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**COMPLEMENT AND ANTIBODY-MEDIATED  
ENHANCEMENT OF RED BLOOD CELL  
INVASION BY PLASMODIUM FALCIPARUM**

A Dissertation in

Microbiology and Immunology

by

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## Abstract

*Plasmodium falciparum* malaria is responsible for close to one million deaths worldwide each year. The *Plasmodium* life cycle involves invasion of hepatocytes and erythrocytes. The repeating cycle of erythrocyte invasion and destruction is the root cause of the morbidity and mortality brought about by the pathogen. Development of naturally acquired immunity against the parasite is an extremely slow process taking years to decades to develop, and it does not seem to result in sterile immunity. Attempts to develop a vaccine to block erythrocyte invasion have proven unsuccessful. Clinical data have demonstrated the development of a strong humoral immune response in vaccinated individuals. Although antibodies raised against vaccine antigens inhibit invasion *in vitro* in the presence of heat-inactivated (HI) serum, there is poor efficacy *in vivo*. The reasons for this discrepancy are unknown. A number of studies, in both humans and animal models, have shown that the complement cascade is activated during malaria infection.

We hypothesize that complement activation and opsonization of merozoites enhances complement receptor 1 (CR1)-mediated invasion of erythrocytes. If we are correct, the parasite can hijack this arm of the innate immune response for its own benefit, avoiding or diminishing the efficacy of antibodies. We tested this hypothesis by studying the effect of mouse monoclonal anti-merozoite surface protein 1 (MSP-1) antibody 5.2 (mAb5.2) and antibodies from recipients of a merozoite vaccine (MSP1<sub>42</sub>) for their ability to enhance or inhibit invasion in a complement-dependent manner. We carried out invasion assays with the above antibodies with appropriate controls in fresh or HI serum, or in complement-depleted or reconstituted serum. In addition, we tested

the relevance of the CR1 pathway by inhibiting with sCR1. Invasion of erythrocytes was enhanced by fresh serum relative to 3 min, 5 min, or 30 min HI serum. Furthermore, addition of mAb5.2 to fresh serum increased the enhancement effect. Addition of C2 and Factor B to 3 min HI serum, but not to 30 min, rescued the enhancement of invasion in the presence of mAb5.2. Likewise, Compstatin, a C3 specific inhibitor, and sCR1 negated the enhancing effects of mAb5.2 in fresh serum but not that of fresh serum alone. Furthermore, soluble CR1 had invasion inhibitory activity in 3 min HI serum that was independent of antibody. Purified antibodies from MSP-1 vaccinees showed inhibitory activity in C3/C4 inactivated serum, but inhibition was drastically reduced in C3/C4 reconstituted serum. Passive transfer of anti-*P. berghei* antibodies into wild type mice enhanced parasitemia in an inverse dose-dependent manner. In addition, C3-deficient mice showed decreased parasite growth relative to wild type mice.

In addition, we utilized erythrocytes from individuals with paroxysmal nocturnal hemoglobinuria (PNH), which results in the absence of glycosylphosphatidylinositol (GPI)-anchored receptors, in an effort to identify novel receptors utilized by the parasite for erythrocyte invasion. We found that PNH RBCs were invaded less effectively than wild type RBCs. Therefore, we carried out growth and invasion assays with anti-GPI erythrocyte receptor antibodies against six potential targets with appropriate controls in fresh or HI serum, or in complement-depleted or reconstituted serum. We identified Semaphorin-7A (SEMA7A) as a potential erythrocyte receptor that may interact with complement in promoting erythrocyte invasion by merozoites.

Our results demonstrate that merozoites can hijack the complement system to their own advantage in a number of ways. Activation of the complement cascade via the classical pathway can allow merozoites to bind to CR1 and invade via this pathway. In addition, in the absence of antibodies, activation of the mannose binding lectin pathway by default may allow merozoites to bind to other complement receptors on the RBC that mediate invasion. Such mechanisms could explain or contribute to the low efficacy of vaccine-induced anti-merozoite antibodies to block invasion *in vivo*. These findings are of great importance to the efforts to develop a merozoite blocking vaccine.

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## Abbreviations

Ab	Antibody
ADCC	Antibody-dependent cell-mediated cytotoxicity
AMA1	Apical membrane antigen 1
AP	Alternative complement pathway
ASP	Apical sushi protein
BSG	Basigin, CD147
CD59	Protectin
CH50	Complement hemolytic activity
CIB1	Calcium and integrin-binding protein 1
CM	Cerebral malaria
CP	Classical complement pathway
CPD	Citrate phosphate dextrose solution
CR1	Complement Receptor 1, CD35
CRP	C-reactive protein
CVF	Cobra venom factor
CyRPA	Cysteine-rich protective antigen
DAF	Decay accelerating factor, CD55
DMR	Detergent-resistant membrane
DV	Digestive vacuoles
EBA	Erythrocyte binding antigens
EMP	Erythrocyte membrane protein
ELISA	Enzyme-linked immunosorbent assay
Fab	Fragment antigen-binding
fB	Factor B
Fc	Fragment crystallizable
FcR	Fc receptor
FcyR	Fcy receptors
fl	Factor I
Flt-1	Fms-like tyrosine kinase-1
FS	Fresh Serum
GIA	Growth inhibition assay
GlcNAc	N-acetyl glucosamine
Gly	Glycophrin
GPI	Glycosylphosphatidylinositol
Hct	Hematocrit
HIS	Heat inactivated serum
IC	Immune complex
ICAM-1	Intercellular adhesion molecule 1

IFN- $\gamma$	Interferon- $\gamma$
IVIg	Intravenous immunoglobulin
LFA3	Lymphocyte function-associated antigen
LP	Lectin pathway
mAb5.2	Anti-merozoite surface protein 1 (MSP-1) antibody 5.2
MAC	Membrane attack complex
MASP	Mannose-binding lectin-associated serine protease
MBL	Mannose-binding lectin
MBLP	Mannose-binding lectin complement pathway
MSP-1	Merozoite surface protein 1
NA	Neuraminidase
NK	Natural killer cells
NO	Nitric oxide
PBMC	Peripheral blood mononuclear cell
PfRipr	<i>P. falciparum</i> Rh5 interacting protein
pLDH	Parasite-derived lactate dehydrogenase
PM	Placental malaria
PMN	Polymorphonuclear granulocyte
PNG	Papua New Guinea
PNH	Paroxysmal nocturnal hemoglobinuria
RAP1A	Ras-Related Protein Rap-1A
RBCs	Erythrocytes, red blood cells
RH	Reticulocyte binding protein [RBP] homologues
ROS	Reactive oxygen species
SA	Sialic acid
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEMA7A	Semaphorin-7A, CD108
SMA	Server malarial anemia
TCC	Terminal complement complex, C5b-9
TF	Tissue factor
TNF- $\alpha$	Tumor necrosis factor- $\alpha$
TRAP	Thrombospondin-related anonymous protein
VEGR1	Vascular endothelial growth factor receptor 1
VSA	Variant surface antigens

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# **Chapter I: Literature Review**

## **Introduction to malaria**

### **Malaria a Severe Public Health Problem**

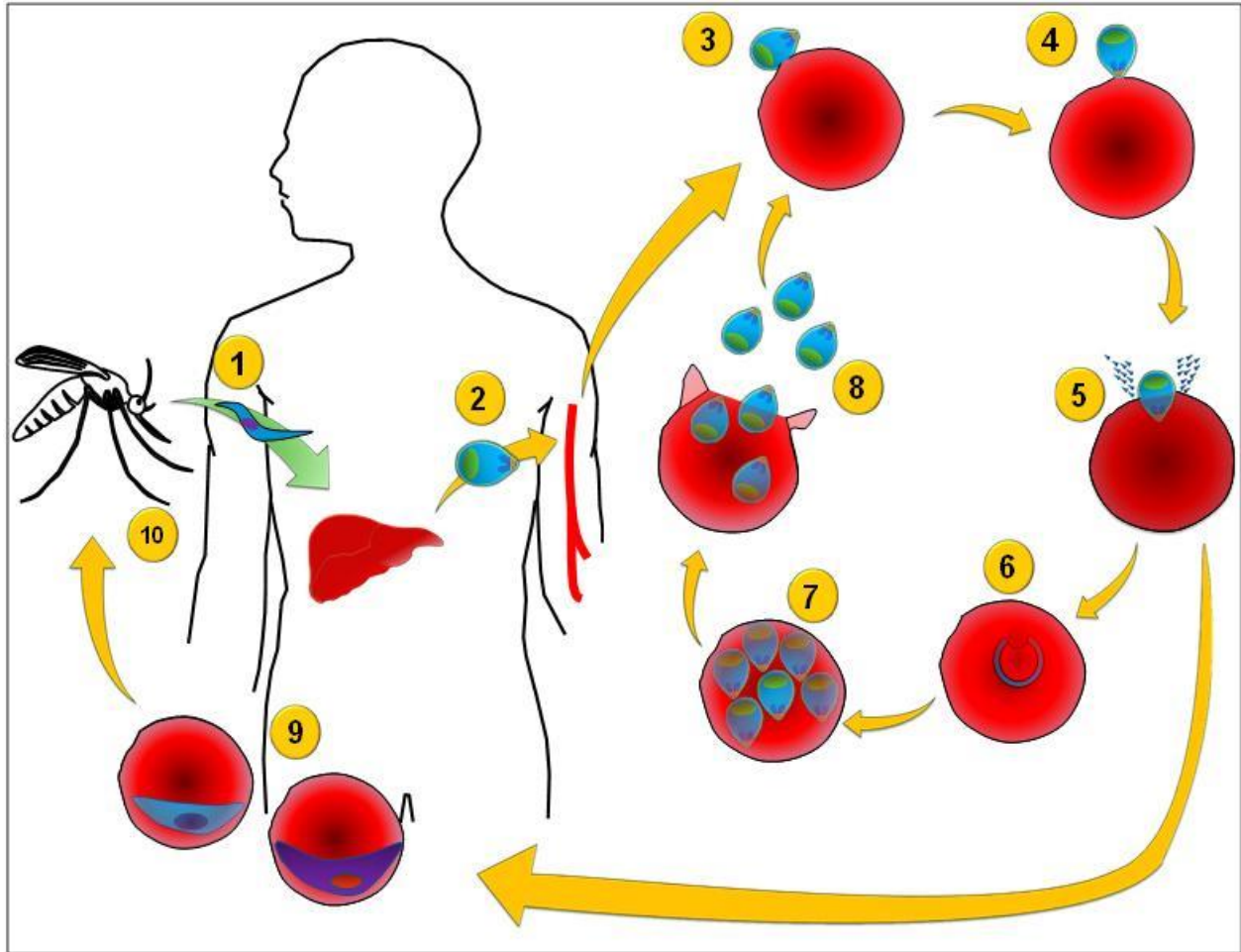
Malaria remains one of the world's most important infectious diseases, and is responsible for close to one million deaths each year (1). Even though major resources over the past century, starting with the creation of the Global Malaria Eradication Program in 1955 by the World Health Organization, have been devoted to eradication of malaria worldwide, malaria still remains a major burden that may be claiming significantly more lives than previously estimated (1). To add to the urgency for development of more efficacious and cheaper control measures, recent reports show the spread of resistance to artemisinin derivatives, which are currently the frontline drugs for malaria treatment (2,3). As with past chloroquine resistance that spread to Africa in late 1970s, the origin of drug resistance is once again in Southeast Asia (3,4). If control measures are not developed and implemented to stop the spread of artemisinin resistant parasites, it will spread to Africa where it will not only have catastrophic effects on morbidity and mortality of the population but also destabilize the political and socio-economic status of most countries in that region. Vector control measures and insecticide-treated bed nets are effective in controlling the spread of malaria in certain geographic areas; yet, these measures offer only temporary reprieve due to the complexity of the malaria vectors and variability in their behavior (5,6). There is a great need for a broad-spectrum malaria vaccine, but due to our lack of

understanding of precise immune correlates of protection and discordance between the *in vitro* vs. *in vivo* vaccine data, the development of a malaria vaccine has failed.

### **Malaria and *Plasmodium falciparum* Lifecycle**

Five apicomplexa protozoan species of the genus *Plasmodium* infect humans: *P. falciparum*, *P. vivax*, *P. ovale*, *P. malaria*, and *P. knowlesi*. Of the five plasmodium species, *P. falciparum* is the deadliest, accounting for most of the malaria-related deaths, the majority of which occur in children under 5 years of age and primigravidae women, due to complications from severe malaria. The life cycle of the parasite is complex involving two hosts, beginning with transmission of sporozoite stage to humans via the bite of infected female *Anopheles* mosquitoes during a blood meal (2). Sporozoites injected by the mosquito travel to the liver where they multiply asexually into merozoites inside hepatocytes; this part of the malaria life cycle is called the exo-erythrocytic cycle. After 10-14 days of growth, the infected hepatocytes lyse, releasing thousands of merozoites that are now free to invade erythrocytes. The *P. falciparum* merozoite stage of the parasite is ~1.5  $\mu\text{m}$  long and 1.0  $\mu\text{m}$  wide and tear-shaped, with a prominent apical complex containing secretory organelles: rhoptries, micronemes and dense granules, which are involved in the erythrocyte invasion process (7). After the initial reversible attachment to the erythrocyte membrane, the merozoite undergoes active reorientation that juxtaposes the apical end of the parasite with the erythrocyte membrane in an irreversible attachment. The expelled contents of the apical secretory organelles and junction formation between the merozoite and the erythrocyte promote merozoite's active invasion into the erythrocyte utilizing the merozoite actomyosin

motor. As the merozoite enters the host erythrocyte it sheds its surface coat and enters the newly formed parasitophorous vacuole. Both the erythrocyte and the parasitophorous vacuolar membranes are resealed once the merozoite is inside the cell (8). *In vitro*, the time between the merozoite egress from the infected erythrocyte and the completion of invasion is 30 to 120 sec (9). Inside the erythrocytes the merozoite, surrounded by the parasitophorous vacuolar membrane, hijacks cellular machinery, and restructures the erythrocyte membrane by expressing and exporting parasite derived ligands. The parasite also produces hemozoin crystals, which are polymerized toxic heme, a byproduct generated by the parasite as it consumes hemoglobin as its main source of amino acids. As the parasite grows it once again divides asexually causing lysis of the erythrocyte 24-72 hrs post-invasion, depending on the species, and the newly formed merozoites are free to infect uninfected erythrocytes. The cyclic infection of erythrocytes by merozoites is called the erythrocytic cycle, which is responsible for all the morbidity and mortality associated with malaria. A small fraction of the infected erythrocytes will undergo differentiation into sexual erythrocytic stages or gametocytes. The gametocytes are ingested by a female *Anopheles* mosquito during a blood meal. Inside the mosquito the parasite undergoes sexual reproduction, genetic recombination and multiplication; eventually the sporozoite stage will migrate to the salivary glands. The parasite's life cycle in the mosquito is called the sporogonic cycle. During the subsequent blood meal the mosquito will inoculate the sorozoites into a new human host. (Figure 1.1)



**Figure 1.1: *Plasmodium* Life Cycle.** The malaria parasite life cycle requires two evolutionarily distant hosts, an invertebrate and a vertebrate. During a blood meal, a malaria-infected female *Anopheles* mosquito inoculates sporozoites into the human host (1). The sporozoites travel through the circulatory system until they reach the liver. Once in the liver the sporozoites migrate through *Kupffer* cells and invade hepatocytes. After 10-14 days of asexual reproduction and metamorphosis, a different parasite stage emerges out of the infected hepatocytes called the merozoite (2). The initial interaction between the merozoite and the erythrocyte may be a random collision. But once contact is made it becomes highly dependent on specific molecular interactions between parasite ligands and receptors on the erythrocyte membrane (3). The merozoite reorients itself such that a junction is formed between the apical end of the merozoite and erythrocyte membrane (4). The merozoite sheds its surface coat as it invades the erythrocyte and enters the newly formed parasitophorous vacuole inside (5). Most of the invaded parasites will mature into ring stage forms (6). Then they undergo asexual replication in the erythrocytes (erythrocytic schizogony) to form 12 -16 daughter merozoites (7). The schizonts, around 48 hours post invasion, burst in near synchrony with other infected erythrocytes, resulting in the characteristic fever cycles that represent the clinical manifestations of the disease. The egressed merozoites invade uninfected erythrocytes (8). A small fraction of newly invaded merozoites develop into sexual erythrocytic stages (gametocytes). The male and female



gametocytes are ingested by a female *Anopheles* mosquito during a blood meal (9). In the mosquito's stomach, the male microgamete penetrates the female macrogamete, generating a zygote. The formation of the zygote accounts for malaria's ability to undergo sexual reproduction, and genetic recombination. The zygote transforms into an ookinete, becoming elongated and motile. The ookinete invades the midgut wall of the mosquito and develops into an oocyst. Within the oocyst the parasite grows, undergoes asexual replication and transforms into sporozoites. The sporozoites egress out of the oocyst and migrate to the mosquito's salivary glands. Once the female mosquito goes in for a subsequent blood meal it injects the sporozoites propagating the malaria life cycle (10).

### ***Plasmodium falciparum* Erythrocyte Invasion Pathways**

Merozoites utilize a multitude of immunologically distinct receptors in the invasion of erythrocytes leading to significant redundancy in the invasion pathways. Even a primed immune system is unable to fully clear the infection, this is a major obstacle to vaccine design. Erythrocyte membranes have high levels of surface glycoproteins that contain sialic acid residues. Previous studies designed to elucidate erythrocyte receptors utilized by the parasite employed genetically deficient cells or enzymatic treatment of erythrocytes in order to remove certain receptors. Treatment of erythrocytes with trypsin or neuraminidase (Nm) reduces *P. falciparum*'s invasion of those erythrocytes (10). When MkMk erythrocytes, cells lacking glycoproteins A and B, or Nm treated erythrocytes are used to determine erythrocyte invasion ligands some stains of *P. falciparum*, such as 7G8, still invade at more than 50% efficiency, such as 7G8, whereas the invasion potential of other stains, such as Camp, is reduced to 1.8% (11). From the distinct invasion patterns of Nm treated erythrocytes two major types of invasion pathways were established for *P. falciparum*: a sialic acid (SA)-dependent pathway and a SA-independent pathway.

Based on this partition, studies revealed other Nm sensitive receptors on the erythrocyte membrane used by the parasite, specifically glycoporphins A, B, C, and Band 4.1, although none have been shown to be essential for invasion(12-14). Progress in identifying Nm resistant erythrocyte receptors employed by the parasite has been lagging. Recently complement receptor 1 (CD35) was identified as the sialic acid-independent receptor utilized by some laboratory strains and field isolates. Sialic acid-independent invasion is blocked by soluble complement receptor 1 (sCR1) as well as by monoclonal and polyclonal antibodies against CR1. Merozoites interact directly with CR1 on the erythrocyte surface and with sCR1-coated microspheres independent of complement activity. Surprisingly both the sialic acid-independent strain 7G8 and the sialic acid-dependent strain Dd2, interact with CR1, based on immunofluorescent microscopy data (15). Increased levels of CR1 expression on the surface of erythrocytes correlate with increased levels of erythrocyte invasion by the parasite (15).

Another Nm resistant receptor, basigin (BSG or CD147) of the Ok antigen blood group, was recently discovered using an avidity-based extracellular interaction screen. This method was designed to detect direct low-affinity protein interactions between erythrocyte receptor ectodomain fragments expressed as biotin-tagged baits, and highly avid pentameric  $\beta$ -lactamase-tagged preys, such as *P. falciparum* receptors. Erythrocyte invasion is inhibited considerably by soluble BSG or by BSG knockdown using lentiviral vectors expressing shRNA targeting *BSG*. Invasion is completely blocked by low concentrations of anti-BSG antibodies, with 50% inhibition observed at 0.5  $\mu$ g/ml of anti-BSG antibody. Of greatest importance, invasion inhibition is observed

across all laboratory-adapted and field strains tested, independent of sialic acid invasion pathway dependence (16).

## **Invasion Ligands Utilized by Merozoites**

Merozoite proteins involved in the invasion process can be subdivided into three discrete functional classes: the merozoite surface proteins (MSPs), the *P. falciparum* erythrocyte binding antigens (PfEBAs) and the *P. falciparum* reticulocyte binding protein [RBP] homologues (PfRHs). The MSPs form the highly polymorphic and structurally complex coat around the merozoite surface, and are exposed to the immune system from the time of merozoite egress from the infected erythrocyte to the completion of uninfected erythrocyte invasion. The PfEBAs and PfRHs are stored in the apical secretory organelles, the rhoptries and micronemes, and it has been proposed that they are released after the initial attachment of the merozoite to the erythrocyte surface (17).

### **The Merozoite Surface Proteins**

The MSPs on the merozoite surface are implicated in forming initial weak and reversible interactions between the merozoite and the uninfected erythrocyte. These MSPs can be subdivided into two main groups. Those that are attached to the merozoite surface via a glycosylphosphatidyl inositol (GPI) anchor are referred to as the membrane-bound proteins, they include MSP1, MSP2, MSP4, MSP5, MSP10, Pf12, Pf38 and Pf92 (18). Most GPI anchored MSPs seem to be enriched in the detergent-resistant membrane (DRM) domains, also known as lipid rafts (19). Three other GPI

anchored merozoite proteins have also been identified; the rhoptry-associated membrane antigen and the Pf34, both localize to the rhoptry, and the apical sushi protein (PfASP) localizes to the micronemes of *P. falciparum* merozoites (20,21). The second group of MSPs, include MSP3, MSP6 and MSP7, are referred to as the peripheral membrane proteins, and they interact with the membrane-bound proteins (22).

One of the most abundant merozoite surface proteins is MSP1. MSP1 is essential for the invasion of erythrocytes and produces a lethal phenotype if disrupted, making it an attractive target for a malaria vaccine (23). The membrane-bound MSP1 forms a non-covalent complex with MSP6 and MSP7 (24,25). Following egress from an infected erythrocyte, MSP1, a ~195 kDa protein, along with MSP6 and MSP7 undergo extensive proteolytic cleavages. Recently, the N-terminal 83-kDa domain of MSP1 has been reported to bind to the neuraminidase (Nm)-sensitive Glycophrin A receptor on the erythrocyte surface (26). As the merozoite enters the erythrocyte, most of the MSP1/MSP6/MSP7 complex is shed and all that remains is the C-terminal GPI-anchored MSP1<sub>19</sub> fragment (27). The anion exchanger (band 3) in the erythrocyte membrane, which allows newly formed bicarbonate to leave the cell in exchange for chloride, is the Nm-insensitive receptor that binds to the MSP1<sub>19</sub> fragment (28). Following erythrocyte invasion, the MSP1<sub>19</sub> fragment is transferred to the developing food vacuole and serves as the first marker for the biogenesis of this organelle. Throughout the duration of the intracellular erythrocytic cycle only the MSP1<sub>19</sub> fragment, which entered on the surface of the merozoite during the erythrocyte invasion, and not the newly synthesized MSP1, is present in the food vacuole, attached to the luminal

side of the membrane. It has been speculated that due to its protease resistant properties, the MSP1<sub>19</sub> fragment serves as a protective coating inside the food vacuole, and/or mechanically stabilizes the food vacuole membrane (29).

### **The *P. falciparum* Erythrocyte Binding Antigens and the *P. falciparum* Reticulocyte Binding Protein Homologues**

After the initial attachment, the parasite utilizes the apical membrane antigen 1 (AMA1) to reorient itself (30). Irreversible secondary interactions follow parasite reorientation and juxtaposition of the apical end of the parasite with the erythrocyte membrane. As the merozoites egress from an infected erythrocyte they encounter low potassium ion concentrations in the blood plasma. Low potassium concentration induces the activation of the phospholipase C pathway and the rise of the cytosolic calcium levels in the merozoite. Influx of calcium prompts the merozoite secretory organelles, the micronemes, to secrete ligands to the merozoite surface, such as the 175 kD erythrocyte binding antigen (EBA175) which binds to the Glycophrin A (Gly A) receptor on the erythrocyte membrane (31-33). The binding of merozoite ligand, EBA175 to the erythrocyte receptor, GlyA, returns cytosolic calcium concentration in the merozoite to its basal levels and prompts the release of auxiliary proteins involved in the erythrocyte invasion from the merozoite rhoptry organelles (33,34).

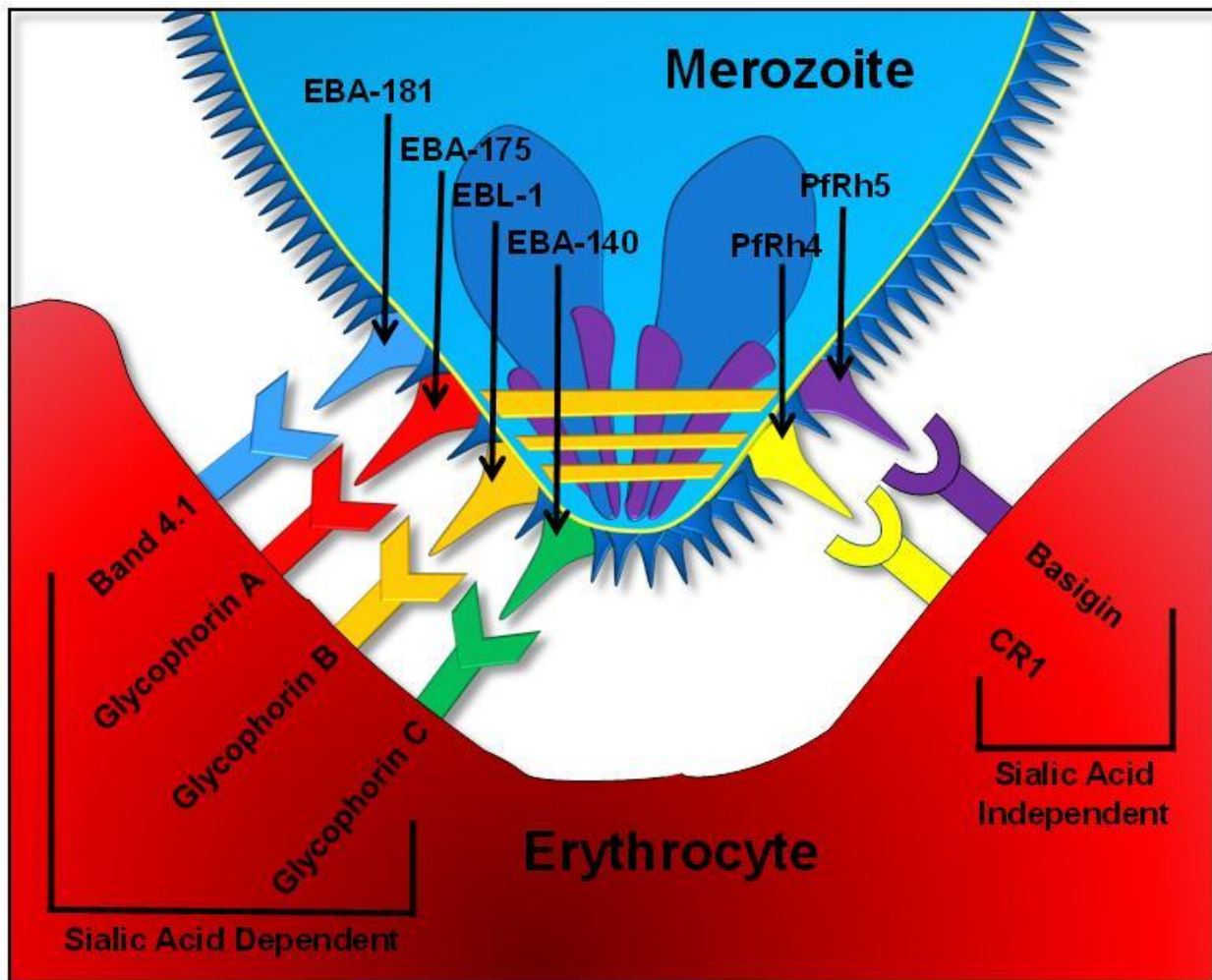
Work utilizing the *P. falciparum* phage display libraries identified a protein-protein interaction between the erythrocyte membrane protein band 4.1 and EBA 181, a paralogue of EBA-175 (13,35). Plasmodium merozoite EBL-1 was identified when erythrocyte-binding domain, region 2 of the EBL-1, expressed on the CHO-K1 cells,

bound to Gly B-sufficient but not glycophorin B-deficient erythrocytes (36). Using erythrocyte ghosts prepared from Melanesian donors with a deletion of exon 3 in the glycophorin C gene, Western blot analysis identified EBA140 binding to Gly C (12).

Of the known PfRh ligands (PfRh1, PfRh2a, PfRh2b, PfRh3, PfRh4, PfRh5 and PfRipr), only two complementary erythrocyte receptors have been identified to play a role in erythrocyte invasion, both mediating sialic acid-independent invasion. PfRh4 binding to CR1 was initially discovered by direct protein-protein interaction and competition assays using recombinant sCR1 to inhibit binding of PfRh4 to the erythrocyte surface. Addition of sCR1 also inhibits invasion of Nm treated erythrocytes by 7G8 and 3D7, both sialic acid-independent strains. But sCR1 is not inhibitory in strains that lack PfRh4 expression (W2mef) or carry a genetic knockout of the PfRh4 gene (W2mef $\Delta$ Rh4) (37). When invasion of Nm-treated erythrocytes by sialic acid-dependent and -independent laboratory strains is compared in the presence of anti-CR1 antibodies or sCR1, sialic acid-dependent strains are less susceptible to the inhibitory effects of these reagents (15).

