IRON DEFICIENCY AND IRON SUPPLEMENTATION CONSPIRE TO MEDIATE SUSCEPTIBILITY TO ERYTHROCYTIC STAGE PLASMODIUM FALCIPARUM INFECTION

Martha Ann Clark

A dissertation submitted to the faculty at the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Microbiology and Immunology in the School of Medicine.

Chapel Hill
2014

Approved by:

Anthony Richardson

Steven Meshnick

Carla Cerami

William Goldman

Raj Kasthuri

Con Beckers

ABSTRACT

MARTHA ANN CLARK: Iron Deficiency and Iron Supplementation Conspire to Mediate Susceptibility to Erythrocytic Stage *Plasmodium falciparum* Infection
(Under the direction of Steven Meshnick)

Malaria and iron deficiency are interconnected public health concerns, which disproportionally affect children and pregnant women. Malaria causes an estimated 250 million infections and 1 million deaths per year. *Plasmodium falciparum* is the most virulent species of the malaria parasite that infects humans. Anemia, predominantly iron deficiency anemia, is the most common nutritional deficiency worldwide, and affects up to 50% of populations in the developing world. The World Health Organization recommends universal iron supplementation in regions where malnutrition is common. This recommendation has been complicated by clinical evidence that iron deficiency protects against malaria infection, and that iron supplementation increases susceptibility to malaria infection. The mechanisms underlying the interaction between malaria, host iron, and iron supplementation remain unclear. Here, I've employed the *in vitro* system for cultivating erythrocytic stage *P. falciparum* to assess first, the impact of extracellular iron on parasite growth as well as the bioavailable iron content of parasitized erythrocytes. I have found that extracellular iron is incorporated into parasitized erythrocytes but does not have affect parasite growth. Second, I assessed the capacity of erythrocytes from iron deficient and iron supplemented donors to support erythrocytic stage *P. falciparum* growth. In these studies I observed that *P. falciparum* propagation is reduced in iron deficient erythrocytes and that reduced parasite propagation is a result of decreased parasite invasion into iron deficient erythrocytes as well as decreased production of infectious daughter

merozoites within iron deficient erythrocytes. I additionally observe that *P. falciparum* propagation is recovered in erythrocytes donated by iron supplemented iron deficient donors. Furthermore, I attribute the recovery of *P. falciparum* erythrocyte propagation to the replacement of iron deficient erythrocytes with young iron-replete erythrocytes that are produced in response to iron supplementation. These results are consistent with clinical observations that iron deficiency is protective against malaria infection and iron supplementation increases the risk of malaria infection. Moreover, my results suggest that iron mediated alterations to erythrocyte physiology and intra-host erythrocyte population dynamics as well as potentially altered serum iron levels contribute to the underlying mechanisms governing the relationship between the malaria parasite, iron deficiency, and iron supplementation.

ACKNOWLEDGMENTS

"These are just physiological dispositions and yet again nature taunts our convention, our intuition, our fear. You have to be brave to study her brilliance."

– Janna Levin, theoretical cosmologist

Above all I would like to thank Carla Cerami for teaching me to be fearless in science as well as life. For teaching me that revelation cannot occur unless you are willing to let go of your assumptions and your biases. Thank you for being an incredible mentor, welcoming me into your family when the distance from my own family was especially painful, and being an invaluable friend and confidante. I will never forget our spirited discussions of a newly discovered paper, a recent result or a new idea. With both sadness and excitement I anticipate leaving the embrace of the scientific family you've created here at UNC with Steve and joining my new scientific family in Boston. I take solace in the fact that I know this is not an end to our brilliant association and friendship, but rather a new chapter. I look forward to sharing my scientific journey with you for many years to come.

I would like to thank Steve Meshnick for sharing his vast expertise and experience in the malaria field. The small little pocket of malaria research you've created at UNC very special and exceptionally unique. You've assembled an excited and talented multidisciplinary team that is fearless bridging the chasm that currently exists between basic science and epidemiology. Thank you for providing me with an exceptional multi-disciplinary environment for my training.

I would like to thank Gail Willsky for planting and nurturing the seed of scientific inquiry and research within me. My life has been transformed in ways I could not have imagined or be more thankful for. Under your tutelage I received the most exceptional of scientific training and

most importantly mentoring. It is people like you who are responsible for inspiring and preparing the next generation of explorers, innovators, and creators; in a word scientists.

I would like to thank Steve Taylor and Jon Juliano for instilling in me an appreciation for the beauty of a clearly communicated scientific story, and helping me find my own scientific voice. The countless lab meetings spent pulling apart presentations and specific aims pages seem like they have been seamlessly incorporated into the very rhythm of my own scientific voice. I guess I've picked up y'alls scientific twang.

I would like to thank my committee for challenging me to develop scientifically, and discover the fine balance between confidently communicating my scientific story and acknowledging the perpetual unknowns that exists in scientific research. Because of you I was able to discover an inner confidence I never knew existed. I would not be where I am today as a scientist without you.

I would like to thank Morgan Goheen, my scientific sister, as well as each of the beautiful smart women I have been blessed to train with and be mentored by here at UNC. Morgan, Cheryl, Blaire, Megan, Caitlin, Maria, Liz, and Eliza thank you for playing and working so hard side by side with me each and every step of the way. Because of you I have been able to discover the sisterhood that exists between each and every woman. I love y'all and look forward to many more long conversations and equally long bike rides, runs, and walks.

I would like to thank my amazing family. I know that more often than not I have stubbornly pushed forward doggedly brandishing my independence. Doubt not though your importance in every aspect and every minute of these past six years. It was learning to live life joyfully and ever rolling with the punches with Mary, Paul, Charlotte, and Daniel that got me to the place I am now. Because of you four, nothing can keep me down long, and I know what it

means to have true comradeship. It was mom day after day demonstrating that rewards that can be found in one's career and the virtue of perseverance. Because of your example mom, I've found a career I love and I learned that to reap the wards one must dig in and push through even in the hardest of times. It was dad repeatedly challenging me to ask questions, and me obviously not doing so (most of the time). Who would have guessed I'd come to the point where I can't stop asking questions. Because of your example dad, I've discovered the transformative gift of wonder. Life has never looked more beautiful.

Finally, I would like to thank Nicholas Spidale, my partner and my anchor. Because of you I was able to keep my sanity in the insanity that is graduate school. It is so easy to forget every other aspect of our lives (food, bills, family and friends) amid the excitement of a new idea or a surprising result. Your daily presence beside me has kept me grounded; ever reminding me nothing is as important as the people (and pups) we love. I cannot express how excited and happy I am to embark with you on the next big adventure of both our professional and personal lives.

PREFACE

This work would not have been possible without the contribution of many talented scientists, skilled nurses, and patient study participants. The experiments outlined in Chapters 2, 3, and 4 were made possible through our collaboration with Dr. Raj Kasthuri. With Raj's assistance we were able to identify and enroll participants into our study.

Chapter 2 represents a publication for which I was the first author. I conducted all the experimentation contained in Chapter 2. Dr. Carla Cerami and Dr. Nancy Fisher provided assistance with experimental design and Dr. Carla Cerami helped write the resulting paper. The paper was published prior to writing this thesis with the following citation.

> Clark, M., Fisher, N. C., Kasthuri, R., and Cerami Hand, C. (2013). Parasite maturation and host serum iron influence the labile iron pool of erythrocyte stage Plasmodium falciparum. *Br. J. Haematol.* 161, 262–269.

Permission to include the article in its entirety in a PhD dissertation was retained from John Wiley & Sons Ltd. (publisher of The British Journal of Haematology) as explained at https://s100.copyright.com/CustomerAdmin/PLF.jsp?ref=17c65dfb-78bf-4cfc-87f0-f86c38ab9926.

As with Chapter 2, I conducted all the experimentation contained in Chapter 3. Dr. Carla Cerami, Dr. Tony Fulford, and Nick Spidale provided assistance with experimental design. Dr. Carla Cerami and Nick Spidale helped write the resulting paper, which has been reviewed by PLOS One.

The experimentation contained within Chapter 4 is primarily my own work. Morgan Goheen provided assistance with experimentation. As mentioned previously Dr. Raj Kasthuri assisted in identifying and enrolling study participants. Michael Nicholoson of Precision Biosciences (Durham, NC) provided access to the Beckman Quanta Flow Cytometer used to analyze RBC volume experiments (Figure 4.7A). Dr. Tony Fulford performed statistical analysis of growth data (Figure 4.3C and D). Dr. Carla Cerami, Dr. Tony Fulford, and Dr. Steve Taylor assisted with experimental design, data analysis, and helped write the resulting paper. The resulting paper has been reviewed by Nature Communications.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS

ACM – Albumax II Complete Media

ATP – Adenosine triphosphate

C – Chymotrypsin treatment

CA-AM – Calcein acetoxymethyl ester

CI – Confidence interval

DFO – Deferoxamine

DNA – Deoxyribonucleic acid

DV – Digestive vacuole

ER – Endoplasmic reticulum

EC – Endothelial cell

Fe^{2+} – Ferrous iron

Fe^{3+} – Ferric iron

G6PD – Glucose-6-phosphate dehydrogenase

Hgb – Hemoglobin

IDA – Iron deficiency anemia

IDA-Fe – Iron deficiency anemia iron supplemented

IR – Iron-replete

IR-Fe – Iron-replete iron supplemented

IRB – Internal review board

IRE – Iron responsive element

IRP – Iron responsive protein

LIP – Labile iron pool

MA – Mature adult

MACS – Magnetic activated cell sorting

MCHC – Mean corpuscular hemoglobin content

MCV – Mean corpuscular volume

N – Neuraminidase treatment

NTBI – Non-transferrin bound iron

O – Old

O+ RBC – O positive RBC

Pf – Plasmodium falciparum

pRBC – Parasitized RBC

RBC – Red blood cell

RDW – RBC Distribution Width

ROS – Reactive oxygen species

RPMI – Roswell park medical institute

SD – Standard deviation

SI – Susceptibility index

T – Trypsin treatment

VY – Very young

WHO – World Health Organization

Y – Young

YA – Young adult

Δ – Change or difference

μ – Micro

Ø – non enzymatically treated

CHAPTER ONE

Introduction

1.1 A relationship revealed – host iron status and malaria

Iron deficiency and malaria are significant co-morbidities in large portions of the developing world, and both maladies disproportionately affect pregnant women and children. Malaria causes an estimated 250 million infections and 500,000 deaths annually. Iron deficiency is estimated to affect one quarter of the world's populations causing substantial morbidity. Fortunately, iron deficiency is easily treated with iron supplementation (Okebe et al., 2011). Accordingly the World Health Organization (WHO) recommends routine iron supplementation for children and adults in areas with high prevalence of iron deficiency (Haider et al., 2013; Low et al., 2013). However, the wisdom of universal iron supplementation campaigns in malaria endemic regions has recently been questioned due to clinical evidence that suggests iron deficiency protects against malaria, and that iron supplementation of women and children may increase the incidence of malaria when given without malaria prophylaxis or access to adequate health care (Sazawal et al., 2006; Tielsch et al., 2006; Veenemans et al., 2011; Zlotkin S et al., 2013; Esan et al., 2013; Nyakeriga et al., 2004; Gwamaka et al., 2012; Jonker et al., 2012; Kabyemela et al., 2008; Senga et al., 2011). This situation has created a dilemma for health policy makers and health care workers in malaria endemic regions of the world (Prentice et al., 2013).

Despite these clinical and epidemiological studies, the extent to which the human host's iron status affects risk to and severity of malaria infection is unknown. Differences in study design and confounding factors (such as acquired immunity to malaria and hemoglobinopathies) have made the clinical and epidemiological studies difficult to interpret (Prentice et al., 2007). Furthermore, though iron and malaria have been and continue to be studied the exact biological relationship between host iron and malaria virulence remains largely unclear.

1.2 Iron deficiency and iron deficiency anemia

Iron deficiency is a condition in which there is insufficient iron in the body to maintain normal physiologic functions. Iron deficiency can be categorized into three stages: iron deficiency without anemia, iron deficiency with mild anemia, and iron deficiency with severe anemia. Iron deficiency anemia occurs when iron stores are exhausted and the supply of iron to tissue is compromised; this condition is defined as anemia with biochemical evidence of iron deficiency. Iron deficiency is most prevalent and severe in young children and women of reproductive age, but can also occur in older children, adolescents, adult men, and the elderly. The WHO estimates that 50% of pregnant women and 40% of preschool children in the developing world are iron-deficient (WHO | Assessing the iron status of populations; Kassebaum et al., 2013) Often, iron deficiency develops slowly and is not clinically diagnosed until severe anemia is apparent (Stoltzfus, 2003).

Studies suggest that iron deficiency impairs the growth, cognition, and neurological development of children from infancy through adolescence, impairs immune function, and is associated with increased morbidity rates (Wang et al., 2013; De-Regil et al., 2011, 2013). Iron deficiency during pregnancy is associated with multiple adverse outcomes for both mother and infant, including increased risk of hemorrhage, sepsis, maternal mortality, perinatal mortality,

and low birth weight (Peña-Rosas et al., 2012a, 2012b). Iron deficiency anemia can be a direct cause of death or contribute indirectly. For example, during childbirth an anemic mother cannot afford to lose more than 150 mL of blood, compared with a healthy mother who can lose up to 1 liter of blood and still survive. Thus, the WHO recommends iron supplementation for all men, women and children in areas where malnutrition is prevalent (WHO | Guidelines on food fortification with micronutrients).

Host iron metabolism is intimately linked to the host response to infection and inflammation. With the high incidence of infection in the developing world, the study and treatment of iron deficiency anemia becomes challenging. In the face of infection and inflammation, the human host protein hepcidin becomes elevated and initiates signaling which results in reduced iron absorption into the body along with the redistribution of body iron stores. As a consequence of the effect of infection and inflammation on human iron metabolism, many of the biomarkers utilized to assess host iron status are sensitive to both iron as well as infection. For example, low serum ferritin (serum ferritin reflects total body iron reservoirs) is indicative of iron deficiency. However, ferritin is also an acute phase protein, which is elevated in the context of infection, and as a result is not a reliable marker of human iron status in the presence of infection or inflammation. Like serum ferritin, transferrin saturation and transferrin receptor levels are biochemical markers of human iron status that are also sensitive to infection and inflammation. As a result evaluating an individual's iron status in the context of infection has proven difficult (Aguilar et al., 2012), and the scientific community has struggled to establish formal guidelines for defining human iron status in the presence of infection.

1.3 Malaria

In 2012 malaria caused an estimated 207 million infections and over 600,000 deaths; 90% of these deaths occurred in sub-Saharan Africa, and 77% occurred in children under five (WHO | World Malaria Report 2013). At least five species of the eukaryotic *Apicomplexan* parasite from the genus *Plasmodium* cause malaria in humans with *Plasmodium falciparum* being the most common and deadly. Malaria parasites are transmitted to the human host by mosquitoes. Following the bite of a malaria parasite infected mosquito, the sporozoite stage of the parasite enters the bloodstream and travels to the liver, where it subsequently infects liver hepatocytes. Malaria replication in the liver is asymptomatic. Next, the merozoite form of the parasite leaves the liver and enters into circulation to infect host red blood cells (RBCs). During the erythrocytic stage of infection, the parasite repeatedly invades, replicates within, and egresses from host RBCs. This erythrocytic stage of infection is responsible for all symptoms of disease (Miller et al., 2013), and the severity of disease is directly associated with parasite burden (Chotivanich et al., 2000; Dondorp et al., 2005).

A wide range of symptoms can be observed in malaria patients. Clinically however, malaria is categorized as either uncomplicated or complicated. Complicated malaria is further divided into three overlapping syndromes: cerebral malaria, severe anemia, and metabolic acidosis. The clinical syndrome observed in each individual patient is influenced by multiple variables: parasite species, host immune status and genetic background, as well as the use and timing of antimalarial drugs (Taylor et al., 2010).

1.4 Clinical studies linking iron and malaria infection

Host iron has received significant attention at the clinical level as a major factor that may regulate malaria virulence. The results of clinical studies conducted prior to 2002 which

examined the relationship between host iron status and malaria risk are reviewed in three meta-analyses (Oppenheimer, 2001; Shankar, 2000; Gera and Sachdev, 2002). In the interim, two large iron supplementation trials as well as several smaller clinical studies have shed further light on the relationship between host iron status and malaria infection (**Table 1**). Clinical trials that have examined the relationship between host iron and malaria fall into two basic categories: those that observe the rate of malaria in individuals with iron deficiency anemia, and those that look at the rate of malaria infection in individuals given iron supplementation. Differences in study design exist within both study types, and include: the definition of study participant iron status, the administration of iron alone or with folate, and access to health care. Despite differences in study design, assessment of the outcome of the clinical studies has led to the general consensus that iron deficiency is protective against malaria, and iron supplementation increases malaria risk in the absence of access to adequate health care (Spottiswoode et al., 2012; Prentice and Cox, 2012; Stoltzfus, 2012).

While these clinical studies and meta-analyses have been indispensable for determining the relationship between host iron status and malaria risk, it is not clear how iron deficiency protects and why iron supplementation increases risk. Immunity to malaria and high prevalence of genetic traits such as Glucose-6-phosphate dehydrogenase (G6PD) deficiency and hemoglobinopathies in the study populations limit the capacity of clinical studies to parse out causation. Furthermore, relatively little is known with regards to the role host iron plays in malaria pathogenesis. Iron impacts a broad range of biological processes that have the potential to shape malaria pathogenesis. As a result, even with the most ideal of clinical study designs; the prerequisite knowledge of which aspects of malaria pathogenesis should be studied is largely absent. A better grasp on the underlying biological principals that govern (i) the protection of

iron deficiency against malaria and (ii) the increased risk of malaria associated with iron supplementation is critical for managing iron supplementation campaigns in malaria endemic regions.

1.5 Biological importance of iron

Iron is an essential nutrient for nearly every living organism including humans and the malaria parasite. Iron impacts a broad range of biological processes; including host and parasite cellular function, erythropoiesis and immune function. The capacity of iron to fluctuate between two oxidation states, ferrous (Fe^{2+}) and ferric (Fe^{3+}), makes it indispensable for many critical biological processes, including DNA replication, cellular respiration, and oxygen transport. However, the same useful biphasic properties of iron, which make it indispensable, also contribute to its high cytotoxicity. As a result the human host tightly regulates iron availability and usage.

Access to iron is particularly important in the context of host-pathogen interactions. When confronted with infection and inflammation the human host reallocates its iron reservoirs in an effort to deprive invading pathogens of iron. As described, the human protein hepcidin – a rheostat of systemic iron homeostasis – signals the body to decrease absorption of iron in the proximal duodenum and orchestrates the movement of iron from serum into storage within the liver and macrophages (Roy, 2013). As a result of reduced serum iron, erythropoiesis—a process exquisitely sensitive to iron levels—slows in the face of infection as well as inflammation. The human host's active reduction in bioavailable iron protects against a wide range of pathogens (Armitage et al., 2011). Not surprisingly, as many pathogens require access to host iron sources to survive and grow, pathogens have evolved sophisticated iron acquisition systems, and the iron

acquisition systems of many bacterial and fungal species have been well described (Skaar, 2010). By comparison how the malaria parasite acquires, regulates, and utilizes iron remains a mystery.

1.6 Iron metabolism in the malaria parasite

Iron is essential for the survival of the malaria parasite. The parasite multiplies 8-32 times in the course of a single intra-erythrocytic lifecycle. Iron is an essential cofactor for the DNA replication enzyme ribonucleotide reductase, and as a result iron is required to fuel this rapid intra-erythrocytic proliferation (Rubin et al., 1993). Iron is also utilized by the parasite for pyrimidine (Krungkrai et al., 1990; van Dooren et al., 2006) and heme biosynthesis (Sato et al., 2004; Nagaraj et al., 2010, 2013, 2009; Sato and Wilson, 2002; Dhanasekaran et al., 2004; Nagaraj et al., 2008). As with the human host, the malaria parasite must also balance its need for iron against the cytotoxicity of iron.

The malaria parasite metabolizes host hemoglobin in its acidic digestive vacuole in order to acquire necessary amino acids; however, as discussed below (**The relationship between intra-erythrocytic iron and erythrocytic stage malaria**) the parasite does not utilize the iron in host heme. *Plasmodium* aspartic and cysteine proteases degrade host hemoglobin and release large quantities of toxic iron-laden heme (Goldberg et al., 1990; Subramanian et al., 2009). Apicoblast parasites neutralize the cytotoxic heme produced during hemoglobin metabolism by sequestering the heme in an inert crystal, hemozoin (Rudzinska et al., 1965; Chugh et al., 2013). Despite neutralizing a substantial portion of host heme into hemozoin, some residual heme remains free and becomes oxidized, generating free oxygen radicals (Francis et al., 1997). The parasite possesses powerful thioredoxin and glutathione systems to maintain intracellular redox equilibrium (Jortzik and Becker, 2012). However, even when these redox systems are functioning at full capacity, oxidative stress significantly increases as the parasite matures and

replicates within host erythrocytes (Fu et al., 2010). In fact, many antimalarials, including artemisinin, appear to target the parasite's ability to detoxify reactive oxygen species (ROS) (Rosenthal and Meshnick, 1996; Klonis et al., 2013; Ariey et al., 2014). For example, it was recently found that mutations in PF3D7_1343700 (Kelch) can confer resistance to artemisinin. The authors speculate that these mutations cause a disruption of the parasite's ability to detoxify reactive oxygen species because the efficacy of artemisinin depends on its ability to generate oxygen radicals and some kelch-containing proteins in other organisms have been shown to be involved in the regulation of cytoprotection (Ariey et al., 2014).

Given the relationship between iron, heme, and ROS, it is possible that perturbations in host iron regulation might also affect the malaria parasite's redox equilibrium. Iron responsive proteins and their accompanying iron responsive elements are critical for maintaining cellular iron homeostasis in the human host. Iron responsive proteins and iron responsive elements are responsible for mobilizing iron when demands are high and moving iron into storage when excess iron may promote ROS formation (Hentze et al., 2010). Loyevsky et al. identified and characterized a *P. falciparum* iron responsive protein (IRP), the expression of which was affected by iron starvation as well as iron supplementation (Hodges et al., 2005; Loyevsky et al., 2001, 2003). However, a search of gene databases failed to identify *Plasmodium* homologues of ferritin, ferroportin, metallothione, a ferrioxamine based transport system or ferredoxin or siderophore biosynthesis pathways – all proteins and processes utilized by other organisms to acquire, regulate, and store iron (Scholl et al., 2005a). Clearly, much remains unknown regarding parasite iron biology.

1.7 Iron chelators and their contribution to the elucidation of malaria iron biology

Realizing the importance of iron for the malaria parasite, researchers have invested extensive time and effort into the investigation of the anti-malarial activity of iron chelating agents. These studies have also provided insight into malaria parasite iron biology. In contrast to mammalian cells, which are sensitive to millimolar concentrations of iron chelators, erythrocytic stage malaria parasites are sensitive to micromolar concentrations of iron chelators *in vitro* and in animal models (Cabantchik et al., 1996). The cytotoxicity of iron chelators is dependent upon the stage of intra-erythrocytic maturation of the malaria parasite and the hydrophobicity of the iron chelator (Lytton et al., 1994). For example, the hydrophilic chelator hydroxamate-based deferoxamine (DFO) has cytostatic activity against the ring stage and cytotoxic activity against the late trophozoite and schizont erythrocytic stages of the parasite (Whitehead and Peto, 1990; Lytton et al., 1994; Cabantchik et al., 1999).

The cytotoxicity of iron chelators against the malaria parasite suggests that the mechanism of action of iron chelators is more complex than simple iron deprivation. Alternative mechanisms have been suggested for some chelators, including the direct inhibition of parasite ribonucleotide reductase activity (Lederman et al., 1984; Lytton et al., 1994). Furthermore, as iron chelators can modulate host immune function, iron chelator anti-malarial activity may be a result of modification of the host immune response (Li et al., 2012; Golenser et al., 2006).

Caution must be taken when considering the use of iron chelators to inform our understanding of the biological relationship between iron deficiency and malaria infection. The evidence that iron chelators do more than merely deprive the parasite of iron introduces potential confounding factors into studies that utilize iron chelators as a model for iron deficiency. Furthermore, most iron chelators cannot chelate iron associated with heme, ferritin, or

transferrin. Because the iron saturation of each of these host iron reservoirs are reduced in iron deficiency, iron chelators are not suitable for studying the effect of host iron reduction on the malaria parasite.

That said, evidence that chelation of chelatable extracellular and intra-erythrocyte iron does not impact erythrocytic stage *P. falciparum* growth, suggests that chelatable host iron is not necessary for the erythrocyte stage of infection (Scott et al. 1990). Furthermore, work by Moormann et al. shows that parasite nuclear and mitochondrial transcripts decrease in the presence of the iron chelator DFO (Moormann et al., 1999). These results are consistent with a normal cellular response to iron deprivation. In conclusion, iron chelators are obviously indispensable in the study of iron biology. However, in the case of malaria caution must be taken.

1.8 Host iron reservoirs available to erythrocytic stage malaria

It is inarguable that iron is essential to erythrocytic stage malaria and therefore possible that alterations in host iron levels may tip the balance between inhibiting or promoting parasite growth and virulence. Consequently, the question of how the parasite acquires host iron becomes central. A healthy iron-replete human has 3 – 4 total grams of iron, which is distributed in hemoglobin contained within circulating RBCs (2.5 g), in iron containing proteins (400 mg), in serum bound to transferrin (3 – 7 mg), and in storage proteins such as ferritin (1 g). Host iron reservoirs available to erythrocytic stage malaria parasite include: (1) transferrin and non-transferrin bound iron (NTBI) in the serum and (2) intra-erythrocytic iron contained within hemoglobin, ferritin, as well as trace amounts freely bioavailable iron in the RBC cytosol (**Figure 1**).

Iron deficiency affects these host iron reservoirs by significantly reducing the availability of both serum iron and intra-erythrocytic iron. Iron supplementation results in brief spikes in serum iron levels (Schümann et al., 2012, 2013), but has little immediate effect on intra-erythrocyte iron. However, approximately two weeks following iron supplementation, average intra-erythrocyte iron levels slowly begin improving as new iron-replete RBCs enter into circulation. It is well documented that virulence of many bacteria is directly associated with the availability of host iron, and as a result iron supplementation can exacerbate infections (Doherty, 2007). Whether described changes in serum and intra-erythrocyte iron stores affect erythrocytic stage malaria infection remains unknown.

1.9 The relationship between serum iron and erythrocytic stage malaria

The relationship between host serum iron and parasitized RBCs (pRBCs) is especially intriguing (**Table 2**). Because transferrin has an extremely high affinity for iron ($10^{23}M^{-1}$ at pH 7.4), NTBI is scarce in healthy individuals. There is strong evidence that transferrin associates with pRBCs but not uninfected RBCs. Work by Pollack et al. shows that pRBCs take up Fe^{59} bound to human transferrin, and a recent publication by our own group demonstrates that incubation of pRBCs with transferrin and ferric citrate increases the bioavailable iron in pRBCs (Pollack and Fleming, 1984; Clark et al., 2013). The idea that the parasite is able to acquire transferrin bound iron is further supported Surolia et al. who demonstrated that gelonin toxicity towards *P. falciparum* is 25 times greater when the gelonin is bound to transferrin (Surolia and Misquith, 1996). Moreover, Fry et al. report transferrin reductase activity associated with pRBCs but not uninfected RBCs (Fry, 1989). Additionally, two groups have reported the identification of a *P. falciparum* transferrin receptor in the RBC membrane of pRBCs (Rodriguez and Jungery, 1986; Haldar et al., 1986). However, a later study by Pollack et al. concluded that transferrin

binding of pRBCs is non-specific (Pollack and Schnelle, 1988), and additional studies were unable to detect any acquisition of transferrin bound iron by pRBCs (Peto and Thompson, 1986; Sanchez-Lopez and Haldar, 1992).

Despite strong evidence that transferrin associates with pRBCs, neither iron depletion nor iron supplementation of malaria culture media has any observable effect on parasite growth (Peto and Thompson, 1986; Sanchez-Lopez and Haldar, 1992; Scott et al., 1990, unpublished data Clark et al.). These results challenge the idea that serum iron, specifically transferrin bound iron, contributes to the protection of iron deficiency from malaria and the increased risk of malaria associated with iron supplementation. Yet, it should be noted that malaria culture media contains tenfold less iron than human sera and all existing studies have utilized culture adapted *P. falciparum* laboratory lines. It is possible laboratory lines have adapted to an iron-starved extracellular environment. Furthermore, because hemoglobin is an essential nutrient for erythrocytic stage malaria, it is impossible to "starve" the parasite of iron *in vitro* and this may in turn limit the ability to study the effect of serum iron on *P. falciparum*.

1.10 The relationship between intra-erythrocytic iron and erythrocytic stage malaria

Much less is known about the ability of the malaria parasite to access intra-erythrocytic iron (**Table 3**). An individual RBC contains 100 fg (20mM) of iron, the majority of which is contained within hemoglobin. It is estimated that if the parasite were able to access only 1% of this hemoglobin iron all of its iron demands would be fulfilled (Gabay and Ginsburg, 1993; Hershko and Peto, 1988). However, as discussed above, the parasite incorporates the majority of heme released as a result of hemoglobin digestion into hemozoin (Chugh et al., 2013). Despite identification of a *Plasmodium* heme oxygenase-like protein, which would facilitate release of iron from host heme (Okada, 2009), the parasite does not exhibit enzymatic heme oxygenase

activity nor possess a canonical heme oxygenase pathway (Sigala et al., 2012). Even without inherent heme oxygenase activity, it remains possible that non-enzymatic mechanisms release enough iron from trace heme to meet the iron requirements of the parasite. Possible mechanisms include heme breakdown by glutathione or hydrogen peroxide, the conditions for which are predicted to exist within erythrocytic stage parasites (Loria et al., 1999; Ginsburg et al., 1998). However, as the parasite synthesizes heme de novo, it does not seem likely that the parasite draws iron from host heme (Nagaraj et al., 2013).

In addition to hemoglobin, RBCs contain residual amounts of bioavailable iron (1-10 μM) as well as iron stored within ferritin (0.7 nM), and it is possible that the parasite is capable of utilizing one or both of these erythrocyte iron reservoirs. Currently, however, there is no reported evidence to either support or refute these possibilities (Scholl et al., 2005). However, despite a lack of evidence that the parasite accesses host intra-erythrocytic iron, recent work by our own group has shown that pRBC bioavailable iron content increases as the parasite matures from ring stage to schizont. This observation suggests that iron is released from some form of storage as the parasite develops within host RBCs (Clark et al., 2013). Whether the iron is released from parasite or host storage remains an open question.

Although the precise host iron source(s) the malaria parasite acquires remains unclear, all the potential host iron reservoirs (serum and intra-erythrocyte) available to erythrocytic stage malaria are affected by iron deficiency as well as iron supplementation. Therefore, it is reasonable to hypothesize that iron deprivation and excess iron contribute to the relationship between host iron and malaria risk observed in the clinical studies discussed earlier. That said, even during iron deficiency, the erythrocytic stage of the parasite inhabits the most iron rich

environment in the human body. As such it is alternatively possible that neither iron deficiency nor iron supplementation perturb iron reservoirs enough to significantly impact the parasite.

1.11 Microcytic iron-deficient RBCs and malaria

In addition to affecting host iron reservoirs, iron deficiency also induces changes in RBC physiology. One such difference between iron-replete and iron-deficient RBCs is the substitution of zinc for iron in hemoglobin when iron is limiting. This results in zinc protoporphoryin IX levels ten times higher in iron-deficient as compared to iron-replete RBCs (Wong et al., 1996). As zinc protoporphoryin IX inhibits hemozoin extension *in vitro*; it is reasonable to hypothesize that that elevated zinc protoporphoryin IX in iron-deficient erythrocytes provides protection against malaria infection by impeding parasite growth (Iyer et al., 2003).

Additional changes to RBC physiology caused by iron deficiency include: microcytosis, greater susceptibility to oxidative stress, reduced ATP content, and decreased deformability (Nagababu et al., 2008; Acharya et al., 1991; Yip et al., 1983; Brandão et al., 2009). Furthermore, iron-deficient RBCs experience enhanced eryptotic cell death (Kempe et al., 2006). The altered physiology of microcytic iron-deficient RBCs may therefore protect against erythrocytic stage malaria infection. Research by Koka et al. indicates that propagation of the erythrocytic stage of *P. falciparum* strain BinH is reduced in iron-deficient RBCs (Koka et al., 2007). However, earlier work by Luzzie et al. observed abnormal parasite morphology but no difference in the growth of *P. falciparum* strain UPO in iron-deficient as compared to iron-replete RBCs (Luzzi et al., 1990). The differences between these studies may be explained by the use of different *P. falciparum* isolates, which feasibly could have different sensitivities to iron-deficient RBCs.

Accelerated host clearance of iron-deficient pRBCs is an additional explanation for the protection afforded by iron deficiency against malaria. Results from two studies that examined malaria infection in iron-deficient mice both observed a higher clearance rate of pRBCs in iron-deficient as compared to iron-replete mice (Koka et al., 2007; Matsuzaki-Moriya et al., 2011). Specifically, Matsuzaki et al. observed elevated phagocytosis of pRBCs in iron-deficient as compared to iron-replete mice, and proposed that the increased phagocytosis rate may be attributable to greater phosphatidylserine levels on iron-deficient pRBCs as compared to iron-replete pRBCs. Koka et al. similarly observed greater phosphatidylserine levels on *P. falciparum* human iron-deficient pRBCs. Ultimately, these limited data suggests that iron deficiency may provide protection against malaria infection by both impeding erythrocytic stage malaria growth and increasing phagocytosis of iron-deficient pRBCs. However, only further investigation will reveal the true relationship between iron-deficient RBCs and *P. falciparum*.

1.12 Perturbations in erythropoiesis and malaria

In the absence of sufficient iron for heme synthesis, the human host's erythropoietic rate decreases. Conversely, iron supplementation of individuals with iron deficiency anemia results in a strong erythropoietic response; because the body attempts to recover RBC numbers and replace less viable iron-deficient RBCs (**Figure 2**). It is well known that *P. vivax* exclusively infects the very youngest RBCs (reticulocytes). However, *P. vivax* is not the only *Plasmodium* species that prefers young RBCs. In fact many species of *Plasmodium*, including *P. falciparum*, preferentially infect young RBCs, and furthermore young RBC support greater parasite replication than more mature RBCs (Wilson et al., 1977; Pasvol et al., 1980; Lim et al., 2013). Thus, significant elevation in the erythropoietic rate could put an individual at increased risk of erythrocytic stage *P. falciparum* infection. Tian et al. have investigated this hypothesis in the

context of pregnant women, who are at greater risk of malaria infection than their non-pregnant counterparts and experience increased erythropoietic rates to meet the oxygen demands of the growing fetus. The authors report that *P. falciparum* growth is significantly greater in the on average younger RBCs taken from pregnant women as compared to the on average older RBCs taken from non-pregnant women (Tian et al., 1998).

Murine models have additionally been used to shed light on the relationship between erythropoiesis and malaria infection. Interestingly, when Chang et al. manipulated the timing of erythropoiesis during the course of a malaria infection it was observed that reticulocytosis early in infection significantly increased infection and morbidity, while reticulocytosis late in infection decreased mortality (Chang et al., 2004). These observations are consistent with recent work by Zhao et al. showing that lipocalin 2, which is elevated during malaria infection, provides protection from malaria infection in mice by limiting reticulocytosis (Zhao et al., 2012).

Furthermore, mathematical modeling by Cromer et al. makes several key predictions that support a role for erythropoiesis in driving the protection from malaria associated with iron deficiency anemia and increased risk associated with iron supplementation. First, their model predicts that low reticulocyte production rate – as would be observed in iron deficiency – in combination with a parasite that prefers reticulocytes, could result in a less severe infection. Second, high reticulocyte production – as would be observed in iron-deficient individuals responding to iron supplementation – could increase severity of malaria infection (Cromer et al., 2009). These results indicate that limiting reticulocytosis early in infection is important for limiting erythrocytic stage malaria infection and further support the hypothesis that iron supplementation-induced reticulocytosis significantly increases the risk of erythrocytic stage *P. falciparum* infection.

Together, these observations provide insight into potential cellular mechanisms contributing to the protection of iron deficiency against malaria, and the increased risk of malaria associated with iron supplementation. With regard to iron deficiency, altered RBC physiology may limit *P. falciparum* propagation within iron-deficient RBCs and increase clearance of iron-deficient pRBCs. Furthermore, the reduced erythropoietic rate and subsequent reduction in an iron-deficient individual's hematocrit may additionally contribute to protection. Conversely, the increased erythropoietic rate triggered by iron supplementation paired with the preference of *P. falciparum* for young RBCs may be partially responsible for the increased risk of malaria infection that is associated with iron supplementation.

1.13 Objectives of this dissertation

The relationship between host iron status and malaria revealed by clinical and epidemiological studies has halted much needed nutritional iron supplementation campaigns in malaria endemic regions. As discussed previously, the biological mechanisms governing the relationship between host iron status and susceptibility to malaria remain largely unknown. As the erythrocytic stage of malaria infections is responsible for all symptoms of disease, I hypothesized that iron deficiency and iron supplementation directly impact the erythrocytic stage of malaria infection. Specifically that iron deficiency limits propagation of erythrocytic stage *P. falciparum* infection while iron supplementation would exacerbate the erythrocytic stage of *P. falciparum* infection.

I have focused on two distinct avenues by which host iron status might impact erythrocytic stage malaria infection: (1) the direct effect of host iron on erythrocytic stage malaria growth and (2) the indirect effect iron has on erythrocyte physiology and erythropoiesis. With regard to determining whether host iron shapes erythrocytic stage malaria growth by either

providing or limiting the availability of essential nutritional iron to the parasite, focused specifically on serum iron bound to transferrin or loosely chelated to citrate. To study the effect iron has on erythrocyte physiology and erythropoiesis, I isolated exclusively RBCs from blood donated by (i) iron-deficient (ii) iron supplemented iron-deficient, and (iii) iron supplemented iron-replete individuals and compared erythrocytic stage *P. falciparum* propagation in the RBCs of each group to propagation in RBCs from iron-replete non-supplemented donors.

The first aim of this thesis was designed to determine the capacity of serum iron to serve as a growth factor for erythrocytic stage *P. falciparum* and affect the bioavailable iron content of pRBCs. The second two aims were constructed to study specifically the effect of exclusively RBCs from iron-deficient and iron supplemented donors on erythrocytic stage *P. falciparum*. Defining the biological parameters of the (i) protection associated with iron deficiency and (ii) the increased risk conferred by iron supplementation on malaria susceptibility is essential for informing public health policy on iron supplementation in malaria endemic areas.

Hypothesis: *Iron deficiency and iron supplementation affect erythrocytic stage P. falciparum infection by either or both (i) altering the availability of host iron, which the parasite requires for growth (ii) altering the physiological parameters of the host RBC population and as a result changing the susceptibility of host RBCs to parasite infection.*

Aim 1: To determine the effect of serum iron on erythrocytic stage *P. falciparum*, I assessed the effect of physiologic levels of transferrin and non-transferrin bound iron on erythrocytic stage *P. falciparum* growth and bioavailable iron.

