stained positive for Annexin V (B) in each population. Erythrocyte membrane ghosts from uninfected mice (UI) and infected mice at the ring stage of infection (RPE) underwent non-reducing SDS-PAGE. High molecular weight bands detected by western blot using anti-C3 (C), anti-IgG (D), and anti-band 3 (E) were quantified and expressed as a ratio to uninfected wild type (+/+). Beta actin was used as a loading control and band intensity of infected samples was corrected for parasitaemia, which was approximately equal between mice. Error bars indicate SEM. * $p < 0.05$ between *Sptb*$^{+/MRI26194}$ or *Sptb*$^{+/MRI53426}$, and wild type. $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$ between wild type UI and RPE. Data is from 3-4 mice per group.

CHAPTER 6

CONCLUSION

The malaria parasite, *Plasmodium*, exists in a delicate relationship with its multiple hosts, developed over hundreds of thousands of years of co-evolution. While natural selection has favoured those parasites best able to survive within a hostile environment, the evolution of the host has favoured those able to disrupt this fragile symbiotic interaction. This study sought to identify novel genetic changes in the host that influence this interaction and alter the course of malaria infection, as well as elucidating the specific biological mechanisms at play.

It was first observed nearly seventy years ago that people with 'sickled' blood cells were less likely to suffer from malaria[51]. Now known as sickle cell trait, this, and many other polymorphisms associated with reduced malaria susceptibility, are the most commonly observed genetic traits in humans[48]. Since their discovery a considerable amount of research has focussed on uncovering the biological mechanisms underlying the survival advantage offered by these conditions. While recent breakthroughs have offered some insights into these mechanisms, particularly in the case of sickle cell trait, much remains unknown. Indeed, while considerable progress has been made in understanding the crucial parasitic factors for *Plasmodium* survival, the host environmental factors required for parasite survival are still poorly understood.

In this study, three novel ENU-induced genetic mutations are described, which shed light on the parasite's dependence on its primary residence within the vertebrate host, the erythrocyte. In the exploration of these mutations two questions were addressed:

1. Do these mutations influence the ability of the parasite to infect its host?
2. What biological changes are introduced by these mutations and how do they influence the parasite's lifecycle?

In order to effectively address these questions, a new methodological approach was developed to allow for the accurate interrogation of the parasite's interaction with its host. The conclusions reached regarding this new method will be outlined first, followed by a discussion of the three ENU-induced mutations.

The parasite's interaction with its host is complex and multifactorial. It is therefore difficult to determine the exact aspects of the parasite's lifecycle that may be affected in a genetically altered host environment. Additionally, the consequences of a genetic mutation can be widespread, affecting multiple biological systems simultaneously, which is further complicated in the context of malaria infection. In developing the flow cytometric assay to quantify invasion of red blood cells by rodent *Plasmodium* parasites *in vivo,* described in chapter 3, it was found that by isolating the interaction of the parasite with the erythrocyte itself, rather than with the entire host, aspects of the parasite's lifecycle being affected could be greatly narrowed down. Furthermore, by maintaining this assay *in vivo*, with precise control of timing, and by the manipulation of host immune factors, this assay allowed for the assessment of specific aspects of the parasite's interaction with the erythrocyte that could not be explored using existing assays. These aspects include merozoite invasion, immune recognition and clearance of infected cells, splenic clearance of cells, and parasite development. Despite some limitations, including the delineation of factors influencing parasite survival and relating to cytoadherence of infected cells, this assay allowed for the investigation of the influence of genetic mutations on the parasite's lifecycle which would not have been previously possible. Therefore, this assay provides a valuable new tool in characterising the parasite's interaction with the erythrocyte *in vivo*.