The other PfRh interaction involves PfRh5, a unique PfRh member due to its absence of a transmembrane and cytosolic region at the C-terminus (38). PfRh5 initially localizes to the rhotyres but later co-localizes to the tight junction during the invasion process and binds to basigin present on the erythrocytes. Efforts to disrupt the PfRh5 gene in 3D7, W2mef, HB3 and D10 strains by using the vector pCC1, that was used previously to knock out *P. falciparum* genes, was unsuccessful, suggesting that its function is essential for parasite survival (38). PfRh5 forms a complex with another transmembrane domain-deficient *P. falciparum* Rh5-interacting protein (PfRipr), and

also the GPI-anchored cysteine-rich protective antigen (CyRPA) that links the complex to the merozoite membrane. The latter also is refractory to knockout, and induces a robust inhibitory antibody response that inhibits invasion of heterogeneous strains (Figure 1.2) (39-41).



**Figure 1.2: *P. falciparum* sialic acid-dependent and -independent ligands with their respective erythrocyte receptors used during invasion.** The *P. falciparum* erythrocyte binding antigens (EBA-181, EBA-175, EBL-1 and EBA-140) and the *P. falciparum* reticulocyte binding protein [RBP] homologues (PfRh 4 and PfRh5) are involved in erythrocyte attachment and invasion. These parasite ligands bind to erythrocyte membrane receptors that can be subdivided into sialic acid containing neuraminidase sensitive receptors (Band 4.1, Glycophorin -A, -B and -C) and neuraminidase resistant receptors (CR1 and Basigin). Of note PfRh5 lacks a

transmembrane region instead forms a complex with PfRipr and GPI-anchored CyRPA (not shown).

## **Characterization of Humoral Immune Response to *Plasmodium* Erythrocytic Stages**

Development of naturally acquired immunity against parasite exposure is an extremely slow process taking years to decades to develop and unfortunately does not result in sterilizing immunity (Figure 1.3). Induction of a robust long term immune response through artificial introduction of the whole *Plasmodium* pathogen or its components through immunization is the definitive solution to this global problem. Unfortunately, after decades of intense research and extensive funding, we have not been able to develop an effective vaccine against malaria. This failure is in part due to the lack of understanding of how natural immunity to malaria is acquired, what effector mechanisms are responsible, and how these mechanisms induce protection. Research on malaria immunity and vaccine development have focused on three different parasite stages: the sporozoite, the merozoite, and the gametocyte. Our focus has been to understand the role of complement on merozoite invasion in the absence or presence of antibody-mediated immunity.

### **Naturally acquired immunity**

Seminal studies on the importance of the humoral response to blood stage malaria infection were performed in 1937 by Coggeshall and Kumm (42). A *Plasmodium knowlesi* infection in *rhesus* monkeys is fatal but can be made chronic for an indefinite period by the administration of antimalarial drugs. When administered to



acutely infected monkeys on the day of infection serum from infected monkeys with chronic infection is protective and in some animals prevents death although in most animals it only prolonged the infection (42). To ascertain the importance of antibodies in human malarial infection, total antibodies were purified from adults living in hyperendemic areas of West Africa. Purified antibodies were then administered to children, aged 4 months to 2.5 years, suffering from severe malaria and high parasitemia. Within 4 days of anti-malarial antibody injection the parasite count dropped to less than 1% of the initial value (43). Surprisingly, purified antibodies from naïve individuals passively transferred to infected children also induced a smaller but significant reduction in parasitemia (43). Despite parasite strain heterogeneity, these same purified antibodies, from West African immune adults, still possessed therapeutic activity when administered to East African children with severe *P. falciparum* malaria (44). In a similar study the protective effects of African immune IgG were determined when passively administered to Thai patients with early recrudescent parasites after quinine treatment failure. Following IgG injection parasitemia decreased by a mean 543 fold and remained low for 7 to 12 days, while gametocyte counts remained unaffected. The kinetics of passively transferred antibodies and parasitemia were monitored for a longer duration than in the previously-mentioned studies. A fast decrease in parasitemia was observed within days. But parasitemia began to increase by day 9 to 12, after the decline of antibody titers from passive transfer. Thus, the antibody was not sterilizing but caused only a partial and transient reduction in parasitemia. Fortunately anti-malaria antibody treatment did not select antigenically different parasites, since when these same individuals with high parasitemias were re-treated, the parasitemia

once again dropped to similarly low levels and remained low for the same duration (45). These transferred antibodies stimulate the naturally acquired immune response by recognizing antigenic targets both on the infected erythrocytes and the merozoites.

Antigens of parasite origin are expressed on infected erythrocytes after merozoite invasion. These parasite derived antigens localize to knob structures formed in late stage infected erythrocytes and are responsible for sequestration of late stage parasites in the microvasculature. This prevents the recirculation of infected erythrocytes through the reticuloendothelial system, minimizing their recognition and clearance by the liver and spleen (46,47). These knob structures also play a role in formation of infected erythrocyte clumps or rosettes, which may cause obstruction in the blood vessels resulting in cerebral malaria. Plasma from Gambian children with mild forms of malaria disrupts rosettes in vitro, but plasma from children with cerebral malaria possesses no anti-rosette activity (48). In haemagglutination tests utilizing *P. knowlesi* trophozoite or schizont-infected erythrocytes from rhesus monkeys, in the presence of heat inactivated anti-sera to homologous and heterologous parasites only agglutinates infected erythrocytes of the same strain that infected the anti-sera donor animal (49).

Clonally variant surface antigens (VSA) in the knob structures on the surface of infected erythrocytes are the main targets of protective antibodies generated from natural infection (50). One VSA family most widely studied contains approximately 60 *var* genes each responsible for encoding a distinct PfEMP1 protein variant. Each VSA has particular antigenic and adhesive properties, and the infected erythrocyte only expresses a single variant at a time (51,52). Along with being one of the targets for the

immune response, the antigenic variability of VSAs accounts for the extremely prolonged acquisition of immunity. The acquisition of immunity eventually leads to suppression of severe malarial disease and inhibition of clinical symptoms. In agglutination assays serum samples of naturally infected children in The Gambia reacted with infected cells from the same donor child, but not with infected cells from the other children demonstrating that each child's immune system recognizes an antigenically limited pool of parasite targets. Sera from Gambian adults on the other hand reacts with the surface of infected cells from all the children, suggesting an expansion of the repertoire of immune recognition of parasite derived erythrocyte surface antigens with age (53). The other possibility is that adults have broadly cross-reactive antibody responses to different variants. This theory is more optimistic for the future of vaccine design, because a vaccine generated from a few conserved crossreactive antigenic determinants should be easier to manage than a vaccine mixture containing all the VSA protein variants. To examine if adult antibodies are cross-reactive or variant specific a study was designed using a modified agglutination assay relying on the multivalent nature of antibody antigen interactions and pre-labeling of two different parasite strains with different colored fluorescent dyes. Cells infected with different parasite laboratory strains and wild isolates that share different antigenic determinants should form mixed color agglutinates only in the presence of cross-reactive antibodies. Most sera from children ages 1-11, formed single color agglutinates, but 2 of 43 sera tested was cross-reactive (54). Contrary to the implications of the previous study, pooled plasma from adults did not produce mixed color agglutinates (54).

One of the most compelling arguments for antibody-mediated protection from natural infection comes from studies performed with placental malaria. Adult women living in malaria endemic areas develop strong immunity, but during first pregnancy, primigravidae develop more frequent and severe malarial infections similar to those observed in infants and children. This spike in infection has been attributed to a specific VSA variant (VAR2CSA) that binds to placentas (55). Anti-VAR2CSA antibody titers are high in third-trimester primigravidae and multigravidae, but those antibodies are absent in children and adult males (56,57). The primigravidae reacquire their pre-pregnancy immunity by third trimester and are immune during consecutive pregnancies (58). Aside from VAR2CSA, VSA targets present on the infected erythrocyte surface are highly antigenically diverse.

Antibody mediated natural immune response to merozoites has also been reported. Invasion potential and potentially intraerythrocytic growth of purified merozoites was reduced in the presence of purified antibodies from immune adults from Kenya and Papua New Guinea (59). A prospective longitudinal study in 3 to 8-year-olds identified an increase with age in prevalence and concentration of antibodies to all regions MSP1. The highest prevalence of antibodies was against the conserved N-terminal block 3 and C-terminal PfMSP1<sub>42</sub> regions of the molecule, which are highly conserved between parasite isolates (60). Antibodies from west African children, specific for an even smaller and terminally processed fragment, PfMSP-1<sub>19</sub>, provided ~40% protection against clinical malaria in children in Sierra Leone (61). Also, antibody responses to one of the epidermal growth factor-like motifs of PfMSP1<sub>19</sub> were strongly associated with resistance to both clinical malaria as well as high levels of parasitemia

in Gambian children (61). The PfMSP1<sub>19</sub> fragment is a promising target for vaccine development due its essential role in erythrocyte invasion and because it is produced from a secondary cleavage site that is a target of a parasite derived-protease. Blocking antibodies that interfere with the antibodies that inhibit this processing step have been identified (62).

Some mAbs but not others that recognize MSP1<sub>19</sub> inhibit the processing of membrane bound MSP1<sub>42</sub> into membrane bound fragments of MSP1<sub>19</sub> and soluble MSP1<sub>33</sub>. These antibodies also inhibit invasion. Non-cleavage inhibitory antibodies successfully enter infected erythrocytes and remain co-localized with the ring stage (63). In a critical follow-up study, antibodies to the post-primary cleavage 83kDa MSP1 fragments were affinity purified from pooled immune sera from Gambian adults. When antibodies that inhibit cleavage of MSP-1<sub>42</sub> were tested in the presence of human 83kDa MSP1 affinity purified antibodies in invasion assays, the invasion inhibitory activity of the mAbs was completely abolished (64). This result was a clear indication that in a naturally acquired immune response to malaria, aside from the production of customary pathogen inhibitory antibodies, a collection of antibodies is generated that help the parasite and suppresses hosts pathogen inhibitory function. It is possible that the parasite's ability to invade erythrocytes, and in turn influence disease severity and outcome, is determined by relative concentrations and affinities of processing inhibitory and blocking antibodies.

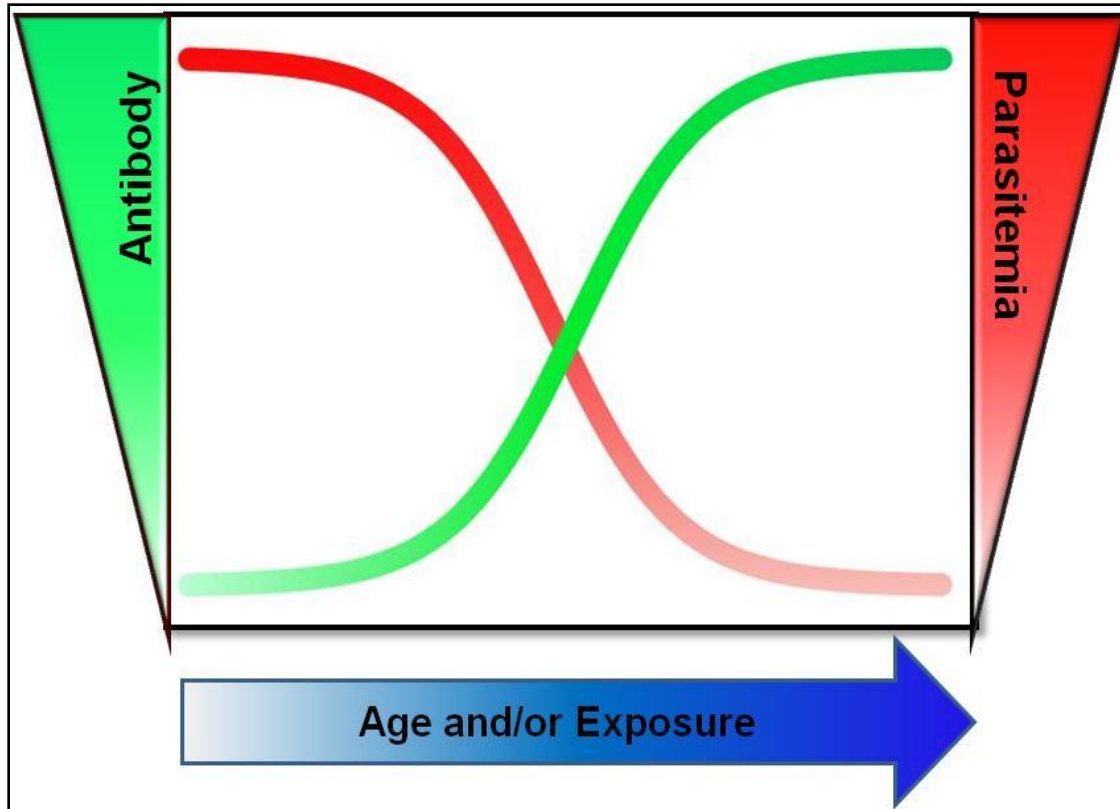
Aside from MSP1 directed naturally acquired immunity, there are antibody responses to other merozoite antigens such as MSP2, MSP3, MSP4, AMA1, and EBA

proteins(65-69). Antibodies to MSP4 in Vietnamese individuals, ages 9 to 55, were mainly of the IgG1 and IgG3 subclasses, potentially playing a role in opsonization and complement-mediated lysis of free merozoites; however no correlation was observed between lack of infection and anti-MSP4 positivity (67). When the antibody response against MSP2 was analyzed in individuals 1 – 75 years of age living in west Africa, predominantly cytophilic IgG1 and IgG3 subclasses were present. Children less than 10 years of age had more IgG1 and adolescents and adults had predominantly IgG3 (65). Overall, predominant IgG subclasses were either IgG1 or IgG3, depending on the merozoite antigen, and IgG3 was most strongly associated with protection. A stronger IgG3-mediated response correlated with increase in age.

Some studies have shown paradoxically that high antibody levels against merozoite antigen in a younger cohorts (ages 1 – 4) are not associated with protection but instead are associated with an increased risk of malaria (70,71). Discordance in these studies could arise from the difference in the ages of the cohorts or the cumulative exposure levels of the individuals to the parasite. Also, geographic location dictates not only the total levels of anti-merozoite antibody response but also antibody affinity and diversity. It's possible that immuno-dominant epitopes that are also extremely polymorphic induce robust anti-merozoite response in children, but antibodies to immune-recessive epitopes, that are much more conserved in sequence, are only acquired later in life after extensive and diverse exposure to the parasite (72).

To further understand the functionality of this antibody-mediated response, purified antibodies from immune African individuals were tested in *in vitro* growth inhibition assays using late stage synchronized or un-synchronized parasite cultures in

the presence or absence of peripheral blood mononuclear cells (PBMCs). The purified immune IgGs did not inhibit intraerythrocytic growth of the parasite but rather sometimes increased it (73). However, when these same antibodies were tested in the presence of PBMCs, there was parasite inhibitory effect. This inhibitory effect disappeared when either immune IgGs or PBMCs were omitted (73). Although there is good experimental support for the involvement of both cell- and humoral immune responses in mediating a robust antibody response to blood stage infection, the effector mechanisms are poorly understood. Antibodies could have multiple protective roles such as inhibition of merozoite invasion into erythrocytes, clearance of infected erythrocytes from the circulation, impeding late stage infected erythrocyte adhesion and sequestration in the microvasculature, inhibiting rosette formation, and preventing rupture of late stage schizonts (48,59,73-75).



**Figure 1.3: Development of natural humoral immune response to *Plasmodium erythrocytic stage* infection.** Individuals residing in malaria endemic regions eventually develop immunity. During initial exposure children and naïve adults will exhibit symptoms, which at time may be extremely severe, attributed to infection with the parasite. Subsequent exposure to the parasite will generally produce less severe symptoms but this is heavily dependent of the age of the individual and exposure frequency, since immunity is short-lived without regular exposure immunity rapidly diminishes. Hence non sterile immunity against the blood stage infection develops slowly, specifically due to extreme antigenic variation as well as allelic exclusion phenomenon, and only after numerous consistent exposures to the parasite. As a robust antibody response develops circulating parasite load diminishes.

### **Conflicting Data Between *in vitro* vs. *in vivo* Blood-stage Studies**

Although naturally immune adults have lower level of parasitemia and show few clinical symptoms, *in vitro* growth inhibition assays demonstrate that few immune adults have significant levels of inhibitory antibodies. For instance the results of ELISA assays



of antibody recognition of schizont antigens, or measurement of the erythrocyte invasion or growth inhibitory properties of sera from clinically immune Gambian adults *in vitro*, are not significantly different from those performed on non-immune European control samples (76). The authors attributed this finding to the use of a parasite isolate that was only present for a limited period in the community and that many older individuals had not been exposed to (76). Also serum inhibition assays measure only one activity of antibodies, binding to its antigen and disrupting its function. These assays fail to capture antibody effects that require other immune components present *in vivo*. Finally, it is possible that the inhibitory effects of antibodies from older individuals are masked *in vitro* by anti-idiotypic antibodies that could bind directly to anti-parasite inhibitory antibodies, and block binding of the latter to the parasite or infected erythrocytes. Also, blocking antibodies may bind to the same parasite derived antigen as the inhibitory antibodies, competing with the latter to prevent disrupting the parasite ligand's function (77).

Malaria vaccines are viewed as the solution for decreasing global morbidity and mortality. One of the prime candidates for a subunit malaria vaccine is the 19 kDa C-terminal fragment of the PfMSP-1. Affinity purified PfMSP1<sub>19</sub>-specific antibodies from malaria immune human sera inhibit parasite growth *in vitro* in the absence of complement and mononuclear cells (78,79). However, when a larger 42-kDa C-terminal fragment of MSP-1, that encompasses the 19kDa fragment, was used to vaccinate children ages 12–47 months, the vaccine offered no protection (80). Furthermore, when MSP1<sub>19</sub>-based vaccines are used in the *P. yoelii* mouse model the level of MSP1<sub>19</sub>-specific inhibitory activity does not correlate with peak parasitemia. Some mice that are

completely protected from infection, with zero parasitemia, have no detectable inhibitory activity in their pre-challenge sera *in vitro*. Also no correlation is observed between the PyMSP1<sub>19</sub>-specific *in vitro* inhibitory activity and the antibody levels measured by ELISA (81).

These inconsistencies extend to other vaccine targets. A promising vaccine candidate directed against apical merozoite antigen 1 (AMA1) shows no protection in 2-3 year olds (82). But contrary to the human data, monkeys immunized with the *Plasmodium fragile* form of the merozoite AMA1 have reduced levels of parasitemia or delays in parasite counts, suggesting protection was achieved. When the animals were re-challenged with *P. falciparum*, unlike their adjuvant controls, none of the animals became infected (83). Another clinical trial with AMA1 produced growth inhibitory antibodies. However, when total antibodies from immune individuals living in Mali were purified to exclude AMA1 specific antibodies and combined with antibodies from U.S. AMA1 vaccinees there was a reduction in growth inhibition. Such a reduction in growth inhibition was not observed when antibodies from immune Mali individuals were mixed with antibodies from U.S. vaccinees purified to exclude AMA1 specific antibodies (84). These puzzling results may be due to the previously mentioned blocking antibodies that bind to regions adjacent to epitopes targeted by parasite inhibitory antibodies, thereby reducing the inhibitory effects from anti-AMA1 antibodies generated by the vaccine. The differences in results from both naturally immune individuals and vaccinees potentially arise due to missing factors in the *in vitro* assays that have a significant impact on disease progression and outcome *in vivo*.

## Common *in vitro* Testing Methods and Antibody Functions

Most methods that assess antibody efficacy against blood stage parasitemia *in vitro* have major limitations that may produce inconsistencies between *in vitro* and *in vivo* data. Microscopic evaluation of blood smears is the gold standard for clinical laboratory detection and identification of *Plasmodium* species, but microscopy is highly laborious, poorly suited for high throughput testing and lacks the sensitivity for low parasitemias. The other popular alternatives are flow cytometric analysis of nucleic acid stained infected erythrocytes or the measurement of enzymatic activity of the parasite-derived lactate dehydrogenase (pLDH), but it is difficult to determine parasite health during its intraerythrocytic development with these methods.

Antibodies *in vivo* modulate and exert their effects through numerous mechanisms. In the *in vitro* setting antibodies from individuals from endemic areas or vaccinees are tested in the presence of heat inactivated (HI) serum or plasma in the absence of immune cells or intact soluble immune effectors such as complement. Only the antibody's direct role on neutralization based on its Fab domain epitope recognition, affinity, and avidity, is tested, where as other effector functions that the antibodies may exert on the parasite are omitted. For instance, the Fc domain of different antibody isotypes and subclasses is responsible for most antibody immune effector functions. The Fc domain binds to Fc receptors (FcRs) linking the cellular and humoral immune responses. Stimulation of immune cells expressing FcRs by immune complexes can induce antibody-dependent cell-mediated cytotoxicity (ADCC), promote phagocytosis, release inflammatory mediators and produce oxidative burst. The Fc domain also activates complement via the antibody dependent classical pathway. Antibodies are

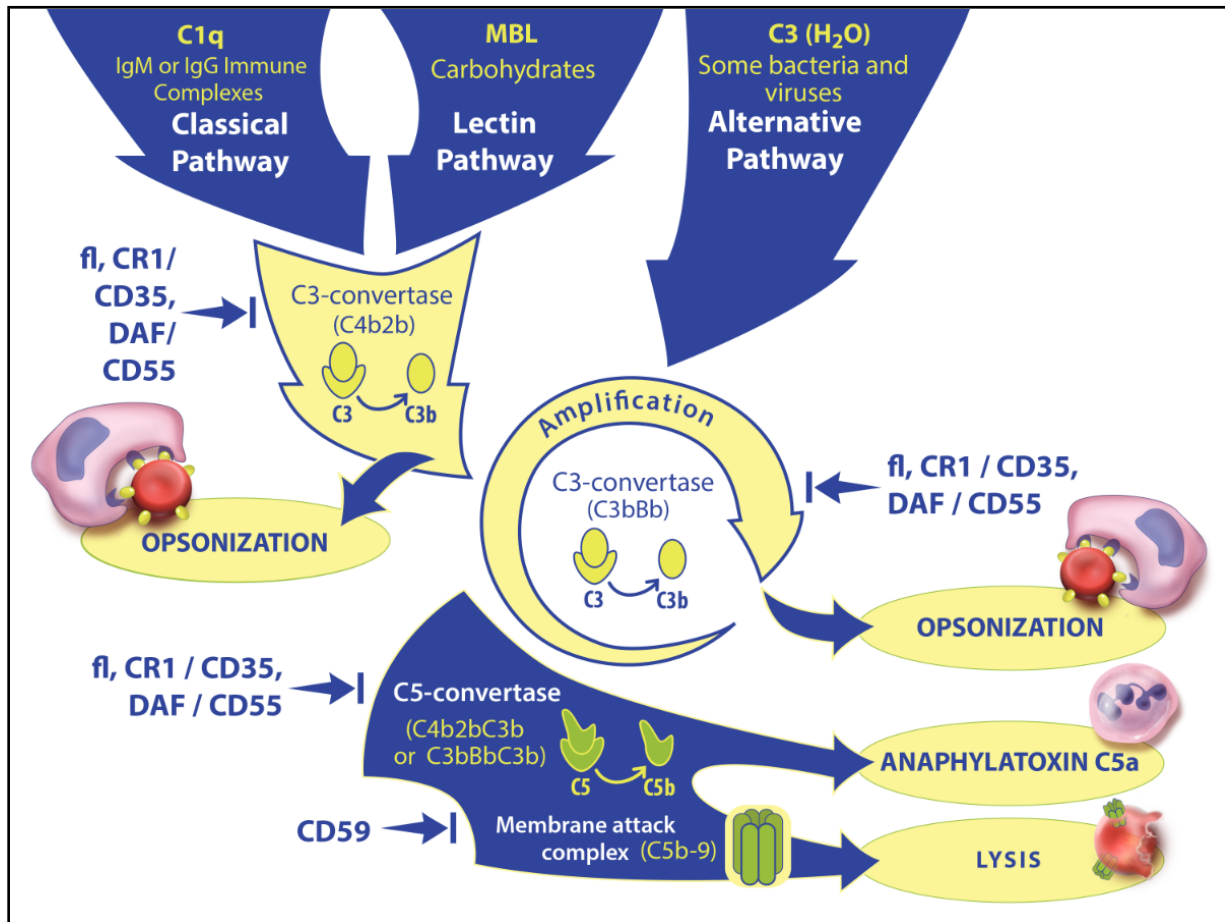
also glycoproteins and the glycans associated with the Fc domain of immunoglobulins influence their function. The level of glycosylation differs by antibody isotype and subclass (85). For instance, all IgG subclasses contain a single conserved N-linked glycosylation site (Asn-297) in each of the CH2 domains that assists in stability and preserves the quaternary structure of the constant region (86,87). Alterations in the glycoforms can alter antibody function. Sugars located at position Asn-297 of CH2 can be subdivided into three subsets: two terminal galactose residues (G2), one galactose and one N-acetyl glucosamine (GlcNAc) (G1) or two terminal GlcNAc (G0). Individuals with rheumatoid arthritis are deficient in antibodies with G2 and G1 glycoforms but have elevated levels of G0 glycoforms (88). Furthermore, antibodies that have sugar residues that terminate with G0 activate complement by the classical pathway but are independent of mannose binding lectin, and promote a pro-inflammatory environment (89). Intravenous immunoglobulin (IVIg) therapy can ameliorate inflammatory diseases. This IVIg anti-inflammatory activity seems to be related to the presence of sialic acid in a 2,6 linkage to a terminal galactose on IgG (90).

These sugar residues also seem to be essential for the antibodies' ability to bind to Fcγ receptors (FcγR) as well as to govern the ADCC activity (91). Expanding the scope of invasion and growth inhibition assays that test antibodies from individuals from endemic areas or vaccinees to encompass different effector mechanisms, such as components of the innate immune response, may prove valuable in deciphering the antibodies true role in anti-malarial response. We know that the host's innate immune response to the parasite partly consists of complement activation and production of pro-inflammatory cytokines. However, this innate response may be largely responsible for

the severe complications associated with malarial infection. The potential role of complement in these complications has recently taken center stage (3). Although complement activation during malaria infection is intended to be harmful to the parasite, it may be even more harmful to the host. Recent evidence illustrates a multitude of different interactions between complement and anti-malarial antibodies *in vitro* in erythrocyte invasion assays (59,92,93).

### **The complement system**

The complement system is composed of soluble proteins circulating in extracellular fluids, notably the blood, as well as membrane-associated cell surface proteins that play a major role in host defense and inflammation. Although many different cell types, such as immune cells and endothelial cells, at extrahepatic sites synthesize complement components, biosynthesis of 90% of plasma complement takes place in liver hepatocytes (94). The classical (CP), mannose-binding lectin (MBLP), and alternative pathways (AP) serve as the three major complement activation routes (Figure 1.4). Upon activation of complement by pathogens, enzymatic reactions drive the sequential cleavage cascade of inactive zymogenic precursors that generate effector fragments. The main effector fragments (C3b) and (C4b) play a role in opsonization that leads to phagocytosis of invading pathogens. Other fragments known as anaphylatoxins (C5a, C4a, and C3a) activate neutrophils and macrophages. If the complement pathway is allowed to progress to completion the final end result is lysis of the pathogen through the formation of a pore in the membrane called membrane attack complex (MAC) or terminal complement complex (TCC), composed of C5b-9 (95).