Aim 2: To study the relationship between different RBC populations and *P. falciparum* infection, I developed an approach for directly comparing *P. falciparum* invasion of different RBC populations.

Aim 3: To determine the susceptibility of erythrocytes from iron-deficient and iron supplemented individuals to erythrocytic stage *P. falciparum* infection, I assessed the propagation of *P. falciparum* in RBCs from iron-deficient donors as well as iron supplemented iron-replete and iron-deficient donors as compared to propagation of the parasite in RBCs donated by iron-replete non supplemented individuals.

Table 1.1 – Summary of clinical studies on iron deficiency, iron supplementation, and malaria				
Children – Interventional studies				
Reference	**Study Design**	**Population**	**Country, malaria info**	**Results**
(Sazawal et al., 2006)	Randomized placebo controlled	7950 children given iron and folic acid 8120 children given iron, folic acid and zinc 8006 control children Ages 1-35 months	Zanzibar, intense malaria transmission	Trial stopped early because of safety concerns. Those who received iron and folic acid with or without zinc were 12% (95% CI 2–23, p=0·02) more likely to die or need hospital treatment for an adverse event and 11% (95% CI 1–23, p=0·03) more likely to be admitted to hospital; there were also 15% (95% CI 7–41, p=0·19) more deaths in these groups.
(Tielsch et al., 2006)	Randomized placebo controlled	8337 children given iron and folic acid 9230 children given iron, folic acid and zinc 8683 control children Ages 1-36 months	Nepal, no malaria	Daily supplementation of young children in southern Nepal with iron and folic acid with or without zinc had no effect on their risk of death, but might protect against diarrhea, dysentery, and acute respiratory illness.
(Veenemans et al., 2011)	2x2 Factorial trial	145 children given zinc only 148 children given both zinc and multi-nutrients (including iron) 146 children given multi-nutrients (including iron) without zinc 148 children given placebo Ages 6-60 months	Tanzania, intense malaria transmission	When data was analyzed by iron status at baseline, multi-nutrient supplementation increased the overall number of malaria episodes in children with iron deficiency by 41%, whereas multi-nutrient supplementation had no effect on the number of malaria episodes among children who were iron-replete at baseline.

(Zlotkin et al., 2013)	Cluster randomized, double blind	967 children given micronutrient powder with iron 991 children given micronutrient powder without iron Ages 6-35 months	Ghana, intense malaria transmission Insecticide treated bed nets provided at enrollment	Malaria incidence was significantly lower in the iron group compared with the no iron group during the intervention period (risk ratio [RR], 0.87; 95% CI, 0.78-0.96). In secondary analyses, these differences were no longer statistically significant after adjusting for baseline iron deficiency and anemia status overall (RR, 0.87; 95% CI, 0.75-1.01) Subgroup analysis of 704 children who had anemia at baseline and for whom additional blood samples were obtained at the end of the intervention period found only a small mean increase in hemoglobin in the iron group (mean change of 0.08 g/dL measured), indicating that the micronutrient powder had limited efficacy in this trial.
(Esan et al., 2013)	2-arm, double-blind, randomized	100 children received multivitamins plus iron 96 children received multivitamins alone HIV infected children aged 6-59 months with moderate anemia (Hgb=7.0-9.9 g/dL); 3 months of treatment, 6 months follow up	Malawi, intense malaria transmission	Children who received iron had a better CD4 percentage response at 3 months, but an increased incidence of malaria at 6 months (incidence rate, 120.2 vs. 71.7; adjusted incidence rate ratio [aIRR], 1.81 [95% CI, 1.04-3.16]; p = .04), especially during the first 3 months (incidence rate, 78.1 vs. 36.0; aIRR, 2.68 [95% CI, 1.08-6.63]; p = .03).

Children – Observational Studies				
Reference	**Study Design**	**Population**	**Country, malaria info**	**Results**
(Nyakeriga et al., 2004)	2 Cross sectional studies	Study 1: Iron-replete (n=95) Iron-deficient (n=78) Study 2: Iron-replete (n=104) Iron-deficient (n=91) Ages 8 months-8 years	Kenya, intense malaria transmission	Incidence of clinical malaria was significantly lower among children with iron deficiency anemia (incidence-rate ratio [IRR], 0.70; 95% confidence interval [CI], 0.51-0.99; P<.05).
(Gwamaka et al., 2012)	Longitudinal	785 children monitored for 3 years	Tanzania, intense malaria transmission	Iron deficiency anemia at routine, well-child visits significantly decreased the odds of subsequent parasitemia (23% decrease, p < .001) and subsequent severe malaria (38% decrease, p = .04). Iron deficiency anemia was also associated with 60% lower all-cause mortality (p = .04) and 66% lower malaria-associated mortality (p = .11).
(Jonker et al., 2012)	Longitudinal	727 children monitored for 1 year	Malawi, intense malaria transmission	Children with iron deficiency anemia at baseline had a lower incidence of malaria parasitemia and clinical malaria during a year of follow-up; adjusted hazard ratios 0.55 (95% CI: 0.41-0.74) and 0.49 (95% CI: 0.33-0.73), respectively.

Pregnant women - observational				
Reference	**Study Design**	**Population**	**Country, malaria info**	**Results**
(Kabyemela et al., 2008)	Cross sectional	445 pregnant women (120 primigravidae, 112 secundigravidae, and 213 multigravidae)	Tanzania, intense malaria transmission	Iron deficiency decreased the risk of placental malaria.
(Senga et al., 2011)	Case-Control	Pregnant women (112 cases with placental malaria, 110 controls with no evidence of placental infection)	Malawi, intense malaria transmission	Iron deficiency decreased risk of acute, chronic and past placental malaria. The association was greater in the multigravidae group.

Table 1.2 – Relationship between host serum iron and *P. falciparum*	
Studies supporting transferrin mediated delivery of iron to pRBCs	
Study	**Major Findings**
(Pollack and Fleming, 1984)	- pRBCs take up more iron from transferrin than uninfected RBCs
(Rodriguez and Jungery, 1986)	- FITC labeled holo-transferrin traverses from the pRBC surface to the parasitophorous vacuole - Internalization of holo-transferrin is most active in early trophozoite stage pRBCs - A 93kD parasite protein inserted into the RBC membrane binds human holo-transferrin
(Haldar et al., 1986)	- A 102kD schizont stage parasite protein inserted into the RBC membrane binds human holo-transferrin
(Pollack and Schnelle, 1988)	- Twice as much human holo-transferrin associates with pRBCs than uninfected RBCs - Human holo-transferrin binding to pRBCs is non-specific
(Fry, 1989)	- RBC membranes of pRBCs possess diferric transferrin reductase activity, uninfected RBC membranes do not - pRBC diferric transferrin reductase activity increases as the parasite matures from the ring to trophozoite stage
(Surolia and Misquith, 1996)	- Human transferrin conjugated to the toxin gelonin selectively binds trophozoite stage pRBCs - Toxicity of gelonin to erythrocytic stage *P. falciparum* is 25 times greater when linked to human transferrin
(Clark et al., 2013)	- Addition of holo-transferrin to trophozoite stage pRBCs increases the bioavailable iron content of pRBCs but not uninfected RBCs
Studies refuting transferrin mediated delivery of iron to pRBCs & Studies showing serum iron does not affect P. falciparum growth	
Study	**Major Finding**
(Peto and Thompson, 1986)	- pRBCs do not acquire iron from holo-transferrin - Depletion of iron from *P. falciparum in vitro* culture media does not reduce parasite growth - Addition of iron to *P. falciparum in vitro* culture media reduced parasite growth
(Scott et al., 1990)	- Restriction of iron chelator DFO to *P. falciparum in vitro* culture media does not affect parasite growth
(Sanchez-Lopez and Haldar, 1992)	- pRBCs do not take up iron from human holo-transferrin - Depletion of human transferrin from culture media does not affect erythrocytic stage parasite growth

Studies supporting acquisition of NTBI by pRBCs	
Study	**Major Finding**
(Peto and Thompson, 1986)	- pRBCs take up NTBI
(Sanchez-Lopez and Haldar, 1992)	- pRBCs take up of free, non-transferrin bound iron (NTBI), but not any more than uninfected RBCs - pRBC NTBI acquisition is time, concentration, and temperature but not energy dependent
(Clark et al., 2013)	- Addition of ferric citrate (NTBI) to trophozoite stage pRBCs increases the bioavailable iron content of pRBCs but not uninfected RBCs

Table 1.3 – Relationship between RBC iron and *P. falciparum*	
RBC hemoglobin	
Study	**Major Findings**
(Rudzinska et al., 1965)	- *P. falciparum* metabolizes host RBC hemoglobin - *P. falciparum* inserts host heme into hemozoin
(Okada, 2009)	- *P. falciparum* has a heme oxygenase homolog
(Sigala et al., 2012)	- *P. falciparum* lacks both heme oxygenase activity and a canonical heme oxygenase pathway
(Loria et al., 1999)	- Hydrogen peroxide degrades host heme under conditions that are analogous to the microenvironment of the parasite food vacuole
RBC ferritin – unknown	
RBC bioavailable iron – unknown	

Figure 1.1 – Host iron available to erythrocytic stage *P. falciparum*. During the erythrocytic stage of infection *P. falciparum* the parasite travels through the hosts vascular system within RBCs. Host iron immediately available to the parasite include serum and RBC iron. Serum iron ranges from 10-27 µM. Iron deficiency anemia is characterized by a significant decline in serum iron. Transferrin bound iron is the predominant form of iron in the serum, though trace amounts of non-transferrin bound iron (NTBI) are present. In some pathologic conditions such as hemochromatosis, NTBI may be significantly greater. In the RBC iron is found within hemoglobin (20mM), ferritin (0.7nM), and as bioavailable iron (1-10µM). Iron deficiency anemia significantly reduces RBC iron, specifically hemoglobin iron. Shown in the figure are: *P. falciparum* (*Pf*), digestive vacuole (DV), parasite nucleus (N), and endothelial cell (EC).

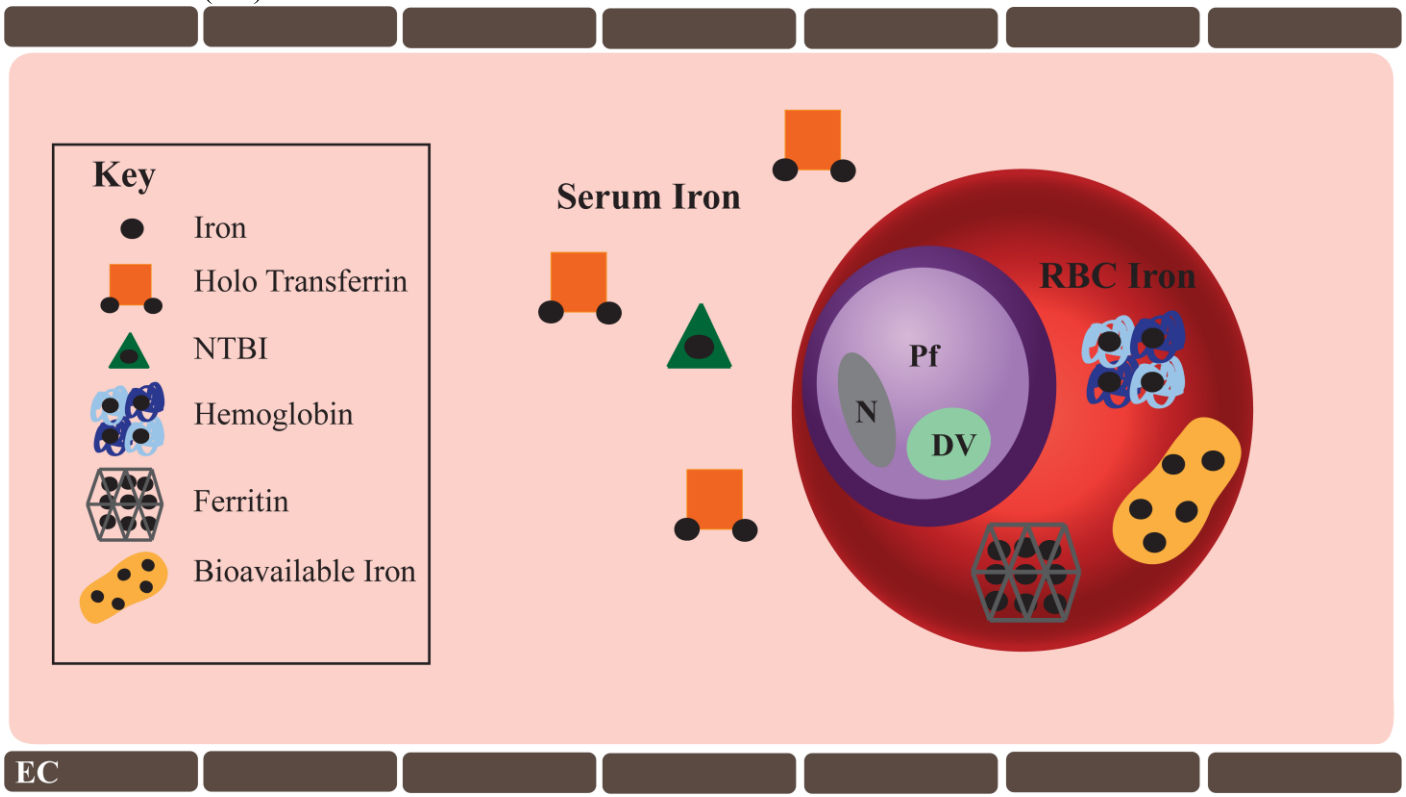
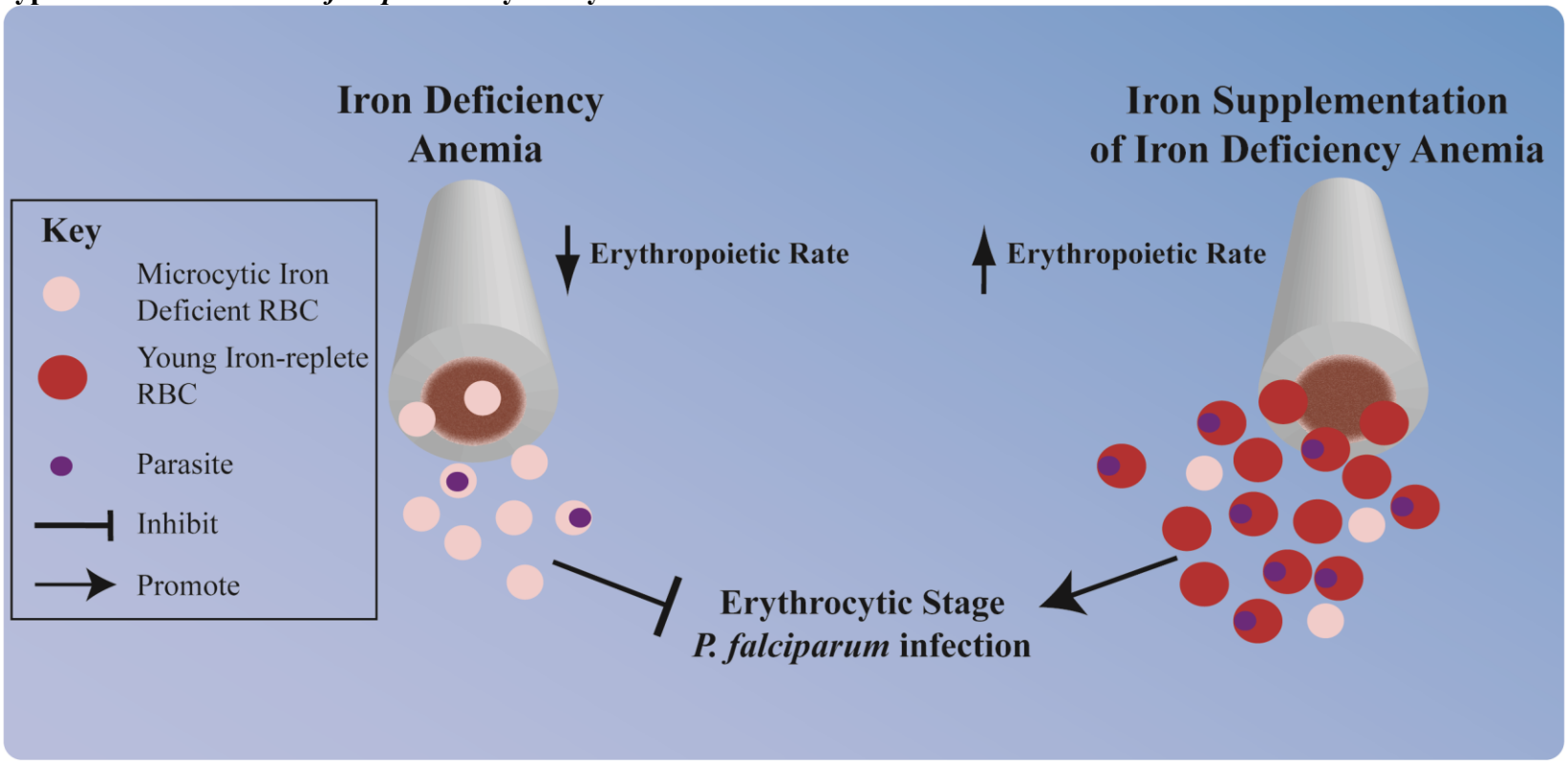


Figure 1.2 – Impact of iron deficiency anemia and iron supplementation on erythropoietic rate and erythrocyte physiology and hypothesized effect on *P. falciparum* erythrocytic infection.



REFERENCES

Acharya, J., Punchard, N. A., Taylor, J. A., Thompson, R. P., and Pearson, T. C. (1991). Red cell lipid peroxidation and antioxidant enzymes in iron deficiency. *Eur. J. Haematol.* 47, 287–291.

Aguilar, R., Moraleda, C., Quintó, L., Renom, M., Mussacate, L., Macete, E., Aguilar, J. L., Alonso, P. L., and Menéndez, C. (2012). Challenges in the diagnosis of iron deficiency in children exposed to high prevalence of infections. *PloS One* 7, e50584.

Ariey, F., Witkowski, B., Amaratunga, C., Beghain, J., Langlois, A.-C., Khim, N., Kim, S., Duru, V., Bouchier, C., Ma, L., et al. (2014). A molecular marker of artemisinin-resistant Plasmodium falciparum malaria. *Nature* 505, 50–55.

Armitage, A. E., Eddowes, L. A., Gileadi, U., Cole, S., Spottiswoode, N., Selvakumar, T. A., Ho, L.-P., Townsend, A. R. M., and Drakesmith, H. (2011). Hepcidin regulation by innate immune and infectious stimuli. *Blood* 118, 4129–4139.

Brandão, M. M., Castro, M. de L. R. B., Fontes, A., Cesar, C. L., Costa, F. F., and Saad, S. T. O. (2009). Impaired red cell deformability in iron deficient subjects. *Clin. Hemorheol. Microcirc.* 43, 217–221.

Cabantchik, Z. I., Glickstein, H., Golenser, J., Loyevsky, M., and Tsafack, A. (1996). Iron chelators: mode of action as antimalarials. *Acta Haematol.* 95, 70–77.

Cabantchik, Z. I., Moody-Haupt, S., and Gordeuk, V. R. (1999). Iron chelators as anti-infectives; malaria as a paradigm. *FEMS Immunol. Med. Microbiol.* 26, 289–298.

Chang, K.-H., Tam, M., and Stevenson, M. M. (2004). Modulation of the course and outcome of blood-stage malaria by erythropoietin-induced reticulocytosis. *J. Infect. Dis.* 189, 735–743.

Chotivanich, K., Udomsangpetch, R., Simpson, J. A., Newton, P., Pukrittayakamee, S., Looareesuwan, S., and White, N. J. (2000). Parasite multiplication potential and the severity of Falciparum malaria. *J. Infect. Dis.* 181, 1206–1209.

Chugh, M., Sundararaman, V., Kumar, S., Reddy, V. S., Siddiqui, W. A., Stuart, K. D., and Malhotra, P. (2013). Protein complex directs hemoglobin-to-hemozoin formation in Plasmodium falciparum. *Proc. Natl. Acad. Sci. U. S. A.* 110, 5392–5397.

Clark, M., Fisher, N. C., Kasthuri, R., and Cerami Hand, C. (2013). Parasite maturation and host serum iron influence the labile iron pool of erythrocyte stage Plasmodium falciparum. *Br. J. Haematol.* 161, 262–269.

Cromer, D., Stark, J., and Davenport, M. P. (2009). Low red cell production may protect against severe anemia during a malaria infection--insights from modeling. *J. Theor. Biol.* 257, 533–542.

Dhanasekaran, S., Chandra, N. R., Sagar, B. K. C., Rangarajan, P. N., and Padmanaban, G. (2004). δ-Aminolevulinic Acid Dehydratase from Plasmodium falciparum; Indigenous versus imported. *J. Biol. Chem.* 279, 6934–6942.

Doherty, C. P. (2007). Host-Pathogen Interactions: The Role of Iron. *J. Nutr.* 137, 1341–1344.

Dondorp, A. M., Desakorn, V., Pongtavornpinyo, W., Sahassananda, D., Silamut, K., Chotivanich, K., Newton, P. N., Pitisuttithum, P., Smithyman, A. M., White, N. J., et al. (2005). Estimation of the total parasite biomass in acute falciparum malaria from plasma PfHRP2. *PLoS Med.* 2, e204.

Van Dooren, G. G., Stimmler, L. M., and McFadden, G. I. (2006). Metabolic maps and functions of the Plasmodium mitochondrion. *FEMS Microbiol. Rev.* 30, 596–630.

Esan, M. O., van Hensbroek, M. B., Nkhoma, E., Musicha, C., White, S. A., Ter Kuile, F. O., and Phiri, K. S. (2013). Iron supplementation in HIV-infected Malawian children with anemia: a double-blind, randomized controlled trial. *Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am.*

Fontecave, M., Nordlund, P., Eklund, H., and Reichard, P. (1992). The redox centers of ribonucleotide reductase of Escherichia coli. *Adv. Enzymol. Relat. Areas Mol. Biol.* 65, 147–183.

Francis, S. E., Sullivan, D. J., Jr, and Goldberg, D. E. (1997). Hemoglobin metabolism in the malaria parasite Plasmodium falciparum. *Annu. Rev. Microbiol.* 51, 97–123.

Fry, M. (1989). Diferric transferrin reductase in Plasmodium falciparum-infected erythrocytes. *Biochem. Biophys. Res. Commun.* 158, 469–473.

Fu, Y., Tilley, L., Kenny, S., and Klonis, N. (2010). Dual labeling with a far red probe permits analysis of growth and oxidative stress in *P. falciparum*-infected erythrocytes. *Cytom. Part J. Int. Soc. Anal. Cytol.* 77, 253–263.

Gabay, T., and Ginsburg, H. (1993). Hemoglobin denaturation and iron release in acidified red blood cell lysate--a possible source of iron for intraerythrocytic malaria parasites. *Exp. Parasitol.* 77, 261–272.

Gera, T., and Sachdev, H. P. S. (2002). Effect of iron supplementation on incidence of infectious illness in children: systematic review. *BMJ* 325, 1142.

Ginsburg, H., Famin, O., Zhang, J., and Krugliak, M. (1998). Inhibition of glutathione-dependent degradation of heme by chloroquine and amodiaquine as a possible basis for their antimalarial mode of action. *Biochem. Pharmacol.* 56, 1305–1313.

Goldberg, D. E., Slater, A. F., Cerami, A., and Henderson, G. B. (1990). Hemoglobin degradation in the malaria parasite Plasmodium falciparum: an ordered process in a unique organelle. *Proc. Natl. Acad. Sci. U. S. A.* 87, 2931–2935.

Golenser, J., Domb, A., Mordechai-Daniel, T., Leshem, B., Luty, A., and Kremsner, P. (2006). Iron chelators: correlation between effects on Plasmodium spp. and immune functions. *J. Parasitol.* 92, 170–177.

Gwamaka, M., Kurtis, J. D., Sorensen, B. E., Holte, S., Morrison, R., Mutabingwa, T. K., Fried, M., and Duffy, P. E. (2012). Iron deficiency protects against severe Plasmodium falciparum malaria and death in young children. *Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am.* 54, 1137–1144.

Haider, B. A., Olofin, I., Wang, M., Spiegelman, D., Ezzati, M., Fawzi, W. W., and Nutrition Impact Model Study Group (anaemia) (2013). Anaemia, prenatal iron use, and risk of adverse pregnancy outcomes: systematic review and meta-analysis. *BMJ* 346, f3443.

Haldar, K., Henderson, C. L., and Cross, G. A. (1986). Identification of the parasite transferrin receptor of Plasmodium falciparum-infected erythrocytes and its acylation via 1,2-diacyl-sn-glycerol. *Proc. Natl. Acad. Sci. U. S. A.* 83, 8565–8569.

Hentze, M. W., Muckenthaler, M. U., Galy, B., and Camaschella, C. (2010). Two to Tango: Regulation of Mammalian Iron Metabolism. *Cell* 142, 24–38.

Hershko, C., and Peto, T. E. (1988). Deferoxamine inhibition of malaria is independent of host iron status. *J. Exp. Med.* 168, 375–387.

Hodges, M., Yikilmaz, E., Patterson, G., Kasvosve, I., Rouault, T. A., Gordeuk, V. R., and Loyevsky, M. (2005). An iron regulatory-like protein expressed in Plasmodium falciparum displays aconitase activity. *Mol. Biochem. Parasitol.* 143, 29–38.

Iyer, J. K., Shi, L., Shankar, A. H., and Sullivan, D. J., Jr (2003). Zinc protoporphyrin IX binds heme crystals to inhibit the process of crystallization in Plasmodium falciparum. *Mol. Med. Camb. Mass* 9, 175–182.

Jonker, F. A. M., Calis, J. C. J., van Hensbroek, M. B., Phiri, K., Geskus, R. B., Brabin, B. J., and Leenstra, T. (2012). Iron status predicts malaria risk in Malawian preschool children. *PloS One* 7, e42670.

Jortzik, E., and Becker, K. (2012). Thioredoxin and glutathione systems in Plasmodium falciparum. *Int. J. Med. Microbiol. IJMM* 302, 187–194.

Kabyemela, E. R., Fried, M., Kurtis, J. D., Mutabingwa, T. K., and Duffy, P. E. (2008). Decreased susceptibility to Plasmodium falciparum infection in pregnant women with iron deficiency. *J. Infect. Dis.* 198, 163–166.

Kassebaum, N. J., Jasrasaria, R., Naghavi, M., Wulf, S. K., Johns, N., Lozano, R., Regan, M., Weatherall, D., Chou, D. P., Eisele, T. P., et al. (2013). A systematic analysis of global anemia burden from 1990 to 2010. *Blood*.

Kempe, D. S., Lang, P. A., Duranton, C., Akel, A., Lang, K. S., Huber, S. M., Wieder, T., and Lang, F. (2006). Enhanced programmed cell death of iron-deficient erythrocytes. *FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol.* 20, 368–370.

Klonis, N., Creek, D. J., and Tilley, L. (2013). Iron and heme metabolism in Plasmodium falciparum and the mechanism of action of artemisinins. *Curr. Opin. Microbiol.* 16, 722–727.

Koka, S., Föller, M., Lamprecht, G., Boini, K. M., Lang, C., Huber, S. M., and Lang, F. (2007). Iron deficiency influences the course of malaria in Plasmodium berghei infected mice. *Biochem. Biophys. Res. Commun.* 357, 608–614.

Krungkrai, J., Cerami, A., and Henderson, G. B. (1990). Pyrimidine biosynthesis in parasitic protozoa: purification of a monofunctional dihydroorotase from Plasmodium berghei and Crithidia fasciculata. *Biochemistry (Mosc.)* 29, 6270–6275.

Lederman, H. M., Cohen, A., Lee, J. W., Freedman, M. H., and Gelfand, E. W. (1984). Deferoxamine: a reversible S-phase inhibitor of human lymphocyte proliferation. *Blood* 64, 748–753.

Li, G., Pone, E. J., Tran, D. C., Patel, P. J., Dao, L., Xu, Z., and Casali, P. (2012). Iron inhibits activation-induced cytidine deaminase enzymatic activity and modulates immunoglobulin class switch DNA recombination. *J. Biol. Chem.* 287, 21520–21529.

Lim, C., Hansen, E., Desimone, T. M., Moreno, Y., Junker, K., Bei, A., Brugnara, C., Buckee, C. O., and Duraisingh, M. T. (2013). Expansion of host cellular niche can drive adaptation of a zoonotic malaria parasite to humans. *Nat. Commun.* 4, 1638.

Loria, P., Miller, S., Foley, M., and Tilley, L. (1999). Inhibition of the peroxidative degradation of haem as the basis of action of chloroquine and other quinoline antimalarials. *Biochem. J.* 339 (Pt 2), 363–370.

Low, M., Farrell, A., Biggs, B.-A., and Pasricha, S.-R. (2013). Effects of daily iron supplementation in primary-school-aged children: systematic review and meta-analysis of randomized controlled trials. *CMAJ Can. Med. Assoc. J. J. Assoc. Medicale Can.* 185, E791–802.

Loyevsky, M., LaVaute, T., Allerson, C. R., Stearman, R., Kassim, O. O., Cooperman, S., Gordeuk, V. R., and Rouault, T. A. (2001). An IRP-like protein from Plasmodium falciparum binds to a mammalian iron-responsive element. *Blood* 98, 2555–2562.

Loyevsky, M., Mompoint, F., Yikilmaz, E., Altschul, S. F., Madden, T., Wootton, J. C., Kurantsin-Mills, J., Kassim, O. O., Gordeuk, V. R., and Rouault, T. A. (2003). Expression of a recombinant IRP-like Plasmodium falciparum protein that specifically binds putative plasmodial IREs. *Mol. Biochem. Parasitol.* 126, 231–238.

Luzzi, G. A., Torii, M., Aikawa, M., and Pasvol, G. (1990). Unrestricted growth of Plasmodium falciparum in microcytic erythrocytes in iron deficiency and thalassaemia. *Br. J. Haematol.* 74, 519–524.

Lytton, S. D., Mester, B., Libman, J., Shanzer, A., and Cabantchik, Z. I. (1994). Mode of action of iron (III) chelators as antimalarials: II. Evidence for differential effects on parasite iron-dependent nucleic acid synthesis. *Blood* 84, 910–915.

Matsuzaki-Moriya, C., Tu, L., Ishida, H., Imai, T., Suzue, K., Hirai, M., Tetsutani, K., Hamano, S., Shimokawa, C., and Hisaeda, H. (2011). A critical role for phagocytosis in resistance to malaria in iron-deficient mice. *Eur. J. Immunol.* 41, 1365–1375.

Miller, L. H., Ackerman, H. C., Su, X., and Wellems, T. E. (2013). Malaria biology and disease pathogenesis: insights for new treatments. *Nat. Med.* 19, 156–167.

Moormann, A. M., Hossler, P. A., and Meshnick, S. R. (1999). Deferoxamine effects on Plasmodium falciparum gene expression. *Mol. Biochem. Parasitol.* 98, 279–283.

Nagababu, E., Gulyani, S., Earley, C. J., Cutler, R. G., Mattson, M. P., and Rifkind, J. M. (2008). Iron-Deficiency Anemia Enhances Red Blood Cell Oxidative Stress. *Free Radic. Res.* 42, 824–829.

Nagaraj, V. A., Arumugam, R., Gopalakrishnan, B., Jyothsna, Y. S., Rangarajan, P. N., and Padmanaban, G. (2008). Unique Properties of Plasmodium falciparum Porphobilinogen Deaminase. *J. Biol. Chem.* 283, 437–444.

Nagaraj, V. A., Arumugam, R., Prasad, D., Rangarajan, P. N., and Padmanaban, G. (2010). Protoporphyrinogen IX oxidase from Plasmodium falciparum is anaerobic and is localized to the mitochondrion. *Mol. Biochem. Parasitol.* 174, 44–52.

Nagaraj, V. A., Prasad, D., Rangarajan, P. N., and Padmanaban, G. (2009). Mitochondrial localization of functional ferrochelatase from Plasmodium falciparum. *Mol. Biochem. Parasitol.* 168, 109–112.

Nagaraj, V. A., Sundaram, B., Varadarajan, N. M., Subramani, P. A., Kalappa, D. M., Ghosh, S. K., and Padmanaban, G. (2013). Malaria parasite-synthesized heme is essential in the mosquito and liver stages and complements host heme in the blood stages of infection. *PLoS Pathog.* 9, e1003522.

Nyakeriga, A. M., Troye-Blomberg, M., Dorfman, J. R., Alexander, N. D., Bäck, R., Kortok, M., Chemtai, A. K., Marsh, K., and Williams, T. N. (2004). Iron deficiency and malaria among children living on the coast of Kenya. *J. Infect. Dis.* 190, 439–447.

Okada, K. (2009). The novel heme oxygenase-like protein from Plasmodiumfalciparum converts heme to bilirubin IXalpha in the apicoplast. *FEBS Lett.* 583, 313–319.

Okebe, J. U., Yahav, D., Shbita, R., and Paul, M. (2011). Oral iron supplements for children in malaria-endemic areas. *Cochrane Database Syst. Rev. Online*, CD006589.

Oppenheimer, S. J. (2001). Iron and its relation to immunity and infectious disease. *J. Nutr.* 131, 616S–633S; discussion 633S–635S.

Pasvol, G., Weatherall, D. J., and Wilson, R. J. (1980). The increased susceptibility of young red cells to invasion by the malarial parasite Plasmodium falciparum. *Br. J. Haematol.* 45, 285–295.

Peña-Rosas, J. P., De-Regil, L. M., Dowswell, T., and Viteri, F. E. (2012a). Daily oral iron supplementation during pregnancy. *Cochrane Database Syst. Rev.* 12, CD004736.

Peña-Rosas, J. P., De-Regil, L. M., Dowswell, T., and Viteri, F. E. (2012b). Intermittent oral iron supplementation during pregnancy. *Cochrane Database Syst. Rev.* 7, CD009997.

Peto, T. E., and Thompson, J. L. (1986). A reappraisal of the effects of iron and desferrioxamine on the growth of Plasmodium falciparum "in vitro": the unimportance of serum iron. *Br. J. Haematol.* 63, 273–280.

Pollack, S., and Fleming, J. (1984). Plasmodium falciparum takes up iron from transferrin. *Br. J. Haematol.* 58, 289–293.

Pollack, S., and Schnelle, V. (1988). Inability to detect transferrin receptors on *P. falciparum* parasitized red cells. *Br. J. Haematol.* 68, 125–129.

Prentice, A. M., and Cox, S. E. (2012). Iron and malaria interactions: research needs from basic science to global policy. *Adv. Nutr. Bethesda Md* 3, 583–591.

Prentice, A. M., Ghattas, H., Doherty, C., and Cox, S. E. (2007). Iron metabolism and malaria. *Food Nutr. Bull.* 28, S524–539.

Prentice, A. M., Verhoef, H., and Cerami, C. (2013). Iron fortification and malaria risk in children. *JAMA J. Am. Med. Assoc.* 310, 914–915.

De-Regil, L. M., Jefferds, M. E. D., Sylvetsky, A. C., and Dowswell, T. (2011). Intermittent iron supplementation for improving nutrition and development in children under 12 years of age. *Cochrane Database Syst. Rev.*, CD009085.

De-Regil, L. M., Suchdev, P. S., Vist, G. E., Walleser, S., and Peña-Rosas, J. P. (2013). Home fortification of foods with multiple micronutrient powders for health and nutrition in children under two years of age (Review). *Evid.-Based Child Health Cochrane Rev. J.* 8, 112–201.

Rodriguez, M. H., and Jungery, M. (1986). A protein on Plasmodium falciparum-infected erythrocytes functions as a transferrin receptor. *Nature* 324, 388–391.

Rosenthal, P. J., and Meshnick, S. R. (1996). Hemoglobin catabolism and iron utilization by malaria parasites. *Mol. Biochem. Parasitol.* 83, 131–139.

Roy, C. N. (2013). An update on iron homeostasis: make new friends, but keep the old. *Am. J. Med. Sci.* 346, 413–419.

Rubin, H., Salem, J. S., Li, L. S., Yang, F. D., Mama, S., Wang, Z. M., Fisher, A., Hamann, C. S., and Cooperman, B. S. (1993). Cloning, sequence determination, and regulation of the ribonucleotide reductase subunits from Plasmodium falciparum: a target for antimalarial therapy. *Proc. Natl. Acad. Sci. U. S. A.* 90, 9280–9284.

Rudzinska, M. A., Trager, W., and Bray, R. S. (1965). Pinocytotic uptake and the digestion of hemoglobin in malaria parasites. *J. Protozool.* 12, 563–576.

Sanchez-Lopez, R., and Haldar, K. (1992). A transferrin-independent iron uptake activity in Plasmodium falciparum-infected and uninfected erythrocytes. *Mol. Biochem. Parasitol.* 55, 9–20.

Sato, S., Clough, B., Coates, L., and Wilson, R. J. M. (2004). Enzymes for heme biosynthesis are found in both the mitochondrion and plastid of the malaria parasite Plasmodium falciparum. *Protist* 155, 117–125.

Sato, S., and Wilson, R. J. M. (2002). The genome of Plasmodium falciparum encodes an active delta-aminolevulinic acid dehydratase. *Curr. Genet.* 40, 391–398.

Sazawal, S., Black, R. E., Ramsan, M., Chwaya, H. M., Stoltzfus, R. J., Dutta, A., Dhingra, U., Kabole, I., Deb, S., Othman, M. K., et al. (2006). Effects of routine prophylactic supplementation with iron and folic acid on admission to hospital and mortality in preschool children in a high malaria transmission setting: community-based, randomised, placebo-controlled trial. *Lancet* 367, 133–143.

Scholl, P. F., Tripathi, A. K., and Sullivan, D. J. (2005). Bioavailable iron and heme metabolism in Plasmodium falciparum. *Curr. Top. Microbiol. Immunol.* 295, 293–324.

Schümann, K., Kroll, S., Romero-Abal, M.-E., Georgiou, N. A., Marx, J. J. M., Weiss, G., and Solomons, N. W. (2012). Impact of oral iron challenges on circulating non-transferrin-bound iron in healthy Guatemalan males. *Ann. Nutr. Metab.* 60, 98–107.

Schümann, K., Solomons, N. W., Orozco, M., Romero-Abal, M. E., and Weiss, G. (2013). Differences in circulating non-transferrin-bound iron after oral administration of ferrous sulfate, sodium iron EDTA, or iron polymaltose in women with marginal iron stores. *Food Nutr. Bull.* 34, 185–193.

Scott, M. D., Ranz, A., Kuypers, F. A., Lubin, B. H., and Meshnick, S. R. (1990). Parasite uptake of desferroxamine: a prerequisite for antimalarial activity. *Br. J. Haematol.* 75, 598–602.

Senga, E. L., Harper, G., Koshy, G., Kazembe, P. N., and Brabin, B. J. (2011). Reduced risk for placental malaria in iron deficient women. *Malar. J.* 10, 47.

Shankar, A. H. (2000). Nutritional modulation of malaria morbidity and mortality. *J. Infect. Dis.* 182 Suppl 1, S37–53.

Sigala, P. A., Crowley, J. R., Hsieh, S., Henderson, J. P., and Goldberg, D. E. (2012). Direct tests of enzymatic heme degradation by the malaria parasite Plasmodium falciparum. *J. Biol. Chem.* 287, 37793–37807.