The first ENU-induced mutation investigated in this study, located in the *Tfrc* gene, provided an opportunity to study the role of iron regulation in malaria infection. It was found that the parasite was more likely to survive to maturation within the abnormal erythrocytes resulting from this mutation, than in normal erythrocytes. The fact that the parasite can be advantaged in an atypical environment is intriguing, and raises two key questions. Is this advantage due to a change that benefits the parasite itself? Or does the mutation hinder the ability of the host to eliminate the parasite? In the first case, it is conceivable that the decreased erythrocyte density, or another, unknown, physiological or biochemical change in the erythrocyte, creates an environment that imparts less stress on the parasite, and therefore increases its likelihood of survival. Alternatively, the

alterations to the erythrocyte may interfere with the host's normal defences. For example, the spleen acts as a natural quality control system by removing erythrocytes unable to meet its criteria for passage, and the reduced size of the abnormal erythrocytes may skew their ability to pass through the spleen, thereby indirectly benefiting the parasite. While the exact reasons for this enhanced survival could not be confirmed within the timeframe of this project, future studies may benefit in exploring these possibilities. The elucidation of these mechanisms would provide clues regarding the mechanisms of resistance provided by other erythrocyte mutations as well as having implications for the management of iron deficiency and supplementation in the context of malaria infection. In the first case, in determining the mechanisms of resistance provided by erythrocyte mutations, it is tempting to postulate that the parasite simply cannot survive within an abnormal environment. However this study highlights that these kind of generalisations are not necessarily true, for example, microcytosis has been suggested as a common cause of reduced parasite growth [i.e. Mockenhaupt *et al.*[412]], whereas this study indicates that, depending on the cause, the opposite can be true. In the second case, following the termination of a major clinical trial in Pemba due to increased malaria incidence in groups receiving iron[379], new guidelines for iron supplementation in malaria endemic areas are being developed. This study highlights the importance of assessing malaria risk due to iron deficiency microcytosis without anaemia, which has not yet been investigated. If malaria risk was found to be increased in this condition (Figure 6.1), this would lend support to the importance of iron supplementation, even in those without anaemia. This is supported by the observation that erythrocytes from people with iron deficiency without anaemia sustain normal *P. falciparum* growth in culture (Martha Clark, personal communication). Of course, it is imperative that malaria incidence is monitored in the short term in these programs, as currently recommended by the WHO[438], however, perhaps in the long term iron supplementation may reduce, rather than increase, malaria susceptibility.