**Figure 1.4: The three major complement activation pathways.** The classical, mannose binding lectin, and alternative pathways are the three major complement activation routes. The classical pathway (CP) is activated by binding of C1q to IgM or IgG-containing immune complexes (ICs) as well as to pentraxins, soluble pattern recognition molecules such as C-reactive protein (CRP) (not shown). The binding of C1q activates the C1r and C1s proteases, which are present as part of the C1 complex, resulting in cleavage of C4 into C4a and C4b. Deposition of C4b on the antigenic surface recruits C2, which is cleaved into C2a and C2b by C1s. The surface bound C4b2b enzymatic complex that is formed is called the C3 convertase, capable of cleaving large quantities of C3 into the anaphylatoxin fragment C3a and the opsonizing fragment C3b. The mannose binding lectin pathway (MBLP) is turned on by the recognition of non-self sugar moieties by mannose binding lectin (MBL) and ficolins. The latter are multimeric lectins that bind N-acetylglucosamine. Binding of MBL or ficolins activates mannose-binding lectin-associated serine proteases (MASPs) that perform similar functions to C1r and C1s, producing the same C3 convertase as in the CP. The alternative pathway (AP) is constantly active at low level through the spontaneous hydrolysis of C3 (C<sub>3H<sub>2</sub>O</sub>), enabling binding of factor B (fB). Bound fB is cleaved by factor D, producing soluble C3 convertase composed of C3bBb. This

soluble C3 convertase is capable of generating short-lived soluble C3b. On non-self antigenic surfaces devoid of complement regulatory proteins, binding of C3b induces the formation of surface bound C3 convertase composed of C3bBb. Generation of C3bBb also enables the AP to serve as an amplification loop for complement activation initiated by the CP and/or MBLP. Addition of one more C3b molecule to either the CP/MBLP convertase or to the AP convertase results in the formation of C5 convertases which cleave C5 into C5a and C5b. C5a is a potent anaphylatoxin that promotes inflammation. C5b recruits factors C6-C9 leading to the formation of the terminal complement complex or membrane attack complex which, when inserted into a cell membrane, can lead to lysis. Complement regulation takes place at different levels. The activity of the convertases is regulated by membrane and soluble regulators. The complement receptor 1 (CR1/CD35) serves as co-factor in the factor I (fI)-mediated degradation of C3b. It also binds C3b or C4b-opsonized ICs allowing transport to the liver and spleen and removal by macrophages. Decay accelerating factor (DAF/CD55) also accelerates the decay of C3 and C5 convertases but in a fI-independent manner.

## **Complement Activation and Malaria**

A number of studies in both humans and animal models show evidence of extensive complement activation during malaria infection leading to reductions in serum C1, C2, C3, C4, and C1q levels, or increased TCC (sC5b-9) during malarial paroxysms (96-103). When merozoites egress from erythrocytes they are exposed to plasma and, in theory, complement activation could take place on the merozoite surface by any pathway. Two studies suggested that merozoites escape complement activation in non-immune serum (92,93), although recently it was demonstrated that direct complement deposition on purified merozoites *in vitro* does occur (59). Other studies have explored the contribution of immune serum to merozoite phagocytosis, but the role of complement in this process was not explored (104). Additional studies are needed to determine the role of complement in immunity against merozoites. Additionally, nonopsonic clearance of ring-infected erythrocytes by monocytes and macrophages,

mediated by CD36, is overshadowed in the presence of intact complement, which greatly enhances erythrophagocytosis of opsonized, ring stage infected erythrocytes (105,106).

Most likely, the predominant mechanism of complement activation during malaria is via the classical pathway by formation of antigen-antibody immune complexes (ICs) containing merozoites or parasite derived circulating antigen (107-109). However, there is both direct and indirect evidence that other pathways are activated. *In vitro* evidence suggests that the food vacuole of *P. falciparum* activates the AP (92,93). The terminal complement complex C5b-9 (TCC) was noted to be deposited only on digestive vacuoles (DV) and not on merozoites, with the authors concluding that the intact digestive vacuole membrane is the primary site for complement activation (92). The AP may be responsible for C5b-9 deposition on the digestive vacuole membrane, because the terminal complement complex is seen on the surface of DV after schizont rupture in the presence of EGTA/Mg<sup>2+</sup>. The latter prevents classic pathway activation by binding calcium which blocks the antibody-dependent classical pathway (92). Also the group claimed that C3c deposition was only observed on the surface of DV, but no data was shown to support this claim. The lack of C3c deposition on merozoites does not exclude the possible presence of upstream opsonins such as C3b and C4b (92). When DVs are incubated with PMN in fresh serum, within 2 min there is complement-driven phagocytosis of intact DV followed by induction of ROS produced by polymorphonuclear granulocytes (PMNs), but phagocytic activity is lost when serum is inactivated (93). Also, when DV membrane is removed no erythrophagocytosis of late – stage-infected erythrocytes or phagocytosis and ROS generation as measured by free



hemozoin occurs in the presence of active serum. Erythrocyte invasion is not inhibited by ROS production by PMNs, and only modest erythrocyte invasion inhibition is produced in the presence of PMNs and antibodies from high-titer immune patients. Phagocytosis of DV by PMNs induces a normal initial ROS response, but drastically reduces the ROS response upon challenge with *S. aureus*, indicating that DV may either inhibit PMN ROS production or lead to exhaustion of PMNs (93). Based on these results complement activation and opsonization is only on the DV membranes and not on the merozoites. Merozoites are therefore free to invade the erythrocytes unobstructed by the opsonic effects of complement components.

Contrary to these studies, immunoblot analysis shows C1q and C3b on merozoites incubated in fresh serum, and enhancement of these complement components was observed in the presence of 25% fresh serum and antibodies from Papua New Guinea (PNG) donors (59). Immuno-fluorescence analysis demonstrates high levels of membrane attack complex (MAC) formation on merozoites that are preincubated with 25% fresh serum and antibodies from PNG donors, where as lower levels of MAC are observed on merozoites preincubated with fresh serum and antibodies from native individuals (59). Invasion inhibitory activity of antibodies from PNG donors remains intact in AP-inactivated 50°C treated serum much like in fresh serum, suggesting that the AP does not play a major role in antibody mediated inhibition (59). Reinforce the CP involvement, antibodies from immune donors possess greater inhibitory activity in C1q-repleted serum than in C1q-depleted serum, where as inhibition is minimal in C5-repleted vs C5-depleted serum, suggesting that MAC formation is not critical for inhibition but mostly depends on C1-C4 opsonization of merozoites.

Surprisingly C1q alone in the presence of immune antibodies is sufficient to induce significant inhibition of erythrocyte invasion (59).

The lack of concordance between these studies may be due to handling of the parasites. *Boyle et al.*, used purified merozoite that may experience extensive damage due to handling and manipulation during extraction of the parasites from the protective environment of the host cell. The other group, *Dasari et al.*, focused on the extraction of intact DV. In the absence of membrane intact DV and ruptured erythrocyte debris, which may preferentially induce complement activation and consumption, purified merozoites are forced to bear the full impact of complement opsonization. There is also evidence of Suchi domain protein in the *Plasmodium* genome, suggesting that complement control proteins made by the parasite inhibit downstream complement activity on the merozoite surface and reduce MAC mediated lysis (110). Of note, Suchi domain proteins contain the complement control protein domain that is also present in a wide variety of complement regulators.

The MBLP may also be activated during malaria infection since MBL has been reported to bind to parasite proteins, such as the 78-kDa glucose-regulated stress protein of *P. falciparum* (111,112). Calcium-dependent binding of MBL to the membrane of *P. falciparum*-infected erythrocytes could be inhibited by mannose (112). Even though MBL recognizes proteins of *P. falciparum*-infected erythrocytes that are parasite-derived and glycosylated as well as immunogenic in humans, it does not inhibit parasite growth when added to *in vitro* cultures of *P. falciparum* (111).

Other evidence for the role of the MBLP in malaria comes from genetic studies. Three polymorphisms of the MBL gene (*MBL2*) exon 1 that result in non-synonymous

substitutions are linked to decreased serum levels of MBL (“B” G54D, “C” G57E, and “D” R52C) (113). Of these alleles, the MBL C allele is found almost exclusively in Africans, suggesting that it provides a survival advantage in that population (114,115). A number of studies have investigated the association of these and other *MBL2* polymorphisms with parasitemia and severe disease. Although some studies have shown associations, others have not (112,116-121). The lack of consistent results among these studies is likely due to differences in experimental design, population demographics such as age and gender, differences in transmission intensity, and statistical rigor. For example, Garred *et al.* reported lack of association between low MBL expressors and severe malaria in a case-control study of children in Accra, Ghana, where transmission is seasonal whereas Holmverg *et al.* reported an association with severe malaria in older children in Northern Ghana where transmission is hyperendemic (112,118). Together, these results suggest that the role of MBL in protection against *P. falciparum* malaria is complex.

Animal studies do not provide clear evidence that complement is critical in protection against malaria. Studies in Rhesus monkeys infected with *P. coatneyi* and treated with cobra venom factor (CVF) to deplete complement show no significant change in parasitemia (122). Likewise,  $C3^{-/-}$  mice infected with the *P. berghei* Antwerpen-Kasapa (ANKA) strain show no more parasitemia than wild type mice (123). *P. chabaudi*-infected mice deficient in C1q exhibited little rise in parasitemia, and only during reinfection is there a significant impairment in the control of parasitemia (124). On the other hand, complement deficiency in rats infected with *P. berghei* increases parasitemia and mortality (125). Mice deficient in the complement receptor 3 (CR3,

CD11b/CD18, MAC-1) do show a small increase in parasitemia than wild type mice, suggesting that complement-mediated phagocytosis may be important in controlling parasitemia (126). Surprisingly, *P. falciparum*, sialic acid-dependent and independent strains invade CR1 transgenic mouse erythrocytes at a higher rate than wild-type mouse erythrocytes, suggesting that both sialic acid-dependent and -independent strains utilize CR1 during the invasion process (15). Also, *P. falciparum* laboratory strains invade high CR1 expressor erythrocytes better than low CR1 expressor erythrocytes when the erythrocytes are pre-treated with neuraminidase. This increase is attributed both to the differential surface expression of CR1 and to lower sialic acid-independent invasion of erythrocytes from low CR1 expressors than to high CR1 expressors (37). Together, these results do not fully support a critical role for complement in the control of parasitemia. The contribution of complement to protection against malaria may be host and parasite species-dependent. On the other hand, merozoites could have developed strategies to regulate complement activation.

### **Role of Complement in Cerebral Malaria**

Cerebral malaria (CM), with coma as the clinical hallmark, is one of the most severe complications of *P. falciparum* malaria (127,128). Depending on the intensity of transmission, CM can affect children or adults. In sub-Saharan Africa, where transmission is generally higher than in most other parts of the world, this disease occurs mostly in children, whereas in Southeast Asia it is seen predominantly in adults (129,130). A large proportion of individuals who recover from CM suffer long-term, and sometimes permanent, neurological and cognitive sequelae of varying degrees (128).

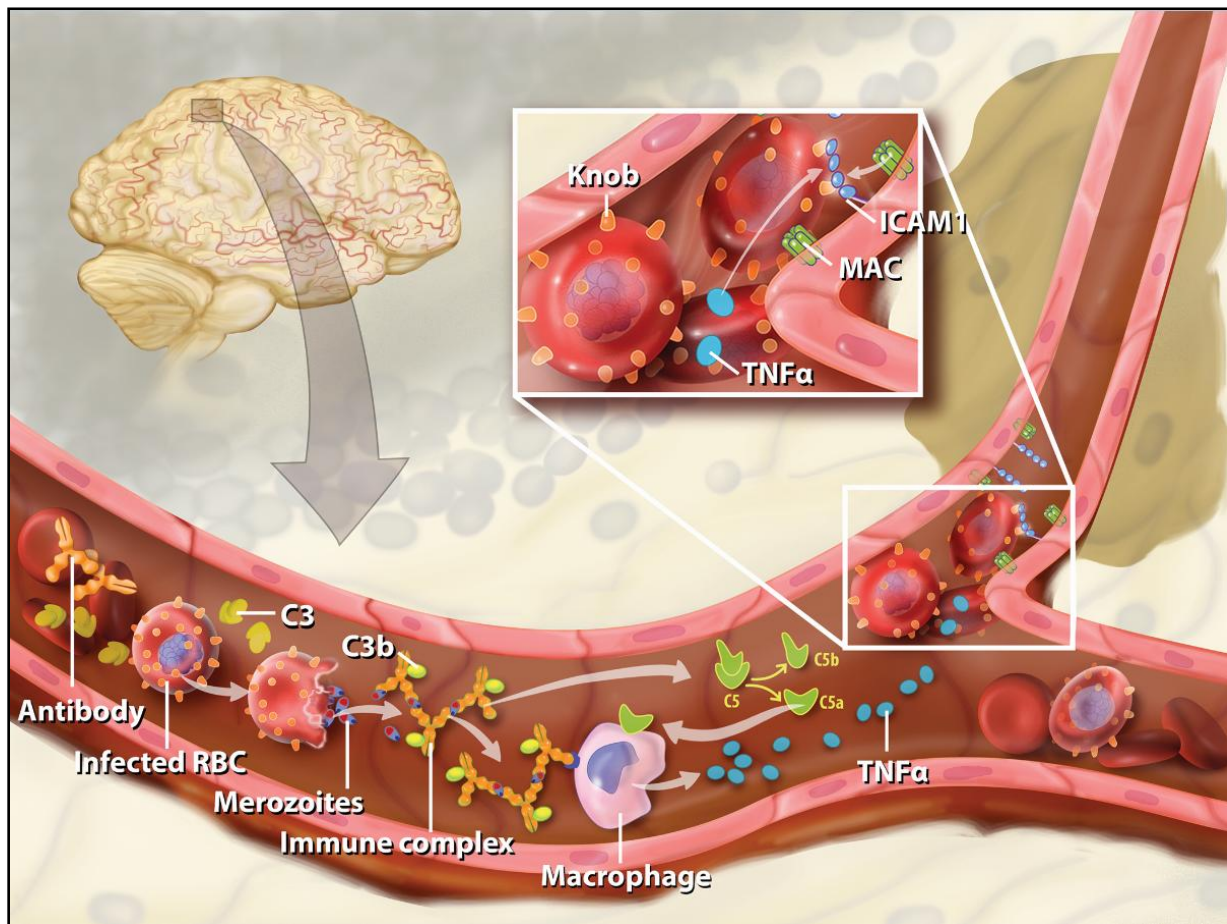
The role of complement in the pathogenesis of human CM is poorly understood. In one study, children with CM had similarly lower levels of C1q, C3, and C4 than children with uncomplicated malaria (100). However, a subsequent study in adult patients with CM showed elevated C1q serum binding activity, probably due to the presence of elevated levels of circulating ICs, and lower levels of C3 and C4 than patients with uncomplicated malaria (109). The differences in the results between these studies could reflect differences in the age of the patients and their level of malaria pre-exposure, determined by the malaria transmission intensity in the different regions of acquisition. Also, extensive immunohistochemical staining of C3d and C5b-9 on brain sections, specifically in the cortical capillaries of patients with severe cerebral malaria, co-localized with hemozoin indicates not only complement activation but also progression to completion with MAC formation (106).

Infection of mice with *P. berghei* ANKA is the most commonly used animal model of CM. One study showed that *P. berghei*-infected mice with CM have higher levels of C1q and C5 in brain homogenates, whereas C3 levels in brain or sera are not altered (131). The susceptibility of mice to *P. berghei*-induced CM correlates with the expression of C5 because all mouse strains that lack functional C5 expression are CM resistant, whereas strains that are susceptible to CM produce functional C5 (132). Transfer of a defective C5 allele from a resistant mouse strain to a susceptible mouse strain confers resistance and, conversely, transfer of the wild type functional C5 allele from a susceptible strain to a resistant strain confers susceptibility (132). Further, mice deficient in C3 (C3<sup>-/-</sup>) show partial resistance to CM, suggesting that the AP C3 convertase makes a contribution to the development of CM. On the other hand, mice

deficient in C4 (C4<sup>-/-</sup>) are fully susceptible to CM, suggesting that the CP and MBLP convertase (C4b2b) can be bypassed (123). However, this result does not mean that complement cannot contribute especially because there is evidence from human and animal studies that ICs play an important role. Treatment of mice with anti-C5a receptor or anti-C5a blocking antibody confers resistance to CM (132). C5a receptor deficient mice (C5aR<sup>-/-</sup>) are partially resistant to CM and produce decreased levels of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interferon- $\gamma$  (IFN- $\gamma$ ) (133). Reports that patients with CM have higher levels of C5a than patients with uncomplicated malaria seem to be in agreement with the rodent data (133). On the other hand, a recent study suggested that susceptibility to mouse CM is due to formation of the MAC/TCC, because anti-C9 antibody delayed the onset of CM (134).

Taken together, these data suggest that activation of the complement cascade by IC formation during malaria infection, leading to activation of the terminal complement components, is necessary for CM. However, because C3<sup>-/-</sup> and C4<sup>-/-</sup> mice show partial or complete susceptibility to CM respectively, C5 activation and generation of C5a may also be occurring via extrinsic pathways, such as the cleavage of C5 by thrombin or by secreted serine proteases from activated neutrophils and macrophages (123,135). These findings are yet to be confirmed in humans and need to be put in the context of other key observations, such as the requirement for infected erythrocyte sequestration in the brain and production of pro-inflammatory cytokines and other inflammatory mediators (129,136). One study showed that MAC/TCC can work synergistically with TNF- $\alpha$  to upregulate expression of the endothelial receptors, E-selectin and intercellular adhesion molecule 1 (ICAM-1) (137). The latter is a key

endothelial receptor responsible for sequestration of parasitized erythrocytes (138). Thus, one potential model for the role of complement in CM involves the deposition of ICs containing malaria antigens in the brain microvasculature, which in turn can activate C5 via the classical and extrinsic pathways. This resulting MAC/TCC formation upregulates ICAM-1 and increases sequestration of parasitized erythrocytes (Figure 1.5).



**Figure 1.5: Role of complement in the pathogenesis of Cerebral Malaria.** Patients with malaria produce immune complexes (ICs) which deposit on the endothelium of capillaries throughout the body, including the brain. ICs activate complement via the classical pathway or via an extrinsic pathway leading to deposition of sublytic concentrations of terminal complement complex or membrane attack complex (MAC/TCC) on endothelium of capillaries. ICs can also induce production of tumor

necrosis factor- $\alpha$  (TNF- $\alpha$ ) from macrophages/monocytes by crosslinking Fc receptors and via the binding of C5a. MAC/TCC and TNF- $\alpha$  upregulate expression of intercellular adhesion molecule 1 (ICAM-1) which leads to cytoadherence and sequestration of parasitized red blood cells.

### **Role of Complement in Placental Malaria**

Placental malaria (PM) is another complication from malaria that usually occurs in primigravid women infected with *P. falciparum*. PM can result in low infant birth weight, along with increased perinatal and maternal mortality (139,140). *P. falciparum* strains expressing erythrocyte membrane protein 1 (PfEMP1) variant VAR2CSA have the capacity to adhere to chondroitin sulfate in the placenta, leading to sequestration of parasitized erythrocytes (140). The histological changes seen in placentas from infected women include the presence of parasitized erythrocytes in intervillous spaces, trophoblastic damage (leading in some cases to exposure of the basement membrane), thickening of the trophoblastic basement membrane, and infiltration by monocytes and macrophages (141,142).

A number of histological, molecular, and genetic studies have shown evidence that complement activation plays an important role in PM. One immunohistological study revealed increased amounts of C1q, C4, C3d, and C9 in all malaria-infected placentas (141). A later study also revealed significant deposition of IgG and C3 in *P. falciparum*-infected placentas (142). Microarray expression analyses of placental samples from primigravid women with PM demonstrate upregulation of C1q, C3, C5aR, and C3aR genes (143). One genetic association study initially revealed no link between the *MBL2* C allele (G57E) and poor pregnancy outcomes in Cameroon (116). However,



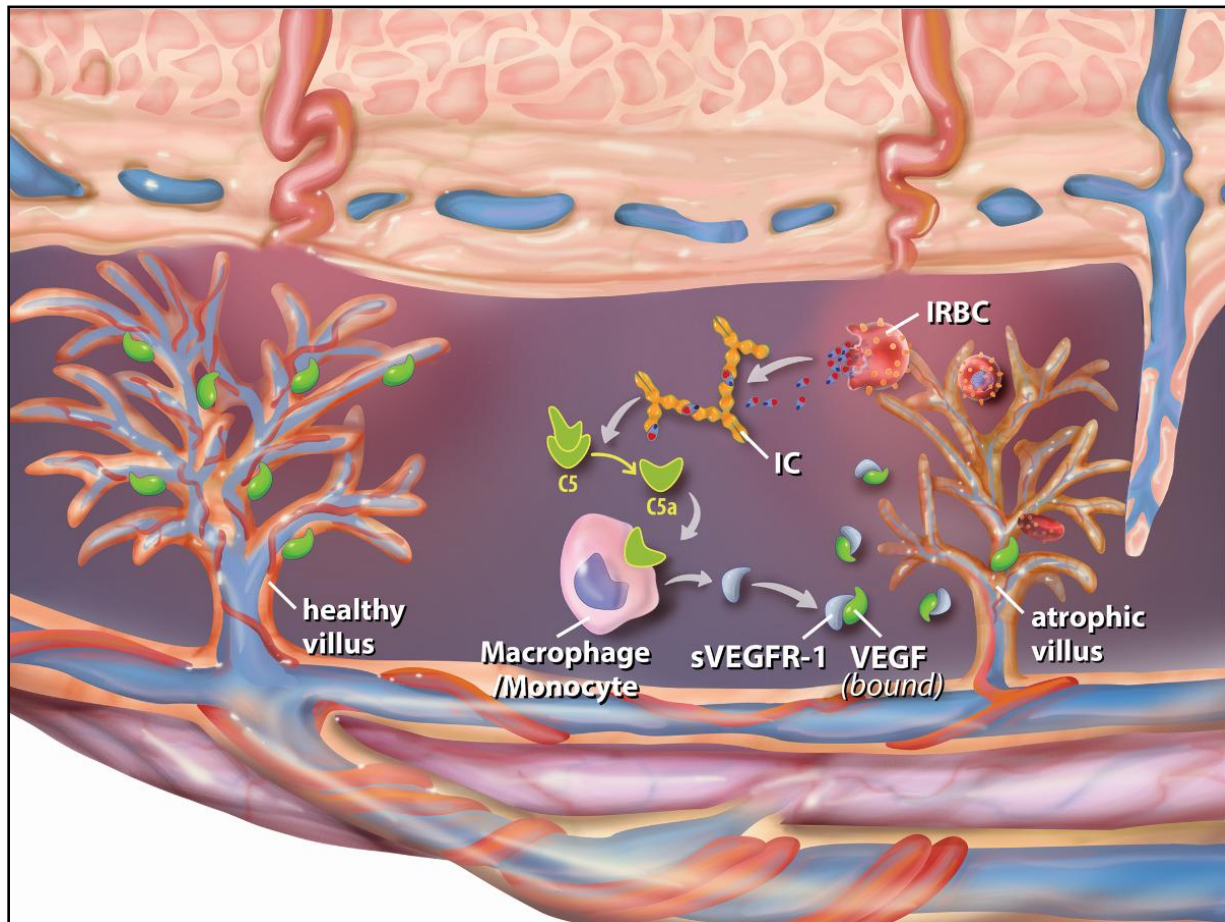
a subsequent study by Holmberg *et al.* identified an association between this polymorphism and increased susceptibility to PM in Ghanaian women (117). Explanations that could account for the contrasting results in the two studies include obvious differences in genetic makeup of the population that result in a higher frequency of the *MBL2* C allele in Ghana (31%) than in Cameroon (17.5%). The study by Holmberg *et al.* also identified an association between a mutation that reduces the enzymatic activity of MBL-associated serine protease 2 (MASP2) and decreased susceptibility to PM (117). How can two mutations that in theory reduce complement activity have opposite effects on PM susceptibility? Increased susceptibility to PM due to the *MBL2* C allele may be related to the complement-independent opsonic activity that has been attributed to MBL and could play a role in phagocytosis of malaria parasites (111,144,145). On the other hand, decreased enzymatic activity of MASP2 may protect by inhibiting downstream activation of the complement cascade.

As in CM, studies of PM in mice point to an important role for C5a. Mating of CBA/J females (C5-sufficient mice) with DBA/2 (C5-deficient mice) males results in an increased frequency of fetal resorption, and hence this cross is used as a model of spontaneous miscarriage and intrauterine growth retardation (IUGR) (146). In this model, production of C5a by cleavage of C5 leads to upregulation of soluble vascular endothelial growth factor receptor 1 (sVEGFR-1), also known as soluble Fms-like tyrosine kinase-1 (sFlt-1), in monocytes. sVEGFR-1 blocks the action of VEGF, preventing appropriate placental vascular development (146). This effect can be blocked by anti-C5a antibodies, anti-factor B antibody, or a C5aR inhibitory peptide (146). Placentas from *P. berghei*-infected mice exhibit a similar phenotype, showing

decreased fetal viability and growth retardation (147). Fetal death is reduced, but not eliminated, in *P. berghei*-infected C5aR<sup>-/-</sup> mice, or by giving injection of anti-C5aR and anti-C5a blocking antibodies to wild-type (C5aR<sup>+/+</sup>) mice (147). In addition, infected placentas from C5aR<sup>-/-</sup> mice show an increased VEGF/sFlt-1 ratio.

In agreement with the animal studies, human studies point to a critical role for C5 and C5a in angiogenic dysregulation of malaria-infected placentas. Placentas of infected primigravid women show upregulation of C5aR and sFlt-1 (143,148). In addition, some sFlt-1 variants appear to confer fetal fitness, which is consistent with the important role of this molecule in the pathogenesis of PM (149). C5a is elevated in peripheral blood from patients with PM and in plasma from infected placentas, and high levels of C5a are associated with fetuses that are small for their gestational age (147,150). Although C5a most likely originates from activation of the complement cascade, an *in vitro* study showed that parasite-derived factors can cleave C5 (150). C5a together with parasite-derived glycosylphosphatidyl inositol (PfGPI) induces production of sFlt-1 in monocytes (150). MAC/TCC is elevated in placental and cord plasma samples of Gabonese primigravidae and patients with a history of malaria during pregnancy (151). However, only primigravid status is associated with an elevated TCC in placental or cord blood in studies using a multiple regression model. Thus, primigravid status seems to be an independent risk factor for complement activation. These results suggest at least an additive effect of complement dysregulation of primigravid status and PM, resulting in full activation of the complement cascade in a fashion reminiscent of CM. Based on the above information, a model of PM where C5a plays a central role has been proposed (152) (Figure 1.6). However,

based on the data from the CM mouse model, the role of the MAC/TCC in PM should be explored as well (134).



**Figure 1.6: Role of complement in the pathogenesis of Placental Malaria.** Normally availability of vascular endothelial growth factor (VEGF) promotes the growth of healthy villi (left). However, in the setting of malaria infection, infected red blood cells (IRBCs) bind to chondroitin sulfate and accumulate in the intervillous spaces. There, they release antigens that form immune complexes (ICs) with maternal anti-malarial antibodies. These ICs activate the complement cascade via the classical pathway. C5a is produced and binds to C5aR on monocytes and macrophages stimulating the production of soluble Fms-like tyrosine kinase-1 (sFlt-1) or soluble vascular endothelial growth factor receptor 1 (sVEGFR-1). sFlt-1 blocks the interaction of vascular endothelial growth factor (VEGF) with fetal vasculature leading to atrophic villi.

## **Role of Complement in Severe Malarial Anemia**

Severe malarial anemia (SMA) is a life threatening complication that is mostly seen in areas of Africa with intense transmission (153). SMA is defined as a hemoglobin level < 5 g/dL (154). Like CM, it also affects predominantly children under five years of age although, on average, children with SMA are one to two years younger than those with CM. In areas of Africa with high malaria transmission, cases of SMA peak at one year of age, whereas in areas with low to moderate transmission, SMA peaks at around two years of age and the risk of SMA decreases with age (153). The pathogenesis of SMA is complex, with contributions from both increased destruction and decreased production of erythrocytes. However, because the lifespan of erythrocytes is 120 days, it is likely that increased destruction plays a more important role, particularly in acute or subacute presentations. A number of observations also suggest that uninfected erythrocytes are destroyed at an accelerated rate during malaria infection. The survival of uninfected erythrocytes is decreased following treatment of *P. falciparum* malaria, and a mathematical model revealed that for every infected erythrocyte 8.5 uninfected erythrocytes are destroyed during malaria infection (155,156). The level of parasitemia cannot account for the degree of anemia since destruction of parasitized erythrocytes accounts for <10% of the blood loss during treatment (157). Hence, destruction of uninfected erythrocytes likely makes a significant contribution to the development of SMA.

There is extensive evidence that complement activation plays a role in the destruction of erythrocytes that leads to SMA. Children with SMA, compared to children with uncomplicated malaria, have considerably lower complement hemolytic activity

(CH50) along with decreased activity of all three major complement pathways, indicating excessive complement consumption, as well as increased presence of cleavage products C3a, C4a, and C5a and increased levels of ICs which can activate the CP (107,158). Release of hematin during the rupture of infected erythrocytes has been proposed to activate the AP, leading to deposition of C3b on the surface of erythrocytes (159). However, *in vivo* evidence is lacking. Patients with SMA and *MBL2* wild type exon 1 have higher levels of MBL on admission than uncomplicated malaria patients (160). In one study, heterozygosity for the *MBL2* exon 1 C allele was associated with SMA relative to healthy controls who were mostly a parasitemic (118). The significance of this finding is uncertain. An earlier study reported that the *MBL2* exon 1 C allele is associated with SMA, but in a subsequent study with a larger cohort by the same group the association did not reach statistical significance (120,160). Hence the role of the *MBL2* in SMA remains uncertain.