Skaar, E. P. (2010). The battle for iron between bacterial pathogens and their vertebrate hosts. *PLoS Pathog.* 6, e1000949.

Spottiswoode, N., Fried, M., Drakesmith, H., and Duffy, P. E. (2012). Implications of malaria on iron deficiency control strategies. *Adv. Nutr. Bethesda Md* 3, 570–578.

Stoltzfus, R. J. (2012). Iron and malaria interactions: programmatic ways forward. *Adv. Nutr. Bethesda Md* 3, 579–582.

Stoltzfus, R. J. (2003). Iron deficiency: global prevalence and consequences. *Food Nutr. Bull.* 24, S99–103.

Subramanian, S., Hardt, M., Choe, Y., Niles, R. K., Johansen, E. B., Legac, J., Gut, J., Kerr, I. D., Craik, C. S., and Rosenthal, P. J. (2009). Hemoglobin cleavage site-specificity of the Plasmodium falciparum cysteine proteases falcipain-2 and falcipain-3. *PloS One* 4, e5156.

Surolia, N., and Misquith, S. (1996). Cell surface receptor directed targeting of toxin to human malaria parasite, Plasmodium falciparum. *FEBS Lett.* 396, 57–61.

Taylor, S. M., Molyneux, M. E., Simel, D. L., Meshnick, S. R., and Juliano, J. J. (2010). Does this patient have malaria? *JAMA J. Am. Med. Assoc.* 304, 2048–2056.

Tian, L. P., Nelson, E. A., Senok, A. C., Yu, L. M., Oppenheimer, S. J., and Li, K. (1998). Red cell age and susceptibility to malaria during pregnancy. *Acta Obstet. Gynecol. Scand.* 77, 717–721.

Tielsch, J. M., Khatry, S. K., Stoltzfus, R. J., Katz, J., LeClerq, S. C., Adhikari, R., Mullany, L. C., Shresta, S., and Black, R. E. (2006). Effect of routine prophylactic supplementation with iron and folic acid on preschool child mortality in southern Nepal: community-based, cluster-randomised, placebo-controlled trial. *Lancet* 367, 144–152.

Veenemans, J., Milligan, P., Prentice, A. M., Schouten, L. R. A., Inja, N., van der Heijden, A. C., de Boer, L. C. C., Jansen, E. J. S., Koopmans, A. E., Enthoven, W. T. M., et al. (2011). Effect of supplementation with zinc and other micronutrients on malaria in Tanzanian children: a randomised trial. *PLoS Med.* 8, e1001125.

Wang, B., Zhan, S., Gong, T., and Lee, L. (2013). Iron therapy for improving psychomotor development and cognitive function in children under the age of three with iron deficiency anaemia. *Cochrane Database Syst. Rev.* 6, CD001444.

Whitehead, S., and Peto, T. E. (1990). Stage-dependent effect of deferoxamine on growth of Plasmodium falciparum in vitro. *Blood* 76, 1250–1255.

WHO | Assessing the iron status of populations *WHO*. Available at: http://www.who.int/nutrition/publications/micronutrients/anaemia_iron_deficiency/9789241596107/en/ [Accessed January 10, 2014].

WHO | Guidelines on food fortification with micronutrients *WHO*. Available at: http://www.who.int/nutrition/publications/micronutrients/9241594012/en/index.html [Accessed January 10, 2014].

WHO | World Malaria Report 2013 *WHO*. Available at: http://www.who.int/malaria/publications/world_malaria_report_2013/report/en/index.html [Accessed January 13, 2014].

Wilson, R. J., Pasvol, G., and Weatherall, D. J. (1977). Invasion and growth of Plasmodium falciparum in different types of human erythrocyte. *Bull. World Health Organ.* 55, 179–186.

Wong, S. S., Qutishat, A. S., Lange, J., Gornet, T. G., and Buja, L. M. (1996). Detection of iron-deficiency anemia in hospitalized patients by zinc protoporphyrin. *Clin. Chim. Acta Int. J. Clin. Chem.* 244, 91–101.

Yip, R., Mohandas, N., Clark, M. R., Jain, S., Shohet, S. B., and Dallman, P. R. (1983). Red cell membrane stiffness in iron deficiency. *Blood* 62, 99–106.

Zhao, H., Konishi, A., Fujita, Y., Yagi, M., Ohata, K., Aoshi, T., Itagaki, S., Sato, S., Narita, H., Abdelgelil, N. H., et al. (2012). Lipocalin 2 bolsters innate and adaptive immune responses to blood-stage malaria infection by reinforcing host iron metabolism. *Cell Host Microbe* 12, 705–716.

Zlotkin S, Newton S, Aimone AM, and et al (2013). Effect of iron fortification on malaria incidence in infants and young children in Ghana: A randomized trial. *JAMA* 310, 938–947.

CHAPTER TWO

Parasite maturation and host serum iron influence the labile iron pool of erythrocyte stage *P. falciparum*[1]

2.1 Overview

Iron is a critical and tightly regulated nutrient for both the malaria parasite and its human host. The importance of the relationship between host iron and the parasite has been underscored recently by studies showing that host iron supplementation may increase the risk of falciparum malaria. It is unclear what host iron sources the parasite is able to access. I developed a flow cytometry based method for measuring the labile iron pool (LIP) of parasitized erythrocytes using the nucleic acid dye SYTO 61 and the iron sensitive dye, calcein acetoxymethyl ester (CA-AM). This new approach allows me to measure the LIP of *P. falciparum* through the course of its erythrocytic life cycle and in response to the addition of host serum iron sources. I found that the LIP increases as the malaria parasite develops from early ring to late schizont stage, and that the addition of either transferrin or ferric citrate to culture media increases the LIP of trophozoites. My method for detecting the LIP within malaria parasitized RBCs provides evidence that the parasite is able to access serum iron sources as part of the host vs. parasite arms race for iron.

[1] First published in *The British Journal of Haematology*, April 2013, Vol. 161, Issue 2

2.2 Introduction

Each year up to 250 million clinical cases of malaria and nearly 1 million deaths from malaria are reported in official statistics (WHO | World Malaria Report 2011). *P. falciparum* malaria is the most deadly of all the species of malaria that infect humans. The malaria parasite has a complex life cycle in the human host. *Anopheles* mosquitoes inject sporozoite stage *P. falciparum* parasites during a blood meal; sporozoites then migrate to the liver where they infect hepatocytes and multiply over a clinically silent 7-10 day period (Sinnis et al., 1996). During the asexual erythrocyte stage of the parasite, merozoites invade RBCs and progress from the metabolically inactive ring stage to the metabolically active trophozoite stage to the schizont stage. DNA replication is initiated during the schizont stage and results in the production of new merozoites that burst from the host RBC into the blood stream and invade new RBCs. The RBC stage of the malaria parasite is responsible for the morbidity and mortality associated with *P. falciparum* infection and is exquisitely sensitive to iron chelators (Ferrer et al., 2012).

Despite the essential role of iron in parasite development, it is unknown what host iron sources *P. falciparum* utilizes during any stage of the human infection. The two principal sources of host iron available to the parasite during the RBC stage are extra-erythrocytic (serum) iron and intra-erythrocytic iron. The intra-erythrocytic iron pool amounts to 100 fg (20 mM) iron, partitioned into hemoglobin, ferritin, and the cytoplasmic labile iron pool (LIP). The erythrocytic LIP consists of residual cytoplasmic bioavailable iron that was not incorporated into hemoglobin or stored within ferritin during the maturation of erythrocyte precursors (Prus and Fibach, 2008b). The majority of host serum iron is bound to host protein transferrin (60-150 μg/dL or 11-27 μM), with a residual amount of iron, non-transferrin bound iron (NTBI), circulating in the serum chelated by low molecular weight molecules such as citrate (Cook and Skikne, 1989). To date there is no evidence that *P. falciparum* is able to release iron from haem or host ferritin. The

malaria parasite is hypothesized to use the host RBC LIP as a source of iron (Scholl et al., 2005). It is unknown how the host LIP impacts the malaria parasite's infectivity and maturation. The ability of the parasite to access serum iron is unclear, and data are conflicting (Sanchez-Lopez and Haldar, 1992; Rodriguez and Jungery, 1986; Haldar et al., 1986; Pollack and Fleming, 1984).

Calcein acetoxymethyl ester (CA-AM) has been widely used to examine the cytoplasmic LIP of mammalian cells (Breuer et al., 1995a, 1996, 1995b; Epsztejn et al., 1997; Tenopoulou et al., 2007). CA-AM is non-fluorescent, non-iron binding, neutrally charged, and easily permeates cell membranes. Upon cellular entry, intracellular esterases cleave CA-AM into the green-fluorescent molecule calcein**,** which is then trapped within the cell. Calcein fluorescence is quenched by 1:1 stoichiometric binding of iron in pH range of 7-7.5 (Breuer et al., 1995a, 1996). The addition of non-fluorescent, high affinity, iron chelators removes iron from calcein and consequently increases calcein fluorescence, providing an effective method for assessing the labile iron content of cells. Alternatively the addition of iron, capable of being incorporated by a cell, quenches calcein fluorescence (Breuer et al., 1995b; Tsien, 1989). Previous investigators have utilized a microscopy based approach for the measurements of calcein fluorescence, to investigate the site of action of anti-malarial iron chelators and gain preliminary insight into the LIP of parasitized human erythrocytes (Loyevsky et al., 1999). More recently, CA-AM has been utilized to assess the LIP of the heterogeneous cell populations of peripheral blood and bone marrow by flow cytometry (Prus and Fibach, 2008a).

In the present study, I have confirmed the apparent variability in results that exists when studying the effect of extracellular iron supplementation on erythrocytic stage *P. falciparum*. To determine whether extracellular iron effects the bioavailable iron content of pRBCs, I adapted

the CA-AM flow cytometry method for the assessment of the LIP of *P. falciparum* infected erythrocytes (Prus and Fibach, 2008a, 2008b). I combined the technique of identifying parasitized erythrocytes with fluorescent DNA dye SYTO 61 (Fu et al., 2010) with the CA-AM method for assessing cellular labile iron to determine the LIP of *P. falciparum* during asexual maturation by flow cytometry. This flow cytometry approach allows for the analysis of the LIP of a mixed population of uninfected and *P. falciparum* infected erythrocytes. Furthermore, I utilized this approach to investigate the effect of extracellular iron sources, transferrin and ferric citrate, on the LIP of the erythrocytic stage of *P. falciparum.*

2.3 Materials and Methods

P. falciparum culture and growth assays

P. falciparum parasite lines 3D7 (MR4, MRA-102), FCR3-FMG (MR4, MRA-736) and Dd2 (MR4, MRA-156) were routinely cultured in iron-replete O-positive (O+) RBCs obtained from healthy individuals at the Clinical and Translational Research Center at the University of North Carolina, Chapel Hill, NC (IRB# 09-0559, approved by the University of North Carolina Institutional Review Board). Cultures were maintained with 2% Hematocrit in complete media containing RPMI 1640 with 10% albumax II, 1 mM hypoxanthine, 20 mM L-glutamine, .45% glucose, and 10 µg/L gentamicin (ACM). Cultures were incubated on a shaker at 37 degrees C in 5% O_2 5% CO_2 and 90% Nitrogen. Parasite density was maintained between 0 .5% and 10% *P. falciparum*-parasitized RBCs (pRBCs). pRBC cultures were synchronized to within 4-6 hours of each other by first treating cultures with 5% D-sorbitol to select for ring stage parasites, followed by MACS (Miltenyi Biotec) isolation of hemozoin containing trophozoite and schizont stage pRBCs 24 hours later. pRBC cultures were next incubated with 30 IU heparin to prevent invasion of merozoites for 18 hours at which point heparin was washed from cultures subsequently incubated under normal culture conditions for an additional 24 hours before a final

MACS isolation of trophozoites and schizonts was performed (Boyle et al., 2010). To obtain a culture with all stages (ring, trophozoite, schizont, and merozoites) synchronized cultures were monitored until approximately 50% of schizonts had ruptured. To assess *P. falciparum* growth, growth assays were started at 0.5% pRBC at 2% Hematocrit in appropriate iron supplemented media. Experiments were monitored for up to 96 hours and media was changed daily. Experiments were analyzed by either microscopy or flow cytometry and the growth rate of *P. falciparum* reflect the fold change in %pRBC from the beginning to the end of experiments.

P. falciparum LIP assay

P. falciparum lines FCR3-FMG or Dd2 at a parasite density of 5-10% pRBCs were washed twice with PBS .5% Albumax II (PBS+), inoculated into a 96 well plate at $2x10^6$ cells per well and subsequently labeled with .125 μM calcein acetoxymethyl ester (CA-AM (Invitrogen)) for 15 minutes in the dark. Following CA-AM labeling, cells were washed twice with PBS+ and allowed to rest for 15 min under standard culture conditions in the dark. Cells were then labeled with .5 μM DNA dye STYO 61 **(**Invitrogen**)** in the presence or absence of 100 μM of iron chelators: deferiprone, dipyridyl, or deferoxamine for 1 hour under standard culture conditions in the dark. Following incubation with STYO 6 and iron chelators, unfixed cells were immediately analyzed by flow cytometry using a Cytek-modified FACS-Calibur under BSL-2 containment. For experiments assessing the impact of extracellular transferrin or ferric citrate on pRBC LIP, *P. falciparum* cultures were incubated with transferrin or ferric citrate prior to CA-AM and SYTO 61 labeling. Individual experiments were performed in triplicate. The statistical significance for each individual experiment was calculated using the Student's t-test. For experiments where data is expressed as delta MFI, delta MFI represents the absolute difference in MFI.

Flow Cytometry Analysis

Flow cytometry was performed at the UNC Flow Cytometry Core Facility, Chapel Hill, NC on a Cytek-modified FACS-Calibur with 2 lasers; a 30 mW 488 Diode Pumped Solid State laser and a 25 mW 637 red diode laser (FACS-calibur; Becton Dickinson, Mount View CA, modified by Cytek Development, Freemont, CA). Channels and fluorescent probes used on the FACS-calibur included: STYO 61 (637nm, 666/27 bandpass), and calcein (488nm, 530/30 bandpass). Detector gain settings were varied between experiments to optimize signal but were kept constant within individual experiments and no compensation was applied to any of the channels. *P. falciparum*-infected RBCs (pRBCs) were gated based on the STYO 61 signal and detector gains for calcein fluorescence were adjusted to achieve a calcein MFI of 10-20 for uninfected RBCs. A minimum 1000 pRBC (STYO 61 +) events were acquired. FACS-Calibur data was collected using FlowJo CE (Treestar, Ashland, OR) and analyzed with Summit v5.1 (Beckman Coulter, Miami, Florida).

Microscopy

Cultures of *P. falciparum* strain FCR3-FMG (5% pRBC, 2% hematocrit) were incubated for 3 hours under standard culture conditions with Alexa fluor 488 conjugated human transferrin (1:20) (Invitrogen). Cells were washed and fixed with 1% paraformaldehyde and 0.0075% Glutaraldehyde in Alsever's Solution for 30 minutes at 4C. Fixative was washed from RBCs before staining cells with Hoescht DNA dye (1:10,000) in PBS for 10 minutes. Cells were washed one last time before being viewed on a Zeiss LSM710 Spectral Confocal Laser Scanning Microscope.

2.4 Results

Assessing the effect of extracellular iron on erythrocytic stage P. falciparum growth

To determine whether extracellular iron effects the growth of erythrocytic stage *P. falciparum in vitro*, I supplemented culture media with physiologic levels of either transferrin or ferric citrate and assessed the growth rate of *P. falciparum* (Dd2 and FCR3-FMG strains). In the course of conducting twenty independent experiments, I observed every possible outcome: (i) decreased; (ii) no change; and (iii) increased growth with the addition of iron to culture media (**Figure 2.1A and 2.1B**). Unable to definitively determine whether extracellular iron influences erythrocytic stage *P. falciparum* growth I proceeded to characterize the LIP of parasitized RBCs (pRBCs) in the effort of ultimately determining whether extracellular iron is incorporated into pRBCs.

Detection of LIP in P. falciparum infected erythrocytes by flow cytometry

To assess the LIP of *P. falciparum* infected erythrocytes, *P. falciparum* (FCR3-FMG strain) infected erythrocyte cultures were loaded with CA-AM, stained with DNA dye SYTO 61 to identify pRBCs and finally incubated with membrane permeable iron chelator deferiprone to allow for the determination of LIP. I first confirmed that neither calcein nor SYTO 61 interfered with the others' fluorescence profile (**Figure 2.2A and 2.2B**), and that SYTO 61 did not interfere with calcein' s sensitivity to either iron chelator or extracellular iron (**Figure 2.2C**). To characterize the basal calcein fluorescence of uninfected and pRBCs, I examined the SYTO 61 profile of stained cells to identify uninfected (SYTO 61 negative, R2- lower region), pRBCs infected with rings (SYTO 61 positive, R3-middle region) and pRBCs infected with trophozoites (SYTO 61 positive, R4-upper region) (**Figure 2.3A**). Each of these three populations was gated upon and the basal calcein fluorescence of each population was determined. I observed that the

pRBCs have greater steady state calcein basal fluorescence than uninfected erythrocytes, and calcein fluorescence increases with increasing parasite maturation. Compared to uninfected RBCs, the calcein fluorescence of ring pRBCs and trophozoite pRBCs is 18% ($p<0.02$) and 153% ($p<0.0002$) greater than uninfected RBCs respectively (**Figure 2.3B**).

Before calcein fluorescence can be made susceptible to iron quenching, cellular esterases must cleave the acetoxymethyl ester group from CA-AM converting the non-fluorescent CA-AM molecule to the fluorescent Calcein. Parasitized RBCs have greater enzymatic activity than uninfected RBCs (Vander Jagt et al, 1982). To assess the impact of iron on calcein fluorescence, I employed the use of iron chelators which when added to calcein loaded cells chelate iron bound to calcein. The resulting increase in calcein fluorescence achieved after adding an iron chelator to calcein-loaded cells represents the cells LIP. To assess the LIP of uninfected and pRBCs, I measured the calcein fluorescence of each of these populations in the absence (red line/vertical hatch) and presence (green line/diagonal hatch) of the iron chelator deferiprone (**Figure 2.4A and 2.4B**). I observed that the addition of deferiprone resulted in significant increase**s** in calcein fluorescence in uninfected as well as in the ring and trophozoite stage parasites**,** indicating the presence of a LIP within pRBCs**.** In addition to deferiprone, I employed a second membrane permeable iron chelator (2,2 bipyridyl) and membrane impermeable iron chelator (deferoxamine) to assess the LIP within uninfected RBCs, ring pRBCs and trophozoite pRBCs (**Figure 2.4C**). Use of the membrane permeable iron chelator deferiprone resulted in ΔMFI in ring pRBCs (ΔMFI=4.55 +/- 0.426,$p<0.005$) and trophozoite pRBCs (ΔMFI=15.02 +/- 1.11,$p<0.005$) as well as uninfected RBCs (ΔMFI=1.93 +/- 0.12, $p<0.005$). Use of a second membrane permeable iron chelator, 2,2 bipyridyl resulted in an increase in ΔMFI in both ring pRBCs (ΔMFI=3.88 +/- 0.44, $p<0.005$) and trophozoite pRBCs (ΔMFI=16.48 +/- 1.10, $p<0.005$) compared to the increase in

ΔMFI observed in uninfected RBCs (ΔMFI=2.22 +/- 0.043, p<0.005). Utilizing membrane permeable iron chelators I consistently observed 15-20% greater ΔMFI (p<.005) in ring pRBCs and 40-50% greater ΔMFI (p<.005) in trophozoite pRBCs as compared to the ΔMFI observed in uninfected RBCs in independent experiments. However with the less permeable chelator deferoxamine, an increase in ΔMFI in both ring pRBCs (ΔMFI=3.1 +/- 0.0.21, p<0.005) and trophozoite pRBCs (ΔMFI=8.67 +/- 0.089, p<0.005), but no ΔMFI was observed in uninfected RBCs (ΔMFI=0.19 +/- 0.32, p>1). This finding is consistent with evidence that pRBCs are more permeable than uninfected RBCs (Pouvelle et al., 1991; Nguitragool et al., 2011).

Characterization of LIP during maturation of P. falciparum within host RBCs

Based upon my observation that pRBCs contain a greater LIP than uninfected RBCs, and that labile iron appeared to increase with the maturation of the parasite from the ring stage to the trophozoite stage, I sought to characterize the dynamics of the LIP during maturation of erythrocyte stage *P. falciparum.* To this end I tightly synchronized parasites to within 4-6 hours of each other by a combination of (i) ring stage selection by sorbitol treatment (ii) hemozoin containing trophozoite and schizont stage isolation by MACS and (iii) merozoite invasion inhibition with heparin. Following synchronization, I allowed the parasite culture to progress to late schizony to the point at which at least 50% of schizonts had ruptured. I was able to observe uninfected, newly invaded ring stage, late stage trophozoites, schizonts, and free merozoites by microscopic analysis of Gyms stained thin blood smears (data not shown) and flow cytometry analysis of STYO 61 stained pRBCs (**Figure 2.5A**). I observed that the addition of iron chelator deferiprone to these calcein loaded parasite cultures resulted in a calcein ΔMFI that increased with parasite maturation. As shown in Figure 3B, compared to uninfected RBCs (ΔMFI =80.61+/-0.99, p<0.0002), the ΔMFI was increased in ring pRBCs (ΔMFI =95.19+/-2.59,

p<0.0002), trophozoite pRBCs (ΔMFI =162.13 +/-6.4, p<0.0002), and schizont pRBCs (ΔMFI =173.91+/-16.5, p<0.0002) (**Figure 2.5B**). Despite differences in cytometer settings between independent experiments, the increase in calcein fluorescence of rings, trophozoites and schizonts was consistently 15-20%, 40-50% and 55-60% greater than the increase observed in uninfected RBCs. There was little to no detectable change in calcein fluorescence in merozoites with the addition of deferiprone **(**ΔMFI =0.963+/-0.076**)** (Figure 3B). These results suggest that as erythrocyte stage *P. falciparum* parasites mature, the level of the LIP increases, in response to increased iron demands as the parasite becomes more metabolically active and begins to replicate DNA.

Investigation of the impact of host serum iron sources on LIP in P. falciparum infected erythrocytes

To determine whether serum iron sources, transferrin bound iron and ferric citrate, can be accessed by erythrocyte stage *P. falciparum* and incorporated into the LIP of pRBCs, I incubated *P. falciparum* (mixed ring and trophozoite stage) infected erythrocyte cultures with increasing physiological concentrations of either holo-transferrin (0.20-1.2g/L) or ferric citrate (7.16 – 26.85µmol/L Fe) for 6 hours. Cells were then loaded with CA-AM and subsequently stained with SYTO 61. The presence of both transferrin and ferric citrate in culture media corresponded with significant decreases in calcein fluorescence in trophozoite pRBC (-ΔMFI calcein), which indicates that additional iron entered the LIP and quenched the calcein fluorescence (**Figure 2.6A**). Compared to untreated trophozoite pRBCs a change of 14% (p<0.005), 15% (p<0.005) and 26% (p<0.0009)was observed in the MFI calcein after the addition of 0.2 g/L, 0.4 g/L and 1.2 g/L human transferrin respectively relative to untreated trophozoite pRBCs. Compared to untreated trophozoite pRBCs a decrease of 10% (p<0.005), 15% (p<0.005) and 16% (p<0.0009) was observed in the calcein fluorescence after the addition of 7.16µmol/L, 11.19µmol/L and

26.85µmol/L ferric citrate. No significant changes in calcein fluorescence were observed for uninfected RBCs or ring stage pRBC. These results demonstrate that the LIP of trophozoite stage *P. falciparum* increases with increasing concentrations of transferrin and ferric citrate. Furthermore, microscopic analysis of the association between human transferrin and pRBCs revealed that human transferrin co-localizes with late stage pRBCs and not with uninfected RBCs (**Figure 2.6B**). Together these data suggest that late stage parasites are capable of accessing serum iron in both the transferrin and non-transferrin bound form (ferric citrate).

2.5 Discussion

During the course of microbial infections, there is an arms race between the pathogen and host for iron. In the course of this arms race pathogens have evolved sophisticated methods of scavenging host iron while the host acute activation of the nutritional immune response effectively limits the availability of iron to invading pathogens (Skaar, 2010). Iron chelating agents suppress the growth of *P. falciparum* in vitro and in vivo (Hershko and Peto, 1988; Gordeuk et al., 1992). In addition, iron chelators also bolster the host innate immune response by synergistically acting with cytokines to increase stimulation of NO production which is protective against severe infection (Fritsche et al., 2001; Weiss et al., 1997). The importance of iron to malaria is additionally demonstrated by clinical studies which have documented an increased susceptibility to malaria infection in individuals given high doses of iron supplementation (Oppenheimer, 2001; Murray et al., 1975; Sazawal et al., 2006; Smith et al., 1989). The sources of host iron used by *P. falciparum* and the strategies used by the parasite to evade host nutritional immunity have remained elusive.

The labile iron pool represents the transition zone for iron between import, cellular utilization, and storage and it is thought to change in response to the metabolic needs of the cell.

As the metabolic demand for iron increases, cells will increase the amount of iron in the labile iron pool. The CA-AM LIP assay measures the LIP present in uninfected RBCs and pRBCs. Calcein fluorescence is sensitive to iron at physiologic pH 7.2-7.4 (Tenopoulou et al., 2007). As RBC precursors mature, all their organelles are lost, producing an anucleated mature erythrocyte with a cytoplasm of pH 7.2-7.4 (Tenopoulou et al., 2007). At this pH, calcein is sensitive to iron and is able to detect the entire LIP. Upon infection of the RBC, *P. falciparum* introduces new organelles and structures including: a nucleus, mitochondria, apicoblast, endoplasmic reticulum (ER) and Golgi apparatus as well as a parasitophorous vacuole and a food vacuole with pH 3.7-6.5 (Hayward et al., 2006). The cytoplasm of pRBCs like uninfected RBCs is maintained between pH 7.2-7.4 (Saliba et al., 1999).

I had initially sought to determine whether extracellular affects erythrocytic stage *P. falciparum* growth. I was unable to definitely determine whether extracellular iron effects *P. falciparum* growth (**Figure 2.1A and 2.1B**). I therefore took a step back and instead worked to characterize the LIP of pRBCs and determine whether extracellular iron affects the LIP of pRBCs. The LIP, which is detected within pRBCs using the CA-AM method, is the bioavailable/labile iron present in the neutral pH regions of the residual RBC cytoplasm, parasitophorous vacuole, and parasite cytoplasm. I defined LIP as the ΔMFI of calcein that occurred with the addition of an iron chelator or iron source. I observed that the basal calcein fluorescence was greater within pRBCs than within uninfected RBCs and that fluorescence increased with parasite maturation. The addition of iron chelators to calcein loaded uninfected and pRBCs resulted in greater ΔMFI within pRBCs than uninfected RBCs, and the ΔMFI further increased with increasing parasite maturation. Interestingly, the extra-erythrocytic merozoite stage of *P. falciparum* had no detectable LIP. My data indicate that the parasite may be able to

access both intra-erythrocytic and serum iron. Since total iron does not differ between uninfected and pRBCs (Marvin et al., 2012), my observation that LIP increases with parasite maturation when it is grown in very low (10-15 μg/dL or 2-3 mM) iron media, suggests that the parasite may be able to release iron from either RBC hemoglobin or ferritin, redistributing but not altering the total cellular iron. Increasing LIP with parasite maturation is consistent with the increasing iron demands of the parasite during the trophozoite and schizont stage as the parasite's metabolic activity dramatically increases and commences DNA replication. Alternatively, changes in intracellular iron levels may not only reflect iron consumption by the parasite but may be due to regulation of iron import/export in infected cells as has been shown in macrophages targeted by intracellular bacteria (Paradkar et al., 2008; Nairz et al., 2007).

To provide new insight into the potential ability of *P. falciparum* to access serum iron, either transferrin or non-transferrin bound iron (ferric citrate), I measured the impact of holo transferrin and ferric citrate on the LIP of uninfected and pRBCs. I observed that the addition of increasing physiological concentrations of either ferric citrate or holo transferrin increased the LIP of trophozoite pRBCs to a significantly greater degree than of ring pRBCs and uninfected RBC. This provides evidence that trophozoite stage pRBC can access serum iron sources. These results do not address whether pRBCs specifically bind or internalize transferrin. Rodriguez et al and Haldar et al. independently postulated the existence of a *P. falciparum* transferrin receptor, however such a receptor has yet to be isolated and cloned (Haldar et al., 1986; Rodriguez and Jungery, 1986). Alternatively, it is well established that pRBCs are able to non-specifically incorporate both micro and macromolecules from the serum (Pouvelle et al., 1991; Nguitragool et al., 2011). Human transferrin, like other abundant serum proteins such as albumin, may be non-specifically internalized into pRBCs (El Tahir et al., 2003).

Our results are relevant to the clinical question of whether host iron status and host iron supplementation affects risk of malarial infection. The relationship between host iron and the malaria parasite is complex and is tightly regulated by both host and parasite. A study published in 2006 by Sazawal et al. conducted in Pemba, Zanzibar, involving more than 24,000 children in a setting where anti-malarial treatment was not readily available, showed that routine supplementation with iron and folic acid increased the rates of severe illness and death from malaria in iron-replete children who took iron supplements (Sazawal et al., 2006). Because non-transferrin bound serum iron transiently increases in iron-replete individuals who are given oral iron supplementation (Schümann et al., 2012), I speculated that *P. falciparum* may scavenge serum iron in order to augment intra-erythrocytic growth and thereby potentiate the risk of malaria.

Our application of the flow cytometry based CA-AM LIP assay has revealed that the LIP content of infected RBCs steadily increases with increasing maturation of the intra-erythrocytic stage of the parasite. Additionally, I demonstrate that the LIP content of late stage trophozoite pRBCs is increased in the presence of extracellular transferrin and ferric citrate. Further studies are needed to elucidate the mechanisms by which the malaria parasite senses, acquires, utilizes, regulates, and stores iron during the erythrocytic stage of its life cycle and on the impact of host serum iron on these processes. Elucidation of parasite iron biology will provide therapeutic insights into how to augment the host innate immune response and may reveal targets for anti-malarial drug development.

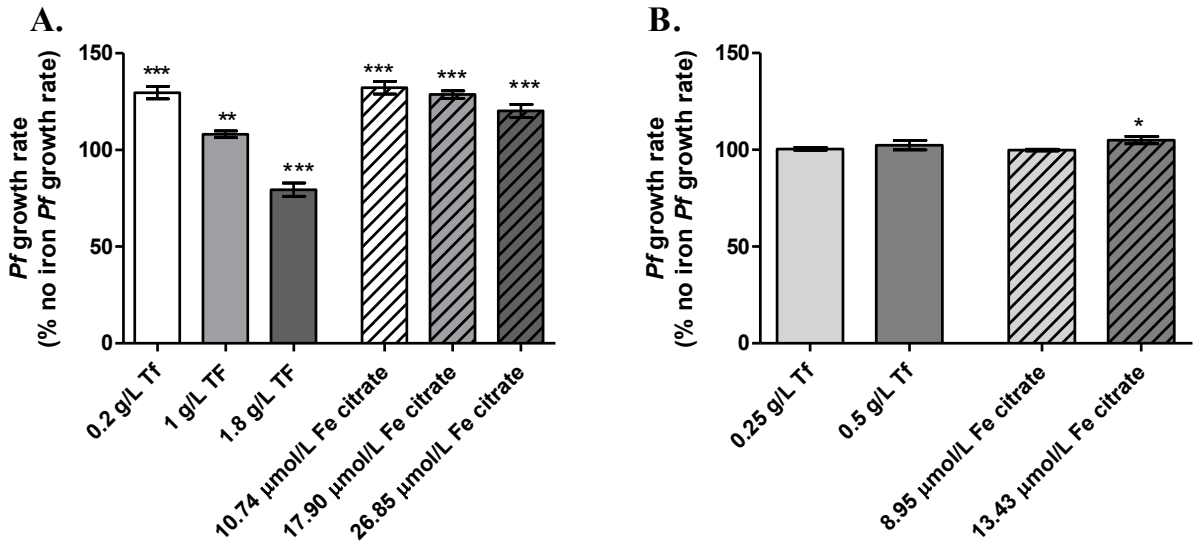


Figure 2.1 – The effect of extracellular iron on erythrocytic stage *P. falciparum* infection is unclear. The effect of extracellular iron (transferrin and ferric citrate) on growth of erythrocytic stage *P. falciparum* growth *in vitro* was assessed by adding physiologic amounts of either transferrin or ferric citrate (60-150 µg/dL or 11-27 µM) to culture media and determining the growth rate of the parasite over the course of 96 hours (two erythrocyte lifecycles). Growth of *P. falciparum* strains 3D7, Dd2, and FCR3-FMG in cultures supplemented with either transferrin or ferric citrate was expressed as a percent of parasite growth in cultures with no additional iron added to culture media. (A and B) are representative data of over 20 independent experiments. The bar graph (mean +/- SD, n=3) shows growth of *P. falciparum* in iron-supplemented culture media (transferrin or ferric citrate) as a percent of parasite growth in non-iron supplemented culture media. Student's t-test statistical analysis was performed comparing the growth rate of *P. falciparum* supplemented with increasing concentrations of transferrin and ferric citrate to the addition of no iron to culture media, *$p<0.05$, **$p<0.002$, and ***$p<0.0005$.

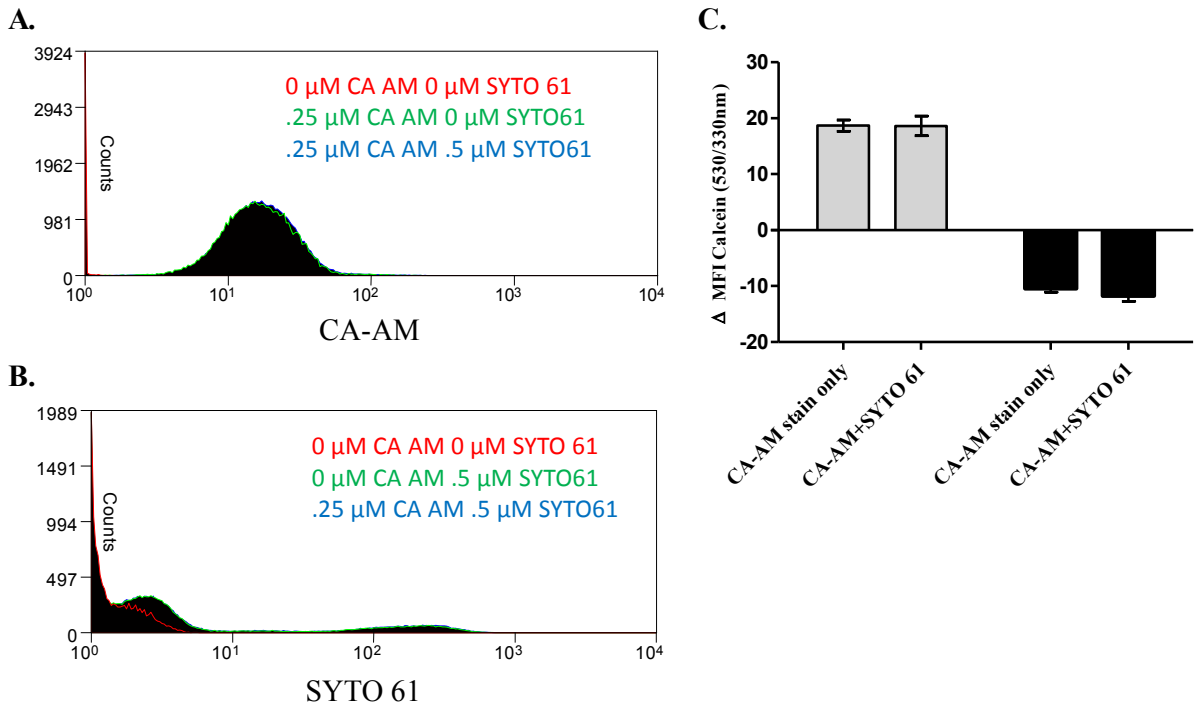


Figure 2.2 – Calcein and SYTO 61 fluorescence does not exhibit spectral overlap, and SYTO 61 does not interfere with Calcein iron binding. (A) Impact of SYTO 61 on Calcein (530/330nm) fluorescence was determined by assessing Calcein fluorescence of Calcein loaded cells in the presence and absence of SYTO 61. Cells from *P. falciparum (*FCR3-FMG strain) infected erythrocyte cultures loaded with CA-AM, and then incubated in the absence or presence of DNA dye SYTO 61 and analyzed by flow cytometry. Histogram depicts the Calcein fluorescence of unstained (red), Calcein stained (green) and Calcein and SYTO 61 stained (blue) cells. No significant difference in Calcein fluorescence was observed. Data is from a single representative experiment, and the experiment was performed three independent times with parasite lines, FCR-FMG and Dd2. (B) Impact of Calcein on SYTO 61 (628/645nm) fluorescence was determined by assessing SYTO 61 fluorescence of SYTO 61 stained cells in the presence and absence of Calcein. Cells from *P. falciparum (*FCR3-FMG strain) infected erythrocyte cultures were incubated in the presence or absence of CA-AM, and then stained with DNA dye SYTO 61 and analyzed by flow cytometry. Histogram depicts the SYTO 61 fluorescence of unstained (red), SYTO 61 stained (green) and SYTO 61 and Calcein stained (blue) cells. No significant difference in SYTO 61 fluorescence was observed. Data is from a single representative experiment, and the experiment was performed three independent times with parasite lines, FCR-FMG and Dd2. (C) Impact of SYTO 61 on Calcein sensitivity to iron chelators and extracellular iron was determined by assessing the change in Calcein fluorescence in response to either deferiprone or transferrin within trophozoite parasitized RBCs in the presence and absence of SYTO 61. Cells from *P. falciparum (*FCR3-FMG strain) infected erythrocyte cultures loaded with CA-AM, and then incubated in the absence or presence of DNA dye SYTO 61. For transferrin experiments cells were incubated with transferrin prior to being loaded with Calcein, while for deferiprone experiments cells were incubated with deferiprone following Calcein loading. The bar graph (mean +/- SD, n=3) shows Calcein ΔMFI of SYTO 61

stained and unstained trophozoite parasitized RBCs. No significant difference in Calcein ΔMFI was observed. Data is from a single representative experiment, and the experiment was performed three independent times with parasite line, FCR-FMG.