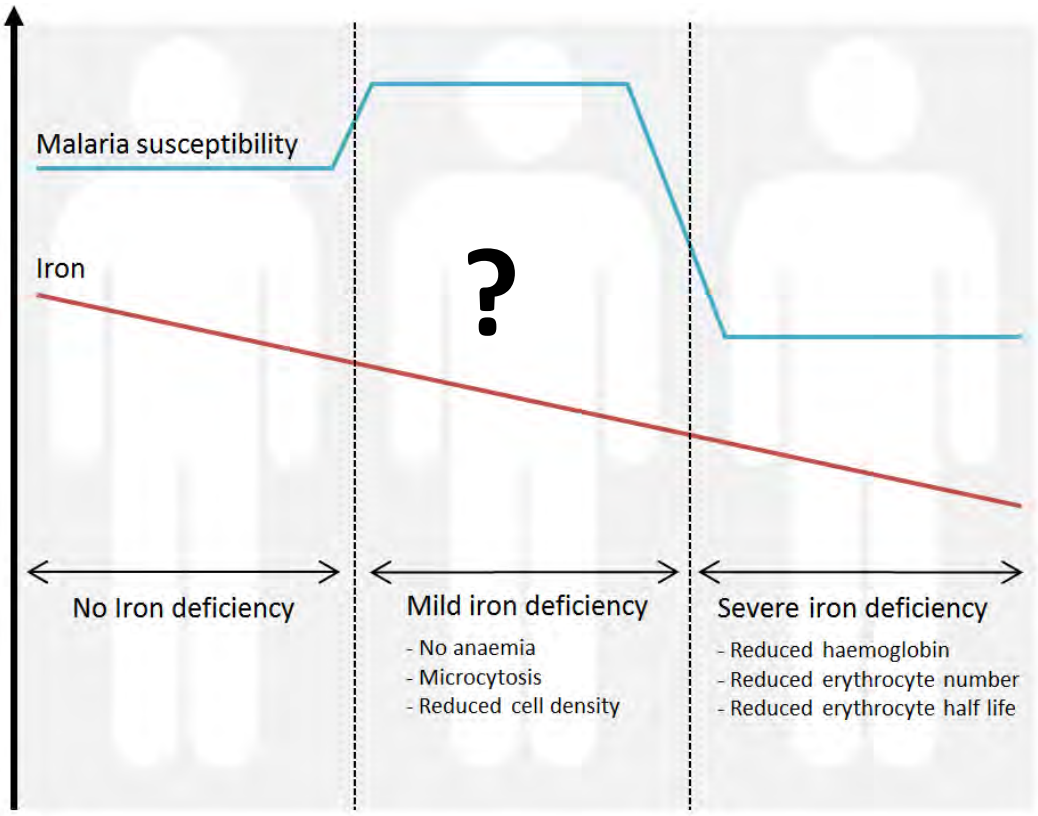


Figure 6.1. Hypothesised model of malaria susceptibility during iron deficiency. Clinical trials and *in vitro* studies indicate that severe iron deficiency reduces malaria susceptibility, however, little is known of the effects of mild iron deficiency. This study has led to the hypothesis that mild iron deficiency may increase malaria susceptibility.

The next two mutations investigated in this thesis were located within the same gene, *Sptb*. Despite the difference in mutation type, the phenotype of these mice, both in terms of erythrocyte properties and decreased malaria susceptibility, was nearly identical. It was found that in these abnormal erythrocytes, in direct contrast to the *Tfrc* mutant erythrocytes, the parasite had a decreased likelihood of survival. In this case, the mechanisms underlying the decreased parasite survival were further narrowed down, and it was concluded that parasites were more susceptible to phagocytosis within mutant erythrocytes. The enhanced phagocytosis of abnormal erythrocytes is a well described process which was noted in sickled erythrocytes over seventy years ago[439]. Likewise, the phagocytosis of parasitised erythrocytes was noted during early studies of malaria, and is recognised as a crucial host defence mechanism[46]. However, the hypothesis that parasitised mutant erythrocytes may be more susceptible to phagocytosis than equivalent, parasitised normal erythrocytes has not been explored until recently. Enhanced phagocytosis of parasitised mutant

erythrocytes has now been reported in several erythrocyte polymorphisms known to convey malaria resistance, and the mechanism for this is reported as either an increase in eryptosis, an erythrocyte specific form of apoptosis, or as accelerated band 3 mediated senescence[68,242]. In this study, it was found that neither of these processes could account for the increased phagocytosis of parasitised mutant erythrocytes. It is therefore likely that a novel mechanism is responsible. One possibility is that non-opsonic phagocytosis, which, in malaria infection, has been reported to primarily occur through CD36 recognition of parasite antigens, is enhanced in these cells[437]. If this is the case, it would have important implications for our understanding of host immunity and parasite virulence factors. The CD36 binding interactions of infected cells have been widely studied due to their role in cytoadhesion, however, the role of CD36 in the immune clearance of parasites is less studied, particularly in abnormal erythrocytes. In general, the observation that mutations in a cytoskeletal protein can influence phagocytosis, without altering the ability of the parasite to invade or growth within the cell, highlights a novel resistance mechanism for this type of mutation, which may inform future studies of the parasite's interaction with the erythrocyte.