Release of DV after rupture of infected erythrocytes *in vitro*, followed by the assembly of the C3- and C5-convertases on the expelled parasitic organelle prompts the deposition of activated C3 and C5b-9 on bystander non-infected erythrocytes that come in direct contact with opsonized DVs (106). Increased opsonins on bystander erythrocytes could promote increased phagocytosis and removal of erythrocytes from the circulation, except that within 4 hours no uptake is observed and 5–8 % erythrophagocytosis occurs only after overnight incubation with neutrophils or macrophages (106). Upon incubation of erythrocytes with DV at a 1:1 ratio, which is presumably more exaggerated than seen physiologically, such low phagocytic activity

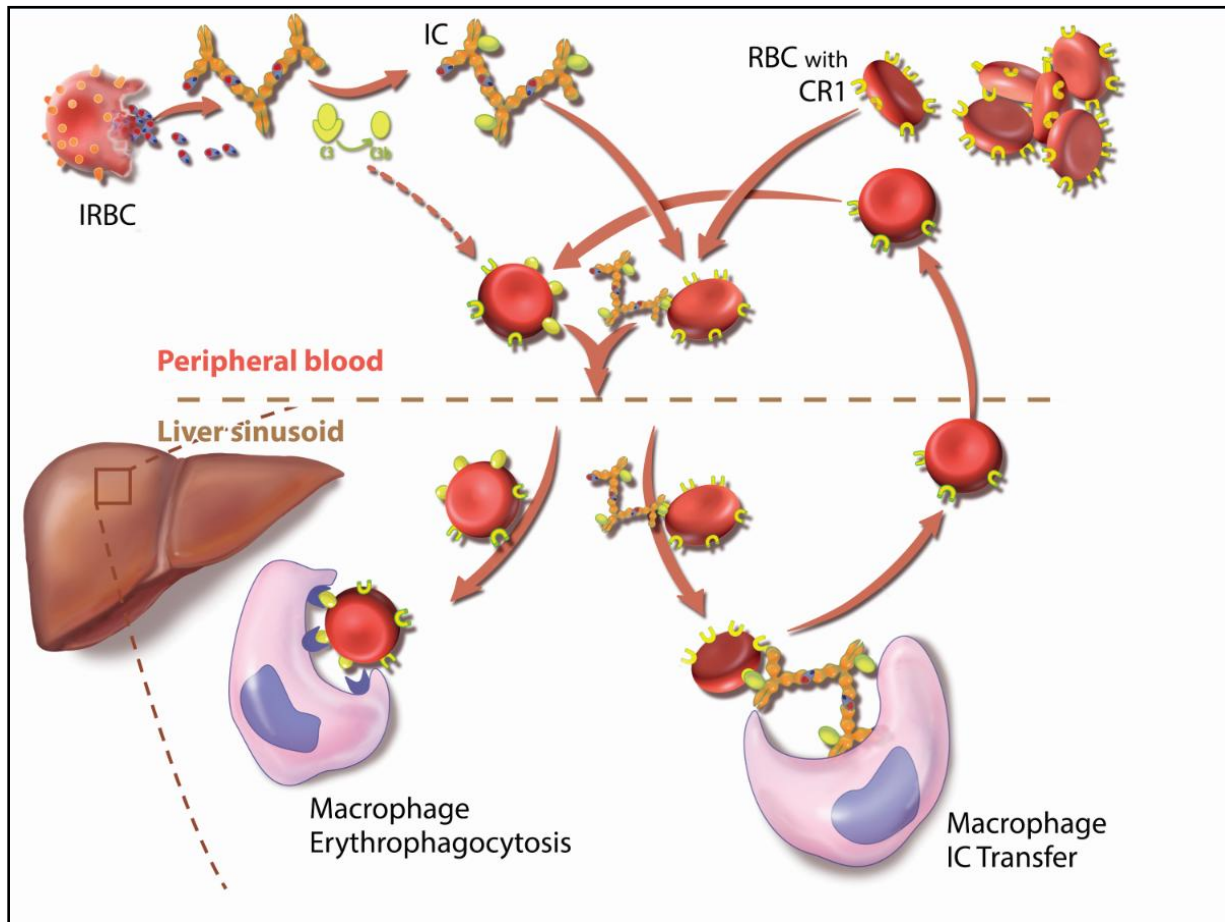
after a very long incubation with phagocytic cells questions the applicability of these *in vitro* findings to the *in vivo* setting.

In addition to defects in complement activation, there is evidence that defects in complement regulation play a role in SMA pathogenesis. Erythrocytes of children with SMA have increased surface IgG deposition and acquire deficiencies of complement regulatory proteins, specifically complement receptor 1 (CR1/CD35) and decay accelerating factor (DAF/CD55) (107,161). Erythrocyte deficiencies of CR1 and CD55 in children with SMA result in reduced IC binding capacity and are associated with increased deposition of C3b on erythrocytes during malaria infection (162,163). These changes in erythrocyte complement regulatory protein expression have been observed by other groups, and similar changes are seen in B cells and monocytes/macrophages of children with SMA (164-167). However, one study was unable to confirm these results, probably due to differences in study design and technical approach (168). In addition to acquired deficiencies in complement regulatory proteins, erythrocytes of children at risk for SMA show age-related variations in the expression of these proteins, with a nadir at around 12 months of age which coincides with the peak age of susceptibility to malaria (163,169).

Most mouse models of SMA are inadequate due to the fact that, contrary to human SMA, mice develop a hyperparasitemia that makes it difficult to separate the contribution of parasite-mediated erythrocyte destruction from non-parasite-related host effects. Recently two models have been developed, based on immune priming by repeated infections, which allows the parasitemia to remain low (170,171). The model developed by Harris *et al.* relies on sequential infection by *P. chabaudi* and *P. berghei*

(171). Erythrocytes show C3b and IgG deposition similar to human malaria. However, C3<sup>-/-</sup> animals develop SMA (171). Hence, C3b deposition by itself cannot account for the destruction of erythrocytes in this model. The bulk of erythrocytes are destroyed by phagocytosis by liver macrophages (171). These findings are consistent with previous observations of the important role that the liver plays in the destruction of IgG-sensitized erythrocytes during *P. berghei* infection (172). A controversial finding by Kawamoto et al. described a potential unknown, parasite-derived complement regulator on *P. berghei* infected erythrocytes that confers greater resistance to complement haemolytic activity than uninfected erythrocytes (173). Based on Western blotting, the molecular weights of CD55 and CD59 on the infected erythrocytes are drastically different than on uninfected erythrocytes. Furthermore, the glycosylation of CD59 is modified in infected erythrocytes (173).

Based on the above data we propose a model of the pathogenesis of SMA whereby repeated episodes of malaria infection lead to IC formation and complement activation via the CP (Figure 7). These ICs bind to CR1 on erythrocytes, and ICs are removed together with surface CR1 by macrophages in the liver and spleen. In this process, CR1 is lost but erythrocytes are recirculated (174). After repeated cycles of IC removal, there comes a point when a significant deficiency of surface complement regulators eventually develops, which, with subsequent malaria infections, leads to C3b deposition on the erythrocytes because the normal regulatory mechanisms are deficient. Erythrocytes are then removed when they encounter macrophages principally in the liver, and also the spleen (Figure 1.7).



**Figure 1.7: Role of complement in the pathogenesis of Severe Malarial Anemia.** Infected red blood cells (IRBC) shed antigens upon lysis. When these antigens are targeted by antibodies, they form immune complexes (ICs) which activate the complement cascade via the classical pathway (CP). C3b is produced, which opsonizes these ICs. The opsonized ICs bind to complement receptor 1 (CR1) on erythrocytes and are removed from the surface by macrophages in the liver and spleen. Erythrocytes are recirculated but lose CR1 and other complement regulators in this process. After several cycles of infection the levels of complement regulators on the erythrocytes reaches a critical level and C3b begins to be deposited indiscriminately on erythrocytes leading to their removal by macrophages (Kupffer cells) in the liver and spleen.



## **Paroxysmal Nocturnal Hemoglobinuria Erythrocyte Invasion by *P. falciparum***

Complex interactions of *P. falciparum* with complement, such as the exploitation of CR1 during erythrocyte invasion, compels the use of complement receptor deficient erythrocytes in the study of invasion and intra-erythrocytic growth. To this end, erythrocytes deficient in two complement regulatory receptors CD55 and CD59 are useful for studying the erythrocyte invasion process by merozoites.

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired hematopoietic disease caused by a somatic mutation of the X-linked phosphatidylinositol glycan anchor biosynthesis, class A (*PIG-A*) gene. The genetic defect results in partial or total loss of the expression of glycosylphosphatidylinositol (GPI) anchored proteins in multi-potential hematopoietic stem cells (175,176). Using an anti-CD59 antibody as a marker for GPI protein expression levels on PNH erythrocytes, three cell population become evident: erythrocytes with close to normal levels of surface GPI-anchored protein (PNH I), erythrocytes with intermediate levels (PNH II) and erythrocytes with no detectable GPI-anchored proteins (PNH III) (177). Of the more than 20 different genes that play a role in GPI anchor biosynthesis in the endoplasmic reticulum, only *PIG-A* is located on the X chromosome, where mutations of a single allele can produce a PNH phenotype (175). Small deletion and insertion mutations distributed throughout the *PIG-A* coding sequence are most common in PNH that result in formation of early stop codons or frameshifts (178). Although missense mutations cluster in exon 2, suggesting that this region encodes for a functionally essential portion of *PIG-A* (179). Even though GPI anchor deficiency is evident in all terminally differentiated cells in males or X-inactivated cell in females of both the myeloid and lymphoid lineages that originate from the

abnormal hematopoietic stem cell clone, it is the GPI deficiency in the erythrocytes that is responsible for clinical manifestations of the disease such as nocturnal hemoglobinuria, chronic hemolytic anemia, and thrombosis (180). The reasons for PNH hematopoietic clonal dominance and expansion are unknown but are most likely the result of a survival advantage, possibly involving extrinsic immunomodulatory mechanisms. First, there could be resistance to natural killer (NK) cell-mediated cytotoxicity due to a deficiency in stress-inducible GPI-linked membrane proteins ULBP1 and ULBP2 on the erythrocyte cell surface which activate NK cells (181). Second there could be beneficial somatic mutations, reminiscent of those found in myeloid neoplasms (181,182).

Two essential GPI linked proteins related to complement are deficient in PNH erythrocytes. Decay accelerating factor (DAF or CD55), disrupts C3 convertase activity by inhibiting formation of the C3 convertase complex and by accelerating C3 convertase decay (183). The presence of CD55 on normal erythrocytes prevents extravascular hemolysis due to phagocytosis by macrophages in the spleen and liver. As few as 70 molecules of CD55 in the erythrocyte membrane are sufficient to prevent classical convertase assembly and significantly reduce opsonin formation (183). Much more prominent erythrocyte destruction in PNH patients is caused by deficiencies in the complement regulator protectin (CD59). Protectin is present at an average of 30,000 molecules/cell and limits the number of C9 molecules that can interact with C5b-8 complex. Consequently C5b-8 catalysed insertion of C9 in the erythrocyte lipid bilayer is inhibited and formation of the membrane attack complex (MAC) is prevented (184). Normally MAC is responsible for complement-mediated lysis through incorporation into

the erythrocyte membrane and formation of a lytic pore. In the absence of CD59 most erythrocytes are destroyed due to intravascular hemolysis.

Excessive erythrocyte lysis in PNH individuals also results in elevated levels of liberated hemoglobin, which binds and depletes circulating nitric oxide (NO) (185). Nitric oxide prevents aggregation and adhesion of platelets to vessel walls thereby limiting thrombosis (186-188). Reduced levels of NO lead to thrombosis, which is one of the leading complications responsible for death of PNH individuals (189). Furthermore, complement activation on the surface of erythrocytes deficient in CD55 and CD59 generates C5a that interacts with its receptor, C5aR, on neutrophils. Neutrophils stimulated by C5a-C5aR interaction produce tissue factor (TF), the primary initiating component of the blood coagulation cascade, thereby significantly enhance the pro-coagulant activity of neutrophils (190).

Of note, *P. falciparum* infected erythrocytes internalize GPI-anchored proteins, such as CD55, CD58 and CD59, that become associated with the parasitophorous vacuolar membrane and may impart a PNH like phenotype to infected erythrocytes (191).

## **Merozoite-specific Thrombospondin-related Anonymous Protein and Semaphorin 7A**

Once the merozoite irreversibly commits to invasion of the erythrocyte, the parasite utilizes a unidirectional actomyosin-powered motor, containing a single-headed class XIV myosin anchored to the inner membrane complex that propels short dynamic actin filaments, to actively penetrate the host cell (192-194). The actin-myosin motor

complex, located between the inner membrane complex and the merozoite plasma membrane, is coupled to aldolase that serves as a bridge between merozoite surface adhesins and the parasite actin cytoskeleton (195,196). The merozoite surface transmembrane adhesin mTRAP, belonging to the TRAP (thrombospondin-related anonymous protein) family, has recently been reported to bind to a GPI-linked protein Semaphorin-7A (SEMA7A or CD108) located on the erythrocyte surface, implicating this process in invasion of erythrocytes by *P.falciparum* merozoites (197,198). Much like other stage-specific cell surface TRAP-family members expressed by the sporozoites disruption of the endogenous *MTRAP* gene has not been achieved, as expected if it is also essential for stage parasite development (197,199,200). Paradoxically, blocking with polyclonal anti-mTRAP or anti-SEMA7A antibodies does not inhibit erythrocyte invasion (198). Aside from SEMA7A binding, TRAP proteins are known to bind to heparin, a clinical and endogenous anticoagulant that possesses an inhibitory effect on complement activation (201,202).

SEMA7A is produced by a variety of lymphoid, myeloid and neuronal cells (203). Immunomodulation through activation of macrophages and monocytes by SEMA7A results in chemotaxis and the production of pro-inflammatory cytokines such as IL-1 $\beta$ , TNF- $\alpha$ , IL-6 and IL-8 (204). SEMA7A also enhances central and peripheral axon growth and is required for axon tract formation and guidance during embryonic development (205). The functional activity of Sema7A on erythrocytes remains elusive.

## **Concluding remarks**

*P. falciparum* merozoites utilize a number of distinct yet highly diverse receptors for the invasion of erythrocytes, resulting in significant robustness in the invasion pathways that should greatly benefit the parasite's survival. This robustness is partially responsible for the parasites ability to bypass a primed immune system which is unable to fully clear the infection even after numerous exposures. Immune evasion and manipulation by the parasite is of course a major obstacle for vaccine design. The complement system is one of the main weapons of the innate immune response and enhances the adaptive response to target pathogens. There is extensive evidence indicating that complement is activated during *P. falciparum* infection. However, there is inadequate evidence that complement contributes significantly to protection from malaria. On the contrary, complement activation during malaria infection appears to be mostly detrimental to the host. The *P. berghei* CM model suggests that, in addition to C5a, generation of MAC/TCC is critical to the development of CM, although evidence from human studies is lacking and the downstream events have yet to be established. Both human and animal data suggest that C5 activation also plays a critical role in the development of PM leading to dysregulation of angiogenesis in the placenta. Finally, there is evidence of complement deposition and of complement regulatory protein deficiencies on the erythrocytes of children with SMA, increasing the susceptibility of uninfected erythrocytes to phagocytosis.

The goal of our research is to investigate whether complement plays a role in immunity against merozoites and whether the parasite has mechanisms to evade and/or subvert the complement system for its own benefit. First, we aim to identify which main complement pathways the parasite could utilize in the invasion of erythrocytes. Second,

we intend to determine if CR1 on the surface of erythrocytes and PfRh4 on the surface of merozoites influence the role of complement during parasite invasion of erythrocytes. Lastly, we aim to determine if the inhibitory activity of human anti-merozoite antibodies from vaccinees is less in the presence than in the absence of complement.

Clinical trials of complement inhibitors, such as the anti-C5 monoclonal Eculizumab and the C3b inhibitor Compstatin, as adjunct treatment for severe *P. falciparum* malaria should be carried out to determine if they have an impact on morbidity and mortality (206,207). Furthermore, identification of cognate erythrocyte surface receptor proteins such as SEMA7A that interact with essential parasite ligands such as mTRAP continues to be critical. Elucidation of the SEMA7A/mTRAP interaction, in the context of innate immune constituents, such as complement may shed light on the question of why *in vitro* attempts to obstruct the interaction has proved unsuccessful in reducing parasite erythrocyte invasion.

## Chapter II: Materials and Methods

### Parasites, Parasite Culture, and RBC Treatment

SA-independent strains (7G8, 3D7, HB3, and Dd2NM) were obtained from the Walter Reed Army Institute of Research. SA-dependent strains (FVO, Camp, Dd2, and FCR3) were obtained from the Malaria Research and Reference Reagent Resource Center (ATCC, Manassas, VA, USA). Parasite cultures were maintained at 1-4% hematocrit (Hct) in O+ blood with 10% HI plasma in RPMI 1640 Medium (Sigma-Aldrich, St. Louis, MO) with 25 µg/ml gentamicin, 20 µg/ml hypoxanthine, and 7.5% w/v NaHCO<sub>3</sub> in malaria gas (5% O<sub>2</sub>, 5% CO<sub>2</sub>, and 95% N) at 37 °C. Cultures were synchronized twice a week by 5% sorbitol lysis (208). Neuraminidase treatment of RBCs was carried out as described (15).

### Antibodies

Polyclonal chicken anti-CR1 was raised against recombinant sCR1 (Celldex Therapeutics, Hampton, NJ) by immunizing chickens (Gallus Immunotech, ON, CA). Total IgY from naïve chickens was used as control. MSP1-specific mouse monoclonal antibody mAb5.2 was produced against purified parasite MSP1 (209). Mouse IgG2b Clone eBMG2b (eBioscience, San Diego, CA, USA) was used as isotype control. IgG from individuals vaccinated with MSP1<sub>42</sub>, comprising the C-terminal 42-kDa portion of the FVO variant of *P. falciparum* MSP1, was purified by protein A/G chromatography (Thermo Scientific, Rockford, IL) (210).

## **Sera, Complement Factors, and CH50/AH50**

Non-hemolyzed whole blood was collected with a 21 gauge needle from human volunteers in glass venous blood collection tubes without additives (Becton Dickinson, Franklin Lakes, NJ) and allowed to clot at room temperature for 50 min. The samples were centrifuged at 1,300xg for 15 min and the serum supernatant was collected and re-spun for 5 min at 1,300xg to pellet any residual RBCs or clot particles. The serum was aliquoted and stored at  $-80^{\circ}\text{C}$ .

Serum was used untreated (fresh) or HI at  $56^{\circ}\text{C}$  for up to 30 min in 200  $\mu\text{l}$  aliquots in 1.5 ml polypropylene microcentrifuge tubes (Denville Scientific, South Plainfield, New Jersey Inc.). Serum C2 and Factor B (fB) were selectively inactivated by incubation of 200  $\mu\text{l}$  serum aliquots in 1.5 ml microcentrifuge tubes in a  $56^{\circ}\text{C}$  water bath for 3 min with constant mixing (211). Purified complement factors and selectively depleted or inactivated sera were obtained commercially (CompTech, Tyler, Texas). CH50 and AH50 assays were performed using the standard methods described in the literature (212).

## ***In Vitro* Invasion and Growth Assays**

On the day of invasion or growth assay setup (Day 0) fresh erythrocytes were added to triplicate wells of a 96-well plate at 2-4% Hct containing sorbitol-synchronized cultures with predominantly late trophozoites or schizonts and a final parasitemia of 0.5% to 2%. HIS, untreated serum, complement-depleted or inactivated serum, and complement reconstituted serum were added to a final concentration of 10%. The cyclical C3 inhibitor peptide compstatin (NH<sub>3</sub>-Ile-Cys-Val-Val-Gln-Asp-Trp-Gly-His-His-

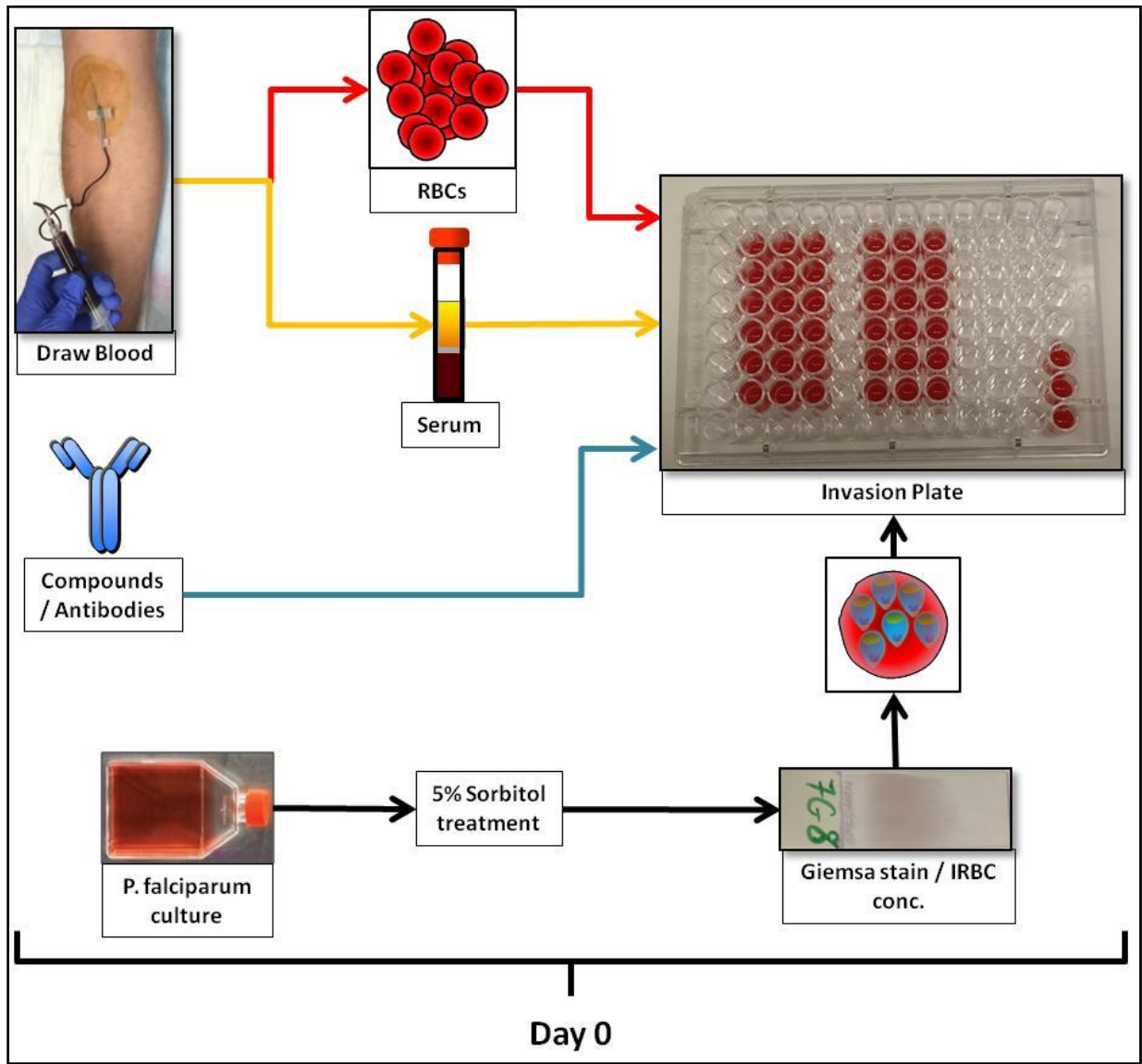


Arg-Cys-Thr-COOH) (Tocris Bioscience, Bristol, UK), was used to determine the effect of blocking C3 (206,213). A peptide derived from the linearized and scrambled sequence of compstatin without cysteins (NH<sub>3</sub>-Arg-Thr-Ala-Trp-Gln-His-Asp-Ala-Ile-His-Val-Gly-Val-COOH) was synthesized and used as a control. sCR1 was used as an inhibitor (AVANT Immunotherapeutics, Inc., Needham, MA, USA) and fetuin (Sigma-Aldrich) was used as negative control protein where appropriate. The plate was placed in a gas-impermeable heat-sealable bag and inflated with malaria culture gas (214). After 20 to 24 hours post invasion or growth assay setup (Day 1), 5  $\mu$ L aliquots of individual wells were added to 100  $\mu$ L 5  $\mu$ g/ml Hoechst 33342 (Life Technologies, Grand Island, NY) in PBS containing 2% paraformaldehyde.

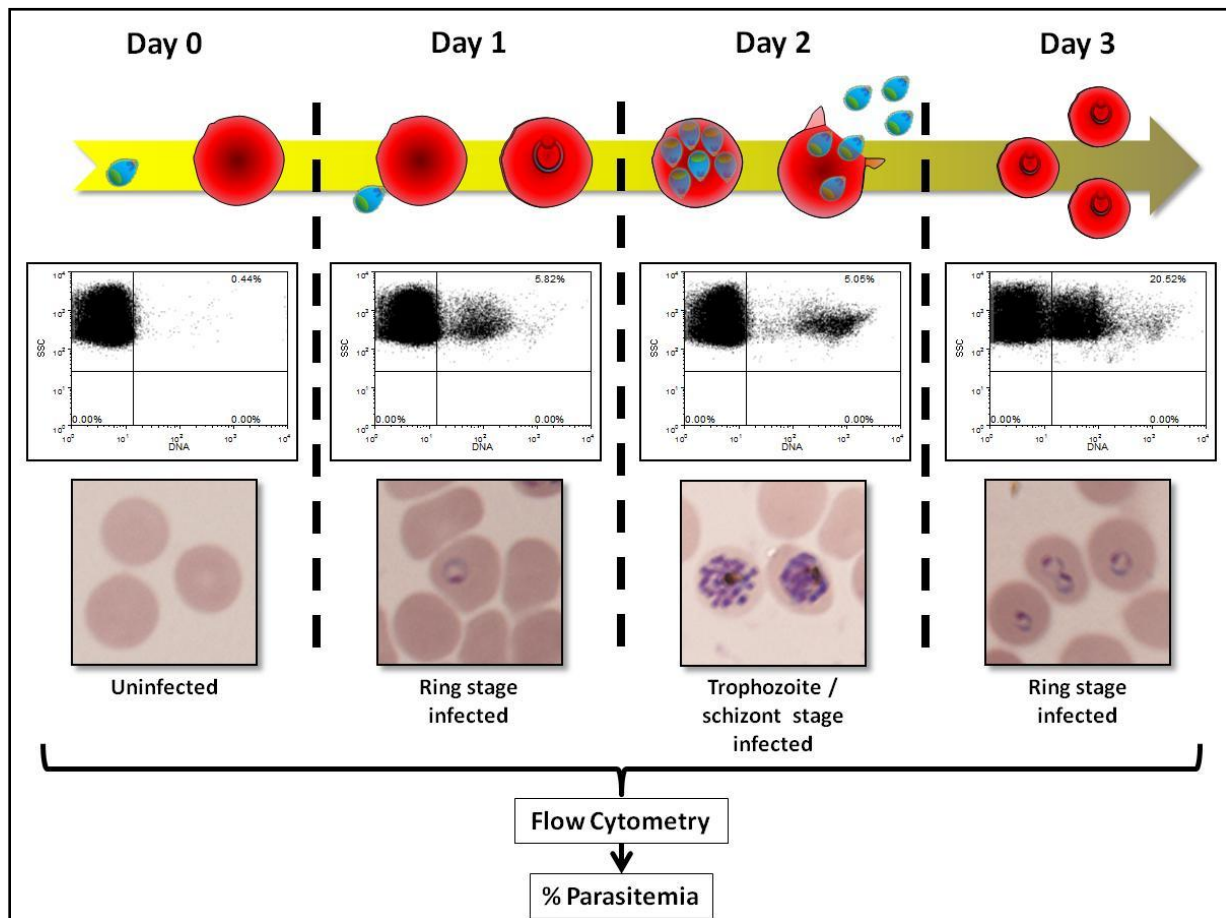
Growth assays were extended for an additional 48 hours (Day 2 and Day 3) for a total of 72 hours post assay setup. Each day a 5  $\mu$ L reaction aliquot was added to 100  $\mu$ L of 5  $\mu$ g/ml Hoechst 33342 in PBS containing 2% paraformaldehyde. Prior to second round of invasion or 48 hours post growth assay setup (Day 2), the 96 well plate was centrifuged at 771xg for 5 min at 21°C and the supernatant was carefully aspirated so as not to disturb the erythrocyte pellet. Fresh reagents were added to the same concentrations as on the day of initial setup and the plate was re-gassed.

At least 100,000 RBCs were acquired for each sample. Acquisition was done using a LSRII flow cytometer (Becton-Dickinson) equipped with a violet laser and analysis was performed using FCS Express (De novo Software). RBCs with Hoechst-positive ring stage parasitemia (early trophozoite) were used as endpoint for 24-hour invasion assays. The background staining of an uninfected RBC sample was subtracted. All experiments were repeated 2-3 times.