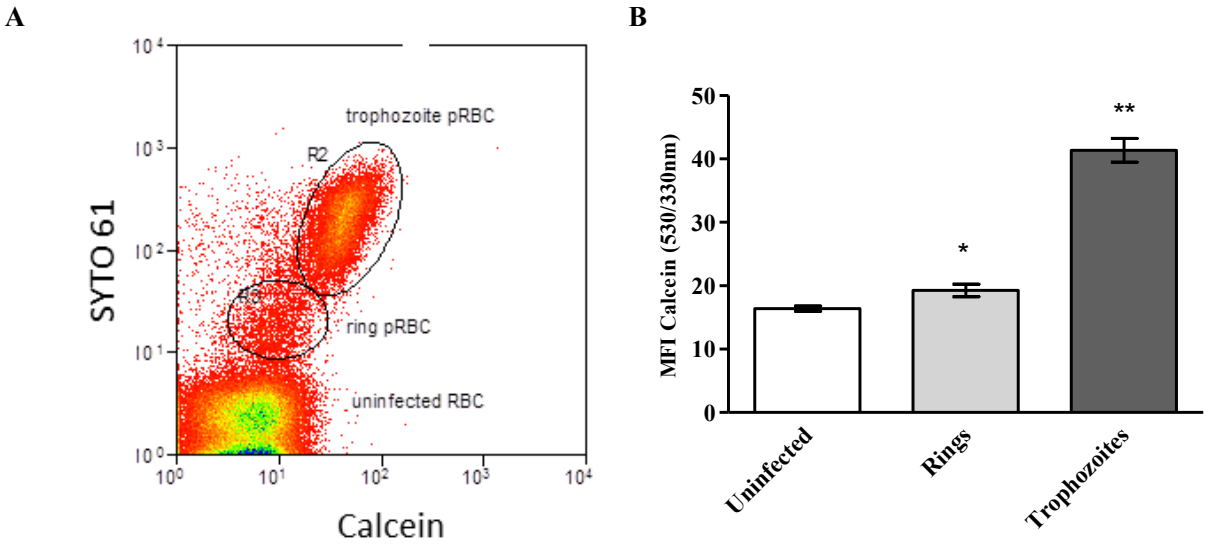


Figure 2.3 – Measurement of the basal levels of CA-AM fluorescence. Calcein fluorescence of parasitized and uninfected RBCs was determined by assessing the calcein fluorescence of SYTO 61- (uninfected) and SYTO 61+ (parasitized) RBCs. Cells from *P. falciparum (*FCR3-FMG strain) infected erythrocyte cultures were seeded in triplicate into a 96 well plate, loaded with CA-AM, and then stained with DNA dye SYTO 61 and analyzed by flow cytometry. (A) Dot-plot of cell distribution of DNA stain SYTO 61 vs. calcein reveals the distribution of uninfected RBCs, rings and trophozoites. (B) Gates were set on uninfected RBCs, rings, trophozoites, schizonts and merozoite populations and mean calcein-fluorescence intensity (MFI) of stained MFI calcein (530/330nm) was assessed for each population. The bar graph (mean +/- SD, n=3) shows calcein fluorescence of uninfected and parasitized RBCs. Student's t-test statistical analysis was performed comparing uninfected to parasitized cells, *p<.02 **p<.0002. Data is from a single representative experiment, and the experiment was performed five independent times with each parasite line, FCR-FMG and Dd2.

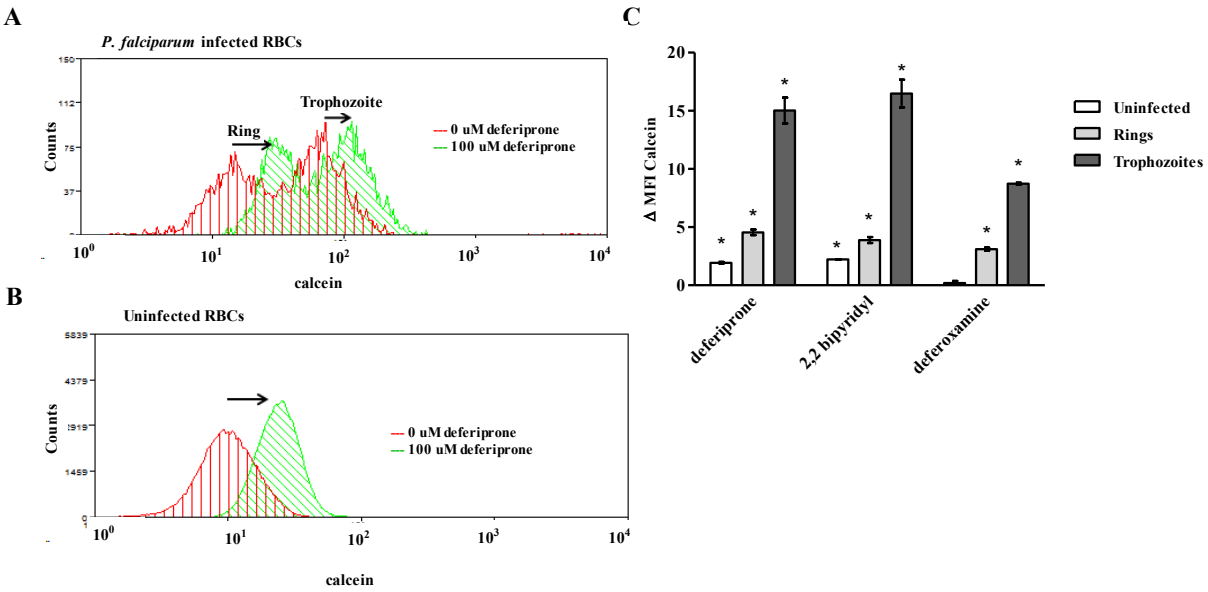


Figure 2.4 – Measurement of LIP in *P. falciparum* infected RBCs using Flow Cytometry. The LIP of uninfected (SYTO 61-) and parasitized RBCs (SYTO 61+) was determined by evaluating the change in MFI of calcein loaded achieved in the presence of different iron chelators. Cells from *P. falciparum* (FCR3-FMG strain) infected erythrocyte cultures were loaded with CA-AM and then incubated with DNA dye SYTO 61 in either the presence or absence of 100 μM of the indicated iron chelator for 1 hour. Cells were immediately analyzed by flow cytometry. (A) RBCs infected with ring and trophozoite stage *P. falciparum* or (B) uninfected RBCs following 1h incubation in the presence (green line/diagonal hatch) or absence (red line/vertical hatch) of iron chelator deferiprone. Black arrows denote the deferiprone-induced shift in calcein fluorescence. The change in the mean fluorescence intensity of calcein (ΔMFI calcein (530/330nm)) following the addition of deferiprone. (C) Change in calcein MFI (530/330nm) with the addition of deferiprone, 2,2 bipyridyl or deferoxamine in uninfected and ring and trophozoite parasitized RBCs. The bar graph (mean +/-SD, n=3) shows calcein ΔMFI of uninfected and parasitized RBCs. Student's t-test statistical analysis was performed comparing MFI calcein before and after the addition of iron chelator to cells, *p<.005. Data is from a single representative experiment using strain FCR3-FMG, and the experiment was performed three independent times with each parasite line, FCR3-FMG and Dd2.

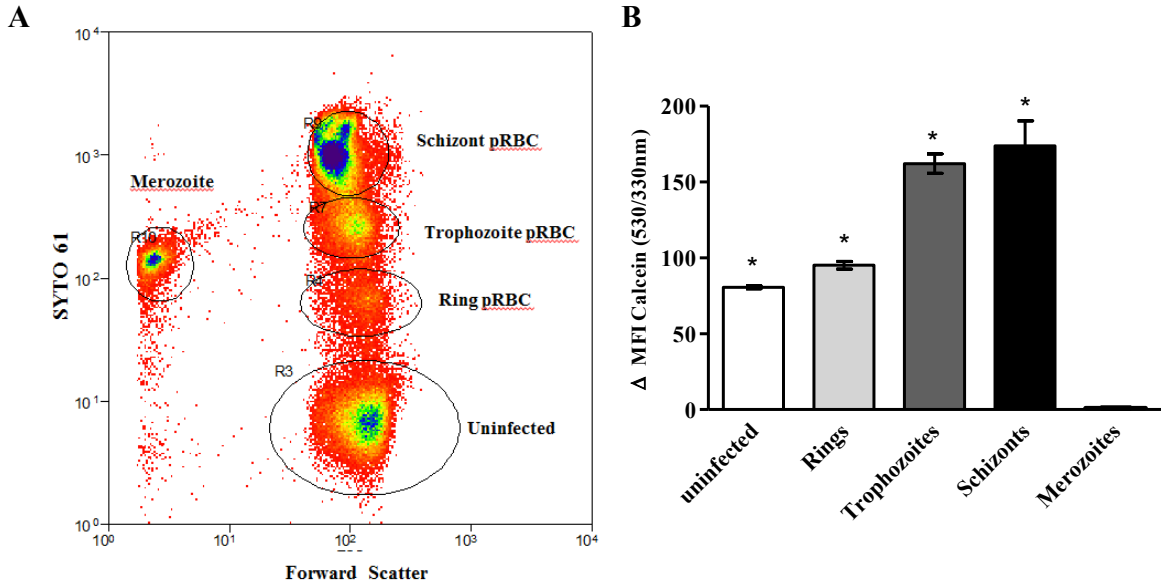


Figure 2.5 – LIP increases as parasite matures inside host red blood cell. The LIP of uninfected and different stage parasitized RBCs was determined by assessing the change in MFI of calcein loaded cells with the addition of iron chelator deferiprone. Cells from *P. falciparum (*FCR3-FMG strain) infected erythrocyte cultures were loaded with CA-AM and then incubated with DNA dye SYTO 61 either in the presence or absence of 100 μM deferiprone for 1 hour and analyzed by flow cytometry. (A) Dot-plot of cell distribution of DNA stain (SYTO 61) vs. Forward scatter showing the distribution of uninfected RBC, ring, trophozoite, schizont pRBCs and merozoites. (B) Change in calcein MFI (530/330nm) with the addition of deferiprone in uninfected RBCs and RBCS infected with ring, trophozoite and schizont stage *P. falciparum* parasites and the extracellular merozoite stage. The change in the MFI of calcein before and after addition of deferiprone represents the LIP of each cell population. The bar graph (mean +/- SD, n=3) shows calcein ΔMFI of uninfected and parasitized RBCs. Student's t-test statistical analysis was performed comparing MFI calcein before and after the addition of iron chelator to cells, *p<.0002. Data is from a single representative experiment, and the experiment was performed three independent times with parasite line, FCR-FMG.

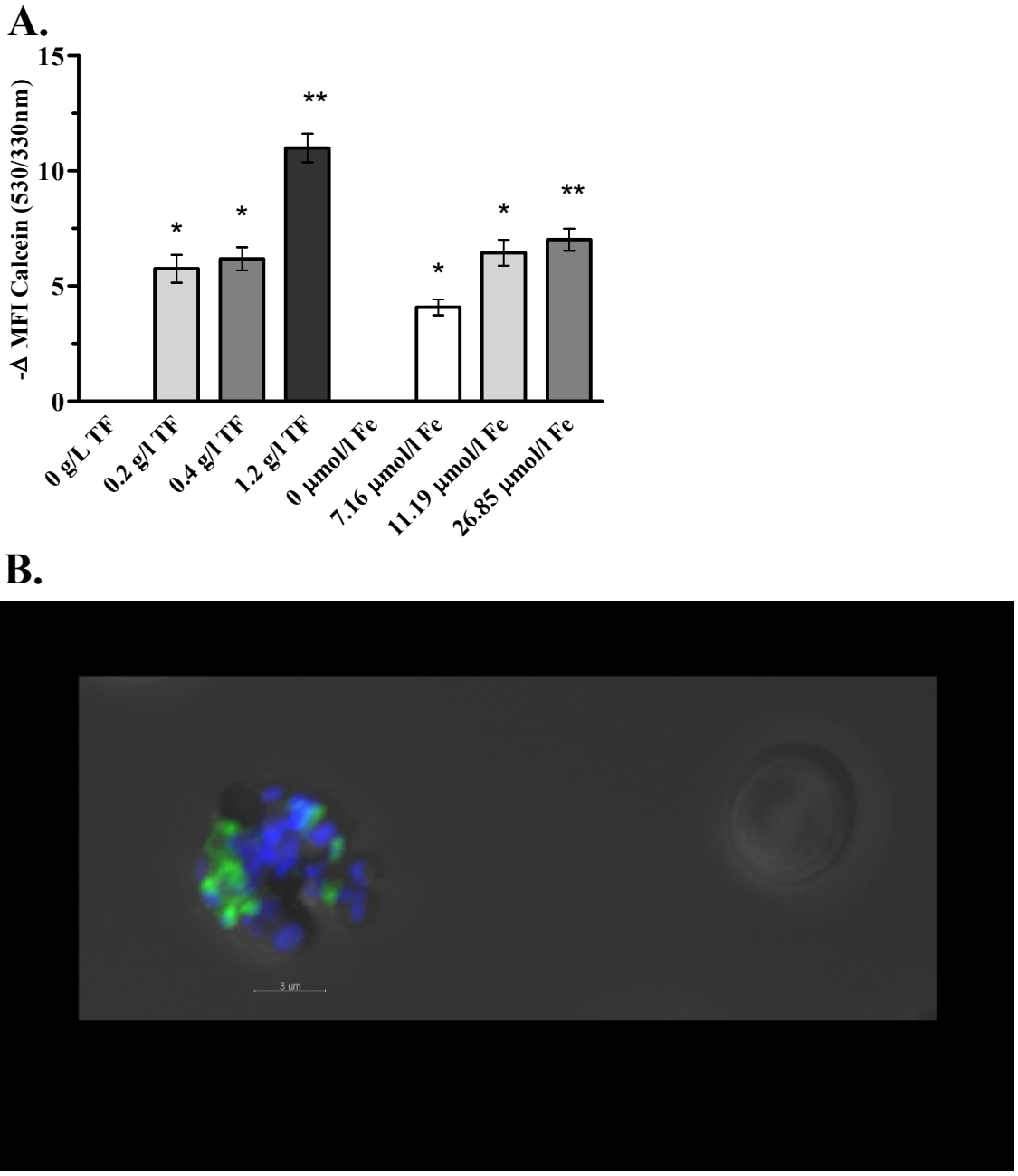


Figure 2.6 – Addition of human transferrin or ferric citrate increases LIP in parasitized RBCs, and transferrin co-localizes with late stage parasitized RBCs. (A) Impact of human transferrin and ferric citrate on the LIP of RBCs infected with trophozoite stage parasites was determined by assessing the MFI of calcein in cells cultured in the absence and presence of increasing concentrations of transferrin or ferric citrate. Cells from *P. falciparum (*FCR3-FMG strain) infected erythrocyte cultures were incubated in the presence or absence of increasing physiological concentrations of human transferrin (200ug/ml-1.2mg/ml) or ferric citrate (40ug/dL-150ug/dL) for 6 hours. Cells were then loaded with CA-AM, subsequently stained with DNA dye SYTO 61 and analyzed by flow cytometry. The LIP is measured by the change in the mean fluorescence intensity of calcein following the addition of the exogenous iron source. The

decrease in calcein fluorescence (-ΔMFI calcein (530/330nm)) indicates the addition of iron to the cellular LIP that results in increased quenching of calcein fluorescence. The bar graph (mean +/- SD, n=3) shows calcein MFI of trophozoite pRBCs with increasing concentrations of either transferrin or ferric citrate. Student's t-test statistical analysis was performed comparing cellular calcein fluorescence of increasing concentrations of transferrin and ferric citrate to the addition of no iron, *p<.005 **p<.0009. Data is from a single representative experiment, and the experiment was performed three independent times with parasite line, FCR-FMG. (B) Association of human transferrin with uninfected RBCs and late stage pRBCs. Cultures of *P. falciparum* strain FCR3-FMG were incubated with Alexa488 conjugated human transferrin (1:20) for 3 hours, fixed, and then stained with DNA dye Hoescht (1:10,000) to identify pRBCs. Cells were viewed on a Zeiss LSM710 Spectral Confocal Laser Scanning Microscope.

REFERENCES

Boyle, M. J., Wilson, D. W., Richards, J. S., Riglar, D. T., Tetteh, K. K. A., Conway, D. J., Ralph, S. A., Baum, J., and Beeson, J. G. (2010). Isolation of viable Plasmodium falciparum merozoites to define erythrocyte invasion events and advance vaccine and drug development. *Proc. Natl. Acad. Sci. U.S.A.* 107, 14378–14383.

Breuer, W., Epsztejn, S., and Cabantchik, Z. I. (1996). Dynamics of the cytosolic chelatable iron pool of K562 cells. *FEBS Lett.* 382, 304–308.

Breuer, W., Epsztejn, S., and Cabantchik, Z. I. (1995a). Iron acquired from transferrin by K562 cells is delivered into a cytoplasmic pool of chelatable iron(II). *J. Biol. Chem.* 270, 24209–24215.

Breuer, W., Epsztejn, S., Millgram, P., and Cabantchik, I. Z. (1995b). Transport of iron and other transition metals into cells as revealed by a fluorescent probe. *Am. J. Physiol.* 268, C1354–1361.

Cook, J. D., and Skikne, B. S. (1989). Iron deficiency: definition and diagnosis. *J. Intern. Med.* 226, 349–355.

El Tahir, A., Malhotra, P., and Chauhan, V. S. (2003). Uptake of proteins and degradation of human serum albumin by Plasmodium falciparum-infected human erythrocytes. *Malar. J.* 2, 11.

Epsztejn, S., Kakhlon, O., Glickstein, H., Breuer, W., and Cabantchik, I. (1997). Fluorescence analysis of the labile iron pool of mammalian cells. *Anal. Biochem.* 248, 31–40.

Ferrer, P., Tripathi, A. K., Clark, M. A., Hand, C. C., Rienhoff, H. Y., Jr, and Sullivan, D. J., Jr (2012). Antimalarial iron chelator, FBS0701, shows asexual and gametocyte Plasmodium falciparum activity and single oral dose cure in a murine malaria model. *PLoS ONE* 7, e37171.

Fritsche, G., Larcher, C., Schennach, H., and Weiss, G. (2001). Regulatory Interactions between Iron and Nitric Oxide Metabolism for Immune Defense against Plasmodium falciparum Infection. *J Infect Dis.* 183, 1388–1394.

Fu, Y., Tilley, L., Kenny, S., and Klonis, N. (2010). Dual labeling with a far red probe permits analysis of growth and oxidative stress in *P. falciparum*-infected erythrocytes. *Cytometry A* 77, 253–263.

Gordeuk, V., Thuma, P., Brittenham, G., McLaren, C., Parry, D., Backenstose, A., Biemba, G., Msiska, R., Holmes, L., and McKinley, E. (1992). Effect of iron chelation therapy on recovery from deep coma in children with cerebral malaria. *N. Engl. J. Med.* 327, 1473–1477.

Haldar, K., Henderson, C. L., and Cross, G. A. (1986). Identification of the parasite transferrin receptor of Plasmodium falciparum-infected erythrocytes and its acylation via 1,2-diacyl-sn-glycerol. *Proc. Natl. Acad. Sci. U.S.A.* 83, 8565–8569.

Hayward, R., Saliba, K. J., and Kirk, K. (2006). The pH of the digestive vacuole of Plasmodium falciparum is not associated with chloroquine resistance. *J Cell Sci* 119, 1016–1025.

Hershko, C., and Peto, T. E. (1988). Deferoxamine inhibition of malaria is independent of host iron status. *J. Exp. Med.* 168, 375–387.

Loyevsky, M., John, C., Dickens, B., Hu, V., Miller, J. H., and Gordeuk, V. R. (1999). Chelation of iron within the erythrocytic Plasmodium falciparum parasite by iron chelators. *Mol. Biochem. Parasitol.* 101, 43–59.

Marvin, R. G., Wolford, J. L., Kidd, M. J., Murphy, S., Ward, J., Que, E. L., Mayer, M. L., Penner-Hahn, J. E., Haldar, K., and O'Halloran, T. V. (2012). Fluxes in "Free" and Total Zinc Are Essential for Progression of Intraerythrocytic Stages of Plasmodium falciparum. *Chem. Biol.* 19, 731–741.

Murray, M. J., Murray, N. J., Murray, A. B., and Murray, M. B. (1975). Refeeding-malaria and hyperferraemia. *Lancet* 1, 653–654.

Nairz, M., Theurl, I., Ludwiczek, S., Theurl, M., Mair, S. M., Fritsche, G., and Weiss, G. (2007). The co-ordinated regulation of iron homeostasis in murine macrophages limits the availability of iron for intracellular Salmonella typhimurium. *Cell. Microbiol.* 9, 2126–2140.

Nguitragool, W., Bokhari, A. A. B., Pillai, A. D., Rayavara, K., Sharma, P., Turpin, B., Aravind, L., and Desai, S. A. (2011). Malaria parasite clag3 genes determine channel-mediated nutrient uptake by infected red blood cells. *Cell* 145, 665–677.

Oppenheimer, S. J. (2001). Iron and its relation to immunity and infectious disease. *J. Nutr.* 131, 616S–633S; discussion 633S–635S.

Paradkar, P. N., De Domenico, I., Durchfort, N., Zohn, I., Kaplan, J., and Ward, D. M. (2008). Iron depletion limits intracellular bacterial growth in macrophages. *Blood* 112, 866–874.

Pollack, S., and Fleming, J. (1984). Plasmodium falciparum takes up iron from transferrin. *Br. J. Haematol.* 58, 289–293.

Pouvelle, B., Spiegel, R., Hsiao, L., Howard, R. J., Morris, R. L., Thomas, A. P., and Taraschi, T. F. (1991). Direct access to serum macromolecules by intraerythrocytic malaria parasites. *Nature* 353, 73–75.

Prus, E., and Fibach, E. (2008a). Flow cytometry measurement of the labile iron pool in human hematopoietic cells. *Cytometry A* 73, 22–27.

Prus, E., and Fibach, E. (2008b). The labile iron pool in human erythroid cells. *Br. J. Haematol.* 142, 301–307.

Rodriguez, M. H., and Jungery, M. (1986). A protein on Plasmodium falciparum-infected erythrocytes functions as a transferrin receptor. *Nature* 324, 388–391.

Saliba, K. J., and Kirk, K. (1999). pH Regulation in the Intracellular Malaria Parasite, Plasmodium falciparum H+ EXTRUSION VIA A V-TYPE H+-ATPase. *J. Biol. Chem.* 274, 33213–33219.

Sanchez-Lopez, R., and Haldar, K. (1992). A transferrin-independent iron uptake activity in Plasmodium falciparum-infected and uninfected erythrocytes. *Mol. Biochem. Parasitol.* 55, 9–20.

Sazawal, S., Black, R. E., Ramsan, M., Chwaya, H. M., Stoltzfus, R. J., Dutta, A., Dhingra, U., Kabole, I., Deb, S., Othman, M. K., et al. (2006). Effects of routine prophylactic supplementation with iron and folic acid on admission to hospital and mortality in preschool children in a high malaria transmission setting: community-based, randomised, placebo-controlled trial. *Lancet* 367, 133–143.

Scholl, P. F., Tripathi, A. K., and Sullivan, D. J. (2005). Bioavailable iron and heme metabolism in Plasmodium falciparum. *Curr. Top. Microbiol. Immunol.* 295, 293–324.

Schümann, K., Kroll, S., Romero-Abal, M.-E., Georgiou, N. A., Marx, J. J. M., Weiss, G., and Solomons, N. W. (2012). Impact of oral iron challenges on circulating non-transferrin-bound iron in healthy Guatemalan males. *Ann. Nutr. Metab.* 60, 98–107.

Sinnis, P., Willnow, T. E., Briones, M. R., Herz, J., and Nussenzweig, V. (1996). Remnant lipoproteins inhibit malaria sporozoite invasion of hepatocytes. *J. Exp. Med.* 184, 945–954.

Skaar, E. P. (2010). The battle for iron between bacterial pathogens and their vertebrate hosts. *PLoS Pathog.* 6, e1000949.

Smith, A. W., Hendrickse, R. G., Harrison, C., Hayes, R. J., and Greenwood, B. M. (1989). The effects on malaria of treatment of iron-deficiency anaemia with oral iron in Gambian children. *Ann Trop Paediatr* 9, 17–23.

Tenopoulou, M., Kurz, T., Doulias, P.-T., Galaris, D., and Brunk, U. T. (2007). Does the calcein-AM method assay the total cellular “labile iron pool” or only a fraction of it? *Biochem. J.* 403, 261–266.

Tsien, R. Y. (1989). Fluorescent probes of cell signaling. *Annu. Rev. Neurosci.* 12, 227–253.

Weiss, G., Thuma, P. E., Mabeza, G., Werner, E. R., Herold, M., and Gordeuk, V. R. (1997). Modulatory potential of iron chelation therapy on nitric oxide formation in cerebral malaria. *J. Infect. Dis.* 175, 226–230.

WHO | World Malaria Report 2011 *WHO*. Available at: http://www.who.int/malaria/world_malaria_report_2011/en/ [Accessed August 13, 2012].

CHAPTER THREE

A three-color invasion assay allows for the study of erythrocyte population dynamics and *P. falciparum* merozoite invasion

3.1 Overview

Plasmodium falciparum invasion of host erythrocytes is essential for the propagation of the blood stage of malaria infection. Additionally, the brief extracellular merozoite stage of *P. falciparum* represents one of the rare windows during which the parasite is directly exposed to the host adaptive immune response. Therefore, efficient invasion of host erythrocyte is necessary not only for productive host erythrocyte infection, but also for evasion of the host immune response. Host traits, such as hemoglobinopathies and differential expression of host erythrocyte invasion ligands, can protect individuals from malaria by impeding parasite erythrocyte invasion. Here I describe a novel three color flow cytometry based *P. falciparum* invasion assay which allows for (i) direct comparison of *P. falciparum* invasion into different erythrocyte populations; (ii) assessment of the impact of changing erythrocyte population dynamics on overall *P. falciparum* invasion; and (iii) determination of how the presence of one erythrocyte population impacts *P. falciparum* invasion of a second erythrocyte population.

3.2 Introduction

Malaria is responsible for significant morbidity and mortality in the developing world, causing an estimated 250 million infections and 1 million deaths annually (WHO | World Malaria Report 2011). Malaria can be caused by any of eleven plasmodia species capable of

infecting humans; of these, *Plasmodium falciparum* is the most malignant. Upon transmission to the human host by *Anopheles* mosquitoes, plasmodia sporozoites migrate to and infect liver hepatocytes. Following the asymptomatic liver stage, merozoites are released into the bloodstream where they infect host red blood cells (RBCs). It is the RBC stage of infection that is responsible for all symptoms of disease. Upon entry into the host RBC the malaria parasite progresses through three distinct stages of development over the course of 48 hours: (i) the metabolically inactive ring stage; (ii) the trophozoite stage distinguished by increased metabolic activity and the digestion of host hemoglobin; and finally (iii) the schizont stage marked by the initiation of DNA replication and formation of daughter merozoites. At the end of the schizont stage the host RBC is ruptured and newly formed merozoites are released into the serum to invade new host RBCs(Bei and Duraisingh, 2012). The extracellular merozoite stage of the malaria parasite is very brief. Invasion occurs within 10 minutes of RBC rupture and the 48 hour intra-erythrocyte cycle begins again (Boyle et al., 2013). Successful invasion of the host RBC is essential for propagation of the parasite. Additionally the merozoite is one of the only parasite stages directly exposed to the host immune system. As a result, merozoite invasion represents a promising point of attack for antimalarial drugs and vaccines.

Conventionally, *P. falciparum* invasion of different RBCs has been studied *in vitro* by inoculating parasitized RBCs (pRBCs) into adjacent wells of culture plates, each containing a single RBC population of interest (target RBCs). Under standard culture conditions, RBC infection generally does not exceed 10-20% pRBCs, and as a result invasion experiments often contain a considerable number of uninfected "contaminant" RBCs in the pRBC inoculums. To specifically investigate invasion into experimental target RBCs and eliminate the influence of "contaminant" RBCs on experimental results, Theron et al. developed a flow cytometry based

two color invasion assay(Theron et al., 2010). In this assay, experimental target RBCs are labeled with a fluorescent cytoplasmic dye before inoculation with pRBCs. Invasion assays are subsequently incubated to allow for the inoculated pRBCs to rupture and release merozoites, which invade the fluorescently labeled target RBCs. Assays are finally labeled with a fluorescent DNA dye, to identify newly invaded pRBCs, and invasion is assessed by flow cytometry.

The RBC population of a given individual is heterogeneous; RBC age, anemia, acidosis, infection, or Glucose-6-phosphate dehydrogenase (G6PD) deficiency heterozygosity are only a few conditions which may contribute to heterogeneity (Bessman, 1977; Brandão et al., 2009; Nagababu et al., 2008; Gifford et al., 2006; Franco et al., 2013; Cordero et al., 2004). Therefore, the most relevant approach to study how RBC heterogeneity influences *P. falciparum* invasion would be to combine different RBC populations in a single culture well and study subsequent *P. falciparum* infection of multiple RBC populations. Pattanapanyasat et al. has described a biotinylation assay for detecting different RBC populations in a single culture condition, which they used to study *P. falciparum* growth in normal and thalassemic RBCs (Pattanapanyasat et al., 1996, 1999). However, the biotinylation assay does not account for the uninfected "contaminant" RBCs in the pRBC inoculums.

Here I describe a three color flow cytometry invasion assay that builds upon the approaches taken by Theron et al. and Pattanapanyasat et al. to directly compare plasmodium invasion of different RBC populations. I have validated this approach using target RBCs that have had host receptors removed by enzymatic treatment with trypsin, neuraminidase or chymotrypsin. The direct comparison of parasite invasion into different RBC populations provides increased sensitivity for detecting differences in merozoite invasion as well as an approach for modeling how different RBC populations interact to determine malaria infection.

3.3 Materials and Methods

P. falciparum culture

P. falciparum parasite lines 3D7 (MR4, MRA-102), Dd2 (MR4, MRA-156), and FCR3-FMG/Gambia (MR4, MRA-736) were routinely cultured in iron-replete O positive (O+) RBCs within two weeks of being obtained from healthy individuals. RBCs were collected at the Clinical and Translational Research Center at the University of North Carolina, Chapel Hill, NC and their use for this study was approved by Institutional Review Board at the University of North Carolina at Chapel Hill (IRB# 09-0559). Written consent was obtained from all donors using a consent form specifically approved by the IRB. Cultures were maintained at 2% hematocrit in complete media containing RPMI 1640 with 10% Albumax II, 1 mM hypoxanthine, 20 mM L-glutamine, 0.45% glucose, and 10 μg/L gentamicin (ACM). Cultures were shaken at 37^{o}C in 5% O_2 5% CO_2 and 90% N. Parasite density was maintained between 0.5% and 10% *P. falciparum* pRBCs. pRBC cultures were synchronized to within 4-6 hours of each other by first treating cultures with 5% D-sorbitol to select for ring stage parasites, followed by Magnetic Activated Cell Sorting (MACS) (Miltenyi Biotec) isolation of hemozoin containing trophozoite and schizont stage pRBCs 24 hours later (Ribaut et al., 2008). A small (0.16%) double positive Violet and DDAO RBC population is typically observed at the end of three-color invasion experiments and is excluded from analysis (data not shown).

Three-color invasion assay

Briefly, RBCs were labeled at 2% hematocrit in RPMI with 5 μM of either CellTrace Violet (RBC^{Violet}) or DDAO-SE ($RBC^{DDAO\text{-}SE}$) (Invitrogen) for two hours with shaking at 37^{o}C, washed twice with ACM, incubated 30 minutes in ACM with shaking at 37^{o}C, and finally washed twice with RPMI and stored in RPMI at 4^{o}C. Invasion assays were performed with fluorescently labeled RBCs within two days of labeling. For three-color invasion assays,

$RBC^{DDAO-SE}$ and RBC^{Violet} were combined in ACM and delivered in triplicate into a 96 well plate and subsequently seeded with MACS purified hemozoin containing pRBCs (trophozoites) from routine iron-replete O+ cultures to achieve 1.5-2% pRBCs. Parasites were maintained for 18-24 hours under standard culture conditions to allow for schizont rupture and subsequent invasion of $RBC^{DDAO-SE}$ and RBC^{Violet} populations. Following merozoite invasion, cells were stained with 1x DNA dye SYBR Green I (Invitrogen), fixed with 1% paraformaldehyde and 0.075% glutaraldehyde in Alsever's Solution (Sigma) as described previously(Clark et al., 2013), and analyzed by flow cytometry or microscopy.

Enzyme treatment of RBCs

RBCs were treated with trypsin, chymotrypsin, or neuraminidase in accordance with the Sanger Institute flow cytometry-based invasion phenotyping protocol (http://www.sanger.ac.uk/research/projects/malariaprogramme-rayner/sop-flow-cytometry-invasion-assay.pdf). Briefly, RBCs labeled with either CellTrace Violet or DDAO-SE were treated with 0.02 U/mL neuraminidase (Sigma), 50 μg/mL trypsin (Sigma), or 0.91 mg/mL chymotrypsin (Sigma) at 2% hematocrit in RPMI for 1 hour with shaking at 37°C, washed twice with ACM, incubated 30 minutes in ACM with shaking at 37°C, and finally washed twice with RPMI. Enzyme treated RBCs were stored in RPMI at 4°C for up to 1 day before being used in experiments.

Microscopy

$pRBC^{DDAO-SE}$ and $pRBC^{Violet}$ were stained with 1x DNA dye SYBR Green I, fixed with 1% paraformaldehyde and 0.075% glutaraldehyde, and then viewed by oil immersion with a 63X/ 1.4 numerical aperture Oil Plan Apo objective lens on a Zeiss CLSM 710 Spectral Confocal Laser Scanning Microscope. Images were captured with Zeiss ZEN 2011 software.

Flow Cytometry Analysis

Flow cytometry was performed at the UNC Flow Cytometry Core Facility on a Beckman-Coulter (Dako) CyAn ADP cytometer. Channels and probes used on the Dako cyan included: CellTrace Violet (405nM excitation, 450/50 bandpass), SYBR Green I (488nm excitation, 530/40 bandpass), and DDAO-SE (635nm excitation, 665/20 bandpass). Detector gain settings varied between experiments to optimize signal but were kept constant within individual experiments and spectral overlap compensation was not necessary with this configuration. *P. falciparum* pRBCs were gated on SYBR Green I signal. Dako Cyan data was collected and analyzed with Summit v4.3.01. Linear amplification of forward scatter was used to set event threshold in order to exclude cell debris, microparticles and doublets. For all experiments samples were diluted to 0.001-0.002% hematocrit and 100,000-500,000 total events were collected.

Statistical Methods

All experiments were performed in triplicate. Results are from either one representative experiment of at least three independent experiments or the combined results of at least three independent experiments. To compare the relative susceptibility of two different RBC populations to *P. falciparum* invasion, I determined the susceptibility index (SI) with an unadjusted Odds Ratio, which allows the determination of the relative risk of the two RBC populations to *P. falciparum* invasion. All statistical analyses for invasion experiments were performed with Stata/IC (v10, Stata Corp, College Station, TX).

3.4 Results

Labeling RBCs with CellTrace dyes DDAO-SE and Violet allows for the direct comparison of P. falciparum invasion into different RBC populations.

To develop a *P. falciparum* RBC invasion assay that would allow the direct comparison of the invasion of the parasite into different RBC populations, I utilized a fluorescent RBC staining method that permits the definitive detection of two distinct RBC populations by flow cytometry. DDAO-SE was used to label the first RBC population ($RBC^{DDAO\text{-}SE}$) (**Figure 3.1A**) and CellTrace Violet was used to label the second RBC population (RBC^{Violet}) (**Figure 3.1B**). Labeling RBCs with DDAO-SE does not affect *P. falciparum* invasion(Theron et al., 2010). Similarly, labeling RBCs with CellTrace Violet does not affect *P. falciparum* invasion (**Figure 3.2A and 3.2B**). I next examined whether $RBC^{DDAO\text{-}SE}$ could be combined with RBC^{Violet} in a three-color invasion assay. An equal number of $RBC^{DDAO\text{-}SE}$ and RBC^{Violet} were combined, inoculated with trophozoite stage pRBCs enriched by Magnetic Activated Cell Sorting (MACS), and incubated for 18-24 hours to allow for schizont rupture and subsequent merozoite invasion of the $RBC^{DDAO\text{-}SE}$ and RBC^{Violet}. $RBC^{DDAO\text{-}SE}$ is readily distinguished from RBC^{Violet} by microscopy (**Figure 3.1E**) and flow cytometry (**Figure 3.1F**). SYBR Green I staining allows for the identification of parasitized $RBC^{DDAO\text{-}SE}$ ($pRBC^{DDAO\text{-}SE}$) and $pRBC^{Violet}$ by both microscopy (**Figure 3.1D & 3.1E**) and flow cytometry (**Figure 3.1G & 3.1H**). Furthermore, no spectral interference of the three fluorescent dyes was detected by flow cytometry or microscopy**.** To assess *P. falciparum* invasion of $RBC^{DDAO\text{-}SE}$ and RBC^{Violet}, the invasion of $RBC^{DDAO\text{-}SE}$ was normalized to the invasion of RBC^{Violet}. The percent invasion of $RBC^{DDAO\text{-}SE}$ was 98.2 (standard deviation [SD] ±7.2), 87.4 (SD ±6.2), and 108.4 (SD ±5.4) that of RBC^{Violet} for *P. falciparum* strains 3D7, Dd2, and FCR3-FMG respectively (**Figure 3.3A**). Additionally, I determined the Susceptibility Index (**SI**), the relative risk of two different target RBC populations to *P.*

falciparum invasion, with an unadjusted Odds Ratio. An SI of 1.0 indicates no difference in the risk of invasion of two target RBC populations. The SI of a one to one combination of $RBC^{DDAO\text{-}SE}$ and RBC^{Violet} to 3D7, Dd2, and FCR3-FMG invasion was 0.97 (confidence interval [CI] 0.96-0.99), 0.90 (CI 0.89-0.91), and 1.08 (CI 1.06-1.09) respectively (**Figure 3.3B**).

The three color P. falciparum invasion assay allows for the direct comparison of P. falciparum invasion into physiologically different RBC populations.

Neuraminidase (N), trypsin (T), and chymotrypsin (C) are commonly used to study *P. falciparum* merozoite RBC invasion. Treatment of RBCs with any of these enzymes reduces merozoite RBC invasion. Different *P. falciparum* laboratory strains as well as clinical isolates exhibit different enzyme sensitivities (Bei and Duraisingh, 2012). I compared the merozoite invasion of *P. falciparum* strains 3D7 and Dd2 into untreated RBCs ($RBC^{Ø}$) and either neuraminidase (RBC^{N}), trypsin (RBC^{T}), or chymotrypsin (RBC^{C}) treated RBCs with the two(Theron et al., 2010) and the three-color invasion assays. The two-color invasion assay compares *P. falciparum* invasion of different RBC populations in adjacent but separate culture wells, while the three-color invasion assay directly compares *P. falciparum* invasion of two different RBC populations in the same culture well. In both assays, the invasion of each parasite strain into RBC^{N}, RBC^{T} and RBC^{C} was normalized to invasion into $RBC^{Ø}$ (**Figure 3.4**). Consistent with previous reports, in both the two- and three-color invasion assays *P. falciparum* strains 3D7 and Dd2 exhibited resistance and sensitivity to neuraminidase, respectively, and similar sensitivity to trypsin. Dd2 was less sensitive to chymotrypsin than 3D7. Furthermore, 3D7 and Dd2 sensitivity to all three enzymes was significantly greater in the three-color than the two-color invasion assay. Specifically, 3D7 invasion was 67.7% (SD±1.8), 65.7% (SD±1.8) and 5.1% (SD±0.4) into RBC^{N}, RBC^{T} and RBC^{C}, respectively, for the two-color invasion assay, and 56.1% (SD±1.9), 33.1% (SD±0.45), and 3.9% (SD±0.1), respectively, for the three-color

invasion assay. Dd2 invasion was 4.5% (SD±0.2), 69.6% (SD±1.0) and 32.9% (SD±0.9) into RBC^N, RBC^T and RBC^C, respectively, for the two-color invasion assay, and 2.6% (SD±0.7), 32.5% (SD±0.9), and 15.6% (SD±1.1), respectively, for the three-color invasion assay (**Figure 3.4**). The SI's obtained from equal combinations of enzyme treated and $RBC^Ø$ populations in the three-color invasion assays (**Figure 3.5C, 3.6C, and 3.7C**) were consistent with the invasion trends shown in Figure 3.4.