Overall, this thesis demonstrates the sensitivity of the parasite to changes within the host erythrocyte. In the past, the study of naturally occurring erythrocyte polymorphisms has provided valuable insights into the nature of the parasite's interaction with its host. However, the ENU mutagenesis screen employed here offered an opportunity to explore this interaction in greater detail. The aim of this study was to identify host factors which influence the ability of the parasite to sustain itself. Ultimately, a greater understanding of host factors important in malaria infection will not only allow for better management of disease, but may also highlight host factors critical for parasite survival. The manipulation of these factors, a so called 'host directed therapy' (HDT), may provide a means to avoid the development of parasite drug resistance, by removing the parasite's genetic control over the drug target. One possible HDT target arising from this study is the binding interaction between beta spectrin and ankyrin. If this interaction could be blocked pharmaceutically, it may produce a malaria resistance phenotype similar to that reported here. Additionally, this study

has led to some important insights regarding the feasibility of the HDT approach. Importantly, this study highlights the possibility of disrupting parasite infection and increasing survival, without directly inhibiting parasite invasion or growth. Indeed, many naturally occurring genetic polymorphisms which convey malaria resistance, including sickle cell trait, which provides up to 90% protection against severe malaria[55], do not influence parasite invasion or growth under normal culture conditions. One way to think of this is that the parasite is essentially 'holding down the lid' on the immune system. In a normal situation the parasite does this incredibly well, the majority of malaria infections proceed unnoticed and are asymptomatic, and sterile immunity rarely occurs, and then only under constant exposure to the parasite. In fact, parasite expressed proteins, such as PfEMP-1, actively supress the immune response[440,441]. In conditions which convey malaria protection, although the parasite may not be directly harmed, its 'hold' on the immune system may be weakened. This can manifest as an increased susceptibility to phagocytosis[68], impaired cytoadherence[261], reduced inflammatory pathologies[442], or possibly by other immune mechanisms which remain unexplored. In terms of HDT, therapies are already being trialled which may be acting on the parasite in this manner. For example, indolone-*N*-oxides are thought to act on the parasite by inducing oxidative stress, which accelerates band 3 mediated senescence, and in turn subjects parasitised erythrocytes to increased phagocytosis[310]. The *Sptb* mutations described here may in fact be acting in a similar manner against the parasite, by creating an environment with increased stress, which ultimately leads to immune clearance of the infected cell. Overall, this study highlights the possibility of 'tipping the balance' in favour of the host immune system, as a HDT for malaria infection.

This study also lends credence to the hypothesis that mutations in cytoskeletal proteins may have an increased prevalence in malaria endemic populations due to a malaria protective effect. The occurrence of cytoskeletal mutations causing SAO and HE is shown to be increased in malaria endemic areas, and associated with malaria protection in the case of SAO[101,107]. However, no studies have investigated the prevalence of cytoskeletal mutations which cause microcytosis without

morphological abnormalities, as seen in the mutations described in this study. This work has indicated that morphological abnormalities are not necessary to influence malaria outcome, therefore, in future studies the influence of asymptomatic microcytosis should be assessed, alongside morphological abnormalities, in determining malaria risk factors. In terms of ENU mutagenesis, this study has demonstrated the utility of a forward genetic screen in identifying novel genes associated with altered malaria susceptibility. However, it should also be noted that in addition to screening mice based on haematological abnormalities mice were also screened based on malaria resistance alone, an approach which has already produced promising results for future studies.

Natural selection has provided proof that the erythrocyte can be altered, without adverse effects, such that the malaria parasite can no longer infect its host. This thesis has provided additional evidence of this possibility. If the critical host changes brought on by these mutations can be identified, it may be possible to mimic these effects using a HDT. This therapy would have many advantages over an equivalent genetically induced change. Therapies would only need to be administered during malaria risk, would likely be more effective against erythrocytes than other targets due to the lack of gene expression in these cells, and would avoid problems associated with compounding effects due to multiple inherited mutations or homozygosity. It is encouraging that nature has succeeded in the face of these challenges and begs the question, can we achieve something similar?

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