A



B



**Figure 2.1: Invasion / Growth Assay.** A) Within 5 days of assay setup, whole blood was drawn with a 21 G needle into a syringe containing citrate-phosphate-dextrose solution with adenine (Sigma-Aldrich) at 1:10 the total volume of the blood draw. Erythrocytes (RBCs) from whole blood were separated from buffy coat and plasma by centrifugation at 771xg for 5 min and washed. Serum was prepared from the same donor as described above. On the day of invasion or growth assay setup (Day 0), late stage *P. falciparum* infected erythrocytes, sorbitol synchronized 24 hours prior to assay setup, were combined with uninfected erythrocytes to a final parasitemia of 0.5% at 2% Hct in a 96 well plate, in the presence of 10% fresh or heat inactivated serum. *P. falciparum* erythrocyte invasion or growth was monitored in the presence of test compounds, such as compstatin, soluble CR1 or anti-*P. falciparum* antibodies. Invasion or growth assay reactions were setup in wells located internally in the 96 well plate in order to minimize any variability due to evaporation. Empty wells around the periphery and internally were filled with 1x PBS to provide humidity and limit overall evaporation from test wells. The 96 well plate was placed in a gas-impermeable heat-sealable bag, with a water saturated paper towel, inflated with malaria culture gas and placed in 37°C incubator (214). B) After 24 hours post invasion or growth assay setup (Day 1), an aliquot from individual wells was stained for DNA content with Hoechst 33342 in PBS

containing 2% paraformaldehyde. Acquisition by flow cytometry of infected erythrocytes with Hoechst-positive (DNA) ring stage parasitemia was used as endpoint for 24-hour invasion assays (Day 1). Growth assays were extended for an additional 48 hours (Day 2 and Day 3) for a total of at 72 hours post assay setup. Each day a reaction aliquot was tested for parasitemia throughout the duration of the growth assay. Prior to second round of erythrocyte invasion or 48 hours post growth assay setup (Day 2), the 96 well plate was centrifuged and the supernatant was aspirated. Fresh reagents were added to the same concentrations as on the day of initial setup (Day 0) and the plate was re-gassed. DNA content of parasites at each erythrocytic stage positively correlated with Hoechst stain intensity. Day 0, uninfected erythrocytes stained negative for DNA. Day 1, newly infected ring stage erythrocytes stained positive for DNA. Day 2, as the parasite matured and asexually replicated within the erythrocyte, late stage trophozoite / schizont infected erythrocytes stained positive for DNA at a greater intensity than ring stage infected erythrocytes. Day 3, after second round of invasion most of the erythrocytes are once again ring stage infected but at a higher parasitemia than what's observed on (Day 1).

### **Merozoite Attachment Assay**

Leupeptin (Sigma-Aldrich) 10 µg/ml was added to highly synchronous late stage parasite cultures and incubated for 6 to 8 hours followed by three washes with incomplete media and harvesting in-between by centrifugation at 770xg for 5 min. Fresh erythrocytes were added to 2% Hct and 100 µL reactions were set up in 96-well well plates in the presence of 2 µM cytochalasin D (Sigma-Aldrich) along with 10% C3/C4-inactivated serum or C3-depleted serum, and 40 µg/ml mAb5.2 or IgG2b isotype control. For repletion of deficient 10% serum we added C4 to a final concentration of 64 µg/ml and/or C3 to a final concentration of 100 µg/ml (CompTech, Tyler, TX, USA). The plates were gassed and incubated at 37 °C for 3-4 hours as before. The reaction plate was centrifuged at 400xg for 1 min 4°C and washed twice with 100 µL of 1% BSA/PBS blocking buffer. Primary mouse anti-MSP1 mAb5.2 (2 mg/ml) and goat polyclonal anti-C3 (MP Biomedicals) antibodies diluted 1:33 were added in blocking buffer for 30 min at 4 °C. Following three washes with blocking buffer, the secondary donkey anti-goat IgG-

PerCP (R&D Systems) and goat anti-mouse-DyLight 488 (KPL, Kirkegaard & Perry Laboratories) antibodies diluted 1:100 were added in blocking buffer for 30 min at 4 °C. Following three washes with blocking buffer the pellets were resuspended in 2% formaldehyde/PBS with 5 ug/ml Hoechst 33342 solution. Acquisition was carried out as above.

### **Immunofluorescence Microscopy of Merozoite Attachment**

For detection of erythrocytes with attached merozoites we followed the same protocol as described above for merozoite attachment assays. However, after the initial assay we resuspended the pellets in 1% BSA/PBS blocking buffer containing 130 µg/ml mouse anti-MSP1 mAb5.2 (if not present during the assay), 190 µg/ml of chicken polyclonal anti-CR1 (Gallus Immunotech), and 30 µg/ml of mouse monoclonal 1H8 Anti-C3 IgG1 (Kerafast) and incubated for 30 min at 4 °C. After three washes with blocking buffer, the pellets were resuspend in 100 µL of blocking buffer containing 1:500 dilutions of goat anti-mouse IgG2b-DyLight 488 (Thermo Fisher Scientific Inc.), goat anti-chicken Alexa Fluor 546 (Thermo Fisher Scientific Inc.), goat anti-mouse IgG1-Alexa Fluor 594 (Thermo Fisher Scientific Inc.), and 30 µM Vybrant DID cell labeling solution (Molecular Probes, Inc, Eugene, OR) and incubated for 30 min at 4 °C. After an additional three washes, the pellets were resuspended in 4% paraformaldehyde with 10 µg/ml Hoechst 33342. Prior to imaging, 10 µL of the cell suspension was transferred into 0.6 ml tubes and centrifuged at 200xg for 1 min. The supernatant was discarded and the pellet was resuspended in 10 µL of VectorShield Hard Set (Vector Labs, Inc.). 4 µL of this suspension was added to wells of a multi-well slide. Fluorescence z-series

were collected on a LEICA SP8 confocal microscope at 40x oil immersion. The images were processed using Imaris (Bitplane) image processing software.

### **Flow Cytometry for Merozoite Surface C3 Deposition**

Sorbitol-synchronized late stage parasite cultures were incubated with 1 mg/ml TPCK-treated trypsin (Sigma-Aldrich) for one hour at 37 °C and washed three times with incomplete media by centrifuging 771xg for 5 min. Leupeptin (Sigma-Aldrich) 10 µg/ml was added and incubated for 6 to 8 hours in complete media. Once majority of the culture matured to schizont stage, as determined by Giemsa staining, the culture was washed three times with incomplete media and centrifuging 771xg for 5 min to washout leupeptin. The culture was resuspended in media containing 20% fresh serum or 30 min HIS, gassed and placed back into 37 °C and monitored for merozoite egress. Once 50% of the merzoites egressed from the IRBCs, the culture was centrifuged for 5 min at 800xg and the supernatant constraining the free merzoites was centrifuged for 15min at 3,300xg. Merozoite pellet was washed once in stain/wash buffer (1%BSA/PBS/10mM EDTA) and incubated for 30 min at RT in 100ul of 1:50 dilution of Goat Anti-Human Complement C3 (MP Biomedicals). After 2 washes, the merzoite pellet was incubated for 30 min at RT in 100ul of 0.25 ug/ml of Donkey Anti-Goat Polyclonal IgG, PerCP (R&D Systems) in stain buffer. Following two more washes, the merozoite pellets were stained with Hoechst 33342 (2 µg/ml in PF) (Life Technologies) for 15 min at RT. Acquisition was carried out as above.

### **Passive Transfer Experiments**

Polyclonal anti-*P. berghei* antibody was generated by intraperitoneal infection of C57BL/6 mice with *P. berghei* ANKA ( $1 \times 10^6$  pRBC). Parasitemia was monitored with Giemsa smears and once parasitemia reached (5%-10%) the mice were treated with sub-curative dose of chloroquine (500  $\mu\text{g}/\text{mouse}$ ) for three consecutive days. The parasitemia was allowed to rebound and the cycle was repeated for a total of 3 times. Plasma was collected from infected mice two weeks after the third cycle in citrate phosphate dextrose solution (CPD) and total antibody was purified using protein A/G columns (GenScript, Piscataway, NJ). Antibody purified from uninfected mice served as control. Wild type and C3 deficient mice of C57BL/6 background (The Jackson Laboratory, Bar Harbor, ME) were injected with  $1.5 \times 10^7$  IRBCs in 100  $\mu\text{L}$  of RPMI 1640 in one retro-orbital plexus and with 100  $\mu\text{L}$  of anti-*P. berghei*, control antibodies, or 100  $\mu\text{L}$  of PBS in the contralateral plexus. Parasitemia was monitored by staining tail vein blood with Hoechst 33342 (2  $\mu\text{g}/\text{ml}$  in PF) (Life Technologies) at pre-determined intervals until day 6 or 7 post-infection. Acquisition and analysis were performed as described above.

### **Enzyme-linked Immunosorbent Assays (ELISA)**

Plates were coated with 0.025  $\mu\text{g}/\text{well}$  of SEMA7A, CIB1, RAP1A (OriGene Technologies, Inc Rockville, MD), sCR1 (AVANT Immunotherapeutics, Inc., Needham, MA, USA) and fetuin (Sigma-Aldrich) in coating buffer (0.015 M  $\text{Na}_2\text{CO}_3$ , 0.035 M  $\text{NaHCO}_3$ , pH 9.6) and incubated overnight at 4°C. Antigen containing wells and black wells were blocked with 100  $\mu\text{L}$  of 0.5% BSA in coating buffer for 2.5 to 3 h at 37°C. Reaction wells were washed 4 times with 100  $\mu\text{L}$  of Tween wash (0.9% NaCl plus

0.05% Tween). FS, HIS, complement depleted or rescued serum were added to the plate and allowed to incubated for 1 h at 37°C. Reaction wells were washed 4 times with 100 uL of Tween wash. For detection of complement deposition 100ul of 10 µg/ml of mouse anti-C3 Clone#1H8 IgG1 (Kerafast Boston, MA), 2 µg/ml mouse anti-C3a Clone# 474 (Thermo Scientific Waltham, MA), or 10 ug/ml rabbit anti-C4 polyclonal (AssayPro, St. Charles, MO) were added to the plate and allowed to incubated for 1 h at 37°C. Reaction wells were washed 4 times with 100 uL of Tween wash. Secondary goat anti-mouse peroxidase labeled (KPL, Gaithersburg, MD) and goat anti-rabbit peroxidase labeled (Thermo Scientific, Waltham, MA) at 1:1000 dilution were added to the plate and allowed to incubated for 1 h at 37°C. Reaction wells were washed 4 times with 100 uL of Tween wash. Reactions were developed with the addition of 100 uL of ABTS Peroxidase Substrate (KPL, Gaithersburg, MD,) and absorbance was read at OD 410 nm.

### **Microspheres Complement Coating**

Incubated 10ul of 2% stock suspension of 1 µm blue fluorescent (365/415) FluoSpheres Carboxylate-Modified Microspheres (Thermo Fisher Scientific, Eugene, OR) with 50% fresh or 30 min HI serum in incomplete malaria media in 100ul reaction for 30 min at 37°C water bath. For a 2% suspension of 1 µm polystyrene microspheres, the number of microspheres per mL =  $[6 \times (0.02 \text{ g/ml}) \times 10^{12}] / [(1.05 \times \pi \times 1^3)] = 3.6 \times 10^{10}$  microspheres / ml. Microspheres were washed three times with 1% (wt/vol) BSA/PBS blocking buffer and centrifuged at 5,000xg for 5 min. For detection of complement deposition, microspheres were stained with 100ul of 20 µg/ml of mouse



anti-C3 Clone#1H8 IgG1 (Kerafast Boston, MA) in blocking buffer for 30 min at 4°C. Followed by three washes with blocking buffer and centrifuged at 5,000xg for 5 min. Microspheres were incubated for 30 min at 4°C in 100ul of 1:100 dilution of goat anti-mouse DyLight 488 (KPL, Kirkegaard & Perry Laboratories). Following three washes with blocking buffer, and centrifuged at 5,000xg for 5 min, the microsphere pellets were resuspended in 2% formaldehyde/PBS solution. Flow cytometric acquisition was carried out as described above.

### **Complement Opsonized Microsphere Erythrocyte Attachment Assay**

Incubated erythrocytes at 1% Hct in 100ul reaction volume in incomplete media with 10ul of complement coated microspheres, prepared as described above, in the presence of 40 ug/ml goat polyclonal anti-C3 (MP Biomedicals), chicken polyclonal anti-CR1 (Gallus Immunotech), goat polyclonal anti-SEMA7A (R&D System), goat polyclonal IgG control (R&D Systems) or control chicken IgY for 30min at 37°C. Erythrocytes were washed three times with 1% (wt/vol) BSA/PBS blocking buffer and centrifuged at 200xg for 1 min. Cell pellets were resuspended in 2% formaldehyde/PBS solution. Flow cytometric acquisition was carried out as described above.

### **Erythrocyte Surface Staining**

Erythrocytes at 5ul of 4% Hct ( $2 \times 10^6$  cells) were washed three times with 1% (wt/vol) BSA/PBS blocking buffer and centrifuged at 400xg for 1 min. For detection of cell surface receptors, erythrocytes were stained with 100ul of 20 µg/ml goat polyclonal anti-SEMA7A (R&D System), 1:100 dilution of rabbit anti-CD59 (USBiological Life Science), 1:100 dilution of anti-CD55 Clone JS11 IgG1 in blocking buffer for 30 min at

4°C. Followed by three washes with blocking buffer and centrifuged at 400xg for 1 min. Erythrocytes were incubated for 30 min at 4°C in 100ul of 1:100 dilution of goat anti-mouse DyLight 488 (KPL, Kirkegaard & Perry Laboratories) goat anti-rabbit FITC (Santa Cruz Biotechnology) and rabbit anti-goat DyLight 488 (KPL). Following three washes with blocking buffer, and centrifuged at 400xg for 1 min, the cell pellets were resuspended in 2% formaldehyde/PBS solution. Flow cytometric acquisition was carried out as described above.

### **Ethics Statement**

All human samples were obtained under protocols approved by the pertinent review boards. Consent was obtained from all participants except when the samples were obtained under non-human subject determination.

### **Statistical Analysis**

Statistical analysis was done with SigmaPlot v11.2 (Systat Software, San José, CA). Differences between groups in variables that showed a normal distribution were evaluated by analysis of variance or t-test, whichever was more appropriate. Post-hoc Holm-Sidak tests were used to correct for multiple comparisons. All tests were two-tailed. A  $P < 0.05$  was considered statistically significant.

## Chapter III: Complement and Antibody-mediated Enhancement of Red Cell Invasion and Growth of *Plasmodium falciparum*

### Abstract:

*Plasmodium falciparum* malaria kills hundreds of thousands of people every year. An effective vaccine that blocks red blood cell (RBC) invasion is still not available. Although anti-merozoite antibodies block invasion *in vitro*, they have no efficacy *in vivo*. Since merozoites utilize the complement receptor 1 (CR1) on RBCs to invade erythrocytes we reasoned that complement activation could enhance invasion. To test this hypothesis we studied the role of complement in RBC invasion *in vitro* and parasite growth in an animal model. Relative to heat-inactivated serum (HIS) fresh serum enhanced RBC invasion. Anti-merozoite monoclonal mAb5.2, directed against MSP1<sub>19</sub>, enhanced invasion that was inhibited by HIS, the C3 inhibitor compstatin, and soluble CR1 (sCR1). Antibody and complement-mediated invasion led to aggregation and colocalization of CR1, C3, and lipids on the RBC surface at the point of merozoite contact. Sialic acid-dependent strains that do not utilize native ligands to bind to CR1 were more dependent on complement for invasion of neuraminidase-treated RBCs than SA-independent strains that do use native ligands. Total IgG from MSP1<sub>42</sub> vaccinees enhanced invasion in a complement-dependent manner. Finally, total anti-*P. berghei* IgG enhanced parasite growth in mice, and C3-deficient mice showed less parasite

growth than wild type mice. Our results demonstrate that merozoites are able to evade the host immune response and hijack the complement system for their own benefit.

### **Significance:**

*Plasmodium falciparum* malaria kills hundreds of thousands of people. The parasite invades and destroys red blood cells (RBCs). The development of a vaccine that blocks RBC invasion has been an elusive. Here we demonstrate that one possible explanation for this failure is that activation of the complement cascade by antibodies against merozoites enhances their attachment to and invasion of RBCs via the complement receptor 1 (CR1). Antibodies that target merozoites and activate complement can paradoxically aid the parasite in binding to and invading RBCs. Thus we demonstrate that malaria parasites can utilize the host immune response for their own advantage.

## Introduction:

Malaria, a mosquito-borne infectious disease caused by eukaryotic intracellular protists of the genus *Plasmodium*, kills over a million people worldwide each year, predominantly children under 5 years of age (1,215). Of the five species of *Plasmodium* that infect humans, *P. falciparum* accounts for the vast majority of deaths worldwide. *Plasmodium's* complex life cycle involves invasion of hepatocytes and red blood cells (RBCs); however, the clinical symptoms arise from the invasion of RBCs by the asexual blood stage parasite. Antibodies play an important role in natural immunity. Antibodies role has been demonstrated by the reduction in parasitemia and clinical symptoms in *P. falciparum*-infected individuals as a result of passive transfer of immunoglobulins from immune donors, but the effector mechanisms are poorly understood (216-218).

A vaccine to block RBC invasion has proven to be elusive. Much of the effort has focused on the merozoite surface protein 1 (MSP1) and the apical membrane antigen 1 (AMA1). Numerous preclinical vaccine studies eliciting antibodies targeting MSP1 and AMA1 of *P. falciparum* have demonstrated reduced RBC invasion *in vitro* and reduced parasite growth in growth inhibition assays (GIA), and protective immunity in different animal models (219-224). Unfortunately, to date these studies have not translated into successful human vaccine trials (80,82,225). Thus, GIA is a poor predictor of blood stage protective immune responses despite the fact that antibodies inhibit RBC invasion. The reasons for this discrepancy are unknown.

One possible explanation for vaccine failure is related to the discovery that the complement receptor 1 (CR1) is a sialic acid (SA)-independent receptor for *P. falciparum* (15). CR1 binds complement factors C1q, C3b and C4b (226,227). The

complement system is part of the innate immune response and is an important effector arm of humoral immunity. The complement system can be activated via three main pathways: The classical pathway (CP); the lectin pathway (LP); and the alternative pathway (AP) (95). Once activated, the complement system induces the formation of opsonins (C3b, C4b) that promote phagocytosis, induce direct killing by means of membrane lysis of microbial organisms, and promote an inflammatory response (95). Once bound to the pathogen, surface C3b and C4b serve as ligands for CR1, which is present on RBCs as well as most leukocytes (226,227).

We hypothesize that *P. falciparum* is capable of exploiting the opsonizing qualities of complement deposition on the merozoite surface to allow the parasite to bind to CR1 and invade via this novel invasion pathway. Complement activation could therefore negate the inhibitory activity of anti-merozoite neutralizing antibodies generated post vaccination or during natural infection.

## Results:

### **Invasion of RBCs is Enhanced in Fresh Serum Relative to Heat-inactivated Serum and in the Presence of an Anti-merozoite Monoclonal Antibody:**

To test the hypothesis that complement can enhance erythrocyte invasion, we tested invasion of RBCs by *P. falciparum* SA-independent laboratory strains 7G8 and 3D7 in fresh or heat-inactivated serum (HIS). Although traditional methods of complement inactivation in serum utilize a condition of 30 min at 56 °C, this protocol likely inactivates multiple enzymes in the complement pathway. To achieve a more selective inactivation, we incubated serum for 3 min or 5 min at 56 °C and confirmed inactivation by measuring 50% complement hemolytic activity of the classical and alternative pathways (CH50 and AH50) (Supplementary Figure 3.1) (211). Figure 3.1A shows the ring parasitemia results of overnight invasion in the presence of 3 min, 5 min, or 30 min HIS or fresh serum. Figure 3.1B shows the enhancement of parasitemia in fresh serum relative to 30 min HIS in the same experiment. The use of fresh serum resulted in a 40% invasion enhancement. A drastic reduction in invasion enhancement was observed with 3 or 5 min HIS (Figure 3.1B). These results suggest that complement may be involved in mediating the enhancement of invasion observed in fresh serum relative to HIS. Subsequent experiments are presented as the relative enhancement over 30 min HIS.

To determine whether activation of complement by anti-merozoite antibodies via the classical pathway could enhance invasion, we used mAb5.2 (MR4, Manasas, VA), raised against the C-terminal 19kD fragment of MSP1 *P. falciparum* Uganda-Palo Alto (209,228). This antibody was selected due to its availability and to the relative ease of

culture of the hybridoma. Figure 3.2 shows that, as before, relative to 30 min HIS invasion was significantly enhanced in fresh serum. Invasion was enhanced by 58% by addition of mAb5.2 but not by the addition of IgG2b isotype control, suggesting that some antibodies that target the parasite enhance erythrocyte invasion (Figure 3.2).

### **Enhancement of Invasion by Anti-merozoite Antibodies is Complement-dependent:**

By measuring CH50 and AH50 we confirmed that addition of C2 and fB reversed the 3 min heat inactivation of complement (211). Therefore, we tested the effect of 3 min HIS on the ability of mAb5.2 to enhance parasite invasion of RBCs. 3 min HIS abolished the antibody-mediated enhancement of invasion (Figure 3.3) (211). Addition of purified C2 and fB to 3 min HIS serum rescued antibody-mediated enhancement of invasion but there was no rescue in the absence of antibody or in the presence of isotype control. C2 or fB added separately also had no effect (Supplementary Figure 3.2).

To confirm that complement is responsible for antibody-mediated enhancement of RBC invasion, we used the C3-selective inhibitor Compstatin, a cyclic 13-amino acid peptide that inhibits C3 cleavage and activation in human serum (229). Once again enhancement of invasion was observed in fresh serum by mAb5.2. The addition of Compstatin prevented this enhancement (Figure 3.4). A scrambled 13-amino acid linear control peptide in fresh serum did not prevent enhancement by mAb5.2. Interestingly, Compstatin had no effect on the enhancement of invasion by fresh serum



alone. These results support the hypothesis that the classical pathway of complement activation, mediated by anti-merozoite antibodies, can enhance RBC invasion.

### **Soluble CR1 Blocks Enhancement of Invasion by Fresh Serum and Anti-merozoite Antibodies:**

CR1 binds C3b and C4b-opsonized immune complexes and was recently shown to be the SA-independent receptor of *P. falciparum* (15,226,227). Therefore, it is likely that CR1 is involved in complement and antibody-mediated enhancement of RBC invasion by *P. falciparum*. To ascertain the role of CR1 in the enhancement of invasion by fresh serum and anti-merozoite antibody, we used soluble CR1 (sCR1) as an inhibitor. Anti-merozoite antibody-mediated enhancement induced by mAb5.2 in fresh serum was not only negated in the presence of sCR1 but produced inhibition of invasion when compared to the isotype control, IgG2b (Figure 3.5). sCR1 had no effect in the absence of mAb5.2 (Figure 3.5).

### **SA-dependent Strains Demonstrate Greater Enhancement of Invasion in the Presence of Complement than SA-independent Strains:**

*P. falciparum* can be subdivided into SA-dependent strains that invade neuraminidase (NA)-treated RBCs at a low level and SA-independent strains that invade NA-treated RBCs at a higher level via CR1 and the merozoite ligand PfRh4 (37). Because SA-dependent strains do not utilize PfRh4 for CR1-mediated invasion, we hypothesized that these strains are more reliant on complement to accomplish this function. Hence, we compared invasion of untreated and NA-treated RBCs by SA-

dependent and SA-independent strains in fresh and 30 min HIS. Figure 3.6A shows that fresh serum enhanced the invasion of NA-treated RBCs by SA-dependent strains to a greater extent than SA-independent strains (median enhancement 54.8% vs. 31.5%,  $P < 0.01$ ). Similar results, although overall enhancement levels were lower, were observed after C3 + C4 were restored to C3/C4 inactivated sera (Figure 3.6B).

### **Anti-merozoite mAb5.2 in the Presence of Complement Enhances Merozoite**

#### **Attachement to RBCs:**

To address whether the presence of complement enhances the binding of merozoites to the RBC surface, we performed attachment assays. To inhibit merozoite invagination we allowed merozoites to egress in the presence of cytochalasin D, in complement deficient or reconstituted serum, and either mAb5.2 or IgG2b isotype control (230). We then detected RBCs with surface merozoites using flow cytometry with mAb5.2. As expected, in the presence of mAb5.2 and complement deficient serum reconstituted with either C3/C4 or C3, a higher percentage of RBCs had attached merozoites and surface C3 than when IgG2b isotype control antibody and complement deficient serum was used (Figure 3.7A & 3.7B). In addition C3b deposition was enhanced on single merozoites after incubation in fresh serum but not in 30 min HIS (Supplementary Figure 3.3).

### **Anti-merozoite Antibody mAb5.2 Induces CR1 Aggregation and Complement**

#### **Deposition at the RBC-merozoite Interface:**

To detect the direct interaction of merozoites with RBCs in the presence of complement and mAb5.2 we performed immunofluorescence microscopy. Attachment

assays were performed in C3 depleted or reconstituted serum, with or without mAb5.2. Figure 3.8 shows that in the presence of C3 and mAb5.2 there was dense aggregation of CR1 and lipids based on DID staining, and C3 was deposited at the site of merozoite contact with the RBC. Also, as previously noted, regardless of the presence of complement CR1 produced classical punctate staining in this case both on the RBCs and on the merozoites (15). This suggests that merozoites could have surface CR1.

### **Complement Decreases Invasion Inhibitory Activity of Human Anti-MSP1**

#### **Antibodies:**

To assess the significance of human antibody-mediated complement activation on RBC invasion we purified total IgG from MSP1<sub>42</sub> vaccinees (210). Using C3/C4-inactivated or reconstituted serum we performed invasion assays in the presence of 2.5 mg/ml total IgG from MSP1 vaccinees or unvaccinated controls. Inhibition was calculated relative to PBS-only since we did not have pre-immunization serum. The median inhibitory activity in C3/C4-inactivated serum was about 6%, but upon reconstitution of serum with the missing complement factors the inhibitory activity was not only eliminated but became negative, indicating enhancement (Figure 3.9). The inhibitory activity with IgG from unvaccinated controls was not affected by the presence or absence of C3/C4.

#### **Passive Transfer of Anti-*P. berghei* Antibodies Enhances Parasitemia in a Dose-dependent Manner:**

To test whether the antibody-mediated complement enhancement of RBC invasion observed *in vitro* could be reproduced *in vivo* we infected C57BL/6 mice with *P. berghei* ANKA and administered varying concentrations of polyclonal total anti-*P. berghei* IgG (anti-Pb), control total IgG from naïve mice, or PBS.

Compared to the PBS control group the anti-*P. berghei* antibody enhanced parasitemia in an inverse dose dependent manner (Figure 3.10). This result is consistent with the previous observation that high doses of human IgG from malaria-exposed individuals reduce parasitemia (45,217,218). Surprisingly, control antibodies inhibited parasitemia, independently of the dose administered.

### **C3 Deficient Mice Show Blunted Parasite Growth:**

To explore further the role of C3 in *in vivo* parasite growth, we infected C3<sup>-/-</sup> mice with *P. berghei* in the presence of anti-*P. berghei*, control antibodies, or PBS. Figure 3.11 shows that C3<sup>-/-</sup> mice supported less parasite growth than their wild type counterparts in each group, although the reduction was statistically significant only in the PBS group. C3<sup>-/-</sup> mice that received anti-Pb antibody showed enhanced parasitemia that did not reach statistical significance. C3<sup>-/-</sup> mice that received control IgG had an intermediate level of reduction.

## Discussion:

The complement cascade is part of the innate immune response. In addition, it potentiates the effect of antibodies that are part of the adaptive immune response. Antibodies that coat pathogens in the circulation induce activation of the complement cascade leading to opsonization with C4b and C3b. Opsonized pathogens bind to CR1 on RBCs via C4b and C3b. When the RBCs traverse the liver or spleen and encounter macrophages, attached pathogens are removed by phagocytosis, and the RBCs are spared and recirculate (231). This process of binding to and removal of opsonized pathogens is beneficial to the host. However, unlike in other pathogens, opsonization of malaria merozoites may facilitate binding to their ultimate target cell, the RBC. Therefore, we reasoned that opsonization of merozoites may enhance RBC invasion. Enhancement of invasion by complement has been reported for other pathogens such as *Leishmania* (232-235).

We showed that fresh untreated serum enhanced RBC invasion compared to serum that was heat inactivated for 3, 5, and 30 min. Although the CH50 activity of 3 min HIS was restored by addition of C2 and fB, addition of these factors did not rescue the RBC invasion enhancing activity of fresh serum. Thus, we suspect that elements of the LP upstream of C2 are also involved. On the other hand, we showed that the invasion enhancing activity of mAb5.2 required C2 and fB. Because C2 is an integral part of the CP and fB of the AP amplification loop, these data provide strong evidence that both of these pathways of complement activation play a role in antibody-mediated enhancement of RBC invasion. The involvement of the CP is not surprising, given its reliance on antibodies. However, the critical role of antibodies in the AP is less well

known. C3b<sub>2</sub>-IgG complexes play an important role in stabilizing the nascent AP convertase (236). Also, even when the classical pathway is the initial source of complement activation the AP serves as the main amplification loop for the majority of complement deposition (237,238). The important role of C3 was further demonstrated by the effect of the C3 specific inhibitor compstatin. Use of this inhibitor eliminated antibody-mediated enhancement of invasion but had no effect on enhancement by serum alone (Figure 3.3). This result is consistent with the observation that inactivation of C2 and fB also inhibits antibody-mediated enhancement of invasion, as these factors are critical for the activation of C3 in the CP and AP.