The relationship between different RBC populations and P. falciparum invasion is revealed by the three-color invasion assay.

I next sought to determine how changing the frequency of enzyme treated and untreated RBC populations affects (i) total infection ($\%pRBC^{total}$) and (ii) contribution of either $pRBC^N$, $pRBC^T$ or $pRBC^C$ and $pRBC^Ø$ to $\%pRBC^{total}$, by combining either RBC^N, RBC^T or RBC^C with $RBC^Ø$ at ratios of 1:10, 1:1, and 10:1. Total 3D7 as well as Dd2 infection of cultures containing either RBC^N, RBC^T or RBC^C with $RBC^Ø$ decreased with increasing frequency of each respective enzyme treated RBC population and decreasing frequency of $RBC^Ø$. Even as total infection decreased, as RBC^N (**Figure 3.5A**), RBC^T (**Figure 3.6A**) or RBC^C (**Figure 3.7A**) increased in frequency, infection of the enzyme treated RBCs accounted for an increasing portion of $\%pRBC^{total}$. Specifically, as RBC^N increased relative to $RBC^Ø$ from 10-50-90% of RBC^{total}, $pRBC^N$ accounted for 5.6% (SD±0.3), 28.2% (SD±0.7), and 73.5% (SD±0.1) of $\%pRBC^{total}$ for strain 3D7 and 0.3% (SD±0.1), 1.9% (SD±0.18), and 16.9% (SD±1.0) for strain Dd2 (**Figure 3.5A**). As RBC^T increased relative to $RBC^Ø$ from 10-50-90% of RBC^{total}, $pRBC^T$ accounted for 2.8% (SD±0.2), 21.3% (SD±1.1) and 67.7% (SD±0.2) of $\%pRBC^{total}$ for strain 3D7 and 2.6% (SD±0.4), 21.1% (SD±0.4), and 70.2% (SD±1.5) for strain Dd2 (**Figure 3.6A**). Finally, as RBC^C increased relative to $RBC^Ø$ from 10-50-90% of RBC^{total}, $pRBC^C$ accounted for 0.5% (SD±0.1),

3.7% (SD±0.2) and 22.1% (SD±1.0) of %pRBC^{total} for strain 3D7 and 1.7% (SD±0.3), 12.8% (SD±0.8) and 54.9% (SD±1.4) for strain Dd2 (**Figure 3.7A**).

To further characterize the relationship between different RBC populations and *P. falciparum*, I examined (i) percent pRBC^{Ø}, pRBC^{N}, pRBC^{T} and pRBC^{C} as the frequency of each decreased (%pRBC in each population) and (ii) the SI of RBC^{Ø} with either RBC^{N}, RBC^{T} or RBC^{C} to parasite infection as the frequency of each population changed. As the frequency of a RBC population decreases there are three possible invasion trends that may be observed, rate of invasion can (i) increase, (ii) remain constant or (iii) decrease. Each of these outcomes was observed in my experiments. %pRBC^{Ø} increased 25-38% as the frequency of RBC^{Ø} decreased in relation to RBC^{N} (**Figure 3.5B**), RBC^{T} (**Figure 3.6B**) and RBC^{C} (**Figure 3.7B**) for both 3D7 and Dd2. For 3D7, %pRBC^{N} similarly increased 30% as the frequency of RBC^{N} decreased; while for Dd2, %pRBC^{N} was unchanged as the frequency of RBC^{N} decreased (**Figure 3.5B**). %pRBC^{T} decreased 30-42% as the frequency of RBC^{T} decreased for both 3D7 and Dd2 (**Figure 3.6B**). %pRBC^{C} was unchanged as the frequency of RBC^{C} decreased for 3D7 and Dd2 (**Figure 3.7B**). However, despite changes in invasion rates with changing frequencies of the different enzyme treated populations and RBC^{Ø}, the SI, which reveals the relative risk of two populations to parasite invasion, was unchanged for 3D7 and Dd2 invasion of both RBC^{T} and RBC^{Ø} (**Figure 3.6C**) and RBC^{C} and RBC^{Ø} (**Figure 3.7C**) as well as Dd2 invasion of RBC^{N} and RBC^{Ø} (**Figure 3.5C**). This reveals that the relative risk of parasite invasion was unchanged. However, the SI of RBC^{N} and RBC^{Ø} to 3D7 invasion decreased from 0.80 (CI 0.75-0.86) to 0.42 (CI 0.40-0.44) as the frequency of RBC^{Ø} decreased and the frequency of RBC^{N} increased. This indicates that reduced risk of RBC^{N} to 3D7 invasion, relative to RBC^{Ø} invasion, is dependent upon the

frequency of the RBC^{N} and $RBC^{Ø}$ and ultimately that reduced risk is obliterated by decreasing the frequency of RBC^{N} (**Figure 3.5C**).

3.5 Discussion

The host RBCs is essential for the erythrocytic stage of malaria infection; consequentially human RBC traits, such as hemoglobinopathies (Taylor et al., 2013) , Glucose-6-phosphate dehydrogenase (G6PD) deficiency (Roth et al., 1983) and differential expression of host RBC invasion ligands (Tham et al., 2010), have arisen in the human population providing protection from malaria. RBCs are not only heterogeneous at the population level but also at the individual level. A given individual's RBC population is phenotypically heterogeneous as a result of differing RBC age, as well as other potential contributing factors such as iron deficiency or exposure to oxidative damage. Another example is found with heterozygous G6PD deficient women, whose RBC population is heterogeneous for G6PD deficiency, as the mutation is found only on one X chromosome (SANSONE et al., 1963). RBC age, oxidative damage, and G6PD status are all known to impact erythrocyte stage *P. falciparum* infection [15–18]. I postulate that the dynamics of an individual's RBC population have the potential to dramatically impact the disease causing the erythrocytic stage of malaria infections.

The study of the influence of different RBC populations on malaria infection necessitates the ability to combine and still identify different RBC populations in a single culture condition. Here I have described the development of a new method for investigating the influence of RBC population dynamics on RBC susceptibility to *P. falciparum* invasion. My novel three-color invasion assay has allowed for (i) the direct comparison of *P. falciparum* merozoite invasion of different RBC populations; (ii) determination of how a changing RBC population structure impacts overall *P. falciparum* infection; and (iii) assessment of how different RBC types impact

one another's invasion susceptibility during a malaria infection. I utilized the enzymes neuraminidase, trypsin, and chymotrypsin, which are commonly used to study *P. falciparum* invasion(Cowman et al., 2012), to establish the capacity of my assay to study the relationship between different RBC populations and *P. falciparum* infection. Specifically, neuraminidase, trypsin and chymotrypsin have been used to define different host RBC ligands utilized by the parasite and to show differential use of host invasion pathways by laboratory strains and clinical isolates (Bei and Duraisingh, 2012; Lopez-Perez et al., 2012; Baum et al., 2005).

I began by showing that the three-color invasion assay reproduces the previously described enzyme sensitivities of *P. falciparum* strains 3D7 and Dd2. I found that the enzyme sensitivities of strain 3D7 and Dd2 obtained from my three-color invasion assay were consistent with those obtained from the two-color invasion assay (Theron et al., 2010) (**Figure 3.4**). Furthermore, I observed 3D7 and Dd2 sensitivity to all three enzymes to be significantly greater in the three-color as compared to the two-color invasion assay. This is consistent with Pattanapanyasat et al.'s conclusion that direct comparison of parasite growth allows for greater sensitivity in detecting small differences in growth rates between physiologically different RBCs than independent growth rate assessment (Pattanapanyasat et al., 1996). I additionally introduce the susceptibly index (SI) as a robust tool for analyzing the relative risk of two different RBC populations to *P. falciparum* invasion.

I next proceeded to demonstrate that changes in RBC population structure impact *P. falciparum* infection. I show that as the frequency of a particular RBC population that is less susceptible to invasion increases, there is a total decrease in the overall parasitemia (**Figure 3.5A, 3.6A, 3.7A,**). These results reveal the loss of merozoites that occurs as the overall risk of invasion of a RBC population decreases, in this case due to increasing frequency of a RBC

population that is refractory to *P. falciparum* invasion. In addition to following the total infection of heterogeneous RBC populations, I also investigated the specific dynamics that exist between the different erythrocyte populations in my study ($RBC^{Ø}$, RBC^{N}, RBC^{T} and RBC^{C}) by assessing (i) the changing rate of invasion of each population with changing population frequency and (ii) the SI of two RBC populations as their frequency changes. In my study, depending on the RBC populations present, I observe each of three trends in the rate of parasite invasion with changing RBC population frequency: (1) increasing rate of invasion with decreasing RBC population frequency, (2) no change in the rate of invasion with changing RBC population frequency and (3) decreasing rate of invasion with decreasing RBC population frequency. $RBC^{Ø}$ invasion by both 3D7 and Dd2 and RBC^{N} invasion by 3D7 consistently followed the first trend, independent of the second RBC population each was paired with (**Figure 3.5B, 3.6B and 3.7B)**. As the $RBC^{Ø}$ is untreated and 3D7 invasion is only very slightly sensitive to neuraminidase treatment, I speculate this is the general trend for parasite invasion of the most viable RBC population available for invasion. Conversely, in circumstances where invasion of a RBC population was dramatically inhibited, as was observed with Dd2 invasion of RBC^{N} and 3D7 invasion of RBC^{C}, no change in invasion rate with changing RBC population frequency was observed (**Figure 3.5B, and 3.7B**). Interestingly, for more intermediate invasion phenotypes, 3D7 and Dd2 invasion of RBC^{T} and Dd2 invasion of RBC^{C}, I observed that the dynamics of parasite invasion of a given RBC population appear to be dependent upon the identity of the RBC population each was combined with. For example, 3D7 and Dd2 invasion of RBC^{T} decreased with decreasing frequency when RBC^{T} was combined with $RBC^{Ø}$ (**Figure 3.6C**). Ultimately these observations reveal that the dynamics of parasite invasion of a single RBC population may be either dependent on or independent of other RBC populations present.

Despite fluctuating rates of invasion with changing RBC population frequency for experiments that combined $RBC^{Ø}$ with either RBC^{N}, RBC^{T}, or RBC^{C}, there was very little effect on the SI (with the exception of 3D7 invasion of cultures containing RBC^{N} and $RBC^{Ø}$ populations) (**Figure 3.5C, 3.6C, and 3.7C**). This indicates that the relative risk of the two populations to invasion remained constant regardless of population frequency. The risk of RBC^{N} to 3D7 invasion relative to invasion of $RBC^{Ø}$ increased with decreasing frequency of RBC^{N} and increasing frequency of $RBC^{Ø}$ (**Figure 3.5C**). These results reveal that the relative risk (the SI) of two populations to invasion may be dependent on the frequency of each RBC population.

I have demonstrated the ability of the three-color invasion assay to study parasite infection of heterogeneous RBC populations. However, the assay may also be used to study the heterogeneity of merozoites. The difficulty of working with the merozoite stage of the parasite and the tradition of studying different RBC populations in isolation has resulted in essentially the exclusive study of invasion competent merozoites, with merozoites unable to invade completely lost to analysis. Here, I am able to observe merozoites that invade both unaltered and enzyme treated RBCs in a single culture, and I clearly show that even in the presence of an unaltered RBC population ($RBC^{Ø}$) some merozoites continue to invade the more resistant enzyme treated RBC populations (**Figure 3.5-3.7**). Conceivably, this assay has the potential to facilitate the study of merozoite heterogeneity and how merozoite heterogeneity changes with continued culture in different invasion conditions.

In conclusion, I have developed and demonstrated the utility of a sensitive, high throughput experimental system which can be used to directly compare the susceptibility of different RBC populations to *P. falciparum* merozoite invasion and assess the impact of changing RBC population dynamics on overall *P. falciparum* invasion. This approach provides a

powerful method for modeling a mixed RBC population in which populations of RBCs with differential susceptibility to infection exist. For example, during a malaria infection, the host often becomes anemic secondary to the destruction of infected and uninfected RBCs; this can result in an increase in the erythropoietic rate and the subsequent release of large numbers of reticulocytes into the circulation. The impact of the release of a population of RBCs preferentially infected by many species of *Plasmodium* including *P. falciparum* (Pasvol et al., 1980; Lim et al., 2013) on the overall infection is unknown. The effects of increasing the number of reticulocytes on the overall *P. falciparum* infection could be modeled using the three-color invasion assay. Future experimentation using this approach will provide invaluable insight into the relationship between *P. falciparum* and its human host.

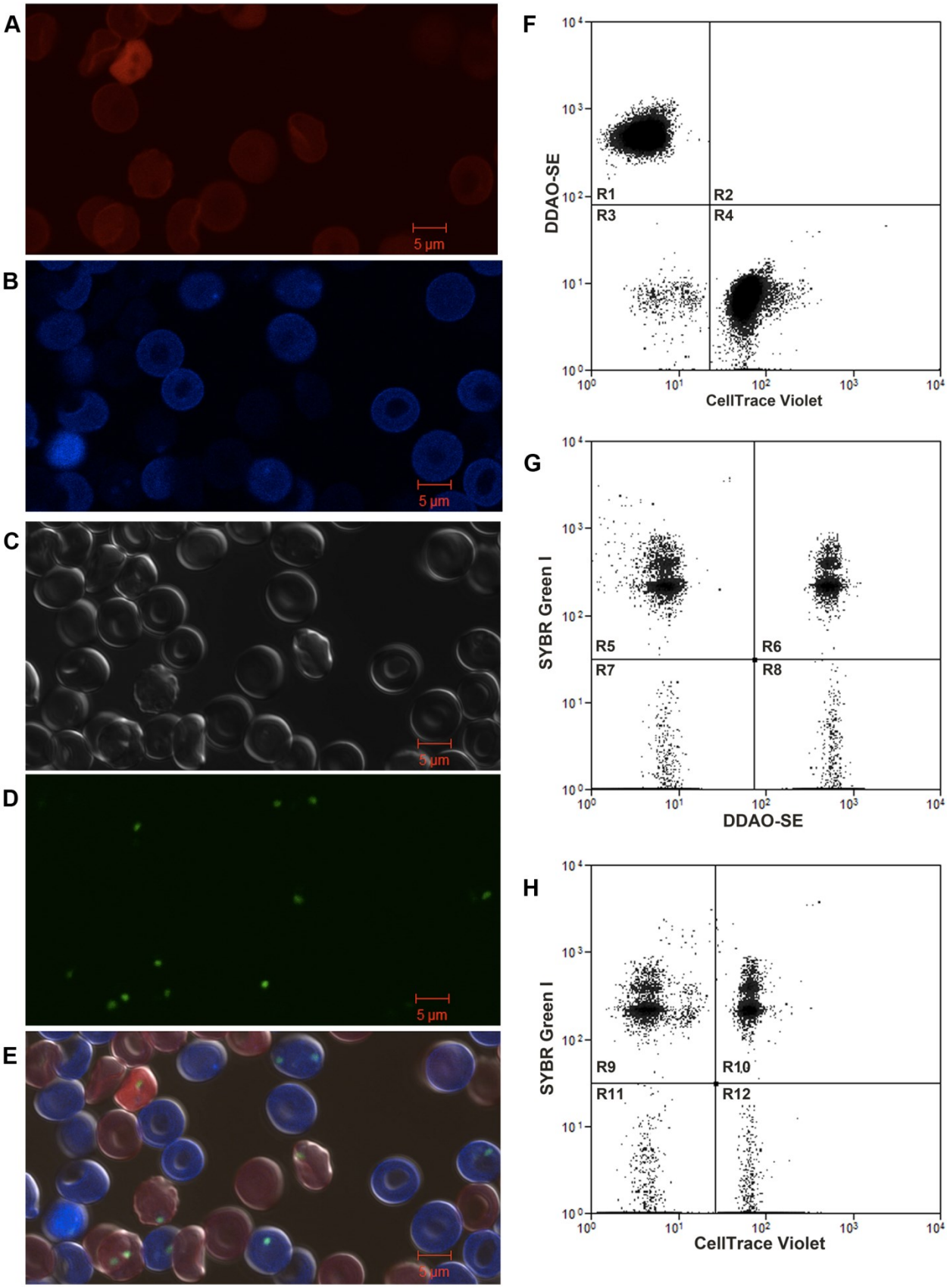

A
5 µm
B
5 µm
C
5 µm
D
5 µm
E
5 µm
F
DDAO-SE
CellTrace Violet
R1
R2
R3
R4
G
SYBR Green I
DDAO-SE
R5
R6
R7
R8
H
SYBR Green I
CellTrace Violet
R9
R10
R11
R12

Figure 3.1 – RBCs stained with CellTrace DDAO-SE and Violet can be combined to directly compare *P. falciparum* invasion in a three-color invasion assay. RBCs were labeled with 5 μM of either DDAO-SE (A) or CellTrace Violet (B). Cells were then combined and infected with unstained *P. falciparum* parasites. Experiments were incubated 18-24 hours, cells were then stained with DNA dye SYBR Green I, fixed, and examined by brightfield (C) and fluorescence (D, E) microscopy and by flow cytometry (F-H). (D) shows green channel only and (E) shows merge of green, red and violet channels. (F) Flow cytometry plot of RBCs stained with CellTrace DDAO-SE (R1) and CellTrace Violet (R4) along with non-stained pRBC (R3). (G) Dot plot shows DDAO-SE negative (unlabeled) pRBCs (R5), DDAO-SE negative uninfected RBCs (R7), DDAO-SE labeled pRBCs (R6) and DDAO-SE labeled uninfected RBCs (R8). (H) Dot plot shows CellTrace Violet negative (unlabeled) pRBCs (R9), CellTrace Violet negative uninfected RBCs (R11), CellTrace Violet labeled pRBCs (R10) and CellTrace Violet labeled uninfected RBCs (R12).

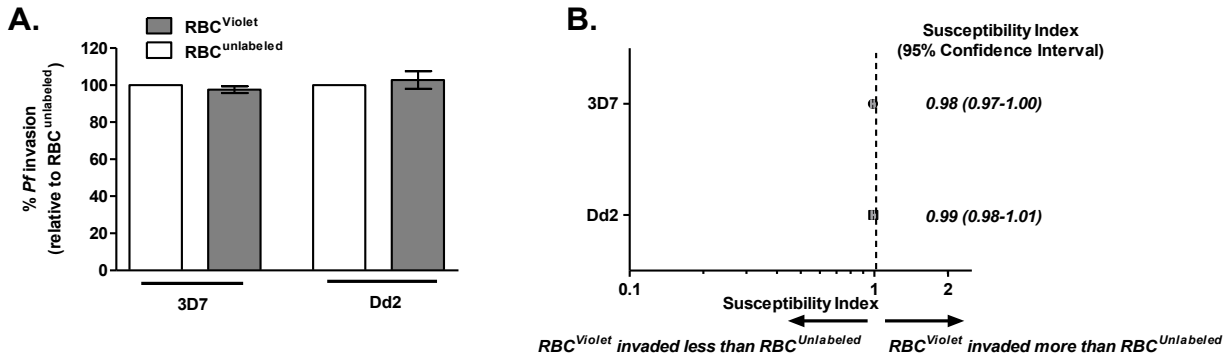


Figure 3.2 – Labeling RBCs with CellTrace Violet does not impact *P. falciparum* invasion. RBC^{Violet} and $RBC^{unlabeled}$ were combined at a ratio of one to one to achieve $2x10^7$ total uninfected RBCs per well, and inoculated with $2x10^5$ MACS enriched hemozoin containing trophozoite stage pRBCs of either *P. falciparum* strain 3D7 or Dd2. Invasion experiments were incubated 18-24 hours to allow for rupture of schizonts and subsequent invasion of merozoites into labeled RBCs, then stained with DNA dye SYBR Green I (to identify pRBCs), fixed and analyzed by flow cytometry. The invasion of *P. falciparum* strains 3D7 and Dd2 into RBC^{Violet} and $RBC^{unlabeled}$ was compared by (A) invasion of RBC^{Violet} as a percent of invasion of $RBC^{unlabeled}$ (error bars represent SD) and (B) the SI, the unadjusted odds ratio assessing the relative risk of RBC^{Violet} and $RBC^{unlabeled}$ to 3D7 and Dd2 invasion. The marker represents the SI point estimate and the bar represents the 95% confidence interval (CI).

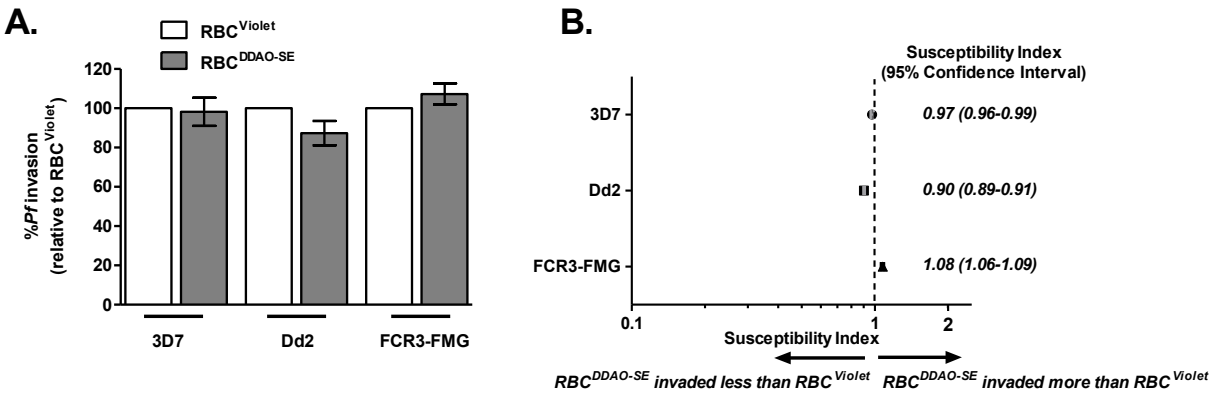


Figure 3.3 – Direct comparison of *P. falciparum* strains 3D7, Dd2 and FCR3-FMG invasion into RBCs labeled with DDAO-SE and CellTrace Violet. RBCs were stained with either 5 μM DDAO-SE or CellTrace Violet and combined at a ratio of one to one to achieve $2x10^7$ total uninfected RBCs per well, and inoculated with $2x10^5$ MACS enriched hemozoin containing trophozoite stage pRBCs of either *P. falciparum* strain 3D7, Dd2, or FCR3-FMG. Invasion experiments were incubated 18-24 hours to allow for rupture of schizonts and subsequent invasion of merozoites into labeled RBCs, then stained with DNA dye SYBR Green I (to identify pRBCs), fixed and analyzed by flow cytometry. (A) 3D7, Dd2, and FCR3-FMG invasion of RBC^{DDAO} normalized to invasion of RBC^{Violet}. Error bars represent standard deviation (SD). (B) The Susceptibility Index (SI), an unadjusted Odds Ratio assessing the relative risk of RBC^{DDAO} and RBC^{Violet} to 3D7, Dd2, and FCR3-FMG invasion. The marker represents the SI point estimate and the bar represents the 95% confidence interval (CI). A SI of 1.0 indicates no difference in parasite invasion of two RBC populations. Data in (A) and (B) are the combination of four, seven, and three independent experiments performed in triplicate with 3D7, Dd2, and FCR3-FMG respectively.

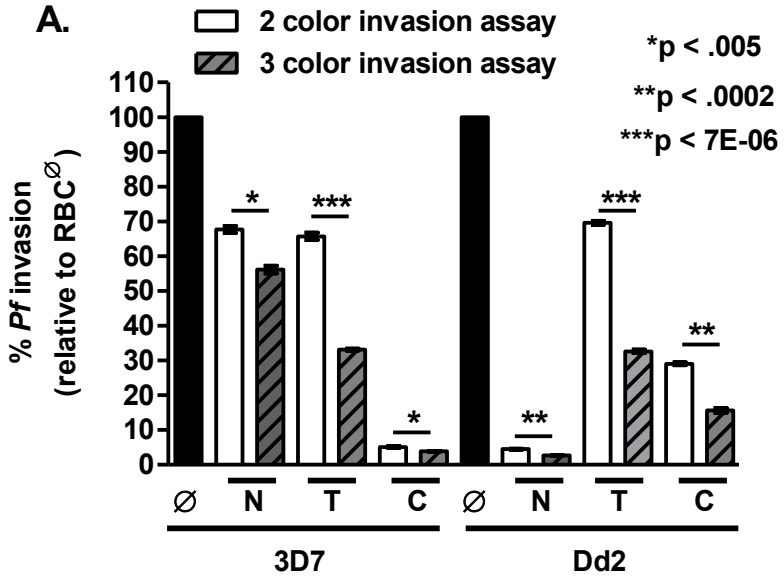


Figure 3.4 – C**omparison of two and three color *P. falciparum* invasion assay.** DDAO-SE stained RBCs were treated with either neuraminidase, trypsin, or chymotrypsin or left untreated and then inoculated at $2x10^7$ RBCs per well for two-color invasion assays, or combined 1:1 with untreated CellTrace Violet labeled RBCs, for a total of $2x10^7$ RBCs per well, for three-color invasion assays. Two and three-color invasion assays were inoculated with $2x10^5$ of either MACS enriched 3D7 or Dd2 *P. falciparum* parasites and invasion assays were further carried out as previously described. Invasion of RBC^N, RBC^T and RBC^C by 3D7 and Dd2 was normalized to invasion of $RBC^Ø$ for both two and three-color invasion assays. Error bars represent the SD. Student's *t*-tests were used to calculate differences in invasion between two and three-color invasion assays.

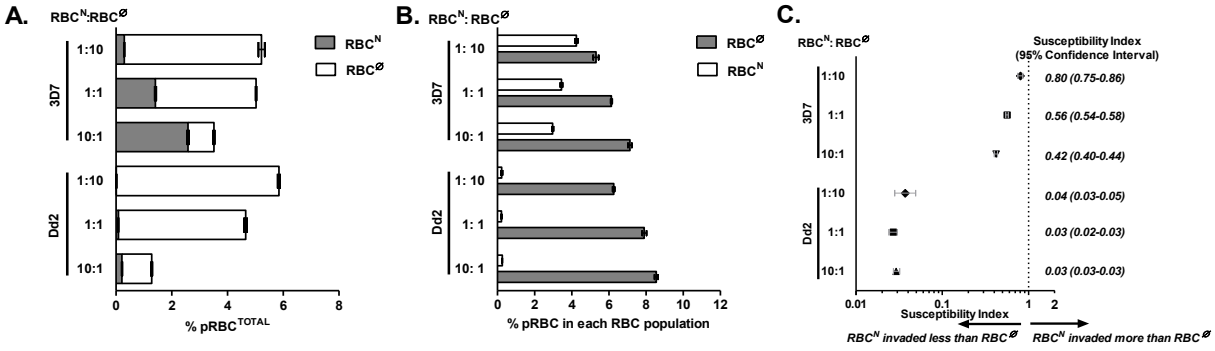


Figure 3.5 – Comparison of invasion of *P. falciparum* into untreated and neuraminidase, treated RBCs. (A) Total 3D7 and Dd2 infection (%pRBC^{total}) of cultures containing 1:10, 1:1, and 10:1 combinations of RBC^{N}:RBC^{Ø}, and the corresponding contribution of each population to %pRBC^{total} was determined. Error bars represent the SD. (B) %pRBC^{N} and %pRBC^{Ø} for both 3D7 and Dd2 in cultures containing 1:10, 1:1, and 10:1 combinations of RBC^{N}:RBC^{Ø}. Error bars represent the SD. (C) The SI for 3D7 and Dd2 infection of 1:10, 1:1, and 10:1 combinations of RBC^{N}:RBC^{Ø}. SI analysis was done with Stata/IC (v10, Stata Corp, College Station, TX). The marker represents the SI point estimate and the bar represents the 95% CI. All data shown is from one representative experiment of three independent experiments performed in triplicate.

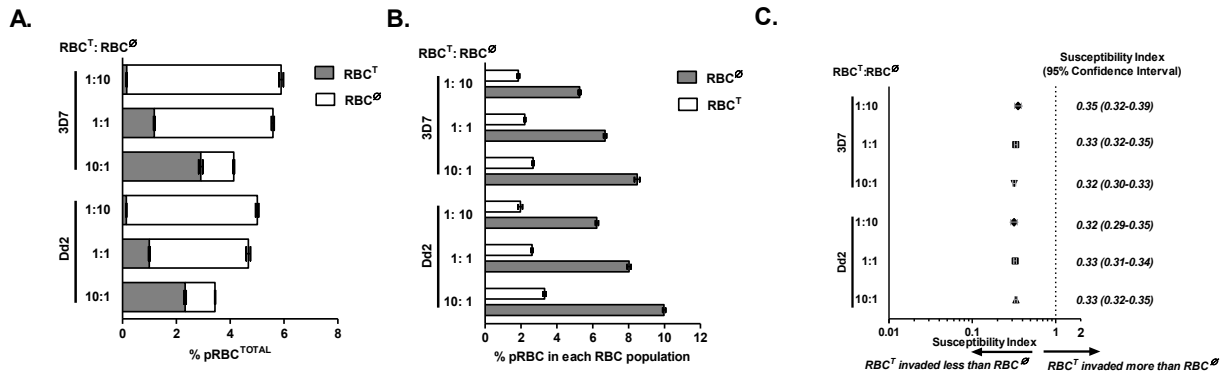


Figure 3.6 – Comparison of invasion of *P. falciparum* into untreated and trypsin treated RBCs. (A) Total 3D7 and Dd2 infection (%pRBC^{total}) of cultures containing 1:10, 1:1, and 10:1 combinations of RBC^{T}:RBC^{Ø} and the corresponding contribution of each population to %pRBC^{total} was determined. Error bars represent the SD. (B) %pRBC^{T} for both 3D7 and Dd2 in cultures containing 1:10, 1:1, and 10:1 combinations of RBC^{T}:RBC^{Ø}. Error bars represent the SD. (C) The SI for 3D7 and Dd2 infection of 1:10, 1:1, and 10:1 combinations of RBC^{T}:RBC^{Ø}. SI analysis was done with Stata/IC (v10, Stata Corp, College Station, TX). The marker represents the SI point estimate and the bar represents the 95% CI. All data shown is from one representative experiment of three independent experiments performed in triplicate.

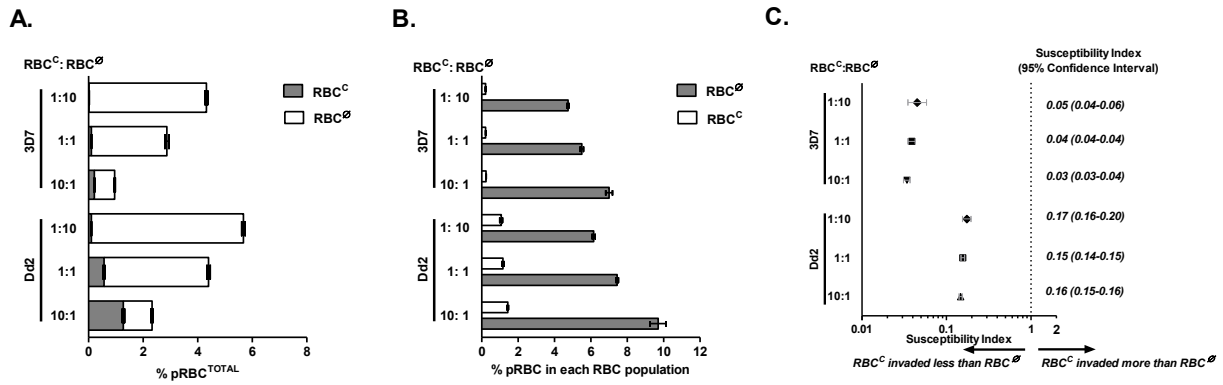


Figure 3.7 – Comparison of invasion of *P. falciparum* into untreated and chymotrypsin treated RBCs. (A) Total 3D7 and Dd2 infection (%pRBC^{total}) of cultures containing 1:10, 1:1, and 10:1 combinations of RBC^{N}:RBC^{Ø}, and the corresponding contribution of each population to %pRBC^{total} was determined. Error bars represent the SD. (B) %pRBC^{C} and %pRBC^{Ø} for both 3D7 and Dd2 in cultures containing 1:10, 1:1, and 10:1 combinations of RBC^{C}:RBC^{Ø}. Error bars represent the SD. The SI for 3D7 and Dd2 infection of 1:10, 1:1, and 10:1 combinations of RBC^{C}:RBC^{Ø}. SI analysis was done with Stata/IC (v10, Stata Corp, College Station, TX). The marker represents the SI point estimate and the bar represents the 95% CI. All data shown is from one representative experiment of three independent experiments performed in triplicate.

REFERENCES

Baum, J., Maier, A. G., Good, R. T., Simpson, K. M., and Cowman, A. F. (2005). Invasion by *P. falciparum* Merozoites Suggests a Hierarchy of Molecular Interactions. *PLoS Pathog* 1, e37.

Bei, A. K., and Duraisingh, M. T. (2012). Functional analysis of erythrocyte determinants of Plasmodium infection. *Int. J. Parasitol.* 42, 575–582.

Bessman, D. (1977). Erythropoiesis during recovery from macrocytic anemia: macrocytes, normocytes, and microcytes. *Blood* 50, 995–1000.

Boyle, M. J., Wilson, D. W., and Beeson, J. G. (2013). New approaches to studying Plasmodium falciparum merozoite invasion and insights into invasion biology. *Int. J. Parasitol.* 43, 1–10.

Brandão, M. M., Castro, M. de L. R. B., Fontes, A., Cesar, C. L., Costa, F. F., and Saad, S. T. O. (2009). Impaired red cell deformability in iron deficient subjects. *Clin. Hemorheol. Microcirc.* 43, 217–221.

Clark, M., Fisher, N. C., Kasthuri, R., and Cerami Hand, C. (2013). Parasite maturation and host serum iron influence the labile iron pool of erythrocyte stage Plasmodium falciparum. *Br. J. Haematol.*

Cordero, J. F., Rodríguez, P. J., and Romero, P. J. (2004). Differences in intramembrane particle distribution in young and old human erythrocytes. *Cell Biol. Int.* 28, 423–431.

Cowman, A. F., Berry, D., and Baum, J. (2012). The cellular and molecular basis for malaria parasite invasion of the human red blood cell. *J. Cell Biol.* 198, 961–971.

Franco, R. S., Puchulu-Campanella, M. E., Barber, L. A., Palascak, M. B., Joiner, C. H., Low, P. S., and Cohen, R. M. (2013). Changes in the properties of normal human red blood cells during in vivo aging. *Am. J. Hematol.* 88, 44–51.

Gifford, S. C., Derganc, J., Shevkoplyas, S. S., Yoshida, T., and Bitensky, M. W. (2006). A detailed study of time-dependent changes in human red blood cells: from reticulocyte maturation to erythrocyte senescence. *Br. J. Haematol.* 135, 395–404.

Lim, C., Hansen, E., Desimone, T. M., Moreno, Y., Junker, K., Bei, A., Brugnara, C., Buckee, C. O., and Duraisingh, M. T. (2013). Expansion of host cellular niche can drive adaptation of a zoonotic malaria parasite to humans. *Nat. Commun.* 4, 1638.

Lopez-Perez, M., Villasis, E., Machado, R. L. D., Póvoa, M. M., Vinetz, J. M., Blair, S., Gamboa, D., and Lustigman, S. (2012). Plasmodium falciparum Field Isolates from South America Use an Atypical Red Blood Cell Invasion Pathway Associated with Invasion Ligand Polymorphisms. *PloS One* 7, e47913.

Nagababu, E., Gulyani, S., Earley, C. J., Cutler, R. G., Mattson, M. P., and Rifkind, J. M. (2008). Iron-Deficiency Anemia Enhances Red Blood Cell Oxidative Stress. *Free Radic. Res.* 42, 824–829.

Pasvol, G., Weatherall, D. J., and Wilson, R. J. (1980). The increased susceptibility of young red cells to invasion by the malarial parasite Plasmodium falciparum. *Br. J. Haematol.* 45, 285–295.

Pattanapanyasat, K., Yongvanitchit, K., Heppner, D. G., Tongtawe, P., Kyle, D. E., and Webster, H. K. (1996). Culture of malaria parasites in two different red blood cell populations using biotin and flow cytometry. *Cytometry* 25, 287–294.

Pattanapanyasat, K., Yongvanitchit, K., Tongtawe, P., Tachavanich, K., Wanachiwanawin, W., Fucharoen, S., and Walsh, D. S. (1999). Impairment of Plasmodium falciparum Growth in Thalassemic Red Blood Cells: Further Evidence by Using Biotin Labeling and Flow Cytometry. *Blood* 93, 3116–3119.

Ribaut, C., Berry, A., Chevalley, S., Reybier, K., Morlais, I., Parzy, D., Nepveu, F., Benoit-Vical, F., and Valentin, A. (2008). Concentration and purification by magnetic separation of the erythrocytic stages of all human Plasmodium species. *Malar. J.* 7, 45.

Roth, E. F., Jr, Raventos-Suarez, C., Rinaldi, A., and Nagel, R. L. (1983). Glucose-6-phosphate dehydrogenase deficiency inhibits in vitro growth of Plasmodium falciparum. *Proc. Natl. Acad. Sci. U. S. A.* 80, 298–299.

SANSONE, G., RASORE-QUARTINO, A., and VENEZIANO, G. (1963). [DEMONSTRATION ON BLOOD SMEARS OF A DOUBLE ERYTHROCYTE POPULATION IN WOMEN HETEROZYGOUS FOR GLUCOSE-6-P DEHYDROGENASE DEFICIENCY]. *Pathologica* 55, 371–375.

Taylor, S. M., Cerami, C., and Fairhurst, R. M. (2013). Hemoglobinopathies: slicing the Gordian knot of Plasmodium falciparum malaria pathogenesis. *PLoS Pathog.* 9, e1003327.

Tham, W.-H., Wilson, D. W., Lopaticki, S., Schmidt, C. Q., Tetteh-Quarcoo, P. B., Barlow, P. N., Richard, D., Corbin, J. E., Beeson, J. G., and Cowman, A. F. (2010). Complement receptor 1 is the host erythrocyte receptor for Plasmodium falciparum PfRh4 invasion ligand. *Proc. Natl. Acad. Sci. U. S. A.* 107, 17327–17332.

Theron, M., Hesketh, R. L., Subramanian, S., and Rayner, J. C. (2010). An adaptable two-color flow cytometric assay to quantitate the invasion of erythrocytes by Plasmodium falciparum parasites. *Cytom. Part J. Int. Soc. Anal. Cytol.* 77, 1067–1074.

WHO | World Malaria Report 2011 *WHO*. Available at: http://www.who.int/malaria/world_malaria_report_2011/en/ [Accessed August 13, 2012].

CHAPTER FOUR

Host iron status and iron supplementation conspire to mediate host susceptibility to the erythrocytic stage of *Plasmodium falciparum*

4.1 Overview

Iron deficiency and malaria have similar global distributions, and frequently co-exist in pregnant women and young children(McLean et al., 2009; Shaw and Friedman, 2011). Where both conditions are prevalent iron supplementation is complicated by observations that iron deficiency anemia protects against falciparum malaria(Kabyemela et al., 2008; Senga et al., 2011; Gwamaka et al., 2012; Nyakeriga et al., 2004; Jonker et al., 2012), and iron supplements increase susceptibility to clinically significant episodes of malaria(Sazawal et al., 2006; Veenemans et al., 2011). This has caused many public health programs aimed at combating iron deficiency anemia to be suspended in malarious regions (Prentice, 2008). The mechanisms by which iron deficiency decreases and supplemental iron enhances susceptibility to malaria remain obscure. Using an *in vitro* culture system with erythrocytes from iron deficient and iron-replete human donors, we demonstrate that *P. falciparum* merozoites exhibit lower invasion and replication rates in microcytic iron-deficient erythrocytes. Additionally, iron supplementation of iron deficient donors reverses the protective effects of iron deficiency. Our results provide experimental validation of field observations reporting protective effects of iron deficiency and harmful effects of iron administration on human malaria susceptibility. Since recovery from anemia must involve transient reticulocytosis our findings imply that iron supplementation should be accompanied by intermittent preventive therapy for falciparum malaria.

4.2 Introduction

The interactions between falciparum malaria and Iron Deficiency Anemia (IDA) are complex and bi-directional. Clinical malaria causes acute anemia by destroying both infected and uninfected red blood cells (RBC) (Price et al., 2001), while persistent sub-clinical infection causes a milder anemia of infection by blocking iron recycling to bone marrow (Nweneka et al., 2010). However, once established, IDA protects against malaria in both pregnancy (Kabyemela et al., 2008; Senga et al., 2011) and childhood (Gwamaka et al., 2012; Nyakeriga et al., 2004; Jonker et al., 2012). Additionally, supplemental iron, given alone or in combination with other micronutrients, predisposes children to malaria(Sazawal et al., 2006; Veenemans et al., 2011) and other serious adverse outcomes(Soofi et al., 2013). Iron homeostasis has been implicated in regulating liver stage *P. falciparum* infection; in murine studies, erythrocytic stage malaria infection initiates hepcidin-mediated hepatic hypoferremia which blocks superinfections by sporozoites from competing plasmodia strains (Portugal et al., 2011) . Mathematical modeling suggests that this can explain the low levels of superinfections in young children(Portugal et al., 2011), but this mechanism cannot account for observed reductions in the risk of primary malaria infection in IDA children. It has also been speculated that transient peaks in non-transferrin bound iron caused by administration of highly absorbable iron supplements(Schümann et al., 2012) could promote intra-erythrocytic parasite growth(Clark et al., 2013) or bacterial septicemia (a common cause of death in malaria patients (Bronzan et al., 2007; Berkley et al., 2009)) but definitive evidence is absent.

I hypothesized that as both IDA, and iron supplementation of IDA individuals profoundly alter erythropoiesis; iron supplementation mediated changes in RBC physiology and RBC population structure alter falciparum malaria susceptibility. In our investigations, we minimize

the confounding factors that have complicated prior field studies of the relationship between host iron status, iron supplementation, and falciparum malaria by utilizing an *in vitro* system with freshly-isolated red blood cells (RBCs) from donors with well-defined, physiologic iron states recruited through our US-based hospital clinic. This approach eliminated the influence of acquired and innate immunity to malaria, hemoglobinopathies and concurrent inflammation. Our study reveals that microcytic iron deficient RBCs (RBC^{IDA}) from donors with IDA are protective against malaria infection due to impairment of *P. falciparum* invasion and intra-erythrocyte propagation, and the replacement of RBC^{IDA} with young normocytic iron-replete RBCs (RBC^{IR}) in IDA donors following iron supplementation increases susceptibility to *P. falciparum* infection relative to infection of RBC^{IDA}. These findings support well-described clinical patterns of differential susceptibility to malaria; taken together, they reveal that the impact of iron on erythropoiesis conspires to mediate host RBC susceptibility to malaria infection by altering the dynamic structure of the host's RBC populations.

4.3 Materials and Methods

Clinical

Study participants included healthy, HIV-negative, non-pregnant donors over 18 years of age with and without iron deficiency anemia (IDA). Exclusion criteria included: on-going inflammation or infection, previous history of malaria, travel to malaria endemic area, malignancy, sickle cell disease (or trait), and thalassemia (or trait for either thalassemia alpha or beta). This study was approved by the UNC Institutional Review Board, Protocol # 09-0559. Study participants with hemoglobin [Hgb] >11g/dL and ferritin >12ng/ml were classified as iron-replete (IR) and those with Hgb< 11g/dL and ferritin<12ng/ml were classified as IDA. Donors with IDA were treated by their personal physicians and made a donation of 40mls of blood at a maximum of 3 time points. Healthy donors were required to take 325 mg of ferrous

sulfate once daily for the 2 month duration of the study. These donors donated 40mL of blood on 3 occasions – at enrollment and on 2 additional occasions 1 month apart. An additional group of healthy donors served as the IR control group. Non-anemic donors with low iron stores (Hgb>11g/dL, ferritin<12ng/ml) were excluded.

Parasite Culture

P. falciparum parasite strains 3D7, Dd2, and FCR3-FMG were cultured in O+ RBCs at 2-3% haematocrit and complete media containing RPMI 1640 (Sigma-Aldrich) with 10% Albumax II (Gibco), 1 mM hypoxanthine, 20 mM L-glutamine (cellgro), 0.45% glucose (cellgro), and 0.01ng/mL gentamicin (Sigma-Aldrich) (ACM). Albumax II was used to supplement the media in place of human serum in order to isolate the effects of the RBCs from different experimental groups. All RBCs used for parasite culture were obtained from well characterized IR O+ donors and used within 14 days of being drawn. Cultured parasite density was maintained between .5% - 10% at 37 degrees in an atmosphere of 5% O_2 5% CO_2 and 90% N_2 with continuous shaking. Early ring stage parasites were synchronized with 5% (w/v) D-sorbitol. Synchronization was repeated 20 h later to achieve a tightly synchronized parasite population [29].

Growth Assays

P. falciparum parasites from routine IR O+ cultures were seeded as rings at 0.5% initial parasitemia in1% hematocrit experimental RBCs in ACM in triplicate in 96 well plates. Parasites were maintained for 96h under standard culture conditions and media was changed daily. At 96h parasite cultures were split back to 0.5% parasitemia and maintained as described for an additional 96h (**Figure 4.1)**. Parasites were stained at all 0h and 96h time points with DNA dye SYBR Green I (Invitrogen) as described in [30], and fixed in 1% paraformaldehyde and 0.0075% glutaraldehyde (Electron Microscopy Sciences) in Alsever's Solution (Sigma-Aldrich) for 30 min at 4 degrees. Fixative was removed and cells were stored in PBS at 4 degrees until

FACS analysis. Parasite growth rates were determined by dividing final % infected RBCs at 96h by initial % infected RBCs at 0h. To identify parasitized reticulocytes, cultures were stained with 0.5uM DNA dye SYTO 61 (Invitrogen)(Clark et al., 2013) and PE conjugated mouse anti-human CD71 antibody (Miltenyi Biotech) and analyzed by FACS.

Invasion assay

RBCs were labeled with 5 uM of either CellTrace Violet or DDAO-SE (Invitrogen) as described (Theron et al., 2010). Violet and DDAO labeled RBCs were combined in ACM and delivered in triplicate into 96 well plates and subsequently seeded with schizonts from routine IR O+ cultures to achieve 1.5-2% infected RBCs. Parasites were maintained for 12-18h under standard culture conditions to allow for schizont rupture and subsequent invasion of Violet and DDAO labeled RBCs. The invasion of *P. falciparum* into Violet and DDAO labeled RBCs was directly compared by measuring the Susceptibility Index (SI), defined as the ratio of the prevalence of infected Violet RBCs to infected DDAO RBCs.

Parasite Erythrocyte Multiplication Rate assay

Trophozoite infected IR O+ cultures were MACS (Ribaut et al., 2008) purified and seeded into experimental RBCs to achieve 1.5-2% infected RBCs and incubated for 12-18 h to allow for invasion of merozoites into experimental RBCs. Experimental RBC parasite density was determined and the same number of infected experimental RBCs were seeded into separate wells containing target IR RBCs to achieve a 1.5-2% infected RBCs. Experiments were then incubated for 12-18 hours to allow merozoites produced within experimental RBCs to invade target IR RBCs; allowing for the comparison of infectious merozoites produced within different experimental RBCs. Following invasion of target RBCs, cells were stained with SYBR Green I and analyzed by FACS to determine total number of ring infected RBCs. The number of ring

infected RBCs represents the total number of infectious merozoites produced within experimental RBCs.

Density Separation

RBCs were separated into five fractions with a modified version of previously described density gradient centrifugation method. Briefly, blood was collected into acid citrate dextrose and plasma was subsequently removed by centrifugation for 15 min at 800 x g. Packed cells were resuspended at 50% haematocrit in RPMI, and passed over a 2:1 (w/w) α-cellulose/microcrystalline cellulose column to remove lymphocytes. Following lymphocyte depletion RBCs were washed twice with 10 mM HEPES (cellgro), 12 mM NaCl, 115 mM KCl, 5% BSA (Sigma-Aldrich) buffer (RBC buffer). 1 mL 50% haematocrit RBCs in RBC buffer were layered onto a 65%, 60%, 55%, 50% discontinuous Percoll gradient and was centrifuged for 25 min at 1075g. Each of the five fractions was removed, washed twice with RBC buffer, and stored at 4 degrees for up to 5 days. Decreasing MCV, reticulocyte content, and Calcein fluorescence of the five fractions confirmed the age separation of RBCs (Bratosin et al., 2005) (**Figure 7.1 C D and E**).

Flow cytometry

Growth, invasion, and infectious merozoite production assays were analyzed by FACS using either a modified FACS-Calibur with 2 lasers 30 mW 488 Diode Pumped Solid State laser and a 25 mW 637 red diode laser (FACS-Calibur; Becton Dickinson, Mount View CA, modified by Cytek Development) or a Beckman-Coulter (Dako) CyAn ADP. Channels and probes used on the FACS-Calibur included: SYTO 61 (637nM, 666/27 bandpass), PE (488nM, 585/42), and SYBR Green I (488nM, 530/30 bandpass). FACS-Calibur data was collected using FlowJo CE and analyzed with Summit v5.1. Channels and probes used on the Dako cyan included: CellTrace Violet (405nM, 450/50 bandpass), SYBR Green I (488nm, 530/40 bandpass), and

DDAO-SE (635nm, 665/20 bandpass). Dako cyan data was collected and analyzed with Summit v4.3.01. Linear amplification of FSC was used to set event threshold in order to exclude cell debris, microparticles and doublets. Electronic volume of uninfected and infected RBCs was assessed on a Beckman Coulter Cell lab Quanta. Channels and probes used on the Quanta included: SYBR Green I (488nM, 525 bandpass). National Institute of Standards and Technologies (NIST) certified beads standard L2 2 μm, L5 5 μm, and L10 10 μm (Beckman Coulter) were used to calibrate electronic volume. Calibrated data was expressed as both electronic volume (μm^3) and diameter (μm). Quanta data was collected with Cell Lab Quanta Collection Software for Instrument Control and analyzed with Kaluza. For all experiments samples were diluted to 0.001-0.002% haematocrit and 100,000-500,000 total events were collected.

Statistical Methods

All experiments were performed in triplicate. Results are from either one representative experiment or the combined results of at least three independent experiments. Growth rates and infectious merozoite production were compared with two-tailed Students *t*-test and one way ANOVA using GraphPad Prism 5. To compare the susceptibility of each RBC type to invasion by *P. falciparum*, an unadjusted Odds Ratio was used to calculate the RBC SI, which compares the susceptibility of two RBC populations to *P. falciparum* invasion. All statistical analyses for invasion experiments were performed with Stata/IC (v10, Stata Corp, College Station, TX). Data from all *in vitro* growth studies was pooled and analyzed using random effects regression[1]. The dependent variable in these models was the logarithm of the ratio of the percent infected RBCs at 96h (final) and 0h (initial). In addition to the usual variation independently affecting each observation we fitted two higher levels of variance: variation between individuals and day-to-day

[1] Dr. Tony Fulford performed random effects regression analysis of growth data.

variation in parasite "preparations" taking account of the fact that in this dataset these two variance components were cross (rather than the more usually encountered nested design). We fitted two exposure variables, iron status and iron supplementation, both as binary variables, and their interaction, focusing on three contrasts: supplemented IDA versus supplement IR donors; supplemented versus non-supplemented IDA donors; supplemented versus non-supplemented IR donors. All experiments were performed in triplicate with three *P. falciparum* strains (3D7, Dd2, and FCR3-FMG) and consisted of three serial 96h growth assays (**Figure 4.1)**; *P. falciparum* strain, growth assay number and their (highly significant) interaction were fitted as binary covariates. We noted that when 0h (initial) percent infected RBCs was greater than 1 the second growth assay always gave anomalous low results (most likely due to a saturation effect). Since these data were uninformative and yet added noise to the analysis we omitted all such cases while noting that although their inclusion increased the standard errors, it did not change the same general conclusions of the study. This model was fitted using Stata's *xtmixed* procedure (v12, Stata Corp, College Station, TX).

4.4 Results

Iron deficient RBCs are protective against P. falciparum infection

To determine the effect of IDA on the growth of erythrocytic stage *P. falciparum,* we enrolled donors from a non-malaria endemic area through our US-based hospital system and assessed growth of three *P. falciparum* strains within microcytic IDA RBCs (RBC^{IDA}) (Hgb range 7.1-10.5 g/dL and MCV range 66-83 fL) *in vitro* (**Table 4.1**). For growth experiments *P. falciparum* strains 3D7, Dd2, and FCR3-FMG were cultured in either iron-replete (IR) RBCs (RBC^{IR}) or RBC^{IDA} in two consecutive 96 hour growth assays, for a total of three *P. falciparum* erythrocyte lifecycles within either RBC^{IR} or RBC^{IDA} cells (**Figure 4.1**). Here we report the resulting growth rate of parasites following the second growth assay. We observed that compared

to RBC^{IR}, parasite growth rate was reduced in microcytic RBC^{IDA} 48% (standard deviation [SD]±23.9), 34% (SD±22.2), and 50.0% (SD±20.4) for strains 3D7, Dd2, and FCR3-FMG, respectively (**Figure 4.2A**).

I next sought to determine at which point propagation of the erythrocytic stage of *P. falciparum* was impeded when cultured in RBC^{IDA} by investigating: (i) invasion, (ii) maturation, and (iii) production of infectious daughter merozoites. To assess invasion of *P. falciparum* into RBC^{IDA}, we utilized our three-color invasion assay (**Chapter 3**) to directly compare invasion *of P. falciparum* strains 3D7, Dd2 and FCR3-FMG into RBC^{IDA} and RBC^{IR}. Here we report the Susceptibility Index (SI), the ratio of the relative risk of RBC^{IDA} and RBC^{IR} to *P. falciparum* invasion. An SI of 1.0 indicates no difference in parasite invasion of two different RBC populations. The mean SI of RBC^{IDA} relative to RBC^{IR} was 0.56 (95% confidence interval [CI] 0.56-0.57), 0.52 (95% CI 0.52-0.53) and 0.72 (95% CI 0.71-0.73) for strains 3D7, Dd2, and FCR3-FMG, respectively (**Figure 4.2B**). With regard to possible disruption of parasite maturation within RBC^{IDA}, analysis of Giemsa blood smears of parasites within RBC^{IDA} and RBC^{IR} revealed normal maturation of parasites within RBC^{IDA} (**Figure 4.2C)**. Finally, we measured the capacity of *P. falciparum* to produce infectious daughter merozoites within RBC^{IDA}. Measurement of the parasitized erythrocyte multiplication rate (PEMR) (Lim et al., 2013; Chotivanich et al., 2000) of *P. falciparum* strains 3D7, Dd2, and FCR3-FMG revealed a respective 48% (SD±12.15), 26% (SD±2.22), and 40% (SD±9.32) decrease in the PEMR within RBC^{IDA} as compared to RBC^{IR} (**Figure 4.2D**). These data indicate that *P. falciparum* matures normally within microcytic RBC^{IDA}, but that invasion into microcytic RBC^{IDA} and production of infectious merozoites within microcytic RBC^{IDA} are significantly reduced.

Erythrocytic stage P. falciparum growth is increased in RBCs from iron supplemented donors.

Given field evidence that iron supplementation of IR children with 12.5mg of iron (1-1.5mg/kg) and 50ng of folic acid may potentiate the risk of malaria(Sazawal et al., 2006), we next investigated the effects of moderate-dose oral iron supplementation of IR donors (IR-Fe) on *in vitro* growth of erythrocytic stage *P. falciparum*. Each individual donated RBCs three times: at enrollment, and then 1 month and 2 months following initiation of daily iron supplementation (325 mg ferrous sulfate orally daily). At each donation the growth rate of three *P. falciparum* strains within RBCs from IR-Fe donors ($RBC^{IR\text{-}Fe}$) was compared to the growth rate within RBCs from a non-supplemented IR donor (RBC^{IR}). We observed a 17.5% SD±16.13, 11.3% SD±15.7, and 6.6% SD±8.1 increase in growth for 3D7, Dd2, and FCR3-FMG respectively between enrolment and 1 month of donors taking iron supplements, and no significant change in parasite growth rate occurred between 1 month and 2 months of donors taking iron supplements (**Figure 4.3A**). Analysis of hemoglobin, hematocrit, MCV, MCHC, transferrin saturation, ferritin, and reticulocyte count of IR-Fe donors revealed no significant change in the iron status of IR donors following iron supplementation (**Table 4.1)**.

I next examined whether iron supplementation of IDA individuals affects propagation of erythrocytic stage *P. falciparum*. To do this, we compared the growth rate of *P. falciparum* strains 3D7, Dd2 and FCR3-FMG within RBCs donated by IDA individuals following iron supplementation ($RBC^{IDA\text{-}Fe}$) to the growth rate of parasites within RBC^{IR}. IDA-Fe donors were identified by their physicians as suitable for the IDA-Fe group based upon meeting the previously described criteria for the study as well as having been prescribed high dose oral ferrous sulfate, 60 mg 9-12.6 mg/kg elemental iron orally three times per day. We observed a 17.3% SD±22.7, 17.6% SD±14.0, and 26.3% SD±16.1 respective increase in 3D7, Dd2, and FCR3-FMG growth in $RBC^{IDA\text{-}Fe}$ as compared to RBC^{IR} (**Figure 4.3B**). All members of the IDA-

Fe group had hemoglobin values between 6.6-9.8 g/dL and MCV values ranged from 75-98 fL. Furthermore, average MCHC, total iron, and ferritin values were greater than that of IDA donors but still lower than IR donor values (**Table 4.1)**. Together these values are indicative of an erythropoietic response to iron supplements, but not full recovery from IDA.

To comprehensively quantify the relative impact of iron deficiency and iron supplementation on the growth rate of *P. falciparum* strains *in vitro*, and to account for variation between study participants and day-to-day differences in parasite growth, we pooled data from all growth experiments and fitted a multilevel random effects model (**Figure 4.3C)**. Growth rates of *P. falciparum* in RBC^{IR} on the Y=X line. Data above the Y=X line indicate growth rates greater than that of parasite growth in RBC^{IR} and data below the Y=X line indicate growth rates lower than that of parasite growth in RBC^{IR}. Based upon this model, we estimate that *P. falciparum* growth is reduced by 59.8% (95% CI 51.9-68.8) in RBC^{IDA} as compared to RBC^{IR}, and that there is a slight increase in the growth of *P. falciparum* in $RBC^{IR\text{-}Fe}$ 18.9% (95% CI 5.0-33.9) and $RBC^{IDA\text{-}Fe}$ 22.8% (95% CI 2.7-46.7) as compared to RBC^{IR} (**Figure 4.3D**). Finally, the model shows no difference between *P. falciparum* growth in $RBC^{IR\text{-}Fe}$ and $RBC^{IDA\text{-}Fe}$ (**Figure 4.3D**). These data clearly indicate that IDA substantially attenuates the growth of *P. falciparum* parasites and that iron supplementation of IDA donors reverses the protection provided by IDA against falciparum infection. Furthermore, these data suggest that iron supplementation of IR and IDA individuals may slightly increase propagation of erythrocytic stage *P. falciparum.*

Changing RBC population dynamics impacts susceptibility to erythrocytic stage P. falciparum infection.

The recovered growth rate of *P. falciparum* observed in $RBC^{IDA\text{-}Fe}$ occurred prior to full recovery of the iron status of IDA-Fe donors (**Table 4.1**). The elevated reticulocyte count and wide RDW observed with IDA-Fe donors indicates an erythropoietic response. We have

explored the hypothesis that elevated erythropoiesis induced by iron supplementation in IDA individuals is responsible for the observed recovery of parasite growth in $RBC^{IDA\text{-}Fe}$ that occurs prior to full recovery from IDA. Unlike *P. vivax* infection, which is restricted to reticulocytes, *P. falciparum* is capable of infecting RBCs of all ages. However, young RBCs are more susceptible to *P. falciparum* infection than older RBCs, with reported reticulocyte preferences ranging from 1.6 – 14 fold(Pasvol et al., 1980; Tian et al., 1998), (Wilson et al., 1977). Furthermore, work in several theoretical modeling papers suggest that an elevated reticulocyte replacement rate leaves the human host at increased risk of hyperparasitemia (Cromer et al., 2009; McQueen and McKenzie, 2004). A critical implication of these observations is that reconstitution of red cell mass in anemic patients would be expected to transiently enhance susceptibility to malaria (**Figure 4.4**). Low incidence of IDA in our study setting, the difficulty of following iron supplemented IDA individuals longitudinally through full recovery from iron deficiency, as well as the inability to use the common surrogates of RBC age (volume and density) in the background of changing host iron status, has prevented us from studying the impact of an elevated erythropoietic rate on erythrocytic stage *P. falciparum* infection *ex vivo*. Therefore, in an attempt to assess the impact of changing RBC population dynamics in the human host in response to iron supplementation on susceptibility to erythrocytic stage *P. falciparum* infection, we explored the effect of changing frequency of physiologically distinct RBC^{IDA} and RBC^{IR} as well as young and old RBCs (RBC^{Y} and RBC^{O}) populations and *P. falciparum* infection.

As RBC^{IDA} are at significantly less risk of being invaded by *P. falciparum* than RBC^{IR} (**Figure 4.2B**), we began by examining the effect of replacing RBC^{IDA} with RBC^{IR} on *P. falciparum* erythrocytic invasion rate. To do this we utilized the three-color invasion assay, which is uniquely suited for studying the relationship between two different RBC populations

and *P. falciparum* invasion. We observed that replacement of RBC^{IDA} with RBC^{IR} steadily increases the invasion rate of *P. falciparum,* and upon replacing 80% of RBC^{IDA} with RBC^{IR} there was no longer any significance in difference from the invasion rate of RBC populations containing exclusively RBC^{IR} (**Figure 4.5A**). As iron supplementation of IDA individuals will result in the release of young IR RBCs (RBC^{IR-Y}) into the circulation, we next investigated the effect of replacing RBC^{IDA} with RBC^{IR-Y} on the growth rate of *P. falciparum in vitro*. Similar to the previous invasion experiments, we observed that replacement of RBC^{IDA} with RBC^{IR-Y} increases the growth rate of *P. falciparum.* However, unlike the invasion experiments we did not observe a complete recovery of *P. falciparum* growth rate to that of the growth of *P. falciparum* in RBC^{IR} (**Figure 4.5B**). It should be noted that in the growth rate experiments however, that only up to 75% of RBC^{IDA} were replaced with RBC^{IR-Y}, and in invasion experiments we did not observe complete recovery of invasion rate until 80% of RBC^{IDA} had been replaced. Together these results support the hypothesis outlined in **Figure 4.4** that replacing an individual's RBC^{IDA} population with RBC^{IR-Y} would increase the susceptibility of the hosts RBC population to *P. falciparum* infection.

Next we assessed the reticulocyte preference of *P. falciparum* in RBC^{IDA-Fe}. To do this we compared the distribution of *P. falciparum* within reticulocytes (CD71+) and RBCs (CD71-) from IDA-Fe donors. We were unable to do similar analysis of RBC^{IR} as we were unable to detect a significant number of CD71+ reticulocytes in IR donors. I observed a parasite prevalence of (8.6% SD±0.1) in CD71+ reticulocytes and (4.5% SD±0.4) in CD71- RBCs (**Figure 4.6A**). However, in the context of the entire parasite infection parasitized reticulocytes accounted for only (3.0% SD±0.1) of the total infection (**Figure 4.6B**). To more fully investigate the relationship between RBC age and *P. falciparum* erythrocytic stage infection, we utilized two

additional proxies for RBC age: RBC volume which decreases with age(Bosch et al., 1992) and is unaffected by parasitization (Esposito et al., 2010); and RBC density, which increases with increasing RBC age (Bosch et al., 1992). We observed that *P. falciparum* prevalence increased with increasing RBC volume in RBC^{IR} (**Figure 4.7A**). Density separation of RBC^{IR} yielded five fractions of increasing RBC age: very young (RBC^{VY}); young (RBC^{Y}); young adult (RBC^{YA}); mature adult (RBC^{MA}); and old (RBC^{O}) (**Figure 4.7B**). Decreasing MCV, reticulocyte content[25], and Calcein fluorescence(Bratosin et al., 2005) of each subsequent fraction confirmed the age separation of RBCs (**Figure 4.7C, D, and E**). In accordance with previous reports (Pasvol et al., 1980; Tiffert et al., 2005; Lim et al., 2013), the RBC^{Y} fraction sustained a significantly greater growth rate than RBC^{YA}, RBC^{MA}, and RBC^{O}, with RBC^{Y} supporting a growth rate 50% greater than RBC^{O} (**Figure 4.7F**), and production of infectious daughter merozoites was reduced by 10% (SD±4.78), 15% (SD±1.16), and 19% (SD±2.23) in RBC^{YA}, RBC^{MA}, and RBC^{O} as compared to RBC^{Y} (**Figure 4.7G**). Furthermore, direct comparison of the invasion of *P. falciparum* into cultures containing equal numbers of RBC^{Y} and either RBC^{Y} (control), RBC^{YA}, RBC^{MA}, or RBC^{O}. The SI of RBC^{YA}, RBC^{MA}, and RBC^{O} as compared to RBC^{Y} was 0.85 (95% CI 0.82-0.89), 0.58 (95% CI 0.56-0.62), and 0.28 (95% CI 0.27-0.30), respectively. (**Figure 4.7H)**. These data clearly reflect the preferential invasion of *P. falciparum* into young RBCs and shows that the risk of RBCs to *P. falciparum* invasion relative to young RBCs decreases with increasing RBC age. All together we have confirmed (i) prevalence of *P. falciparum* infection positively correlates with RBC age and (ii) the increased capacity of young RBCs to support *P. falciparum* growth.

As RBC age appears to more significantly affect risk of invasion than the ability of the parasite to produce infectious daughter merozoites (**Figure 4.6G and 4.6H)**, we hypothesized

that changing the age dynamics of a RBC population would impact susceptibility to *P. falciparum* invasion. To study the effect of changing age dynamics of a RBC population on *P. falciparum* invasion we took the same approach we had taken to study the effect of replacing RBC^{IDA} with RBC^{IR} (**Figure 4.5A**). RBC^{Y} were replaced with RBC^{O} in three-color invasion assays and *P. falciparum* invasion rate as well as the contribution of each RBC population to $\%pRBC^{Total}$ was assessed. We observed that as RBC^{Y} decreased in frequency from 100% to 50% of RBC^{Total} the total rate of invasion remained relatively constant, decreasing by only 4.9% SD±0.7, but then as RBC^{Y} frequency fell from 50% to 0% of RBC^{Total} *P. falciparum* infection decreased steadily, ultimately falling by 45.7% SD±1.9 (**Figure 4.8A**). As RBC^{Y} represents the RBC population most susceptible to *P. falciparum* infection, we speculated that *P. falciparum* infection had plateaued as a result of merozoites being limited. However, three-color invasion experiments with double the inoculum of merozoites also resulted in a plateau in the rate of *P. falciparum* invasion when RBC^{Y} accounted for more than 50% of RBC^{Total} (**Figure 4.8B**). As the rate of *P. falciparum* infection decreased in experiments with a greater merozoite inoculum, this data suggests that *P. falciparum* infection does not increase proportionally to increasing RBC^{Y} abundance *in vitro* (**Figure 4.8A and B**). That said, the rate of *P. falciparum* invasion of RBC populations containing 20-100% of RBC^{Y} to *P. falciparum* was significantly greater than the rate of invasion of unseparated RBC^{IR}, with the rate of invasion being up to 29.9% SD±1.9 greater. These experiments further support the hypothesis outlined in **Figure 4.4** that following replacement of RBC^{IDA} cells with $RBC^{IR\text{-}Y}$ an individual's RBC population would be at greater risk of *P. falciparum* infection.

Together these results confirm that *P. falciparum* infection is skewed towards young RBCs, and that the rate of *P. falciparum* invasion increases with increasing prevalence of RBC^{Y}

and RBC^{IR}. Additionally, we show that *P. falciparum* produces fewer infectious daughter merozoites within RBC^{IDA} than RBC^{IR} and confirm that the capacity to produce infectious daughter merozoites decreases with increasing RBC age (Lim et al., 2013). These results are consistent with the hypothesis that the effect of iron deficiency and iron supplementation on RBC physiology and erythropoiesis determines an individual's risk of malaria infection (**Figure 4.4**).

4.5 Discussion

Iron supplementation has clear nutritional benefits for children and pregnant women, but iron is also an essential nutrient for most pathogens and as a result is a critical mediator of host-pathogen interactions (Drakesmith and Prentice, 2012). Activation of the host innate immune system by the malaria parasite or other infectious organisms triggers reduction in iron absorption, redistribution of existing iron stores, and decreased erythropoiesis, which effectively limits the availability of iron to invading pathogens. The iron sources utilized by *P. falciparum* during any stage of its development and the strategies used by the parasite to circumvent the host's attempt to restrict iron remain unclear (Scholl et al., 2005). It has been previously postulated that iron deficiency inhibits *P. falciparum* infection via iron deprivation as is reported to occur with other pathogens (Skaar, 2010). While the malaria parasite may find iron less readily available in the context of iron deficiency, our work reveals an alternate mechanism by which iron deficiency is protective against malaria. This investigation highlights how the impact of both iron deficiency and iron supplementation influence the dynamics of the host RBC population and thus alter propagation of the malaria parasite. We have clearly shown that iron deficient RBCs are more resistant to malaria infection and that this resistance is lost following host iron supplementation. Additionally, we have investigated the hypothesis that there is a transient period during the

response to iron supplementation that may put patients at an increased risk of malaria infection relative to iron-replete patients.

Clinical studies in different field sites have demonstrated the protective effect of iron deficiency on malaria. In Malawian children, baseline iron deficiency was associated with significant reductions in the subsequent risks of both parasitemia (45% reduction) and malaria (51% reduction) (Jonker et al., 2012). Similarly, in Tanzanian children, baseline iron deficiency significantly decreased the odds of subsequent parasitemia (23% reduction) and severe malaria (38% reductions) (Gwamaka et al., 2012). Additionally, in two studies of pregnant women, iron deficiency was associated with a decreased prevalence of placental malaria (Senga et al., 2011; Kabyemela et al., 2008), a major cause of neonatal and maternal morbidity. Our results that IDA RBCs impair parasite propagation *in vitro* are consistent with these clinical findings, and provide valuable insight into cellular mechanisms governing the observations made in the clinical setting. There are multiple physiological differences between iron-deficient and iron-replete RBCs that may contribute to the impaired invasion of IDA RBCs including: greater osmotic fragility and membrane rigidity and accelerated ageing in vivo (Yip et al., 1983; Yermiahu et al., 1999; Brandão et al., 2009; Bunyaratvej et al., 1992). Additionally, IDA RBCs have a lower hemoglobin content and smaller size compared to iron-replete RBCs, and therefore may be deficient in essential metabolites required by the parasite for merozoite production. Our results exclusively focus on the influence of host iron on the RBC and malaria infection, and do not address the potential effect of host serum iron (Clark et al., 2013) or additional protective effects of iron deficiency which may function *in vivo,* including impaired cytoadherence to the endothelium, accelerated clearance of parasitized RBCs (Koka et al., 2007; Matsuzaki-Moriya et

al., 2011), restricted growth of the hepatic stage of the parasite (Goma et al., 1996), or effects of hepcidin (Portugal et al., 2011) and lipocalin 2 (Zhao et al., 2012).

We have previously reported that the addition of extracellular transferrin or ferric citrate increases the bioavailable iron pool of trophozoite infected RBCs but not that of uninfected RBCs (Clark et al., 2013). It is possible that parasite growth may be enhanced by the transient increase in serum iron that is observed in iron-replete individuals who are given oral iron supplementation (Schümann et al., 2012). Future investigations should further explore the possibility that *P. falciparum* scavenges extracellular iron in order to augment intraerythrocytic growth, as has been observed in other human pathogens (Haley and Skaar, 2012). However, in this study we have focused exclusively on the impact of iron supplementation on host RBCs and their subsequent effect on erythrocytic stage *P. falciparum* propagation. As previously discussed we've clearly demonstrated that microcytic iron deficient RBCs are protective against erythrocytic stage *P. falciparum* infection (**Figure 4.2**), and that iron supplementation of IDA donors reverses the protection afforded by IDA **(Figure 4.3**). We subsequently went on to experimentally investigate the capacity of changing the iron status and age distribution of a RBC population on erythrocytic stage *P. falciparum* infection. Our experiments support the hypothesis that the replacement of IDA RBCs with IR RBCs is responsible for the recovered growth rate of *P. falciparum* in RBCs from iron supplemented IDA individuals (**Figure 4.5**), and that upon replacement of IDA RBCs an individual may be at increased risk of malaria infection due to an on average younger RBC population than IR counterparts (**Figure 4.8**).

Our findings, in the context of those from field studies, raise the important medical and public health question: How can iron supplementation be safely administered to IDA children in malarious areas? A critical implication of these observations is that reconstitution of red cell

mass in anemic patients would be expected to transiently enhance susceptibility to malaria (**Figure 4.4**), which may inform the ongoing debate as to whether fortification with iron would be safer than supplemental iron. Our data imply that, where *P. falciparum* is endemic, effective treatments for anemia should be accompanied by preventive treatment against malaria. Additional questions raised by this study are: (1) does IDA in African children represent an evolutionarily advantageous phenotype that derives from polymorphisms in iron homeostasis?; and (2) what molecular mechanisms confer protection from malaria in the setting of microcytosis, and can these protective mechanisms be exploited by medical interventions?. Future clinical and translational studies will be needed in order to design safe and effective interventions to address the twin burdens of iron deficiency and falciparum malaria.

Growth assay	# replications in RBCs of interest	Time (h)												
		*0**	*24*	*48***	*72*	*96*	*120*	*144*	*168*	*192*	*216*	*240*	*264*	*288*
1	**1**	*Rings*	*Trophs*	*Rings*	*Trophs*	*Rings*								
2	**3**					*Rings*	*Trophs*	*Rings*	*Trophs*	*Rings*				
3	**5**									*Rings*	*Trophs*	*Rings*	*Trophs*	*Rings*

Figure 4.1 – Serial *in vitro* growth assay design for *P. falciparum* Synchronized *P. falciparum* parasite lines (3D7, Dd2, FCR3-FMG) cultured in iron-replete O+ RBCs were seeded as rings at 0.5% initial parasitemia at 1% haematocrit in ACM in triplicate into 96 well plates. Parasites were maintained for 96 h at 37 degrees in 5% O2, and media was changed daily. At 96 h, the parasite cultures were split back to 0.5% parasitemia and maintained for an additional 96 h in Growth Assay 2. At 192 h, the parasite cultures were split back again to 0.5% parasitemia and maintained for an additional 96 h in Growth Assay 3. Growth rate was determined for each growth assay. *experimental cultures seeded with 0.5% ring infected RBC. **parasites invade experimental cells of interest.