Because our data support a critical role for C3 in antibody-mediated enhancement of RBC invasion, we investigated the role of CR1 in this process. When sCR1 was used as a competitor not only was antibody-mediated enhancement in fresh serum completely reversed, but compared to the isotype there was significant inhibition (Figure 3.5). The inhibitory activity of sCR1 may arise from any of its diverse functions. Firstly, sCR1 competes with erythrocyte surface CR1, which may lead to large C3b-sCR1 complexes on the merozoite surface and produce steric hindrance of ligand receptor interactions. Secondly, sCR1 binds PfRh4 on the merozoite surface, thereby inhibiting the direct interaction of erythrocyte surface CR1 with PfRh4. Third, sCR1 possesses complement regulatory activity that destabilizes membrane assembled C3 convertases through its intrinsic decay accelerating activity as well as by acting as a cofactor for Factor I, which breaks down C3b into iC3b.

Because SA-dependent strains cannot use PfRh4 as a CR1 ligand, we reasoned that these strains may rely more on complement for CR1-mediated invasion than SA-

independent strains that can utilize PfRh4. To test this hypothesis, we used NA-treated RBCs for which invasion is almost totally CR1-mediated (15). The overall invasion of NA-treated RBCs by SA-dependent and independent strains was reduced in both complement sufficient and deficient serum. Although for both SA-dependent and independent strains, invasion of NA-treated RBCs was enhanced in complement sufficient serum to a greater extent than that of untreated RBCs. The enhancement occurred either when fresh serum was compared to HIS, or C3/C4-reconstituted serum was compared to C3/C4-inactivated serum (Figure 3.6). However, SA-dependent strains exhibited levels of invasion of NA-treated RBCs that were higher than SA-independent strains. Thus, it is likely that inability to make use of PfRh4 or other ligands for RBC invasion increases the reliance on complement.

We showed that complement activation via the CP increases the attachment of merozoites to RBCs. Confocal microscopy of this interaction led to a number of novel observations. In the presence of mAb5.2 and complement, the RBC membrane contained what appeared to be aggregates of CR1 and lipids, as suggested by the intense fluorescence of the lipid soluble dye DiD at the point of merozoite attachment. The colocalization of C3, CR1, and increased DiD fluorescence suggests that the aggregation of CR1 is an active process brought on by engagement with C3. We did not observe these changes in the absence of C3. The merozoites also appear to be coated with CR1. The possibility that merozoites are coated with CR1 could explain their relative resistance to complement-mediated lysis. In addition, there could be CR1-CR1 mediated interactions between merozoites and the RBC. If the merozoites bind opsonized ICs, that could lead to engagement of CR1 on the RBC.

To explain the relevance of antibody and complement-mediated enhancement of RBC invasion to clinical malaria we purified total IgG from serum collected from MSP1<sub>42</sub> vaccine recipients two weeks after three doses of vaccine (210). We measured the RBC invasion inhibitory activity of total IgG in C3/C4-inactivated or reconstituted serum. Consistent with previous results, we found very little inhibitory activity in total IgG (210). However, serum reconstituted with C3/C4 no longer inhibited RBC invasion but enhanced it in the presence of total IgG from vaccine recipients whereas IgG from non-immunized control did not. Thus, these results demonstrate that enhancement of RBC invasion can occur with human anti-merozoite antibodies by means of complement activation.

Lastly, our passive transfer experiments in mice show that anti-malaria antibody and complement can benefit parasite growth. Administration of Anti-Pb IgG enhanced parasite growth in an inverse dose-dependent manner in infected animals. These results appear to contradict previous studies that show that antibodies from immune rodents inhibit parasite growth when given to infected animals (239-243). However, the milligram doses of antibody in those studies were much higher than those we used, sometimes administered several times before and after infection. Passive transfer of total IgG from individuals living in endemic areas to acutely infected children can reduce parasitemia, but this reduction was achieved at 5-10 fold higher amounts per Kg than the doses in our studies (45,217,218). It is unlikely that these levels of antibodies are achieved during natural infection. Thus, it is possible that low doses of anti-merozoite antibodies enhance parasite growth *in vivo*. Our studies suggest that one mechanism may be complement activation. In contrast, the inhibitory activity of control polyclonal



antibody may be due to its complement scavenging properties (244,245). Consistent with this hypothesis, C3<sup>-/-</sup> mice consistently had less parasitemia than wild type mice. Yet, anti-Pb IgG still enhanced parasite growth in C3<sup>-/-</sup> mice in response to anti-Pb IgG probably due to residual C4b opsonizing activity.

While this work was in progress, Boyle *et al.* reported that anti-merozoite antibodies from humans inhibit invasion by activating complement, and thus, their results partially contradict our findings (59). The differences may be methodological in origin. In our studies the merozoites are allowed to egress naturally from RBCs, where are Boyle *et al.* use protease-synchronized trophozoites and filter-purified merozoites (59). Because of this manipulation, the merozoites may be less healthy than freshly egressed merozoites and, hence, more susceptible to complement deposition. The time it takes for merozoites to egress from RBCs to the time of RBCs invasion is around 30-120 sec (9). If the parasites are less fit, the merozoites will be susceptible to adverse complement effects, such as MAC formation, for longer period of time during their protracted invasion of RBCs. We also limited our complement concentration to the standard 10% serum or plasma level that was previously used in invasion and growth inhibition assays. Boyle *et al.* utilized higher concentrations, anywhere from 25% to 50% which may have resulted in their observed RBC invasion inhibition rather than RBC invasion enhancement that we observed. It remains feasible that some anti-merozoite antibodies are capable of enhancing invasion, whereas others are inhibitory. The final outcome would then depend on the relative amounts of the two antibodies. We support the proposal by Boyle *et al.* that complement activation should be included in GIA (59).

In summary, we have shown that fresh untreated serum contains heat labile components that enhances RBC invasion. The invasion can be inhibited by as little as 3 minutes of heat treatment of serum, which inactivates C2 and fB. Also anti-MSP1<sub>19</sub> mAb5.2 IgG<sub>2b</sub> and total IgG from MSP1<sub>42</sub> vaccinees enhance RBC invasion in a complement-dependent manner. The antibody and complement-dependent invasion appears to be CR1-mediated. This hypothesis is supported by our *in vivo* studies that showed a critical role for anti-Pb antibody and C3 in parasite growth in mice. We demonstrate that enhancement of RBC invasion by malaria merozoites can be complement mediated which allows the parasite to not only evade the immune response of the host but also hijack it for its own advantage. Our results have important implications for merozoite vaccine development. Based on our studies, merozoite vaccines may be more effective if they induce non-complement fixing antibodies.

### **Acknowledgments:**

We thank Dr. Henry Marsh, Celldex Therapeutics, for the gift of soluble CR1.

We thank Mr. Wade Edris and the microscopy core facility at Penn State College of Medicine for assistance with confocal microscopy. This work was funded by Grant P131040 from the Congressionally Directed Medical Research Program, PI José A. Stoute.

## Figure Legends:

Figure 3.1: Enhancement of *P. falciparum* invasion of RBCs with fresh serum relative to heat-inactivated serum (HIS). A) Two SA-independent strains of *P. falciparum*, 7G8 and 3D7, were tested in the presence of 10% fresh serum, 3 min HIS, 5 min HIS, or 30 min HIS. Invasion of RBCs decreased with progressively longer heat inactivation times. P is for the comparison with 30 min HIS, One-way ANOVA with post-hoc comparisons. B) Invasion of RBCs in the same experiment expressed as percent enhancement relative to 30 min HIS. P is for the comparison with 5 min HIS. Error bars represent standard deviations.

Figure 3.2: Anti-merozoite monoclonal antibody mAb5.2 enhances *P. falciparum* invasion of RBCs in the presence of fresh serum. Invasion of *P. falciparum* strain 7G8 was tested in the presence of monoclonal anti-merozoite surface protein 1(MSP1) mAb5.2 (40 µg/ml), an isotype control antibody (IgG2b), or PBS in 10% fresh serum or 30 min HIS. Invasion of erythrocytes is expressed as percent enhancement in fresh serum relative to 30 min HIS. Error bars represent standard deviations. \*P <0.001 is for one-way ANOVA with post-hoc comparisons.

Figure 3.3: Addition of C2 and fB rescues RBC invasion enhancement properties of mAb5.2 in 3 min HIS. Invasion of *P. falciparum* strain 7G8 was tested in the presence of 40 µg/ml anti-MSP1 mAb5.2, 40 µg/ml IgG2b isotype control, or PBS in the presence of 10% fresh serum, 3 min HIS, or 30 min HIS. C2 and fB were added to a final concentration of 25 µg/ml and 200 µg/ml respectively, or the addition of 225 µg/ml fetuin

as a control. Error bars represent standard deviations. P is for the comparison between mAb5.2 and IgG2b or PBS.

Figure 3.4: The C3 inhibitor compstatin inhibits enhancement of RBC invasion by mAb5.2. Invasion of *P. falciparum* strain 7G8 was tested in the presence of 40 µg/ml anti-MSP1 mAb5.2, 40 µg/ml IgG2b isotype control, or PBS in 10% fresh serum or 30 min HIS with the addition of C3 inhibitor peptide compstatin or control peptide. Invasion of erythrocytes is expressed as percent enhancement in fresh serum relative to 30 min HIS. Error bars represent standard deviations. P is based on the t-test.

Figure 3.5: Enhancement of RBC invasion by mAb5.2 is abolished in the presence of sCR1. Invasion of *P. falciparum* strain 7G8 was tested in the presence of 40 µg/ml anti-MSP1 mAb5.2 or 40 µg/ml IgG2b isotype control in the presence of 10% fresh serum or 30 min HIS. The final concentration of sCR1 or fetuin was 80 µg/ml. Error bars represent standard deviations. P is based on t-test.

Figure 3.6: Differences in enhancement of RBC invasion in NA-treated and untreated RBCs between SA-dependent and independent strains. A) SA-dependent strains show greater enhancement of invasion into NA-treated RBC (median 54.8%) in the presence of fresh serum relative to 30 min HIS than SA-independent strains (median 31.5%),  $P < 0.01$  by t-test. B) Enhancement of invasion into NA-treated RBCs in the presence of C3/C4 reconstituted serum relative to C3/C4-inactivated serum. P values are based on t-test.

Figure 3.7: Antibody and complement-mediated enhancement of merozoite attachment to RBCs. Attachment of *P. falciparum* strain 3D7 was tested in the presence of anti-MSP1 mAb5.2 or IgG2b isotype control, in the presence of A) C3/C4-inactivated or C3/C4-reconstituted serum and B) C3-depleted serum or C3-reconstituted serum.

Figure 3.8: Interaction of merozoites with RBCs in the presence of anti-MSP1 mAb5.2 and in the presence or absence of C3. C3-reconstituted serum and anti-MSP1 mAb5.2 bind to RBCs via CR1 (yellow) which shows intense aggregation at the site of merozoite contact. This area is also positive for C3 (cyan) and shows lipid accumulation by DiD staining (red). Hoechst 33342 was used to stain DNA (blue) and MSP1 staining shows green. On the other hand, using C3-depleted serum there is little or no C3 deposition, and absence of CR1 aggregation or lipid accumulation. Of interest, we observed the characteristic speckled staining of CR1 (yellow) on the surface of all the merozoites. Scale bar is 2  $\mu\text{m}$ .

Figure 3.9: Antibodies from MSP1<sub>42</sub> vaccine recipients enhance invasion in a complement-dependent manner. A) Invasion of *P. falciparum* strain FVO in the presence of purified total IgG (2.5 mg/ml) from MSP1<sub>42</sub> vaccine recipients and either C3/C4-inactivated or C3/C4-reconstituted serum, N = 13. B) Invasion in the presence of total IgG from malaria naïve non-vaccinee controls, N = 14. Horizontal markers represent medians. P is by paired t-test.

Figure 3.10: Anti-*P. berghei* antibodies enhance endpoint parasitemia in an inverse dose-dependent manner. Wild-type (C57BL/6) mice infected with *P.berghei* ANKA ( $1.5 \times 10^7$  pRBC/100  $\mu$ l) and injected with anti-*P. berghei* antibodies or control antibodies at 250  $\mu$ g, 500  $\mu$ g, 1000  $\mu$ g and 1,500  $\mu$ g (per 100  $\mu$ l injection). Control mice were injected with 100ul PBS. Tail vein blood sample six days post infection was stained with Hoechst 33342 for flow cytometric acquisition. Enhancement in parasitemia relative to PBS control group. Error bars represent standard deviations. \*P <0.001 is for one-way ANOVA with post-hoc comparisons.

Figure 3.11: C3<sup>-/-</sup> mice have decreased parasite growth relative to wild type mice. C3<sup>-/-</sup> or wild type mice infected with *P.berghei* ANKA ( $1.5 \times 10^7$  pRBC/100  $\mu$ l) and injected with 250  $\mu$ g of anti-*P. berghei* IgG, control IgG, or an equivalent volume of PBS on the day of infection. The y axis shows the enhancement of day 6 post-infection parasitemia relative to wild type mice that received PBS. This is a representative experiment of three. The P value was obtained using t-test.

Supplementary Figure 3.1: Selective inactivation of complement hemolytic activity of the classical and alternative pathways (CH50 and AH50) by heat inactivation. A) Hemolytic activity of the classical pathway (CH50) was measured in fresh serum (FS) as well as after heat inactivation for 3 min, 5 min and 30min (HIS) at 56 °C. Full hemolytic activity of 3 min HIS was rescued with the addition of C2, while 5 min HIS was only partially rescued when compared to FS. B) Inactivation of hemolytic activity of the alternative pathway (AH50) was verified for 3 min HIS at 56 °C and compared to fresh serum.

Supplementary Figure 3.2: Addition of C2 or fB partially rescues RBC invasion enhancement properties of mAb5.2 in 3 min HIS. Invasion of *P. falciparum* strain 7G8 was tested in the presence of 40 µg/ml anti-MSP1 mAb5.2, 40 µg/ml IgG2b isotype control, or PBS in the presence of 10% fresh serum, 3 min HIS, or 30 min HIS. C2 or fB were added to a final concentration of 25 µg/ml and 200 µg/ml respectively, or the addition of 200 µg/ml fetuin as a control. Error bars represent standard deviations. P is for the comparison between mAb5.2 and IgG2b or PBS.

Supplementary Figure 3.3: C3 deposition on merozoites in the presence of fresh serum or 30 min HIS. Flow cytometry plots show the gating strategy for merozoite population identified by SSC vs FSC stained with Hoechst 33342. Of the gated Hoechst 33342 positive merozoites 7.53% were positive for C3 staining in the presence of fresh serum while 0.58% were positive for C3 staining in 30 min HIS.

# Figures:

Figure 3.1

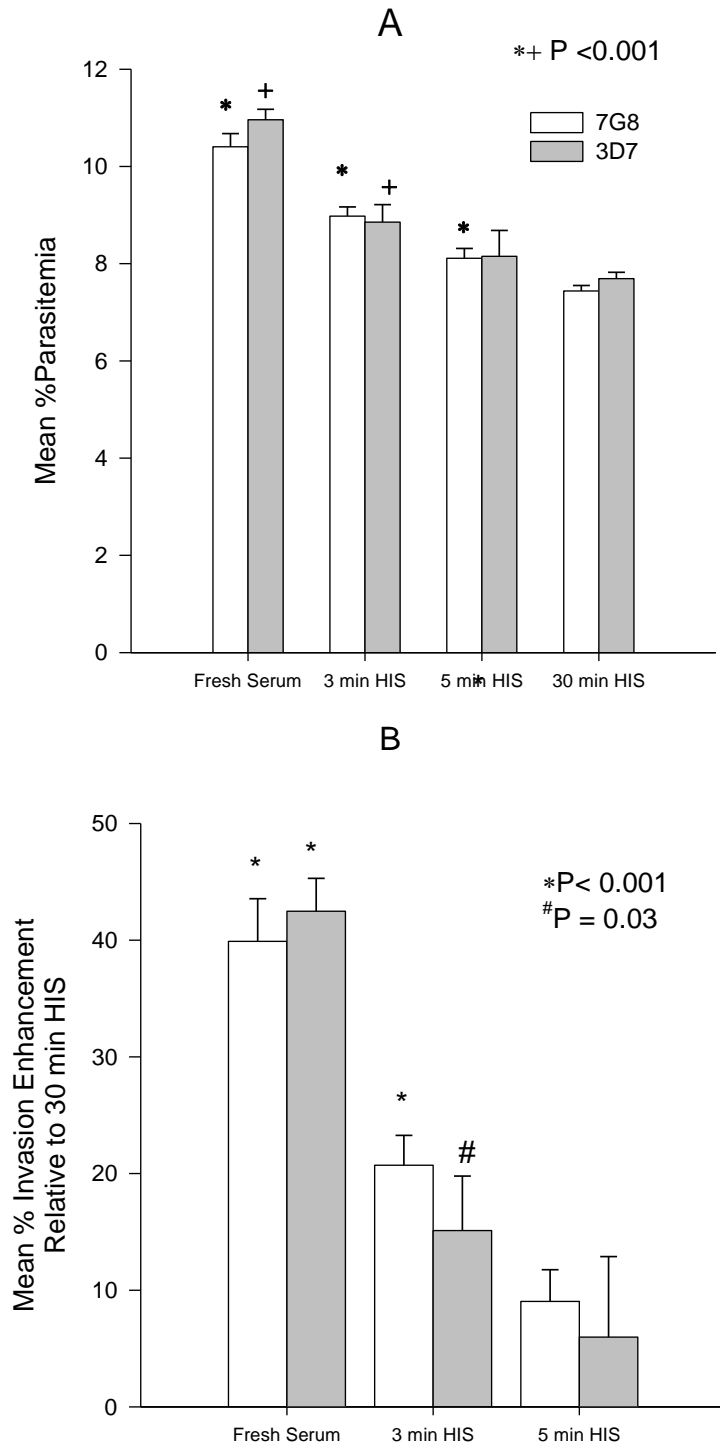




Figure 3.2

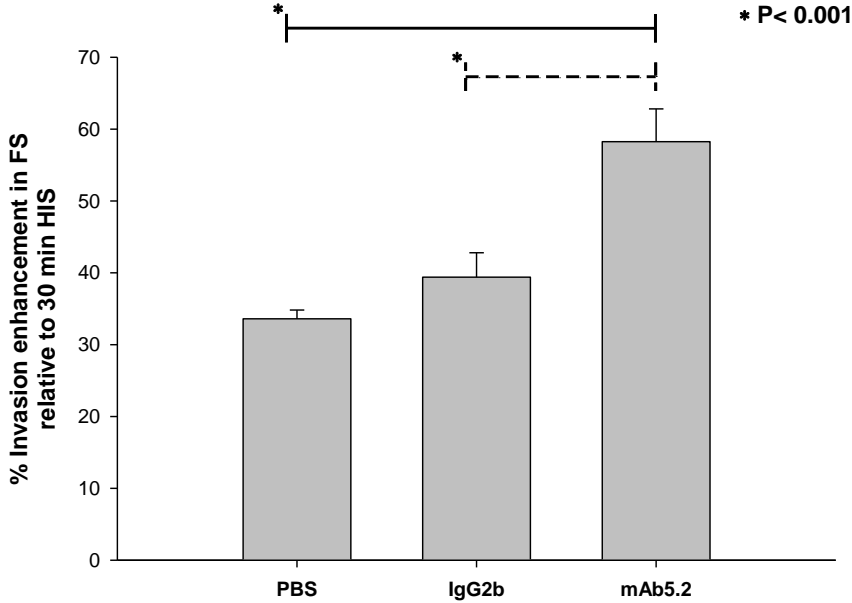


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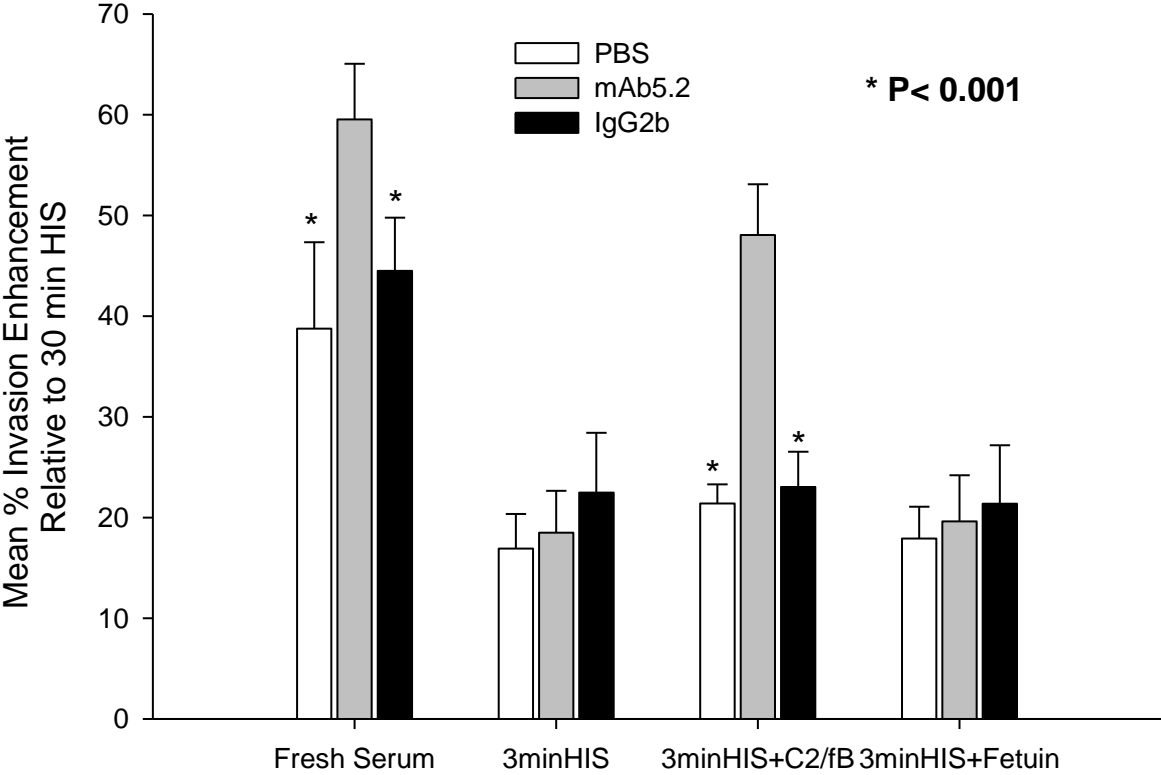


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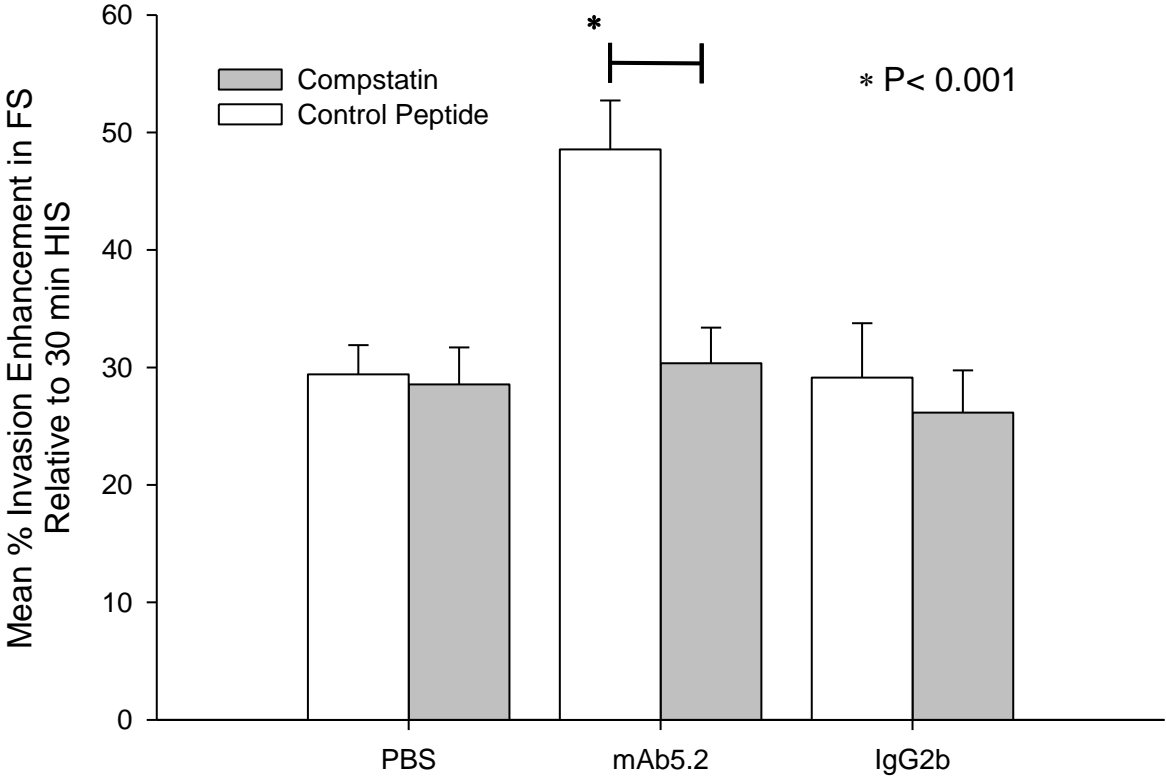


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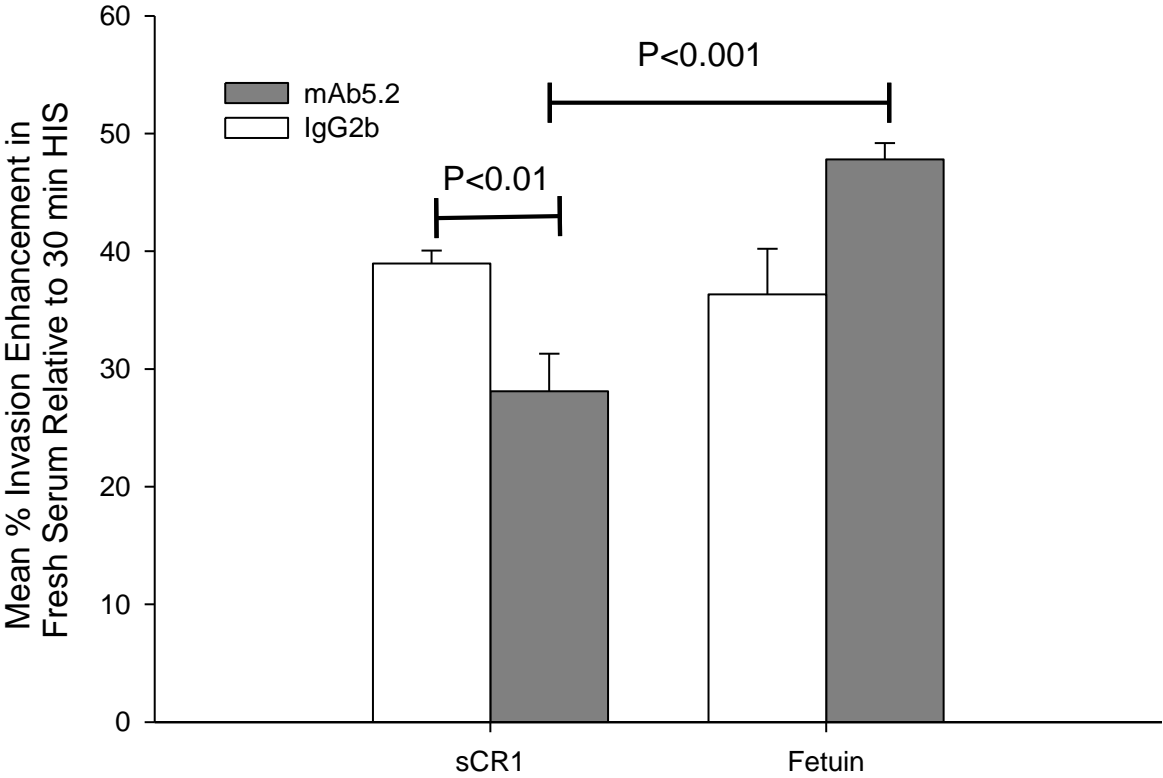


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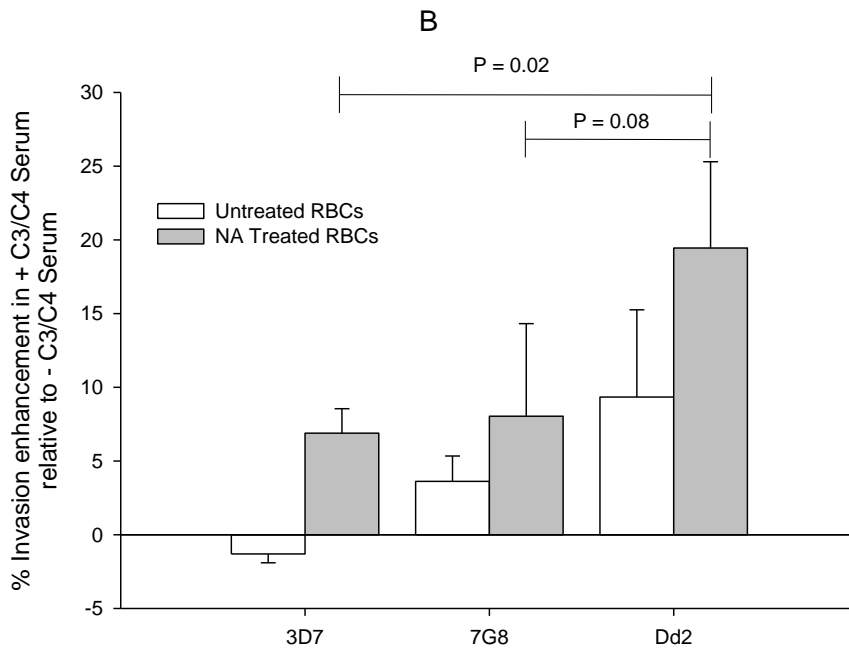
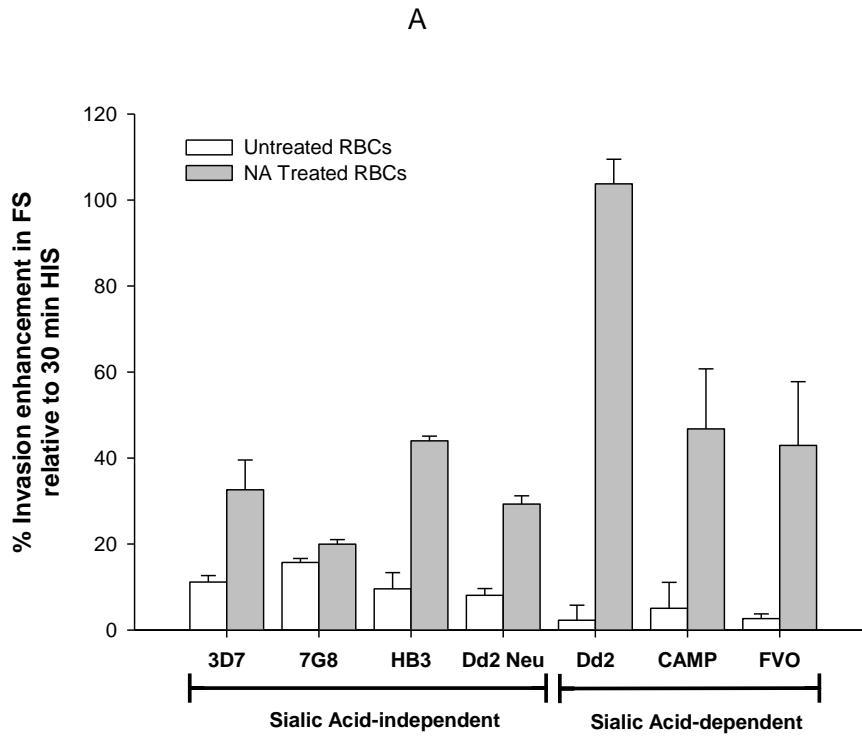


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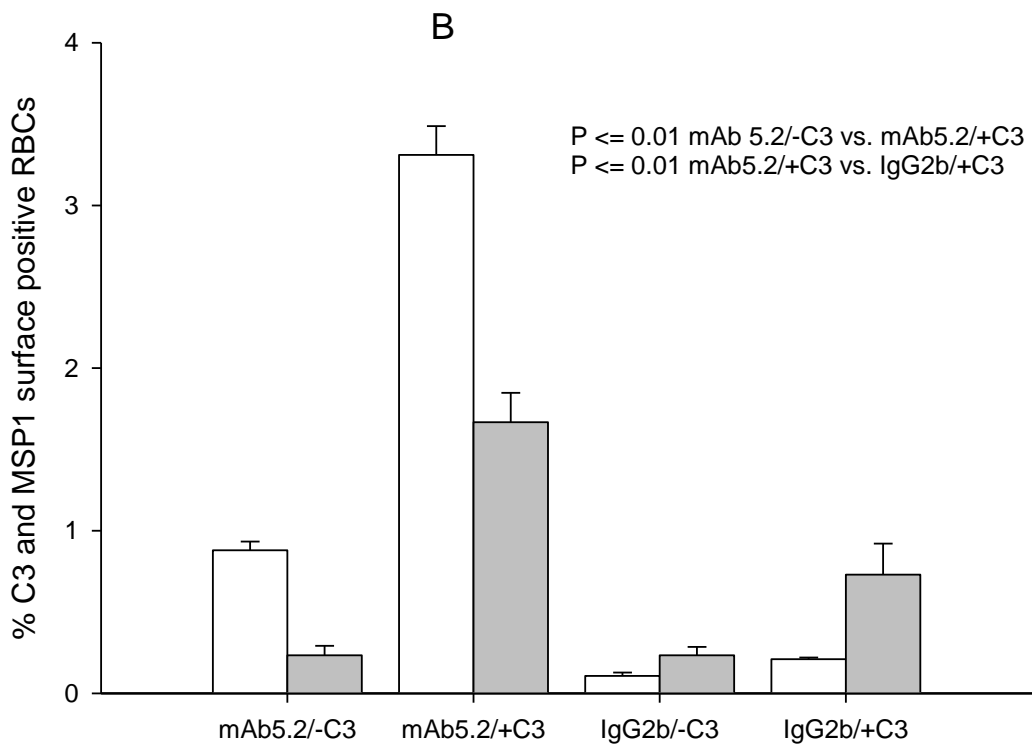
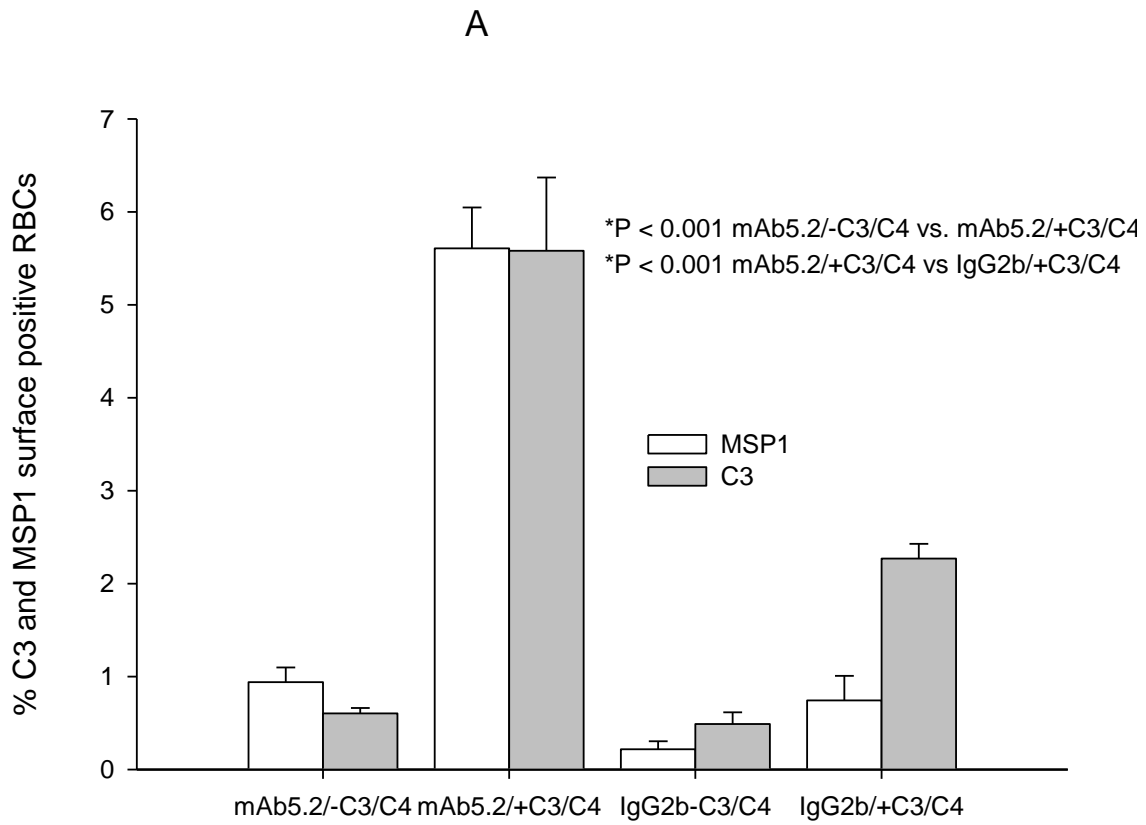


Figure 3.8

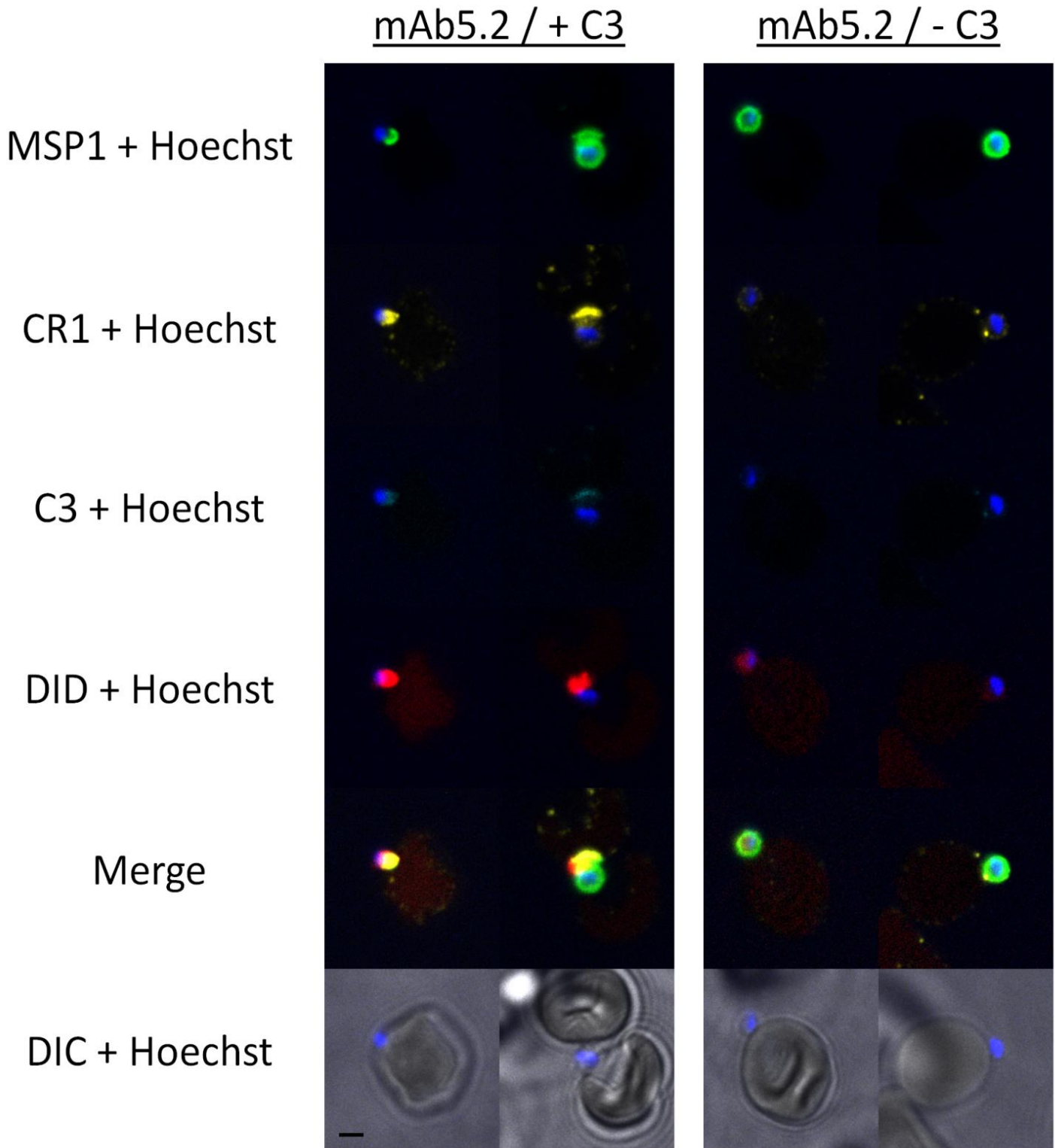


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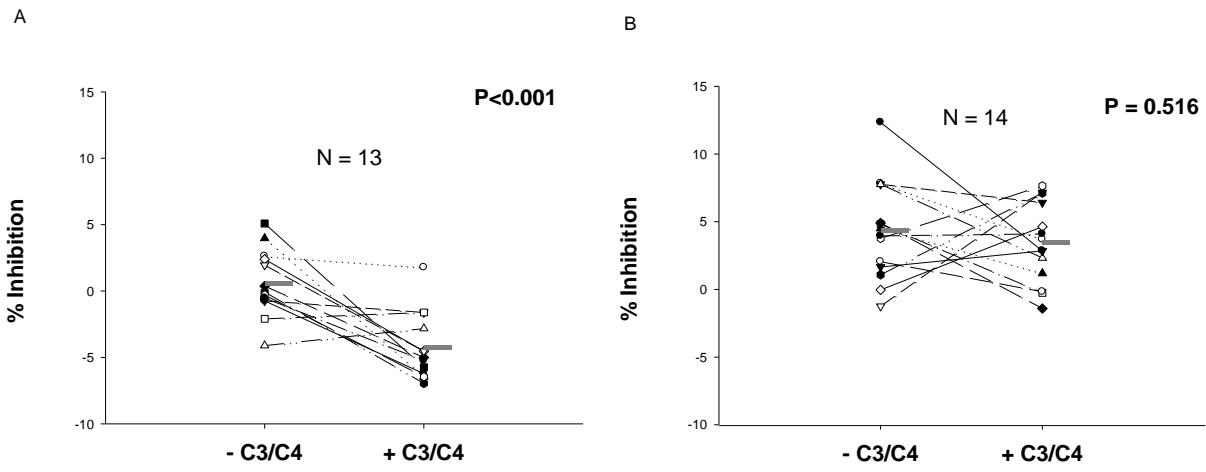




Figure 3.10

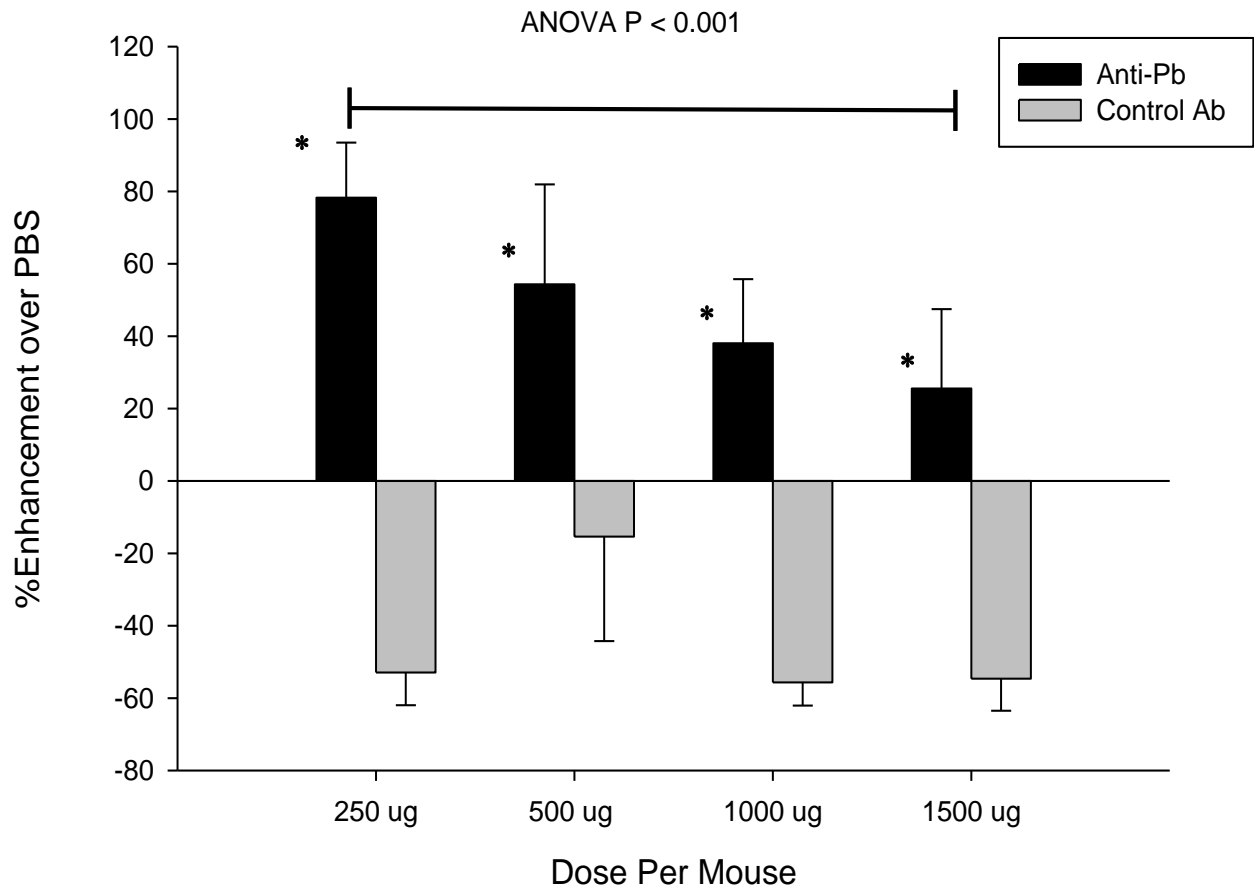
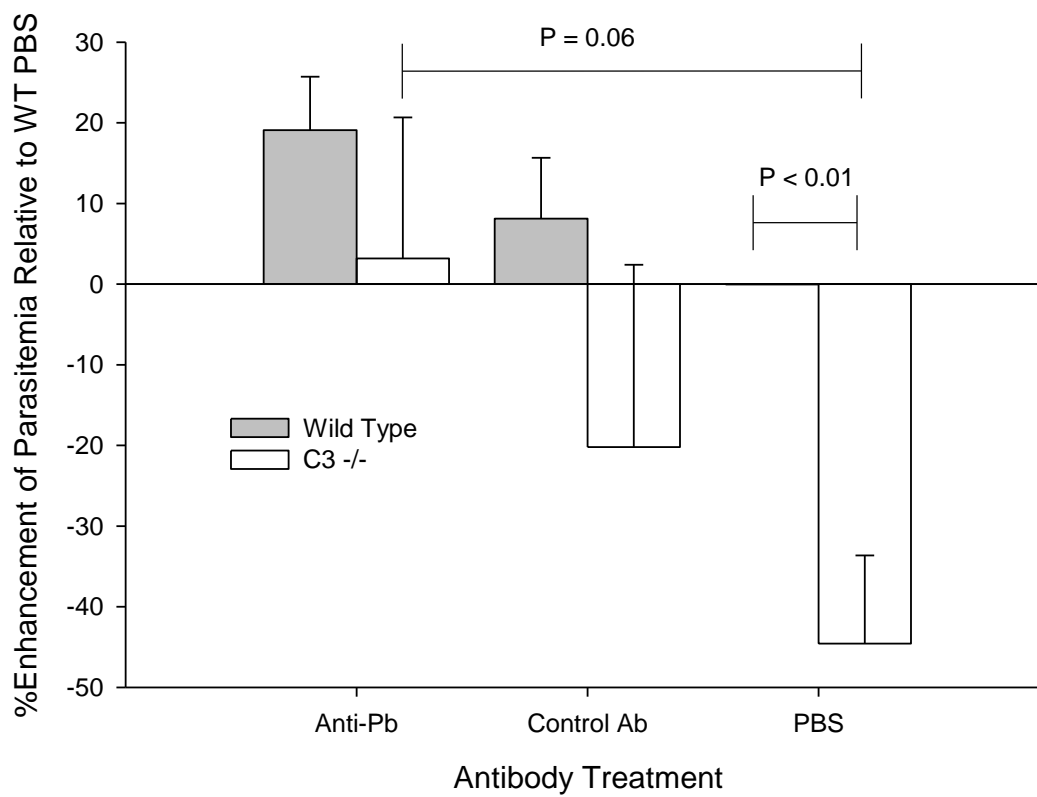
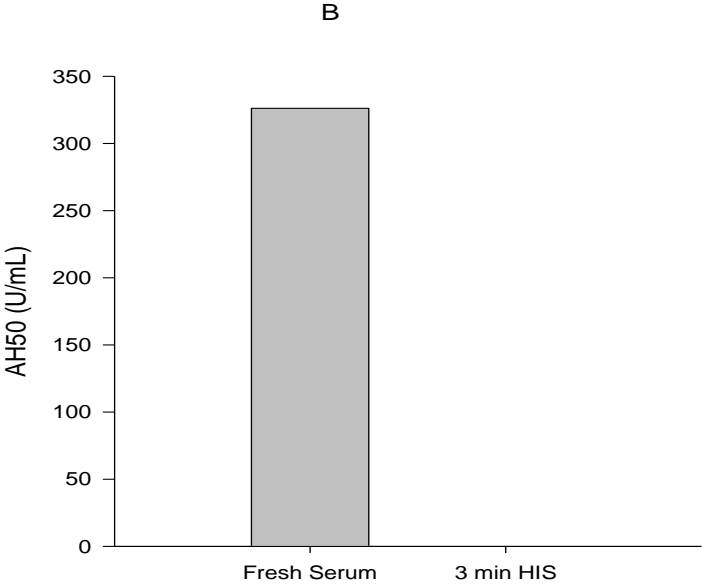
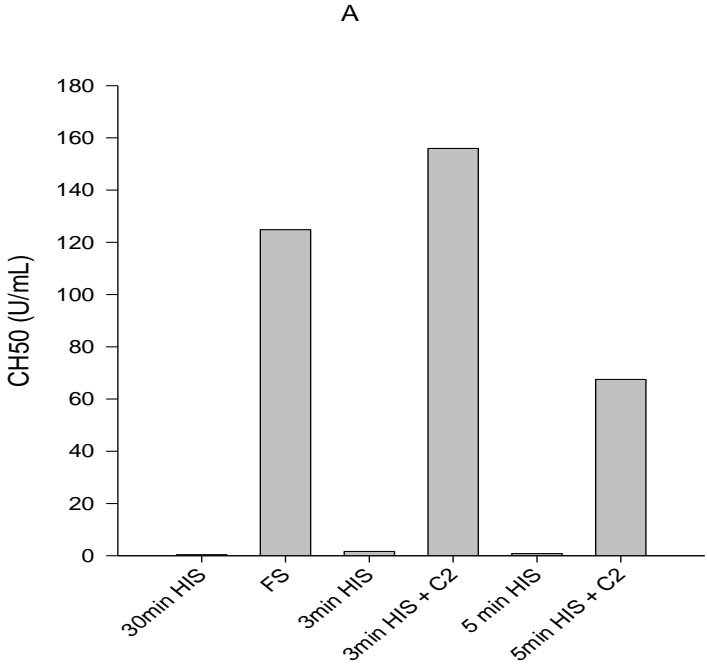


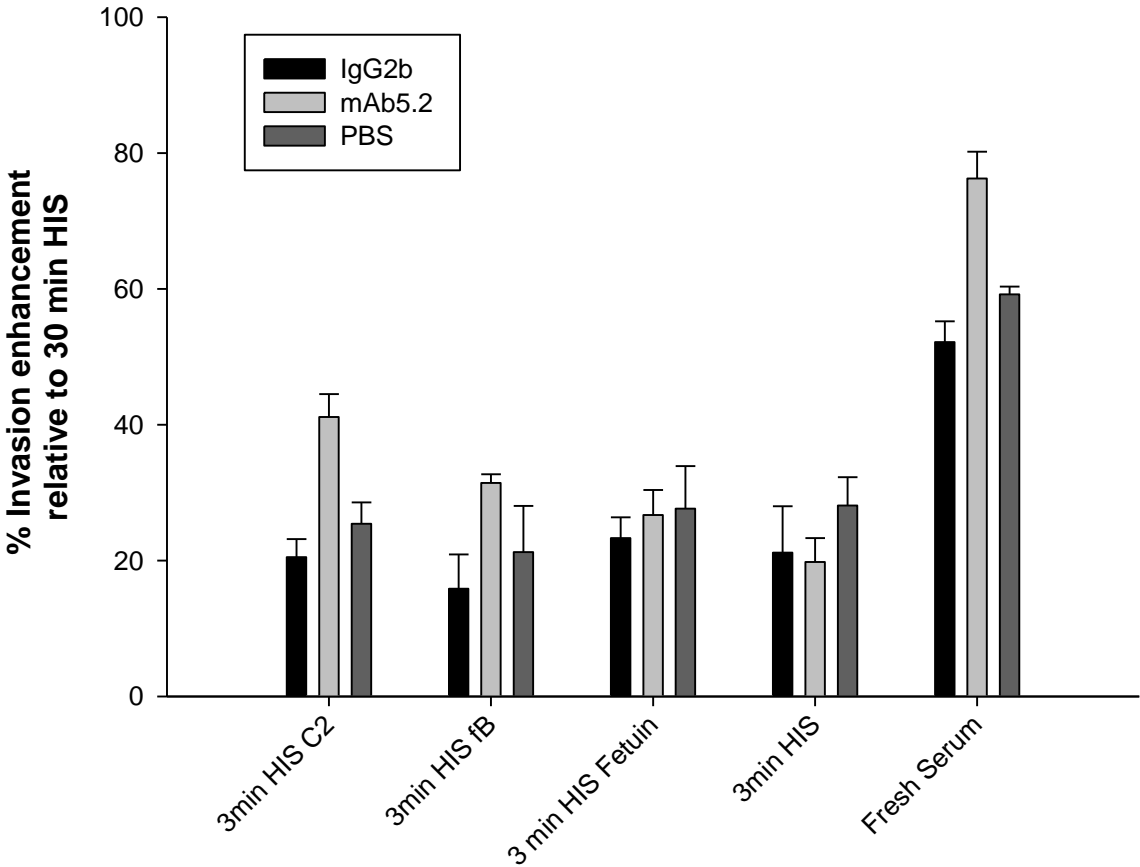
Figure 3.11



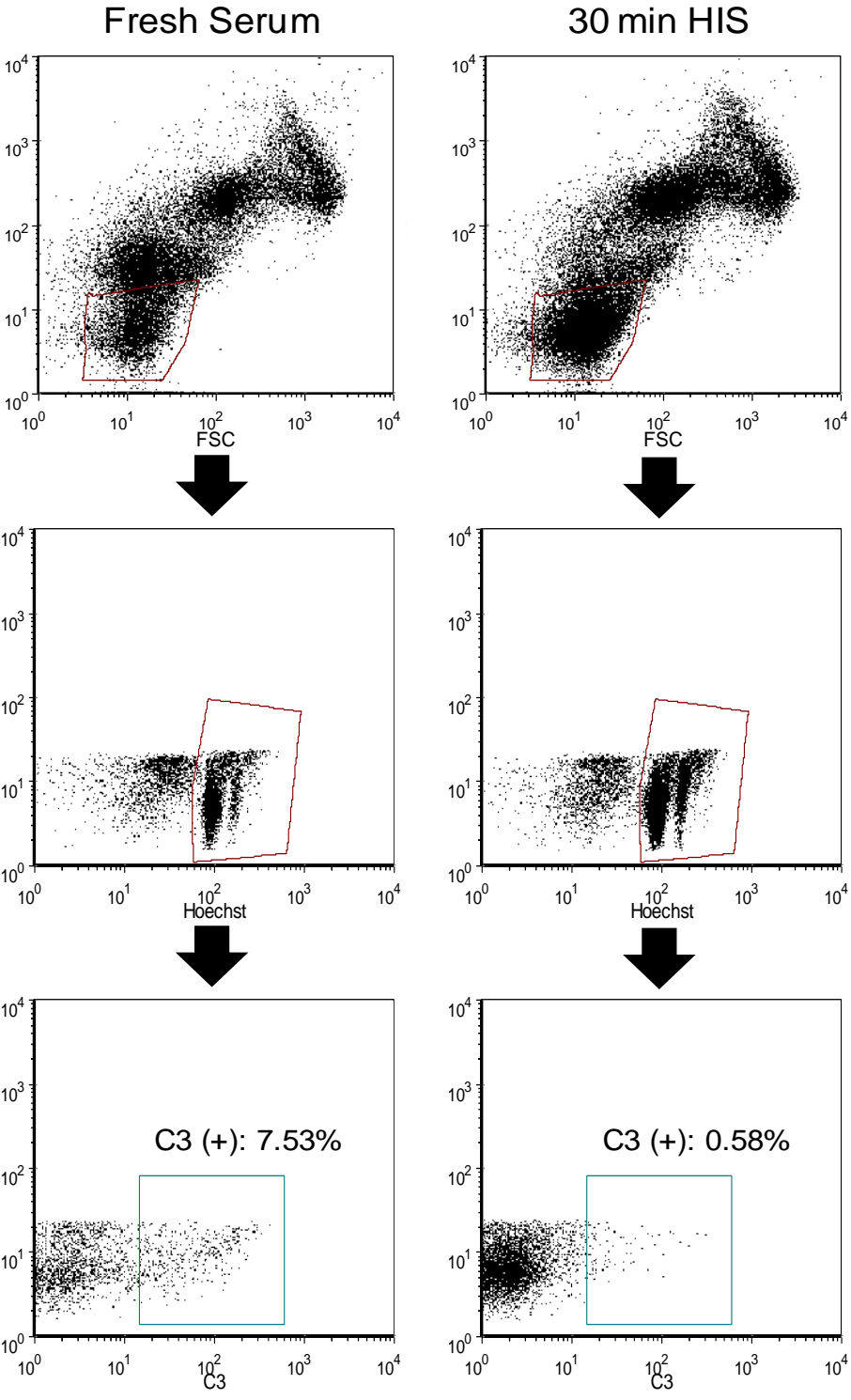
Supplementary Figure 3.1



Supplementary Figure 3.2



Supplementary Figure 3.3



## **Chapter IV: Inhibition of erythrocyte invasion by *Plasmodium falciparum* due to Semaphorin 7A and complement interaction**

### **Introduction:**

With over half a million deaths each year, predominantly in children under 5, malaria remains one of the main causes of childhood mortality worldwide (215). Reoccurring *Plasmodium* infections lead to the development of naturally acquired immunity, with rapid attainment of protection against most severe forms of malaria. But the development of an immune response that can confer asymptomatic status requires uninterrupted lifelong heavy exposure to the parasite that never results in sterilizing immunity (246,247). After inoculation of *Plasmodium* sporozoites via the bite of female *Anopheles* mosquitoes the parasite travels to the liver and undergoes dramatic metamorphoses into the merozoite stage. This erythrocytic form is responsible for malaria attributed morbidity and mortality. The merozoite stage utilizes many different receptor-ligand interactions to invade the host erythrocyte and evade host immune responses (14,248).