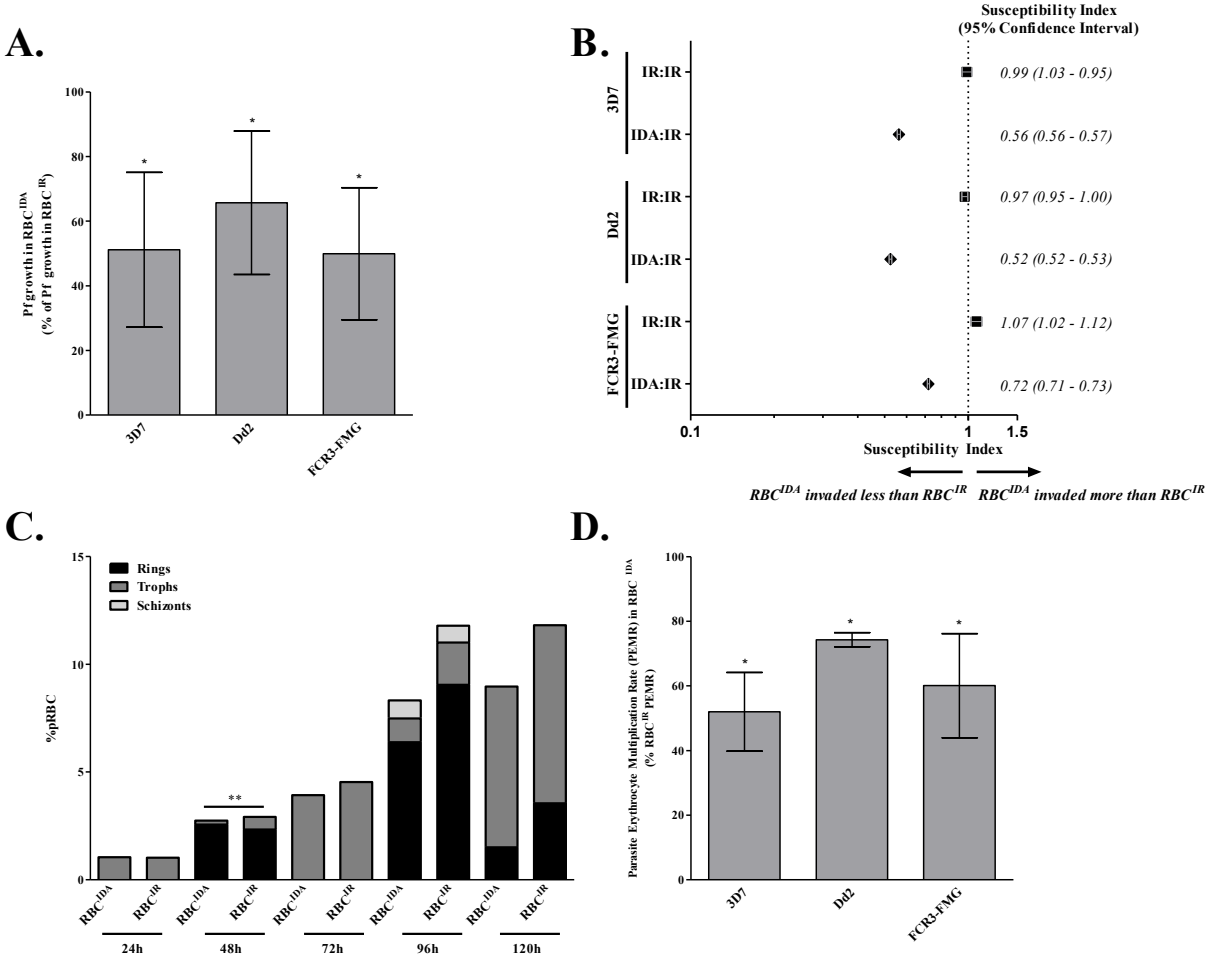


Figure 4.2 – *P. falciparum* erythrocytic stage propagation is attenuated in microcytic IDA RBCs as compared to normocytic iron-replete RBCs *in vitro.* Study participants with hemoglobin [Hgb] >11g/dL and ferritin >12ng/ml were classified as IR and those with Hgb< 11g/dL and ferritin<12ng/ml were classified as IDA (**Table 1**). (A) Growth of *P. falciparum* strains (3D7, Dd2, and FCR3-FMG) in RBCs from IDA donors (RBC^{IDA}) (n=7) normalized to growth in RBCs from IR donors (RBC^{IR}). Growth experiments were performed in triplicate and growth rates (the fold increase in the number of *P. falciparum* infected RBCs between 0 and 96 h) were determined for each of up to three consecutive growth assays (five total *P. falciparum* life cycles) (**Figure 1**). Experiments for all seven IDA donors were performed independently and included an IR control. Bars represents the mean growth rate of parasites after a minimum of two 48h life cycles in RBC^{IDA} as a percent of parasite growth rate in RBC^{IR} of seven independent experiments. The error bars represent the standard deviation. Significance was determined by two-tailed paired Students *t* test (GraphPad Prism 5, La Jolla, CA). *p<3E-10, compared to growth in IR RBCs. (B) Invasion of *P. falciparum* strains (3D7, Dd2, and FCR3-FMG) into either RBC^{IDA} or RBC^{IR}. RBC^{IDA} and RBC^{IR} were differentially labeled with CellTrace Dyes (DDAO-SE or Violet), combined, inoculated with trophozoite stage infected 3D7, Dd2, or FCR3-FMG RBCs, and incubated for 12-18 h to allow for rupture of schizonts and subsequent invasion of merozoites into either RBC^{IDA} or RBC^{IR}. Following invasion, wells were stained with DNA dye SYBR Green I to identify invaded RBCs. The Susceptibility Index (SI) (RBC

susceptibility to invasion) was determined with an unadjusted Odds Ratio. Analyses were performed with Stata/IC (v10, Stata Corp, College Station, TX). A SI of 1.0 indicates no difference of parasite invasion into two RBC populations. For each RBC^{IDA} and RBC^{IR} invasion experiment, RBCs from two IR donors were included as a control. Invasion experiments for each RBC^{IDA} donor were performed independently and each experiment was performed in triplicate. Data show the mean SI of RBC^{IDA} relative to RBC^{IR} of seven independent experiments. The marker represents the SI point estimate and the bar represents the 95% CI. (C) *P. falciparum* maturation is un-impeded within RBC^{IDA} as compared to development within RBC^{IR}. Synchronized *P. falciparum* strain 3D7 RBC^{IR} cultures were seeded as rings a 0.5% initial parasitemia at 1% haematocrit in duplicate into 24 well plates containing either RBC^{IDA} or RBC^{IR}. Giemsa stained thin blood smears were made every 24 h and 1000 RBCs were counted by light microscopy to determine the percent of infected RBCs as well as parasite intra-erythrocytic stage of maturation. **parasites invade experimental cells of interest. (D) Production of infectious daughter merozoites (Parasite Erythrocyte Multiplication Rate [PEMR]) of *P. falciparum* strains 3D7, Dd2 and FCR3-FMG within RBC^{IDA} and RBC^{IR} (n=3). *P. falciparum* merozoites were allowed to invade RBC^{IDA} and RBC^{IR}, and upon maturation to the trophozoite stage, equal numbers of $pRBC^{IDA}$ and $pRBC^{IR}$ were sub-cultured into separate wells containing RBC^{IR} target RBCs and incubated for 12-18 h to allow merozoites within RBC^{IR} and RBC^{IDA} to invade target RBC^{IR}. Experiments were stained with DNA dye SYBR Green I to identify pRBCs. The number of ring infected target RBCs represents the number of infectious daughter merozoites produced per $pRBC^{IDA}$ or $pRBC^{IR}$. Data is the mean of three independent experiments performed in triplicate. Error bars represent the standard deviation. Significance determined by two-tailed paired Student's *t* test (GraphPad Prism 5, La Jolla, CA).*p<3E-6, compared to infectious daughter merozoites produced within RBC^{IR}.

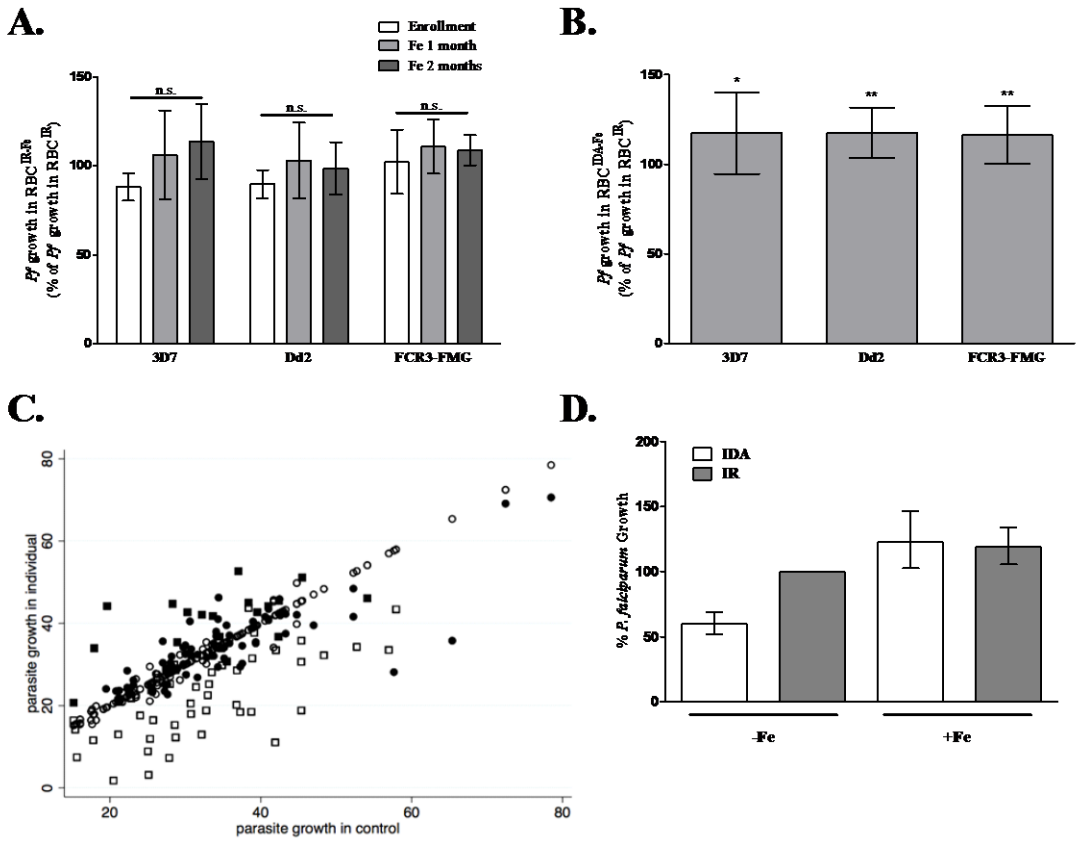


Figure 4.3 – Effect of iron supplementation of IR and IDA donors on erythrocytic stage *P. falciparum* growth *in vitro.* (A) Growth of *P. falciparum* strains (3D7, Dd2, and FCR3-FMG) in RBCs from IR donors (n=4) at enrollment, 1 month and 2 months following prescription of daily iron supplementation (ferrous sulfate, 325 mg, 3-4.2 mg Fe/kg) for 60 days (IR-Fe)($RBC^{IR\text{-}Fe}$). Analysis of hemoglobin, haematocrit, MCV, MCHC, transferrin saturation, ferritin, and reticulocyte count of IR-Fe donors revealed no significant change in haematocrit, hemoglobin, MCV, total iron, or ferritin following iron supplementation of IR donors (**Table 1)**. Growth experiments were performed as previously described, and data represents the growth rate for parasite strains after three 48 h life cycles within RBC from IR donors at enrollment, 1 month and, 2 months following iron supplementation ($RBC^{IR\text{-}Fe}$) as a percent of the growth rate of parasite strains within IR non-supplemented RBCs (RBC^{IR}). Experiments were performed independently and each experiment was performed in triplicate. Bars represent the mean parasite growth rate within RBCs from four IR-Fe donors at enrollment, 1 month on iron, and 2 months on iron as a percent of the corresponding growth rates of parasites within RBC^{IR}. Error bars represent the standard deviation. One way ANOVA analysis (GraphPad, Prism, v. 5.04, La Jolla, CA) show no significant change in parasite growth following iron supplementation (p=0.08). (B) Growth of *P. falciparum* strains (3D7, Dd2, and FCR3-FMG) in RBCs from IDA donors following iron supplementation (n=5) ($RBC^{IDA\text{-}Fe}$) with either oral iron (325 mg ferrous sulfate three times per day) or intravenous iron (at a dosage determined by their personal physician using the following equation: Dose = 0.0442 [desired Hgb - observed Hgb) x LBW + (0.26 x LBW); Desired hemoglobin (Hgb)=14.8 g/dL; LBW = Lean body weight in kg] (IDA-Fe). The

same criteria for classification as IDA were used as previously described. The average MCHC, iron total, and ferritin values for IDA-Fe donors were greater than that of IDA donors but still lower than IR donor, indicative of an erythropoietic response to iron supplements (**Table 1)**. Growth experiments were performed as previously described, and data represents the growth rate for parasite strains after three 48 h life cycles within $RBC^{IDA\text{-}Fe}$ as a percent of the growth rate of parasite strains within non supplemented RBC^{IR}. Experiments were performed independently and each experiment was performed in triplicate. Bars represent the mean parasite growth rate within $RBC^{IDA\text{-}Fe}$ relative to growth of parasites within RBC^{IR} of five independent experiments. Error bars represent the standard deviation. Significance was determined by two-tailed paired Student's *t* test (GraphPad, Prism, v. 5.04, La Jolla, CA). *p<0.003 **p<3E-6, compared to growth within RBC^{IR}. (C^2) *P. falciparum* growth after two erythrocyte life cycles within RBC^{IR}, RBC^{IDA}, $RBC^{IDA\text{-}Fe}$, and $RBC^{IR\text{-}Fe}$. Data are the mean growth rate of parasites in individual donor RBCs calculated from growth assay 2 for *P. falciparum* strains (3D7, Dd2, and FCR3-FMG) within RBC^{IR} (O), RBC^{IDA} (□), $RBC^{IR\text{-}Fe}$ (●), and $RBC^{IDA\text{-}Fe}$ (■) plotted against growth in corresponding control non-supplemented RBC^{IR}. Data was analyzed by mixed effects regression. Growth rates of *P. falciparum* in RBC^{IR} on the Y=X line. Data above the Y=X line indicate growth rates greater than that of parasite growth in RBC^{IR} and data below the Y=X line indicate growth rates lower than that of parasite growth in RBC^{IR}. (D) Graphical summary of the mixed effects regression analysis of the logarithm of *P. falciparum* growth after two erythrocyte life cycles within RBC^{IR}, RBC^{IDA}, $RBC^{IDA\text{-}Fe}$ and $RBC^{IR\text{-}Fe}$. Donor and parasite preparation were fitted as crossed random effects while growth assay number (**Figure 1)** and parasite strain differences were treated as fixed covariates. The model was fitted using Stata's *xtmixed* procedure (v12, Stata Corp, College Station, TX). The bars show the estimated parasite growth in RBC from the different donor groups expressed as a percentage of that in IR donors and error bars are the 95% CI for this percentage.

[2] Dr. Tony Fulford performed random effects regression analysis of growth data.

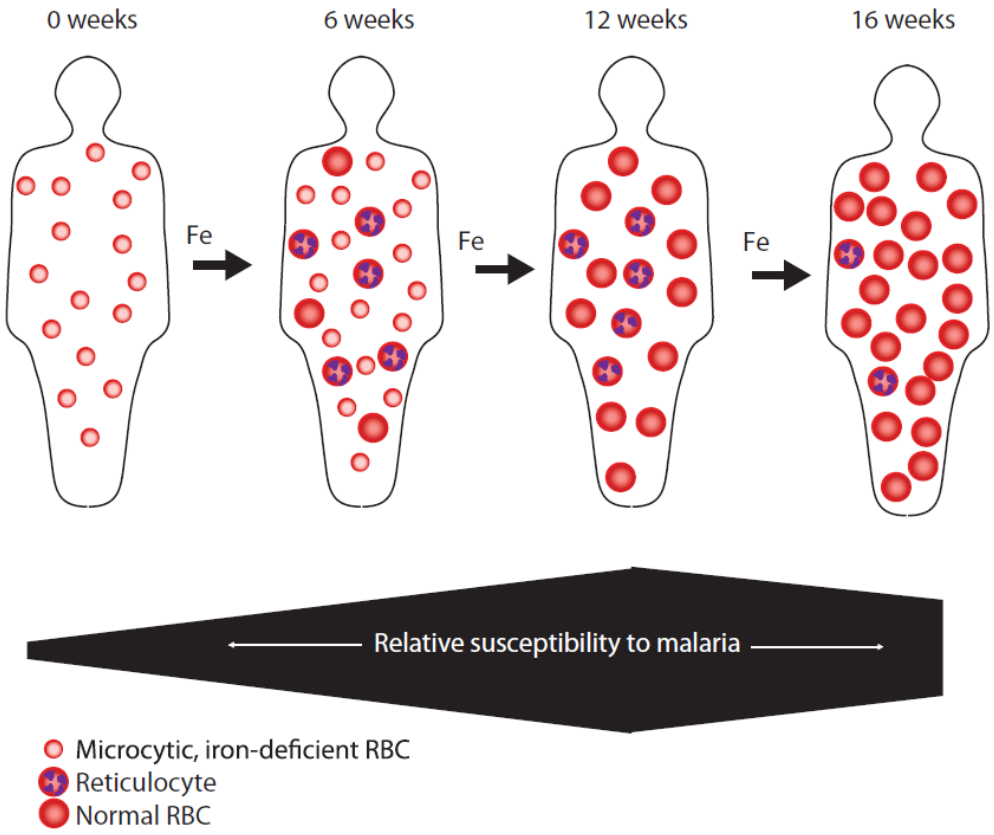


Figure 4.4 – The potential effect of altered host RBC population dynamics that result from iron deficiency and iron supplementation, on susceptibility to erythrocytic stage malaria infection. The image shows a hypothesized model for the effect of iron supplementation on the erythrocyte population dynamics of an individual with IDA, and susceptibility to erythrocytic stage *P. falciparum* infection. Recovery from iron deficiency is a complex process, which will vary between individuals based on initial transferrin saturation levels, levels of circulating hepcidin, erythropoietic rate, and genetic factors. Under ideal conditions iron supplementation of an IDA individual (0 weeks) results in reticulocytosis and the production of IR young RBCs ($RBC^{IR\text{-}Y}$) (6 weeks). By 12 weeks after the initiation of supplementation, the majority of RBC^{IDA} (90 day lifespan) are cleared from circulation. A normal distribution of the individual's RBC^{IR} (120 day life span) population is achieved by week 16. Increasing thickness of the black shape corresponds with increasing susceptibility of the individual to malaria infection. Reduced growth of *P. falciparum* in RBC^{IDA} and *P. falciparum* preferential infection of young RBCs (RBC^{Y}) leads to the hypothesis that IDA individuals will be less susceptible erythrocytic stage malaria. The induction of erythropoiesis in the IDA individual by iron supplementation and subsequent replacement of the individuals RBC^{IDA} with $RBC^{IR\text{-}Y}$ will increase the susceptibility of the individual to erythrocytic stage malaria infection; with susceptibility to infection being predicted to peak at the point when all RBC^{IDA} have been replaced but the age distribution of RBC^{IR} is on average younger than a fully recovered IR individual.

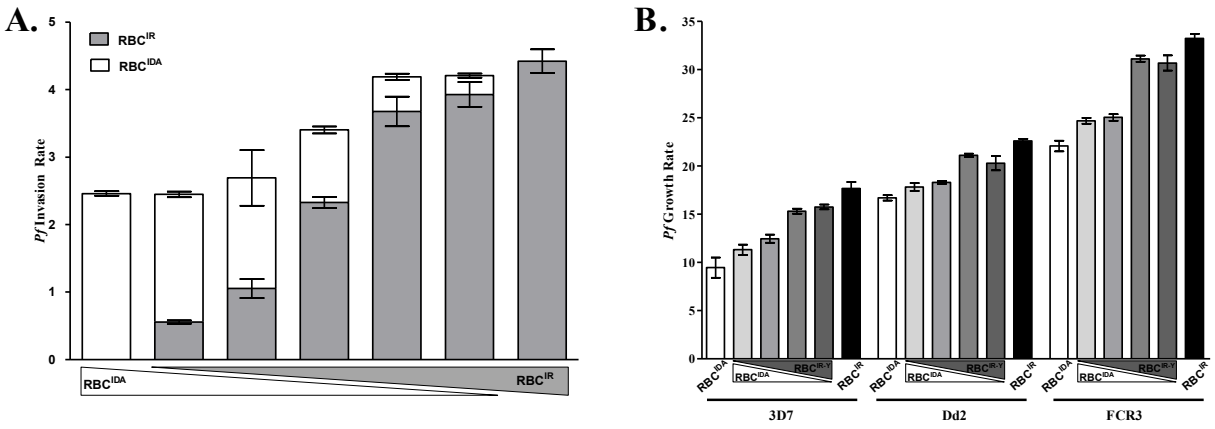


Figure 4.5 – Replacement of RBC^{IDA} with RBC^{IR} increases the invasion and growth rate of erythrocytic stage *P. falciparum*. (A) Invasion experiments performed as previously described with RBC^{IR} and RBC^{IDA}. RBC^{IR} and RBC^{IR} were inoculated in isolation as well as in combination at ratios of 1:10, 1:5, 1:1, 5:1, and 10:1 with 0.3x10^6 pRBCs of *P. falciparum* strain FCR3-FMG. Data is from a representative experiment of three independent experiments with three different RBC^{IDA} and two different RBC^{IR} donors infected with *P. falciparum* strains 3D7, Dd2, and FCR3-FMG. Bars represent the rate of *P. falciparum* strain FCR-FMG invasion into each respective RBC condition, with the contribution of RBC^{IDA} and RBC^{IR} to the total rate of invasion denoted by the white and gray bars respectively. Error bars represent the standard deviation. (B) Growth rate of *P. falciparum* strains 3D7, Dd2, and FCR3-FMG within cultures containing of 20x10^6 RBC^{IDA} or RBC^{IR} in isolation as well as RBC^{IDA} combined 10:1, 4:1, 1:1 and 1:4 with density separated RBC^{IR-Y} for a total of 20x10^6 RBCs per well. Growth experiments were performed as previously described and data presented is a representative experiment of two independent experiments performed in triplicate with *P. falciparum* strains 3D7, Dd2, and FCR3-FMG and two different RBC^{IDA} as well as RBC^{IR} donors. Data represents parasite growth rates in each respective RBC condition after one 96 hour growth assay. Error bars represent the standard deviation.

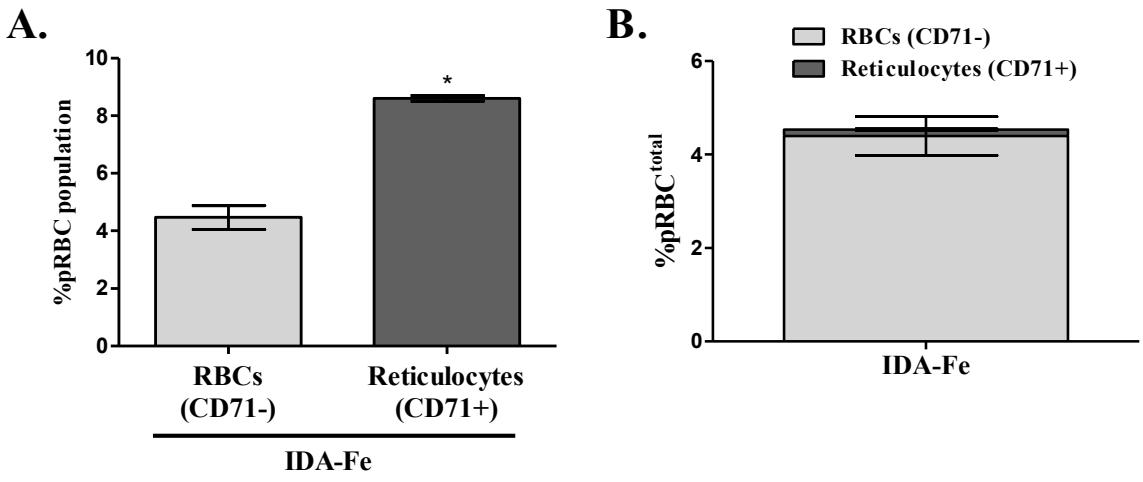


Figure 4.6 – *P. falciparum* prevalence is higher in CD71+ reticulocytes from iron supplemented iron deficient donors. (A) Frequency of *P. falciparum* strain 3D7 in early reticulocytes (CD71+) as compared to RBCs (CD71-). (B) Total *P. falciparum* 3D7 infection of $RBC^{IDA\text{-}Fe}$, and the contribution of parasitized reticulocytes (CD71+) and parasitized RBCs (CD71-). Following invasion of *P. falciparum* 3D7 merozoites into RBCs, cells were stained with a PE conjugated anti-human antibody against the early reticulocyte marker CD71 and DNA dye SYTO 61 to detect infected RBCs. The percent infected reticulocytes (CD71+ SYTO 61+) and infected mature RBCs (CD71- SYTO 61+) was determined by flow cytometry. Data is from a representative experiment of three independent experiments performed in triplicate with *P. falciparum* strains 3D7, Dd2, and FCR3-FMG. Error bars represent the standard deviation. Significance determined by two-tailed paired Student's *t* test (GraphPad, Prism, v. 5.04, La Jolla, CA). *p<8E-5, compared to percent infected RBCs (CD71-).

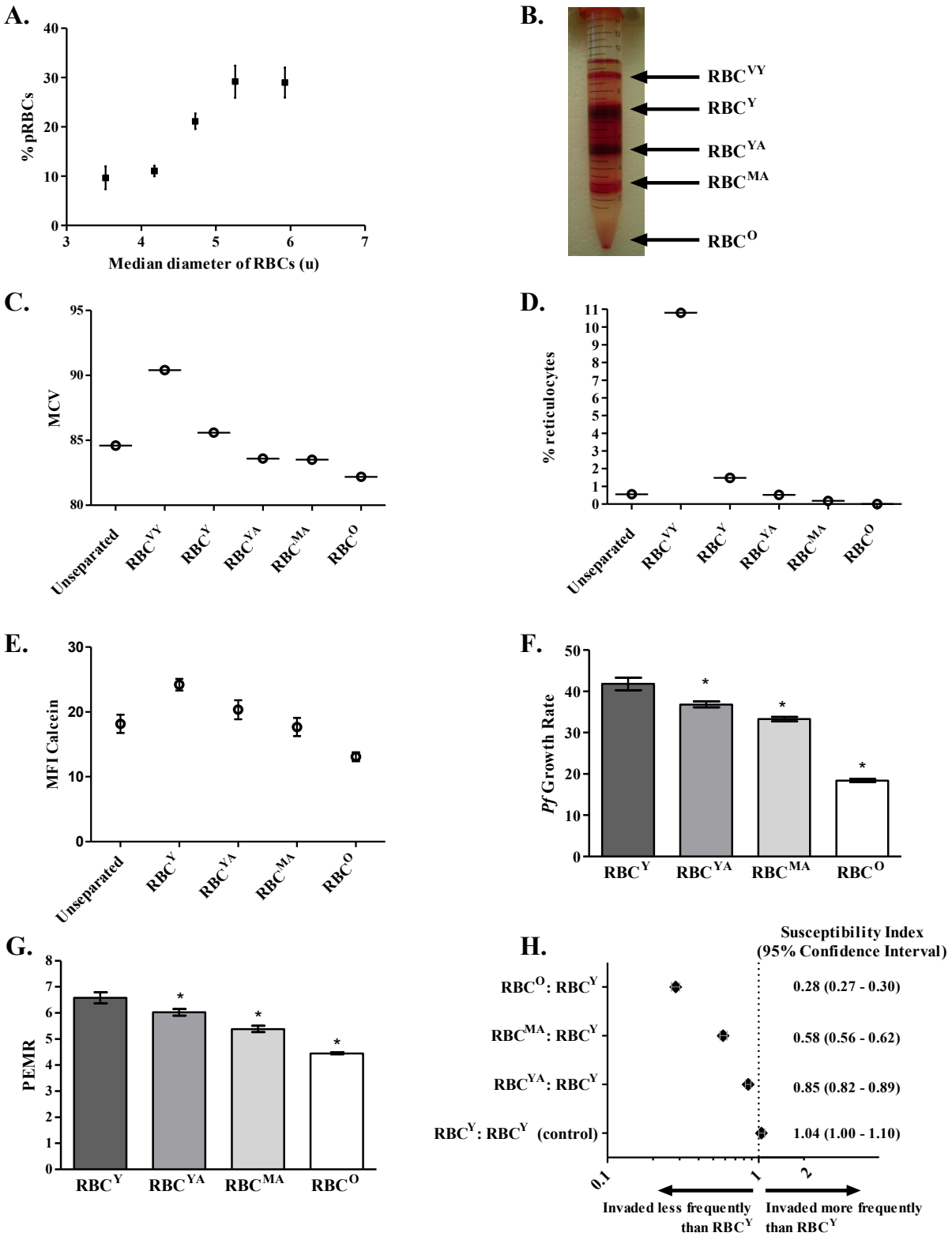

A.
% pRBCs
Median diameter of RBCs (u)
B.
RBC^VY
RBC^Y
RBC^YA
RBC^MA
RBC^O
C.
MCV
Unseparated
RBC^VY
RBC^Y
RBC^YA
RBC^MA
RBC^O
D.
% reticulocytes
Unseparated
RBC^VY
RBC^Y
RBC^YA
RBC^MA
RBC^O
E.
MFI Calcein
Unseparated
RBC^Y
RBC^YA
RBC^MA
RBC^O
F.
Pf Growth Rate
RBC^Y
RBC^YA
RBC^MA
RBC^O
G.
PEMR
RBC^Y
RBC^YA
RBC^MA
RBC^O
H.
Susceptibility Index
(95% Confidence Interval)
RBC^O: RBC^Y
0.28 (0.27 - 0.30)
RBC^MA: RBC^Y
0.58 (0.56 - 0.62)
RBC^YA: RBC^Y
0.85 (0.82 - 0.89)
RBC^Y: RBC^Y (control)
1.04 (1.00 - 1.10)
Invaded less frequently than RBC^Y
Invaded more frequently than RBC^Y

Figure 4.7 – Young RBCs sustain greater *P. falciparum* growth due to increased risk of young RBCs to invasion and greater capacity of *P. falciparum* produce infectious daughter merozoites within young RBCs. (A) Frequency of *P. falciparum* strain (3D7) in RBCs of increasing diameter, a proxy for increasing RBC age. Following invasion of *P. falciparum* (3D7) merozoites into RBC^{IR}, cells were stained with DNA dye SYBR Green I to identify $pRBC^{IR}$. The percent infected RBCs within RBCs of increasing diameter was assessed by quantitating the frequency of infected RBCs within RBCs gated upon increasing electronic volume as assessed by flow cytometry. Data is from a representative experiment of two independent experiments performed in triplicate with *P. falciparum* strains 3D7, Dd2, and FCR3-FMG. Error bars represent the standard deviation. (B) Percoll density separation of RBC^{IR} into five fractions of increasing RBC age. (C) MCV as measured by Beckam Coulter AcT diff2 (Brea, CA), (D) percent reticulocyte as determined by new methylene blue, and (E) calcein fluorescence of density separated RBCs stained with 5 μM calcein-AM for 30 min and assessed by flow cytometry. Decreasing MCV, reticulocyte content[25], and Calcein fluorescence(Bratosin et al., 2005) of each fraction confirmed the age separation of RBCs. (F) Growth of *P. falciparum* strain (FCR3-FMG) within age separated RBC^{IR}. Growth experiments were performed as previously described. Growth rates were determined for each age separated RBC fraction and data represents parasite growth rate after one 96 hour growth assay. Data is from a representative experiment of three independent experiments performed in triplicate with *P. falciparum* strains 3D7, Dd2, and FCR3-FMG. Error bars represent the standard deviation. Significance determined by two-tailed paired Student's *t* test (GraphPad, Prism, v. 5.04, La Jolla, CA). *$p<0.001$, compared to *P. falciparum* growth rate in RBC^{Y} fraction. (G) *P. falciparum* strain FCR3-FMG production of infectious daughter merozoites (PEMR) within RBCs of increasing age. Infectious merozoite production assays were performed as previously described. Briefly merozoites were allowed to invade RBC^{Y}, RBC^{YA}, RBC^{MA}, and RBC^{O}. Following invasion the same number of $pRBC^{Y}$, $pRBC^{YA}$, $pRBC^{MA}$, and $pRBC^{O}$ inoculated into separate wells containing non-age separated RBC^{IR} and cultured for 12-18 hours to allow merozoites within RBC^{Y}, RBC^{YA}, RBC^{MA}, and RBC^{O} to be released and invade target RBC^{IR}. The number of ring infected target RBC^{IR} represent PEMR within RBC^{Y}, RBC^{YA}, RBC^{MA}, and RBC^{O}. Data is from a representative experiment of three independent experiments performed in triplicate with *P. falciparum* strains 3D7, Dd2, and FCR3-FMG. Error bars represent the standard deviation. Significance determined by two-tailed paired Student's *t* test (GraphPad Prism 5, La Jolla, CA).*$p<0.001$, compared to infectious merozoites produced within young RBCs. (H) Direct comparison of *P. falciparum* strain FCR3-FMG invasion into RBC^{Y} and either RBC^{YA}, RBC^{MA}, or RBC^{O}. Invasion experiments were performed as previously described. The SI, an Odds Ratio (OR) was calculated to estimate the relative risk of *P. falciparum* invasion into RBCs of increasing age as compared to RBC^{Y}. For age invasion experiments RBC^{Y} combined with RBC^{Y} served as the control. Data represents the SI of *P. falciparum* invasion into RBC^{Y} combined with increasingly older RBC fractions. Data is from a representative experiment of three independent experiments performed in triplicate with *P. falciparum* strains 3D7, Dd2, and FCR3-FMG. The markers represent the SI point estimate and the bar represents the 95% CI.

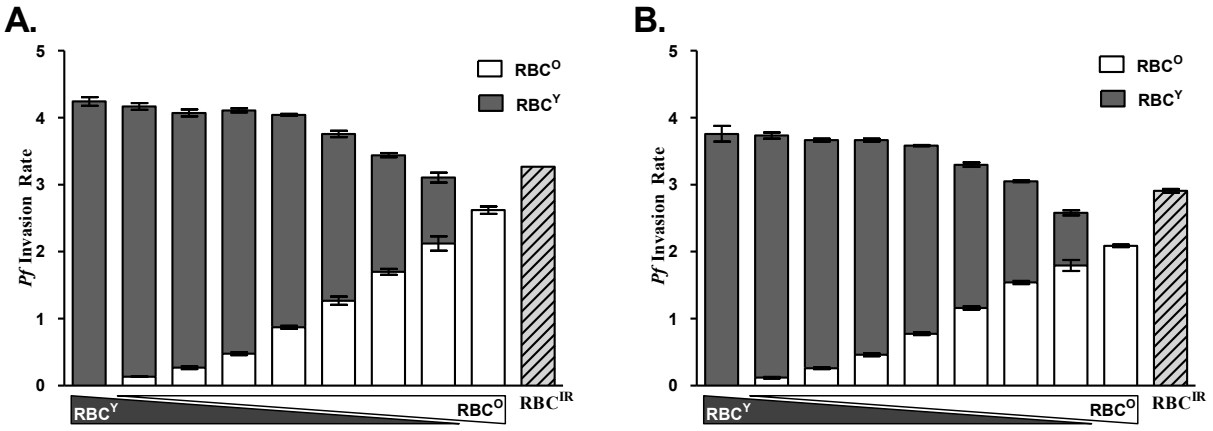


Figure 4.8 – Elevated frequency of RBC^{Y} in a RBC population sustains an elevated *P. falciparum* invasion rate, and replacement of RBC^{Y} with RBC^{O} decreases invasion rate. RBC^{Y} and RBC^{O} were isolated and invasion experiments performed as previously described with RBC^{Y} and RBC^{O}. RBC^{IR}, RBC^{Y}, and RBC^{O} were inoculated in isolation and RBC^{Y} and RBC^{O} were additionally combined at ratios of 1:10, 1:5, 1:3, 1:1, 3:1, 5:1, and 10:1. Data is from a representative experiment of three independent experiments with *P. falciparum* strains 3D7 and FCR3-FMG. Bars represent the rate of *P. falciparum* strain FCR-FMG invasion with parasite inoculums of (A) $0.3x10^{6}$ and (B) $0.6x10^{6}$ pRBCs into each respective RBC condition, with the contribution of RBC^{O} and RBC^{Y} to the total rate of invasion denoted by the white and gray bars respectively. Error bars represent the standard deviation.

Variable	Iron Deficiency Anemia (IDA) (N = 7)	Iron Deficiency Anemia after Iron supplementation (IDA-Fe) (N = 6)	Iron Replete (IR) (N = 10)	Iron Replete after 1 month Iron Supplementation (IR-Fe) (N = 4)	Iron Replete after 2 months Iron Supplementation (IR-Fe) (N = 4)
White Blood Cell ($x10^9$/L)	4.84 (1.78)	4.45 (1.15)	6.40 (1.61)	6.3 (1.41)	6.2 (1.12)
Red Blood Cell ($x10^{12}$/L)	3.96 (0.60)	3.42 (0.35)	4.78 (0.52)	4.94 (0.52)	4.91 (0.39)
Hemoglobin (g/dL)	8.2 (1.56)	8.73 (1.23)	14.60 (1.40)	14.9 (0.57)	14.63 (0.53)
Haematocrit (%)	28.13 (4.32)	29.11 (4.25)	42.80 (4.31)	43.85 (2.62)	42.88 (2.81)
Mean Corpuscular Volume (fL)	71.20 (6.63)	84.78 (8.28)	89.80 (2.74)	89.67 (4.24)	87.75 (2.50)
Mean Corpuscular Hemoglobin (Pg)	20.79 (2.54)	25.50 (2.35)	30.70 (0.95)	30.5 (2.12)	30.0 (1.83)
Mean Corpuscular Hemoglobin Content (g/dL)	29.20 (1.50)	30.27 (0.86)	34.20 (0.63)	34.50 (0.71)	34.25 (0.96)
Red Cell Distribution Width (%)	17.41 (1.44)	18.13 (2.71)	13.22 (0.85)	13.90 (0.28)	13.2 (0.45)
Mean Platelet Volume (fL)	8.56 (1.05)	8.78 (0.65)	7.63 (0.54)	8.45 (0.92)	8.15 (0.52)
Platelet Count ($x10^9$/L)	294.57 (59.70)	344.0 (168.63)	251.80 (36.70)	212.5 (89.80)	256 (79.31)
Iron Total (mg/dL)	21.60 (10.55)	32.0 (16.79)	103.60 (43.34)	105.75 (32.71)	87.5 (17.99)
Transferrin (mg/dL)	343.60 (74.72)	305.20 (63.55)	264.30 (41.33)	275 (29.50)	276.75 (31.83)
TIBC (mg/dL)	438.67 (92.08)	384.60 (80.29)	333.0 (52.20)	346.0 (37.43)	348.75 (40.07)
Transferrin Saturation (%)	4.71 (3.15)	8.0 (7.45)	31.60 (13.04)	31.0 (9.49)	25.75 (7.63)

Ferritin (ng/mL)	5.71 (2.75)	17.17 (25.44)	42.01 (24.28)	46.75 (38.75)	33.33 (18.45)
Reticulocyte (%)	1.42 (0.55)	3.52 (1.62)	1.48 (0.48)	2.4 (0.71)	1.4 (0.38)
Reticulocyte Hemoglobin (Pg)			32.03 (1.34)	31.05 (0.35)	32.25 (1.23)

Table 4.1 – Iron status of donors enrolled in IDA, IDA-Fe, IR, and IR-Fe study groups. Data are presented as means with standard deviation. Tests were performed by McClendon Clinical Laboratory on samples taken from each donor at the same time points that the blood samples were drawn for the assays described in Figures 1 (IDA individuals) 2A (IR individuals on iron supplementation) and 2B (IDA individuals on iron supplementation).

REFERENCES

Berkley, J. A., Bejon, P., Mwangi, T., Gwer, S., Maitland, K., Williams, T. N., Mohammed, S., Osier, F., Kinyanjui, S., Fegan, G., et al. (2009). HIV infection, malnutrition, and invasive bacterial infection among children with severe malaria. *Clin. Infect. Dis.* 49, 336–343.

Bosch, F. H., Werre, J. M., Roerdinkholder-Stoelwinder, B., Huls, T. H., Willekens, F. L., and Halie, M. R. (1992). Characteristics of red blood cell populations fractionated with a combination of counterflow centrifugation and Percoll separation. *Blood* 79, 254–260.

Brandão, M. M., Castro, M. de L. R. B., Fontes, A., Cesar, C. L., Costa, F. F., and Saad, S. T. O. (2009). Impaired red cell deformability in iron deficient subjects. *Clin. Hemorheol. Microcirc.* 43, 217–221.

Bratosin, D., Mitrofan, L., Palii, C., Estaquier, J., and Montreuil, J. (2005). Novel fluorescence assay using calcein-AM for the determination of human erythrocyte viability and aging. *Cytometry A* 66, 78–84.

Bronzan, R. N., Taylor, T. E., Mwenechanya, J., Tembo, M., Kayira, K., Bwanaisa, L., Njobvu, A., Kondowe, W., Chalira, C., Walsh, A. L., et al. (2007). Bacteremia in Malawian children with severe malaria: prevalence, etiology, HIV coinfection, and outcome. *J. Infect. Dis.* 195, 895–904.

Bunyaratvej, A., Butthep, P., Sae-Ung, N., Fucharoen, S., and Yuthavong, Y. (1992). Reduced deformability of thalassemic erythrocytes and erythrocytes with abnormal hemoglobins and relation with susceptibility to Plasmodium falciparum invasion. *Blood* 79, 2460–2463.

Chotivanich, K., Udomsangpetch, R., Simpson, J. A., Newton, P., Pukrittayakamee, S., Looareesuwan, S., and White, N. J. (2000). Parasite multiplication potential and the severity of Falciparum malaria. *J. Infect. Dis.* 181, 1206–1209.

Clark, M., Fisher, N. C., Kasthuri, R., and Cerami Hand, C. (2013). Parasite maturation and host serum iron influence the labile iron pool of erythrocyte stage Plasmodium falciparum. *Br. J. Haematol.*

Cromer, D., Stark, J., and Davenport, M. P. (2009). Low red cell production may protect against severe anemia during a malaria infection--insights from modeling. *J. Theor. Biol.* 257, 533–542.

Drakesmith, H., and Prentice, A. M. (2012). Hepcidin and the iron-infection axis. *Science* 338, 768–772.

Esposito, A., Choimet, J.-B., Skepper, J. N., Mauritz, J. M. A., Lew, V. L., Kaminski, C. F., and Tiffert, T. (2010). Quantitative imaging of human red blood cells infected with Plasmodium falciparum. *Biophys. J.* 99, 953–960.

Goma, J., Rénia, L., Miltgen, F., and Mazier, D. (1996). Iron overload increases hepatic development of Plasmodium yoelii in mice. *Parasitology* 112 (Pt 2), 165–168.

Gwamaka, M., Kurtis, J. D., Sorensen, B. E., Holte, S., Morrison, R., Mutabingwa, T. K., Fried, M., and Duffy, P. E. (2012). Iron deficiency protects against severe Plasmodium falciparum malaria and death in young children. *Clin. Infect. Dis.* 54, 1137–1144.

Haley, K. P., and Skaar, E. P. (2012). A battle for iron: host sequestration and Staphylococcus aureus acquisition. *Microbes Infect.* 14, 217–227.

Jonker, F. A. M., Calis, J. C. J., van Hensbroek, M. B., Phiri, K., Geskus, R. B., Brabin, B. J., and Leenstra, T. (2012). Iron status predicts malaria risk in Malawian preschool children. *PLoS ONE* 7, e42670.

Kabyemela, E. R., Fried, M., Kurtis, J. D., Mutabingwa, T. K., and Duffy, P. E. (2008). Decreased susceptibility to Plasmodium falciparum infection in pregnant women with iron deficiency. *J. Infect. Dis.* 198, 163–166.

Koka, S., Föller, M., Lamprecht, G., Boini, K. M., Lang, C., Huber, S. M., and Lang, F. (2007). Iron deficiency influences the course of malaria in Plasmodium berghei infected mice. *Biochem. Biophys. Res. Commun.* 357, 608–614.

Lim, C., Hansen, E., Desimone, T. M., Moreno, Y., Junker, K., Bei, A., Brugnara, C., Buckee, C. O., and Duraisingh, M. T. (2013). Expansion of host cellular niche can drive adaptation of a zoonotic malaria parasite to humans. *Nat Commun* 4, 1638.

Matsuzaki-Moriya, C., Tu, L., Ishida, H., Imai, T., Suzue, K., Hirai, M., Tetsutani, K., Hamano, S., Shimokawa, C., and Hisaeda, H. (2011). A critical role for phagocytosis in resistance to malaria in iron-deficient mice. *Eur. J. Immunol.* 41, 1365–1375.

McLean, E., Cogswell, M., Egli, I., Wojdyla, D., and de Benoist, B. (2009). Worldwide prevalence of anaemia, WHO Vitamin and Mineral Nutrition Information System, 1993–2005. *Public Health Nutrition* 12, 444–454.

McQueen, P. G., and McKenzie, F. E. (2004). Age-structured red blood cell susceptibility and the dynamics of malaria infections. *Proc. Natl. Acad. Sci. U.S.A.* 101, 9161–9166.

Nweneka, C. V., Doherty, C. P., Cox, S., and Prentice, A. (2010). Iron delocalisation in the pathogenesis of malarial anaemia. *Trans. R. Soc. Trop. Med. Hyg.* 104, 175–184.

Nyakeriga, A. M., Troye-Blomberg, M., Dorfman, J. R., Alexander, N. D., Bäck, R., Kortok, M., Chemtai, A. K., Marsh, K., and Williams, T. N. (2004). Iron deficiency and malaria among children living on the coast of Kenya. *J. Infect. Dis.* 190, 439–447.

Pasvol, G., Weatherall, D. J., and Wilson, R. J. (1980). The increased susceptibility of young red cells to invasion by the malarial parasite Plasmodium falciparum. *Br. J. Haematol.* 45, 285–295.

Portugal, S., Carret, C., Recker, M., Armitage, A. E., Gonçalves, L. A., Epiphanio, S., Sullivan, D., Roy, C., Newbold, C. I., Drakesmith, H., et al. (2011). Host-mediated regulation of superinfection in malaria. *Nat. Med.* 17, 732–737.

Prentice, A. M. (2008). Iron metabolism, malaria, and other infections: what is all the fuss about? *J. Nutr.* 138, 2537–2541.

Price, R. N., Simpson, J. A., Nosten, F., Luxemburger, C., Hkirjaroen, L., ter Kuile, F., Chongsuphajaisiddhi, T., and White, N. J. (2001). Factors contributing to anemia after uncomplicated falciparum malaria. *Am. J. Trop. Med. Hyg.* 65, 614–622.

Ribaut, C., Berry, A., Chevalley, S., Reybier, K., Morlais, I., Parzy, D., Nepveu, F., Benoit-Vical, F., and Valentin, A. (2008). Concentration and purification by magnetic separation of the erythrocytic stages of all human Plasmodium species. *Malar. J.* 7, 45.

Sazawal, S., Black, R. E., Ramsan, M., Chwaya, H. M., Stoltzfus, R. J., Dutta, A., Dhingra, U., Kabole, I., Deb, S., Othman, M. K., et al. (2006). Effects of routine prophylactic supplementation with iron and folic acid on admission to hospital and mortality in preschool children in a high malaria transmission setting: community-based, randomised, placebo-controlled trial. *Lancet* 367, 133–143.

Scholl, P. F., Tripathi, A. K., and Sullivan, D. J. (2005). Bioavailable iron and heme metabolism in Plasmodium falciparum. *Curr. Top. Microbiol. Immunol.* 295, 293–324.

Schümann, K., Kroll, S., Romero-Abal, M.-E., Georgiou, N. A., Marx, J. J. M., Weiss, G., and Solomons, N. W. (2012). Impact of oral iron challenges on circulating non-transferrin-bound iron in healthy Guatemalan males. *Ann. Nutr. Metab.* 60, 98–107.

Senga, E. L., Harper, G., Koshy, G., Kazembe, P. N., and Brabin, B. J. (2011). Reduced risk for placental malaria in iron deficient women. *Malar. J.* 10, 47.

Shaw, J. G., and Friedman, J. F. (2011). Iron Deficiency Anemia: Focus on Infectious Diseases in Lesser Developed Countries. *Anemia* 2011. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3124144/ [Accessed November 2, 2012].

Skaar, E. P. (2010). The battle for iron between bacterial pathogens and their vertebrate hosts. *PLoS Pathog.* 6, e1000949.

Soofi, S., Cousens, S., Akhund, T., Khan, J., Ahmed, I., Zaidi, A., and Bhutta, Z. (2013). Effect of provision of daily zinc and iron with several micronutrients on growth and morbidity among young children in Pakistan: a cluster-randomised trial. *Lancet* in press.

Theron, M., Hesketh, R. L., Subramanian, S., and Rayner, J. C. (2010). An adaptable two-color flow cytometric assay to quantitate the invasion of erythrocytes by Plasmodium falciparum parasites. *Cytometry A* 77, 1067–1074.

Tian, L. P., Nelson, E. A., Senok, A. C., Yu, L. M., Oppenheimer, S. J., and Li, K. (1998). Red cell age and susceptibility to malaria during pregnancy. *Acta Obstet Gynecol Scand* 77, 717–721.

Tiffert, T., Lew, V. L., Ginsburg, H., Krugliak, M., Croisille, L., and Mohandas, N. (2005). The hydration state of human red blood cells and their susceptibility to invasion by Plasmodium falciparum. *Blood* 105, 4853–4860.

Veenemans, J., Milligan, P., Prentice, A. M., Schouten, L. R. A., Inja, N., van der Heijden, A. C., de Boer, L. C. C., Jansen, E. J. S., Koopmans, A. E., Enthoven, W. T. M., et al. (2011). Effect of supplementation with zinc and other micronutrients on malaria in Tanzanian children: a randomised trial. *PLoS Med.* 8, e1001125.

Wilson, R. J. M., Pasvol, G., and Weatherall, D. J. (1977). Invasion and growth of Plasmodium falciparum in different types of human erythrocyte. *Bull World Health Organ* 55, 179–185.

Yermiahu, T., Ben-Shalom, M., Porath, A., Vardi, H., Boantza, A., Mazor, D., and Meyerstein, N. (1999). Quantitative determinations of microcytic-hypochromic red blood cell population and glycerol permeability in iron-deficiency anemia and beta thalassemia minor. *Ann. Hematol.* 78, 468–471.

Yip, R., Mohandas, N., Clark, M. R., Jain, S., Shohet, S. B., and Dallman, P. R. (1983). Red cell membrane stiffness in iron deficiency. *Blood* 62, 99–106.

Zhao, H., Konishi, A., Fujita, Y., Yagi, M., Ohata, K., Aoshi, T., Itagaki, S., Sato, S., Narita, H., Abdelgelil, N. H., et al. (2012). Lipocalin 2 bolsters innate and adaptive immune responses to blood-stage malaria infection by reinforcing host iron metabolism. *Cell Host Microbe* 12, 705–716.

CHAPTER FIVE

Discussion

5.1 Where we stand in the grand scheme of things

Iron supplementation campaigns effectively address iron deficiency, a significant cause of morbidity and mortality. However, the confounding relationship between malaria, iron deficiency, and iron supplementation has disrupted iron supplementation campaigns in regions where malaria is endemic. I have attempted to gain insight into the biological processes that result in the reduced incidence of malaria in iron deficient individuals and the subsequent increased risk associated with iron supplementation. My studies have focused on the relationship between host iron status and the pathogenic erythrocytic stage of malaria infection.

5.2 Determining the impact of host iron status on virulence of erythrocytic stage *P. falciparum*

Transferrin and ferric citrate, which are found in human serum, contribute to the bioavailable iron of erythrocytic stage P. falciparum.

Nearly every living organism, including humans and most human pathogens, requires iron to survive Furthermore, iron availability is a determinant of virulence for many human pathogens (Drakesmith and Prentice, 2012). As a result, iron sequestration is a primary innate immune response to microbial insult (Ganz, 2012). Although iron is also essential for the malaria parasite, whether the availability of host iron influences erythrocytic stage *P. falciparum* growth and virulence is unclear. Following in the footsteps of previous studies, I began by assessing the effect of extracellular iron (in the form of either transferrin or ferric citrate) on *P. falciparum*

growth *in vitro*. The approach taken by previous groups had primarily been to deplete *in vitro* culture conditions of iron (Peto and Thompson, 1986c; Scott, 1990; Schaze-Lopez, 1992b). However, upon realizing that malaria culture media contains only ten percent of the iron of human sera, I decided to study the effect of supplementing culture media with physiologic levels of either transferrin or ferric citrate on *P. falciparum* growth *in vitro*. In the course of nearly twenty independent experiments, our investigation resulted in each possible outcome: increased *P. falciparum* growth, no effect on *P. falciparum* growth, and decreased *P. falciparum* growth (**Figure 2.1**). Summarily leaving as much in the dark as when I had begun.

Not being able to confidently make heads over tails of the effect of transferrin or ferric citrate on *P. falciparum* growth, I decided to take a step back. From the literature and our own observations (**Figure 2.6B**), I knew without a doubt that transferrin specifically associates with pRBCs but not uninfected RBCs (Pollack and Fleming, 1984b; Rodriguez, 1986b; Haldar, 1986; Pollack and Schnelle, 1988b; Fry, 1989; Surolia, 1996b). It was not however as clear whether extracellular iron (in the form of transferrin or ferric citrate) was actively incorporated into pRBCs. Therefore, to investigate whether host serum iron is accessed by erythrocytic stage *P. falciparum*, I developed a flow cytometry method for assessing the bioavailable iron content of pRBCs *in vitro* (**Figure 2.4**). Under standard culture conditions, I observed that pRBCs contain more bioavailable iron than uninfected RBCs and that bioavailable iron levels increase with parasite maturation (**Figure 2.5)**. Additionally, when physiologic levels of either transferrin or ferric citrate were added to culture media, the bioavailable iron content of trophozoite stage pRBCs further increased. The bioavailable content of early ring stage pRBCs or uninfected RBCs did not change (**Figure 2.6A**). This work clearly demonstrates that the bioavailable iron content of pRBCs is dynamic and changes with parasite maturation. As to the relationship

between extracellular iron and *P. falciparum* I for the first time show that both transferrin and ferric citrate extracellular iron are incorporated into the bioavailable iron pool of pRBCs.

Unraveling erythrocytic stage P. falciparum iron biology and the parasites dependence on host iron.

With regards to the clinical observations linking host iron with susceptibility to malaria infection; it remains unclear whether the availability of host iron dictates *P. falciparum* growth and virulence. Some researchers in the field hypothesize that transient increases in serum NTBI that occur with iron supplementation exacerbate *P. falciparum* infection. The inability to observe an effect of either iron depletion or iron supplementation of culture media on *P. falciparum* growth; would appear to refute this hypothesis. That said, it is important to note that every study to date has been performed *in vitro* with culture adapted strains of *P. falciparum*, and as discussed previously this may be inhibiting our ability to detect an effect of host iron on *P. falciparum* growth and virulence. It is my humble opinion that only more careful investigation in the spirit of my own bioavailable iron work will reveal the true relationship between host iron and erythrocytic stage *P. falciparum*.

Despite being unable to definitively determine whether specifically extracellular iron promotes erythrocytic stage *P. falciparum* growth, my work provides important insight into *P. falciparum* iron biology. Our discovery the bioavailable iron content of pRBCs increases with parasite maturation leads to several intriguing questions (**Table 5.1**). Of these, two lines of inquiry stand out as most pertinent for determining the impact of host iron on *P. falciparum* pathogenesis: (1) what host iron is accessed by erythrocytic stage *P. falciparum* and how does the parasite utilize this iron; and (2) do the increased bioavailable iron levels of pRBCs reflect an increased demand for iron by the parasite, or are they a result of the massive breakdown of host

hemoglobin by the parasite? Although I have not yet experimentally pursued these questions, I have several ideas for doing so.

To determine which host iron sources are accessed and utilized by erythrocytic stage *P. falciparum*, I would employ an approach based on the one taken by Skaar et al. in which isotope-labeled iron nutrient compounds added to *in vitro* parasite cultures are followed by inductively coupled plasma mass spectrometry (ICP-MS)(Skaar et al., 2004). I would begin by focusing on transferrin and hemoglobin, each containing different iron isotopes. Adding transferrin to culture media would be sufficient, while generating RBC ghosts and resealing in the presence of isotype-labeled hemoglobin would be necessary to replace endogenous hemoglobin. The ratio of transferrin to hemoglobin iron in the parasite would then be followed from ring to schizony as well as the merozoite stage of the parasite to shed light on the importance of these host iron sources in parasite growth. Furthermore, to determine the subcellular localization of any incorporated host iron, I would isolate parasite organelles (food vacuole, mitochondria, apicoblast, and nucleus) and use ICP-MS to determine the isotopic ratio in each compartment. I would couple these experiments with RNAseq analysis of parasite gene expression in order to begin mapping the erythrocytic stage *P. falciparum* transcriptional response to iron.

To determine whether the elevated bioavailable iron content of trophozoite and schizont stage pRBCs reflects (i) increased parasitic iron demands, or (ii) excess free iron that is contributing to the elevated ROS observed in late stage parasites, I would begin by expanding on the work of Loyevsky et al. that identified *P. falciparum* iron responsive protein (IRPs) and iron responsive elements (IREs) (Hodges et al., 2005; Loyevsky et al., 2001, 2003). IRPs and IREs are the primary mediators of cellular iron homeostasis in mammalian cells (Hentze et al., 2010) Specifically, I would monitor (i) PfIRP and PfIRE binding activity, and (ii) PfIRP iron sulfur

complexes and PfIRP proteosomal degradation of synchronized parasites as they mature from ring stage to schizony. Increased binding of PfIREs by PfIRP would support a hypothesis that parasite iron demands are elevated, and that the parasite is working to orchestrate iron uptake and most likely release of iron from storage. However, an observation of either increased PfIRP iron sulfur complexes or PfIRP proteosomal degradation would support a hypothesis that plenty of iron is available and in fact the parasite is actively attempting to limit the cytotoxic effects of excess iron that accumulates presumably from the parasite breakdown of host hemoglobin. Similar study of PfIRPs and PfIREs in the context of iron deficiency (RBC^{IDA} vs. RBC^{IR}) and iron supplementation (addition of excess extracellular iron) would inform our understanding of whether iron in and of itself a critical factor in the protection conferred by iron deficiency and the increased risk associated with iron supplementation with regards to erythrocytic stage *P. falciparum* infection.

Ultimately, these two approaches would be invaluable in their ability to shed light on how the parasite regulates iron, whether the parasite is able to store iron, and most critically, whether host iron directly influences parasite virulence. Towards the goal of unraveling the details of parasite iron biology, it may be pertinent to study recent clinical isolates in parallel with well-characterized traditional lab strains of *P. falciparum.* As mentioned previously, malaria culture media contains tenfold less iron than human sera. Therefore, it will be important to determine whether lab isolates have adapted to an iron deficient extracellular environment so that ultimately only the most relevant model systems are used in further studies of the relationship between host iron status, parasite iron biology, and malaria pathogenesis.

5.3 Defining the influence of changing host RBC population dynamics on erythrocytic stage *P. falciparum* infection, specifically in the context of host iron deficiency and iron supplementation.

Microcytic iron deficient RBCs are inhospitable to erythrocytic stage P. falciparum.

The erythrocyte is absolutely essential for propagation of *P. falciparum* infection. As a result of the pressure malaria has placed on the human population, a wide range of heritable genetic mutations have arisen (Duffy negative, hemoglobinopathies, G6PD deficiency) which protect the human hosts RBCs from malaria infection. Giving testament to the importance of the human RBC in malaria infection (Taylor et al., 2013). Iron deficiency and iron supplementation both alter RBC physiology as well as the dynamics of the total RBC population. It has been speculated that it is the effect iron deficiency and iron supplementation have on erythropoiesis and RBC physiology which is responsible for the observed relationship between iron deficiency, iron supplementation, and severity of malaria infection (Oppenheimer et al., 1986; Cromer et al., 2009). To experimentally assess this possibility, I began by assessing the growth of *P. falciparum* in RBCs donated by individuals with iron deficiency anemia. I observed that *P. falciparum* growth is attenuated in iron deficient RBCs due to reduced RBC invasion of iron deficient RBCs as well as reduced production of invasive daughter merozoites within iron deficient RBCs (**Figure 4.2A**). These findings are in direct support of the clinical observations that iron deficiency is protective against malaria infection, and support the hypothesis that iron deficiency is protective in part because of the effect of iron deficiency on RBC physiology.

Changing age and iron status distribution of a RBC population directly effects erythrocytic stage P. falciparum infection in vitro.

I have additionally sought to determine the impact of iron supplementation on erythrocytic stage *P. falciparum.* To this end I compared the growth of *P. falciparum* within RBC donated by either iron supplemented iron-replete or iron supplemented iron deficient

donors to growth within RBCs from non-supplemented iron-replete donors. I observed that *P. falciparum* growth was slightly elevated within RBCs from both iron supplemented iron-replete as well as iron supplemented iron deficient donors (**Figure 4.3D**). Examination of the iron status of the iron deficient iron supplemented donors revealed that individuals in this group were not yet fully recovered from their iron deficiency anemia and were undergoing reticulocytosis (**Table 4.1**). This information prompted us to speculate that the replacement of iron deficient RBCs with young iron-replete RBCs may be responsible for the increased growth of the parasite.

Like many species of *Plasmodium, P. falciparum* preferentially infects young RBCs and mathematical modeling has predicted that elevated reticulocytosis increases the risk of hyperparasitemia and severe infection (Cromer et al., 2009) In order to investigate the effect of changing iron status and age distribution of a RBC population on susceptibility to erythrocytic stage *P. falciparum* infection, I developed a three-color invasion assay, which allows for study of the relationship between different RBC populations and *P. falciparum* infection (**Figure 3.1 and 3.3**). With the three-color invasion assay, I examined the relationship between (i) iron deficient and iron-replete RBCs and (ii) young and old iron-replete RBCs. These studies clearly demonstrated that changing the iron status and age distribution of a RBC population significantly impacts erythrocytic stage *P. falciparum* infection (**Figure 4.5 and 4.8)**. Together these data are consistent with clinical observations and furthermore support the hypothesis that the protection provided by iron deficiency against malaria and the increased risk of malaria associated with iron supplementation is cell mediated (**Figure 4.4**).

Pursuing mechanisms by which microcytic iron deficient RBCs are protective against P. falciparum infection.

Any new observation is a treasure trove for new avenues of investigation. Some of the questions I find most intriguing with regards to my finding that iron deficient RBCs are

protective against malaria infection are outlined in **Table 5.2**. Probably the most obvious line of investigation to pursue is the identification of the characteristics of iron deficient RBCs which (i) protect the cells from *P. falciparum* invasion and (ii) limit the parasites capacity to produce infectious daughter merozoites. In order to pursue the mechanisms governing reduced rate of invasion of *P. falciparum* into iron deficient RBCs, I would begin with careful microscopic analysis of *P. falciparum* invasion into iron deficient RBCs . This approach would allow me to determine which step of parasite invasion (RBC attachment, parasite reorientation, or tight junction formation and RBC entry) is being impeded. The results of such a study would inform the direction of further experimentation, specifically whether iron deficient RBC surface ligands or the mechanical properties of iron deficient RBCs should be further pursued.

With regard to growth of the parasite within iron deficient RBCs, I would begin with RNAseq analysis of the gene expression of parasites within iron deficient as compared to parasites within iron-replete RBCs. This approach would allow for the potential identification of metabolic factors such as iron that are limited within iron deficient RBCs or parasite systems such as the redox system that are unable to operate optimally within iron deficient RBCs, and ultimately limit intra-erythrocytic growth of the parasite. It is alternatively possible that the smaller volume of iron deficient RBCs limits the ability of *P. falciparum* to produce daughter merozoites. To assess this possibility, I would utilize methods involving resealed RBC ghosts to systematically increase the MCV of iron deficient RBCs. If daughter merozoite production is recovered in larger iron deficient RBCs, I would conclude the smaller size of iron deficient RBCs limits the number of *P. falciparum* daughter merozoites.

Finally, it is important to consider that *P. falciparum* has considerable genetic diversity and that *P. falciparum* isolates may exist that exhibit normal invasion of and growth within iron

deficient RBCs. The identification of such a parasite isolate would be highly useful, as it would allow for a genetic cross of an iron deficient RBC sensitive parasite with an iron deficient RBC resistant parasite and subsequent identification of genes that confer either sensitivity or resistance to iron deficient RBCs. Alternatively, a resistant parasite strain could be generated by adapting a parasite to growth in iron deficient RBCs, and would similarly allow for identification of genes that determine the sensitivity of the malaria parasite to iron deficient RBCs.

Pursuing the effect of changing RBC population dynamics in the human host on susceptibility to malaria infection in vivo.

Clinical studies suggest iron supplementation in malaria endemic areas may increase the risk of malaria infection in previously iron deficient individuals beyond that of a normal iron-replete subjects (Veenemans et al., 2011; Esan et al., 2013). Here I provide experimental evidence to support the hypothesis that replacing iron deficient with iron-replete RBCs supports the recovery of *P. falciparum* infection, and that individuals with on average younger iron-replete RBC population are at increased risk of *P. falciparum* infection (**Figure 4.5 and 4.8**). A caveat to our studies is that they all rely upon *in vitro* experimentation to study the relationship between these different RBC populations and *P. falciparum.* To address this limitation, I would begin by focusing on the identification of biomarkers that would allow for the classification of intact *P. falciparum* infected RBCs by age and iron status by flow cytometry. This would allow for *ex vivo* assessment of parasite distribution in RBCs from *P. falciparum* infected individuals and the definitive determination of the impact of iron deficient and young iron-replete RBCs on susceptibility to erythrocytic stage *P. falciparum* infection.

Evidence has begun to build that additional unique RBC populations may be in play in the context of iron deficiency and iron supplementation. The data suggests that RBCs produced during steady state erythropoiesis are physiologically different than RBCs produced during stress

erythropoiesis, as occurs following iron supplementation of individuals with iron deficiency anemia (Ramos et al., 2013). Therefore, I would be extremely interested in studying the susceptibility of RBCs produced during stress erythropoiesis to *P. falciparum* infection. Determining whether stress erythropoiesis differentially affects malaria susceptibility may be critical in definitively explaining the risk of iron supplemented individuals to malaria infection.

Finally, our approach of directly comparing *P. falciparum* infection of two different RBC populations with a three-color invasion assay has revealed for the first time that *P. falciparum* infection of a RBC population can be dependent upon the other RBC populations present. First and foremost I would want to determine whether this occurs *in vivo*. I would begin this line of investigation by applying our three-color invasion assay to the murine model of malaria infection. If in fact our *in vitro* observations holds up *in vivo*, I would next focus every effort on determining how and why different RBC populations affect one another's susceptibility to malaria infection, and finally how this fact shapes host susceptibility to *P. falciparum* infection (**Table 5.3**).

5.4 Beyond iron as a growth factor and mediator of RBC population dynamics – exploration of alternate mechanisms governing the relationship between iron deficiency, iron supplementation and malaria.

Through the course of my dissertation I have focused exclusively on two hypotheses: (i) iron deficiency and iron supplementation affect *P. falciparum* infection by respectively limiting and providing nutritional iron, which is essential for parasite propagation; and (ii) the effect of iron deficiency and iron supplementation on erythropoiesis and erythrocyte physiology are what shape the host's respective resistance and increased susceptibility to *P. falciparum* erythrocytic stage infection. Though our investigation of both hypotheses has shed considerable light on possible mechanisms governing the dynamics of host iron status and malaria susceptibility, there

are very likely other factors at play in the relationship between iron deficiency, iron supplementation, and malaria infection. For instance, work by several groups has clearly shown that host iron status also shapes the liver stage of *P. falciparum* infection (Portugal et al., 2011; Goma et al., 1995, 1996). In the context of the erythrocytic stage of infection alone there are several factors beyond parasite growth that determine virulence. Moreover, in malaria endemic areas, host iron status is additionally affected by chronic infections as well as heritable RBC disorders (hemoglobinopathies and G6PD deficiency). The respective contribution of anemia of chronic disease, hemoglobinopathies, and nutritional iron deficiency to the protection from malaria associated with iron deficiency and increased risk associated with iron supplementation is completely unknown.

Probing the effect of iron deficiency and iron supplementation on important mediators of malaria pathogenesis – phagocytosis of pRBCs and pRBC endothelial cytoadhesion

With respect to the erythrocytic stage of infection, there are several additional processes beyond erythrocytic parasite propagation that are affected by host iron status and have the potential to shape malaria pathogenesis (**Table 5.4**). Two processes I would be extremely interested in investigating are (i) phagocytosis of iron deficient pRBCs and (ii) endothelial cytoadherence and rosetting of iron deficient pRBCs. Macrophages, the human host's primary phagocytic cell, are critical in both malaria infection and the reticuloendothelial system – where macrophages recover and recycle iron from senescent RBCs (Drakesmith and Prentice, 2012; Chua et al., 2013; Ganz, 2012). Furthermore, iron levels can directly impact macrophage activity (Recalcati et al., 2012; den Haan and Kraal, 2012). Work in a murine model of iron deficiency and malaria infection reported elevated phagocytosis of iron deficient pRBCs (Koka et al., 2007). To build upon this line of investigation, I would begin by determining whether human iron deficient pRBCs are phagocytosed to a greater extent than iron-replete pRBCs. I would

additionally want to characterize macrophage polarization and function in the context of iron deficiency and subsequent malarial infection.

The second mediator of malaria pathogenesis I would be extremely interested in examining is parasite sequestration -- infected RBCs sequester in the microvasculature of the human host by adhering to the host endothelium. *P. falciparum* export of proteins that mediate cytoadherence is attenuated in RBCs from individuals with hemoglobinopathies (Taylor et al., 2013). As iron deficiency anemia has similar physiologic effects on RBCs as hemoglobinopathies, I would speculate that iron deficient pRBCs may similarly exhibit defects in the export and display of parasite cytoadherence proteins. To assess this possibility, I would compare the abundance and distribution of parasite cytoadherence proteins on the surface of iron deficient and iron-replete pRBCs by microscopy and flow cytometry. I would additionally probe functional endothelial cytoadherence of iron deficient pRBCs by quantitatively comparing cytoadherence of iron deficient and iron-replete pRBCs to a human endothelial cell line. It is additionally possible that host iron status may influence endothelial function. To assess this possibility I would also perform endothelial cytoadherence assays with iron starved as well as iron supplemented endothelial monolayers.

5.5 Looking forward

The body of work pertaining to human iron biology produced in the past ten years has revealed iron regulation to be a major component of the human host's response to infectious and non-infectious disease (Roy and Andrews, 2005; Drakesmith and Prentice, 2012). In the course of its infection of the human host, *P. falciparum* takes up residence within the two most iron rich environments of the body, the liver (where iron is stored) and the RBC. Furthermore, *P. falciparum* propagation and subsequent transmission are directly dependent on erythropoiesis,

which is itself directly dependent upon iron. I know that host iron status is linked to malaria susceptibility and that during infection the parasite interacts intimately with the exact organs and cell types most closely involved in iron regulation in the human host. I also know malaria has placed significant pressure on human evolution, as evidenced by the emergence of hemoglobinopathies and other RBC mutations. Considering all the evidence together, the question arises whether malaria may in fact have shaped the evolution of the human iron regulatory and erythropoietic systems. In addition to being an exceptionally interesting area of study, further research on the relationship between host iron status and malaria will help the global health community reach the fundamental goal of devising a strategy to safely implement iron supplementation campaigns in malaria endemic regions.

Table 5.1 – Questions for future investigation of erythrocytic stage *P. falciparum* utilization of host iron.	
Observation	Questions
pRBCs contain more bioavailable iron than uninfected RBCS, and bioavailable iron content increases with parasite maturation from ring stage to schizony.	What intra-erythrocytic host iron sources are utilized by the malaria parasite?
	Where is the bioavailable iron localized to in pRBCs?
	Do changes in the amount of intra-erythrocytic iron (as would be observed in iron deficiency, iron supplementation or iron overload) affect malaria parasite growth and virulence?
	Can malaria merozoites sense host intra-erythrocytic iron?
	How does the malaria parasite regulate iron?
	Does the malaria parasite store iron?
Addition of transferrin or ferric citrate (NTBI) culture media increases the bioavailable iron content of trophozoite stage pRBCs but not ring stage pRBCs or uninfected RBCs.	How does the malaria parasite access and incorporate extracellular iron?
	Do changes in serum iron levels (as would be observed in iron deficiency, iron supplementation, or iron overload) affect malaria parasite growth and virulence?
	Is the association of transferrin with trophozoite and schizont stage pRBCs receptor mediated?
Neither depletion nor addition of iron to culture media affects erythrocytic stage *P. falciparum* propagation.	Have lab parasite isolates adapted to a low iron extracellular environment?
	Would field isolates be more sensitive to extracellular iron?

Table 5.2 – Questions for future investigation of the protection provided by microcytic iron deficient and old RBCs against erythrocytic stage *P. falciparum* infection.	
Observation	Questions
P. falciparum invasion of microcytic iron deficient and old RBCs is reduced.	What step of *P. falciparum* RBC invasion is impeded during invasion of microcytic iron deficient and old RBCs?
	What are the RBCs factors that result in reduced *P. falciparum* invasion of microcytic iron deficient and old RBCs?
	What merozoite invasion factors are being affected during invasion of IDA and old RBCS?
	Can *P. falciparum* merozoites differentiate between IDA and IR or young and old RBCs?
	Does decreased hematocrit affect *P. falciparum* infection?
P. falciparum production of invasion competent merozoites is reduced within microcytic iron deficient and old RBCs	Is hemoglobin a limiting resource in IDA RBCs?
	Does the smaller size of IDA and old RBCs limit parasite growth?
	Does the gene transcription profile of *P. falciparum* differ between IDA and IR or young and old RBCs?

Table 5.3 – Questions for future investigation of the relationship between host RBC population dynamics and erythrocytic stage *P. falciparum* infection.	
Observation	Questions
Replacement of microcytic iron deficient RBCs with young iron-replete RBCs increases the growth rate of erythrocytic stage *P. falciparum* infection.	What is the relationship between IDA and young IR RBCs in the context of *P. falciparum* invasion?
	Is the reduced hematocrit of IDA also protective against erythrocytic stage *P. falciparum* infection?
	How does hematocrit affect parasite biomass?
Elevated frequency of young iron-replete RBCs increases the risk of an iron-replete RBC population to erythrocytic stage *P. falciparum* invasion.	Are young RBCs formed during stress erythropoiesis different from young RBCs formed during steady stage erythropoiesis in their susceptibility to *P. falciparum* infection?
	Why does *P. falciparum* invasion rate of young iron-replete RBCs plateau?
What physiological properties may be utilized to differentiate IR from IDA RBCs and young from old RBCs on a cell by cell basis?	
Are effects of changing RBC population dynamics on erythrocytic stage *P. falciparum* infection that I observe in vitro also present in vivo?	

Table 5.4 – Future Questions.
Questions
Are parasitized IDA and old RBCs more likely to be cleared by the host?
Does iron deficiency and iron supplementation affect parasite cytoadhesion to host endothelium?
How are the host innate and adaptive immune responses to malaria affected by iron deficiency and iron supplementation?
What is the effect of host iron deficiency and iron supplementation on *P. falciparum* gametocytogenesis?
Is anemia of inflammation protective against malaria?
How does the presence of iron deficiency anemia modify the effects of HbS, HbC, or HbE on parasite growth, maturation, microvascular adhesion, or endothelial cell activation?

REFERENCES

Chua, C. L. L., Brown, G., Hamilton, J. A., Rogerson, S., and Boeuf, P. (2013). Monocytes and macrophages in malaria: protection or pathology? *Trends Parasitol.* 29, 26–34.

Cromer, D., Stark, J., and Davenport, M. P. (2009). Low red cell production may protect against severe anemia during a malaria infection--insights from modeling. *J. Theor. Biol.* 257, 533–542.

Drakesmith, H., and Prentice, A. M. (2012). Hepcidin and the iron-infection axis. *Science* 338, 768–772.

Esan, M. O., van Hensbroek, M. B., Nkhoma, E., Musicha, C., White, S. A., Ter Kuile, F. O., and Phiri, K. S. (2013). Iron supplementation in HIV-infected Malawian children with anemia: a double-blind, randomized controlled trial. *Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am.*

Ganz, T. (2012). Macrophages and systemic iron homeostasis. *J. Innate Immun.* 4, 446–453.

Goma, J., Renia, L., Miltgen, F., and Mazier, D. (1995). Effects of iron deficiency on the hepatic development of Plasmodium yoelii. *Parasite Paris Fr.* 2, 351–356.

Goma, J., Rénia, L., Miltgen, F., and Mazier, D. (1996). Iron overload increases hepatic development of Plasmodium yoelii in mice. *Parasitology* 112 (Pt 2), 165–168.

Den Haan, J. M. M., and Kraal, G. (2012). Innate immune functions of macrophage subpopulations in the spleen. *J. Innate Immun.* 4, 437–445.

Hentze, M. W., Muckenthaler, M. U., Galy, B., and Camaschella, C. (2010). Two to Tango: Regulation of Mammalian Iron Metabolism. *Cell* 142, 24–38.

Hodges, M., Yikilmaz, E., Patterson, G., Kasvosve, I., Rouault, T. A., Gordeuk, V. R., and Loyevsky, M. (2005). An iron regulatory-like protein expressed in Plasmodium falciparum displays aconitase activity. *Mol. Biochem. Parasitol.* 143, 29–38.

Koka, S., Föller, M., Lamprecht, G., Boini, K. M., Lang, C., Huber, S. M., and Lang, F. (2007). Iron deficiency influences the course of malaria in Plasmodium berghei infected mice. *Biochem. Biophys. Res. Commun.* 357, 608–614.

Loyevsky, M., LaVaute, T., Allerson, C. R., Stearman, R., Kassim, O. O., Cooperman, S., Gordeuk, V. R., and Rouault, T. A. (2001). An IRP-like protein from Plasmodium falciparum binds to a mammalian iron-responsive element. *Blood* 98, 2555–2562.

Loyevsky, M., Mompoint, F., Yikilmaz, E., Altschul, S. F., Madden, T., Wootton, J. C., Kurantsin-Mills, J., Kassim, O. O., Gordeuk, V. R., and Rouault, T. A. (2003). Expression of a recombinant IRP-like Plasmodium falciparum protein that specifically binds putative plasmodial IREs. *Mol. Biochem. Parasitol.* 126, 231–238.

Oppenheimer, S. J., Gibson, F. D., Macfarlane, S. B., Moody, J. B., Harrison, C., Spencer, A., and Bunari, O. (1986). Iron supplementation increases prevalence and effects of malaria: report on clinical studies in Papua New Guinea. *Trans. R. Soc. Trop. Med. Hyg.* 80, 603–612.

Portugal, S., Carret, C., Recker, M., Armitage, A. E., Gonçalves, L. A., Epiphanio, S., Sullivan, D., Roy, C., Newbold, C. I., Drakesmith, H., et al. (2011). Host-mediated regulation of superinfection in malaria. *Nat. Med.* 17, 732–737.

Ramos, P., Casu, C., Gardenghi, S., Breda, L., Crielaard, B. J., Guy, E., Marongiu, M. F., Gupta, R., Levine, R. L., Abdel-Wahab, O., et al. (2013). Macrophages support pathological erythropoiesis in polycythemia vera and β-thalassemia. *Nat. Med.* 19, 437–445.

Recalcati, S., Locati, M., Gammella, E., Invernizzi, P., and Cairo, G. (2012). Iron levels in polarized macrophages: regulation of immunity and autoimmunity. *Autoimmun. Rev.* 11, 883–889.

Roy, C. N., and Andrews, N. C. (2005). Anemia of inflammation: the hepcidin link. *Curr. Opin. Hematol.* 12, 107–111.

Skaar, E. P., Humayun, M., Bae, T., DeBord, K. L., and Schneewind, O. (2004). Iron-source preference of Staphylococcus aureus infections. *Science* 305, 1626–1628.

Taylor, S. M., Cerami, C., and Fairhurst, R. M. (2013). Hemoglobinopathies: slicing the Gordian knot of Plasmodium falciparum malaria pathogenesis. *PLoS Pathog.* 9, e1003327.

Veenemans, J., Milligan, P., Prentice, A. M., Schouten, L. R. A., Inja, N., van der Heijden, A. C., de Boer, L. C. C., Jansen, E. J. S., Koopmans, A. E., Enthoven, W. T. M., et al. (2011). Effect of supplementation with zinc and other micronutrients on malaria in Tanzanian children: a randomised trial. *PLoS Med.* 8, e1001125.