Complement is a major part of the innate immune system, and it is one of the main effectors of antibody-mediated immunity that plays a role in merozoite invasion of erythrocytes (59). Situated at the interface between the innate and the adaptive immune systems, complement exerts its effector function through the opsonization of pathogens, the disposal of immune complexes, and the production of inflammatory mediators. Recently complement receptor 1 (CR1), located on the erythrocytes, was

identified as the sialic acid-independent receptor that provides some laboratory strains and wild type isolates the ability to utilize this pathway for entry, especially when the predominant sialic acid-dependent receptor pathways are obstructed (15,37,249-251). The postulated role of CR1 is to bind directly to the PfRH4 ligand on the merozoite surface during the sialic acid-independent erythrocyte invasion (37). The intrinsic function of CR1 is that of a complement receptor and regulator (37,226,227). CR1 interact with C3/C4-derived fragments, thereby promoting immune complex clearance. CR1 also inhibits both the classical and the alternative pathways of complement activity by means of its C3/C5 convertases decay-accelerating activity and co-factor activity for factor I cleavage of C3b/C4b (252). There is also evidence that opsonizing fragments, specifically C4b, interact with CR1 during PfRh4 mediated erythrocyte invasion as well as for deposition of complement proteins on the merozoite surface (59,253).

The erythrocyte surface contains two other complement regulatory receptors, the decay accelerating factor (CD55) and protectin (CD59). CD55 inhibits complement activation by accelerating the decay of the C3/C5-convertase of the classical and alternative pathways (254,255). CD59 inhibits membrane attack complex assembly by preventing the binding of C9 to the C5b-8 complex (184). Both of these complement regulatory receptors are glycosylphosphatidylinositol (GPI)-anchored receptors. Many pathogens, including echoviruses and E coli, exploit these receptors for host cell entry (256,257).

Erythrocytes from individual with paroxysmal nocturnal hemoglobinuria (PNH) are deficient in a number of receptors including CD55, CD59 and Semaphorin-7A, due

to a somatic mutation of the X-linked phosphatidylinositol glycan anchor biosynthesis, class A (*PIG-A*) gene (175,176). Since both CD55 and CD59 are glycosylphosphatidylinositol (GPI)-anchored receptors, erythrocytes from patients with PNH are opportune for studying the role these receptors in erythrocyte invasion by *P. falciparum*. Because merozoites activate, and become opsonized, by complement fragments, we predict that complement receptors CD55 and CD59, play a role in parasite invasion of erythrocytes. Of course other GPI anchored receptors such as Semaphorin-7A may also play a role in the erythrocyte invasion process.

## **Results:**

### ***P. falciparum* Invades Normal Erythrocytes More Efficiently than PNH**

#### **Erythrocytes**

To determine if *P. falciparum* merozoites utilize CD55 and CD59 receptors on erythrocytes for invasion we performed invasion assays with erythrocytes from patients with paroxysmal nocturnal hemoglobinuria (PNH). PNH disorder is caused by an acquired somatic mutation in the hematopoietic stem cell, which results in the loss of glycosylphosphatidylinositol (GPI)-anchored receptors, such as CD55 and CD59. The result is haemolytic anaemia from the increased sensitivity of erythrocytes to complement mediated lysis (Figure 4.1) (258-260). We compared erythrocytes from normal or PNH individuals in fresh serum or 30 min heat inactivated serum (HIS) for invasion by *P. falciparum*. Fresh serum contains an active complement cascade, which may engage with GPI linked complement receptors on the erythrocytes and affect invasion. Incubation of serum at 56°C for 30 min inactivates complement activity. Invasion of normal erythrocyte by *P. falciparum* was significantly greater than into PNH



erythrocytes (Figure 4.2A). To determine if the parasite selectively preferred invasion of normal rather than PNH erythrocytes within the same reaction (intra reaction), we stained either the PNH or normal erythrocytes with a lipid tracer (DID) and compared the level of invasion within the same reaction to the unstained cells. As with invasion in separate cultures, in intra reactions merozoites preferentially invaded normal erythrocytes (Figure 4.2B). Levels of invasion was consistently higher in fresh serum than in 30 min HIS independent of erythrocytes phenotype.

To test specifically for the role of complement in invasion sera that were depleted in C3 or inactivated in C3 and C4, or subsequently repleted with the depleted or inactivated factors were used in the invasion assays. Again invasion of PNH erythrocytes was reduced in both complement active and inactive serum. Restoration of C3 or C3 and C4 slightly inhibited invasion of PNH erythrocyte, but slightly enhanced invasion of normal erythrocytes (Figure 4.3A, 4.3B, 4.3C, & 4.3D). The reduction of invasion of PNH erythrocytes by the complement rescued serum was the opposite of the enhancement of invasion that fresh serum provided. Heat is likely to inactivate serum factors, other than complement, that have an effect on invasion.

Erythrocytes from PNH individuals vary in the number of GPI receptors on their surface, from nearly normal (PNH I) to non detectable levels (PNH III) (Figure 4.1) (177). We compared invasion of PNH erythrocyte with high and low CD55 levels from the same individual. As observed previously with erythrocytes from normal and PNH individuals, *P. falciparum* invaded CD55 high PNH erythrocytes at high levels than CD55 low erythrocytes in fresh or 30 min HI serum (Figure 4.4).

## **Blocking Semaphorin 7A in the Presence of Fresh Serum Results in Inhibition of *P. falciparum* Invasion and Growth of Erythrocytes**

Based on the increased *P. falciparum* invasion of CD55 high PNH erythrocytes we postulated that the loss of CD55 and/or CD59 may be responsible for the reduced invasion of PNH erythrocytes. Using anti-CD55 and anti-CD59 antibodies to block accessibility of the receptors on normal erythrocyte we measured invasion in growth inhibition assays after two cycles of invasion. During growth of the parasite for 72 hours, neither antibody inhibited invasion (Figure 4.5A & 4.5B). When anti-CD55 and anti-CD59, antibodies were added in combination, 72 hours post assay setup some growth inhibition was observed in the presence of fresh serum (Figure 4.5C).

In addition to CD55 and CD59, four other GPI linked receptors on erythrocyte membranes; Ras-Related Protein Rap-1A (RAP1A), calcium and integrin-binding protein 1 (CIB1), lymphocyte function-associated antigen (LFA-3) and Semaphorin 7A (SEMA7A), have been identified (261,262). In fresh serum containing anti-SEMA7A, anti-LFA3, or anti-RAP1A antibodies, some inhibition of parasitemia was observed, where as anti-CIB1 had no inhibitory activity (Figure 4.6A, 4.6B, 4.6C & 4.6D). No inhibitory activity was observed for any of the antibodies in 30 min HIS serum. As with the other GPI linked receptors, CD55 and CD59, PNH erythrocytes had lower levels of SEMA7A than normal erythrocytes (Figure 4.7).

## **Activated C3b in Fresh Serum Binds to Semaphorin-7A**

Although blocking CIB1, LFA-3 and SEMA7A on erythrocytes produced parasite growth inhibition after 72 hours in fresh serum, only anti-SEMA7A antibodies produced

a rapid and significant invasion inhibition of erythrocytes after only 24 hours (Figure 4.6 & 4.8). Furthermore, recently Semaphorin-7A (SEMA7A) was identified as a novel erythrocyte receptor used by the parasite to invade host cell (198). *Bartholdson et al.* demonstrated receptor ligand interaction between SEMA7A and mTRAP (198). mTRAP is a blood stage, micronemes localized, invasion adhesin that bridges the interaction between the erythrocyte cell surface and the merozoite motor complex (197). Attempts to block the interaction in invasion assays *in vitro* have been unsuccessful (198).

We hypothesized that complement deposition and interaction with SEMA7A blocks merozoite invasion of erythrocytes. To this end, we analyzed the invasion of erythrocytes by *P. falciparum* in C3 depleted or reconstituted serum containing anti-SEMA7A antibodies. Anti-SEMA7A antibodies inhibited invasion only in C3 reconstituted and not C3 depleted serum (Figure 4.8). These results were similar to those obtained with anti-SEMA7A antibodies and fresh serum only (Figure 4.6D). Anti-mTRAP antibodies were tested in the presence of C3 had no effect on invasion inhibition (Figure 4.9) which was in agreement with *Bartholdson, S. et al* (198).

### **Activation of C3 in Fresh Serum is Required to Promote Opsonization of Semaphorin-7A**

To determine if complement interacts with SEMA7A, we performed ELISA assays. Complement components were incubated with recombinant SEMA7A (rSEMA7A), the immobilized target, and probed with anti-C3 antibody for complement deposition. C3 deposition was observed in fresh serum or C3 reconstituted serum, but not 30 min HIS or C3 depleted serum (Figure 4.10A & 4.10B). Unlike sCR1 which binds

to pure C3b and moderately to intact C3 and iC3b in the absence of other complement components, SEMA7A coated wells did not bind to purified C3, C3b, iC3b, or C4 (data not shown). Addition of C3 cleavage products C3b or iC3b to C3 depleted serum did not result in their binding to SEMA7A (Figure 4.10B & 4.10C). This suggests that one of the three complement pathways is critical for C3b deposition on SEMA7A. These results also imply that C3 deposition involves complement recognition of rSEMA7A as a non-self antigen and subsequent C3 opsonization. These results indicate that C3 has to be activated through cleavage to interact with SEMA7A. No C3a binding to SEMA7A was observed with fresh serum (Figure 4.10D) and only the active C3b fragment is deposited on the surface or the vicinity of the rSEMA7A receptor.

Proteins made *in vitro* may differ from those made *in vivo* such as in different glycosylation patterns (263-266). To see whether native erythrocyte surface bound SEMA7A recognizes complement coated particles, complement opsonized 1 $\mu$ m latex beads were preincubated in fresh or HI serum (Figure 4.11) and then added to erythrocytes in the presence of anti-C3, anti-CR1 or anti-SEMA7A antibodies. Anti-C3 antibodies, as well as antibodies against the erythrocyte surface receptor CR1; completely abrogated the binding of FS coated beads to erythrocytes. Anti-SEMA7A antibodies or species-specific control antibodies, did not inhibit binding of FS coated beads (Figure 4.12). These results support the notion that complement does not recognize native surface bound SEMA7A.

**Potential Pattern Recognition Proteins of the Mannose-binding Lectin Pathway Recognize SEMA7A and Promote Complement Activation and C3b Deposition.**

To establish which complement pathways recognize rSEMA7A protein and promote C3 activation and C3b deposition, we carried out ELISA assays with rSEMA7A as the target antigen. Elevated C3b deposition was observed whether C1q in serum was depleted or not implying that C1q of the classical pathway does not recognize rSEMA7A (Figure 4.13). If so, the intact complement pathways still active in C1q depleted serum must be involved in SEMA7A recognition. C3b binding was detected in fB depleted serum but the addition of fB amplified C3b binding (Figure 4.13). This result suggests that, in fB depleted serum, the MBL pathway recognizes rSEMA7A and activates complement deposition. Upon addition of fB increased C3b deposition may be caused by the induction of the alternative pathway amplification loop. Attempts to induce C3 deposition on rSEMA7A by purified C3b/fB/C3/fD proteins in the alternative pathway, which are necessary for C3 convertase assembly, was unsuccessful. Therefore other pattern recognition proteins in serum are required to initiate rSEMA7A recognition (Figure 4.13).

Depletion of properdin (fP) from serum had no effect on observed elevated levels of C3b deposition on rSEMA7A (data not shown). Therefore, initial recognition of rSEMA7A is alternative pathway independent. Depletion of C4 prevented C3b deposition providing further evidence against the alternative pathway as the main recognition pathway of rSEMA7A (Figure 4.10D & 4.13). These results indicate that the MBL pathway is probably responsible for recognition of rSEMA7A.

Recognition of rSEMA7A by complement in the absence of anti-SEMA7A antibodies is unexpected because like other self molecules SEMA7A should not induce an innate immune response. We thus tested for C3 deposition on human recombinant

GPI-anchored proteins from erythrocytes that were expressed in HEK293 cells. As with rSEMA7A, C3 deposited on the erythrocyte GPI linked receptors, CIB1 and RAP1A in the presence of C4 rescued serum (Figure 4.14A). Also, C3 deposition also occurred on LFA3 and RAP1A expressed in HEK293 cells or RAP1A expressed in E coli (data not shown). Complement also recognized fetuin, a bovine derived protein, used as a non-self antigen, to the same extent as it recognized rSEMA7A (Figure 4.14B).

## **Discussion:**

To evade the immune response *P. falciparum* merozoites utilize numerous host receptors, which appear to be functionally redundant, in the invasion of erythrocytes. CR1 an immune specific receptor on the erythrocyte surface, reduces the pathogen load by binding to C4b- and C3b-opsonized pathogens and delivering them to macrophages in the liver or the spleen for removal by phagocytosis (231). Recently CR1 was identified as the sialic acid-independent receptor that is subverted by *P. falciparum* merozoites from its inherent role in host protection to allow parasite invasion of red blood cells (15,37). Furthermore, we and others showed complement opsonization and C3b deposition on merozoites (Figure 3.8 and Supplementary Figure 3.3) (59). The erythrocyte surface contains two additional complement receptors, CD55 and CD59 (184,254,255). We hypothesized that these complement receptors also play a role in erythrocyte invasion by merozoites. Opsonization of merozoites by complement fragments could bridge the interaction between the parasite and CD55 and/or CD59, thereby facilitating erythrocyte invasion. A role for CD55 and CD59 in

erythrocyte invasion by *P. falciparum* may have escaped detection in standard invasion inhibition assays performed in complement inactivated mediums.

Here we demonstrate that, compared to normal erythrocytes, erythrocytes deficient in GPI-anchored receptors from a PNH individual were invaded by merozoites to a lesser extent. Thus reduction in invasion of PNH cells was in part complement-independent, as significant reduction was observed in both fresh serum and heat inactivated serum. The hallmark of PNH erythrocytes is the loss of GPI-anchored proteins, due to a mutation in the gene encoding PIGA that leads to susceptibility to complement mediated lysis. However, recent evidence indicates that PNH individuals have many additional somatic mutations. The expansion of the nonmalignant GPI-deficient hematopoietic stem cell clone that results in PNH cells with mutations in genes such as *TET2*, *SUZ12*, *U2AF1*, and *JAK2*, which are known to be involved in myeloid neoplasm pathogenesis (182). Because these cells have a growth advantage, they preferentially accumulate additional mutations that may confer to erythrocytes resistance to parasite invasion and growth. Also, *P.falciparum*-infected erythrocytes internalize GPI-anchored proteins, such as CD55, CD58 and CD59, imparting a PNH-like phenotype on the infected erythrocytes. Reduced invasion of PNH erythrocyte may arise if merozoites recognize these cells as already infected and extent their search for uninfected erythrocytes (191).

We observed some inhibition of invasion of PNH erythrocytes that depended on functional complement, rather than the invasion enhancement that occurred in normal erythrocytes. PNH erythrocytes may bind more complement and extensive opsonization could obstruct parasite-ligand interactions with host receptors. Also, PNH

erythrocytes may be more susceptible to the elevated  $\text{Ca}^{+}$  influx induced by sublytic complement C5b-9.  $\text{Ca}^{+}$  influx promotes increased vesiculation in erythrocytes and minimizes lysis, while at the same time limiting the availability of erythrocyte membrane receptors to merozoites (267). In contrast to our results, Iida et al. showed that *P.falciparum* invades PNH erythrocytes at similar if not greater levels than normal erythrocytes (268). They used a strain from Thailand, TM267TR, that is isogenic with the sialic acid-dependent FCR3, whereas we tested invasion in the sialic acid-independent Brazilian 7G8 strain. Differences in receptor utilization by these parasite strains may be responsible for the differing results.

To determine if loss of GPI-anchored receptors reduces invasion of PNH erythrocytes, we examined the role of complement in blocking invasion by antibodies directed against these receptors. Invasion of normal erythrocytes was reduced in fresh serum only by anti-SEMA7A, anti-RAP1A or anti-LFA3 antibodies but not anti-CD55, anti-CD59 or anti-CIB1. We used antibodies raised in different species that may differ in their ability to activate human complement and thereby inhibit erythrocyte invasion (269). Rabbit and mouse antibodies seem to fix human complement similarly to human antibodies (270). Unlike most mammalian antibodies, chicken antibodies do not activate human complement; likely accounting for the lack of inhibition by anti-CD55 (270). Future work should address this potential species specificity.

Because Bartholdson et al. identified SEMA7A as an erythrocyte receptor for merozoites and of all the anti-GPI-anchored receptor antibodies we tested for the ability to inhibit invasion of erythrocytes anti-SEMA7A antibodies showed the largest and fastest inhibitory effect (198). Anti-SEMA7A antibodies inhibited erythrocyte invasion



only in the presence of functional complement pathways, while in the absence of complement activity there was no inhibition of invasion which was in accordance with Bartholdson *et al.* findings (198). Anti-SEMA7A antibodies may reduce the enhancing effects of complement during invasion of erythrocyte if SEMA7A has an affinity towards complement opsonic fragments that deposit on the merozoite surface thereby stabilizing merozoite binding to and/or invasion of erythrocyte. Furthermore, like Bartholdson *et al.*, we could not block invasion of erythrocytes with anti-mTRAP antibodies in the presence or absence of complement (198). This lack on inhibition with anti-mTRAP antibodies could be due to the role of mTRAP in the final steps of erythrocyte invasion, where mTRAPs location within the moving junction potentially makes anti-mTRAP antibodies inaccessible for proper neutralization of their target (197). Also, it is unlikely that the lack of anti-mTRAP antibody inhibition is due to redundancy in mTRAPs functionality since previous attempts at disruption of endogenous *MTRAP* gene proved unsuccessful (197).

To demonstrate the potential interaction of SEMA7A with complement opsonic fragments we resorted to ELISA based binding assays. Sera deficient in specific complement components or purified complement proteins was allowed to interact with rSEMA7A. Binding of C3b to rSEMA7A was only detected in the presence of active complement cascades indicating that complement pathways recognize rSEMA7A, activate C3 through cleavage and mediate covalent attachment of C3b to rSEMA7A through intramolecular thioester bond. Also, unlike sCR1 which recognized C3b and C4b, pure complement cleavage products, in the absence of complement activation, rSEMA7A did not bind to any downstream opsonic C3 cleavage fragments. This

nonspecific deposition of opsonins on rSEMA7A could be due to recognition of rSEMA7A as a non-self antigen by complement. Additionally, flow cytometric analysis revealed that complement coated bead attachment to erythrocytes is blocked by anti-C3 and anti-CR1 antibodies but anti-SEMA7A antibodies did not. Confocal microscopy revealed no co-localization of surface CR1 with SEMA7A in the presence or absence of complement coated beads attached to erythrocytes excluding potential interaction between the two receptors (data not shown).

Although we were unable to definitively show which complement pathway recognized rSEMA7A, we eliminated some of the main complement recognition mechanisms. The presence of C1q in serum did not affect C3b deposition on rSEMA7A, eliminating the role of the classical pathway in rSEMA7A recognition. Also, in the absence of the alternative pathway, only diminished C3b deposition on rSEMA7A was evident. This reinforces the functionality of the alternative pathway amplification loop post-complement activation but excludes the alternative pathway ability to recognize rSEMA7A. No C3b deposition was present in C4 depleted serum but upon reconstitution with C4, elevated level of C3b on rSEMA7A was observed. We postulate that the MBL pathway recognizes and activates complement on rSEMA7A. Although, when MBL deficient serum was used in the presence or absence of MBL no C3b deposition was detected (data not shown). This does not exclude the MBL pathway, since complement activity in our MBL deficient serum might have diminished with time, as a result severing the complement cascade and impeding its ability to cleave C3. In addition, the source of MBL deficient serum is a donor with MBL genotype B/B. The gene frequency of the mutant allele has been estimated at 0.13 in some populations,

making it one of the most frequent immunodeficiencies (271). Garred et al. propose that MBL protein deficiency is maintained in populations because this deficiency reduces the infectivity of some opsonization dependent intracellular pathogens (271). Of course since the MBL protein deficient serum comes from a single donor other MBL pathway activators, such as ficolins, that may be responsible for rSEMA7A recognition and complement activation could also be diminished (272).

Besides rSEMA7A, other GPI-anchored recombinant surface proteins, such as rRAP1A, rLFA3, and rCIB1, are also recognized by complement, promoting opsonization by C3b. Since all proteins tested are expressed in HEK293 cells it is possible that unusual post-translational modifications that promoted recognition by complement are cell line dependent (266). Although rRAP1A, expressed in *E.coli* and HEK293, induced the same level of complement deposition (data not shown). Many factors influence protein glycosylation in cell culture including the accessibility of sugar-nucleotide donors, the enzyme repertoire of the expression cell as in the case of CHO cells not expressing *N*-acetyl glucosaminyltransferase III, the tertiary structure of the protein and the rate of translation as well as the duration of time the protein remains in the Golgi (266,273). In mammalian cells high-mannose glycan structures are normally produced as intermediates but very low glutamine or glucose concentrations can lead to decrease in sialic acid content and increased presence of glycan species high in mannose (274). A decrease in sialic acid and an increase in mannose residue may be a non-self or eat-me signature on the recombinant protein, thereby promoting complement recognition and activation, similar to signals produce by apoptotic cells (275).

In summary, invasion of PNH erythrocytes by *P.falciparum* are significantly reduced in comparison to normal erythrocytes. This may be due to considerably low levels of GPI-anchored receptors on PNH erythrocytes, specifically SEMA7A, RAP1A and LFA3. The potential role of these receptors in the invasion of erythrocytes was determined by using antibodies directed against these receptors in the presence or absence of complement. Only in the presence of complement was merozoites invasion enhancing activity reduced when these receptors are blocked. This suggests that complement opsonized merozoites favorably interact with GPI-anchored receptors on erythrocytes. Although, data from ELISA based binding assays are inconsistent with erythrocyte invasion and growth assay results. This discrepancy may be attributed to drastically different glycosylation patterns in native proteins compared to their recombinant cell culture expressed counterparts. The recombinant proteins could be recognized as non-self by endogenous complement pathways. It is possible to implement complement dependent ELISA based assays for fast, cheap and high throughput initial screening of recombinant proteins to ascertain the level of protein structure homology relative to native protein counterparts. This will ultimately assist in modification of recombinant proteins in an effort to reduce their recognition by the immune system thereby prolonging the serum half life of protein therapeutics. Continued research into the role of GPI-anchored receptors on erythrocyte during the invasion by *P.falciparum* may pave the way for identification of critical parasite host interactions that may be exploited for therapeutic purposes.

## Figure Legends:

Figure 4.1: Reduced expression of CD55 and CD59 on PNH but not normal erythrocytes. Erythrocytes were isolated from whole blood obtained from a healthy (normal) and PNH donor and stained for CD55 and CD59 expression levels. Flow cytometry plots show that of the gated normal erythrocytes, 91.92% and 92.29% were positive for CD55 and CD59 respectively. Only 18.99% and 19.08% of PNH erythrocytes were positive for CD55 and CD59 respectively.

Figure 4.2: Invasion of normal erythrocytes by *P. falciparum* was significantly greater than of PNH erythrocytes in fresh and heat inactivated serum. An invasion assay of *P. falciparum* 7G8 strain was tested in the presence of 10% fresh serum or 30 min HIS into healthy (normal) or PNH erythrocytes. Invasion of PNH compared to normal erythrocytes was reduced in A) separate invasion reactions (inter-reactions) as well as B) intra reaction invasion. For intra reaction invasion, normal or PNH erythrocyte were stained with DID and erythrocyte invasion was compared to the unstained erythrocyte population within the same well. P is for the comparison of 7G8 invasion into normal and PNH erythrocytes in fresh or HIS serum. Error bars represent standard deviations. P values are based on t-test.

Figure 4.3: Invasion of normal erythrocytes by *P. falciparum* was significantly greater than of PNH erythrocytes in complement sufficient and deficient serum. A) Invasion of healthy (normal) or PNH erythrocytes by *P. falciparum* 7G8 strain was tested in the presence of 10% C3 depleted and C3 rescued serum. Dashed lines (●●● and — — —) demarcate erythrocyte invasion enhancement or inhibition relative to C3 depleted

serum. B) Invasion of erythrocytes is expressed as percent enhancement in C3 rescued relative to C3 depleted serum. Invasion enhancement is observed into normal erythrocyte while inhibition is observed into PNH erythrocytes by *P. falciparum* in C3 rescued serum. C) Invasion of healthy (normal) or PNH erythrocytes by *P. falciparum* 7G8 strain was tested in the presence of 10% C3/C4 inactivated and C3/C4 reconstituted serum. Dashed lines (●●● and — — —) demarcate erythrocyte invasion enhancement or inhibition relative to C3/C4 inactivated serum. D) Invasion of erythrocytes is expressed as percent enhancement in C3/C4 reconstituted relative to C3/C4 inactivated serum. Invasion enhancement is observed into normal erythrocyte while inhibition is observed into PNH erythrocytes by *P. falciparum* in C3/C4 reconstituted serum. Error bars represent standard deviations. P values are based on t-test.

Figure 4.4: *P. falciparum* invasion of PNH erythrocytes expressing normal levels of CD55 is greater than PNH erythrocytes expressing low levels of CD55. Invasion of PNH erythrocytes by *P. falciparum* 7G8 strain was tested in the presence of 10% fresh serum or 30 min HIS serum. Infected erythrocytes stained with Hoechst 33342 were subdivided and distinct populations enumerated based on CD55 staining by flow cytometric acquisition. Error bars represent standard deviations. P values are based on t-test.

Figure 4.5: *P. falciparum* growth is diminished in the presence of fresh serum with both anti-CD55 and anti-CD59 antibodies, but not when antibodies are used individually. *P. falciparum* growth was monitored over two cycles of invasion in the presence of 10% fresh or 30 min HI serum and 40 µg/ml of A) anti-CD55, B) anti-CD59, C) anti-CD55 and

anti-CD59 or control antibodies. Error bars represent standard deviations. \*P <0.05 is for one-way ANOVA with post-hoc comparisons.

Figure 4.6: *P. falciparum* growth is diminished in the presence of anti-RAP1A, anti-LFA3 and anti-SEMA7A but not anti-CIB1 antibodies in fresh serum. *P. falciparum* growth was monitored over two cycles of invasion in the presence of 10% fresh or 30 min HI serum and 40 µg/ml of A) anti-RAP1A, B) anti-CIB1, C) anti-LFA3 and D) anti-SEMA7A or control antibodies. Error bars represent standard deviations. \*P values are for one-way ANOVA with post-hoc comparisons.

Figure 4.7: Reduced expression of SEMA7A on PNH but not normal erythrocytes. Erythrocytes were isolated from whole blood obtained from a healthy (normal) and PNH donor and stained for SEMA7A (FITC) expression levels. Flow cytometry plots show significantly higher overall expression of SEMA7A on gated normal erythrocytes (Geometric Mean: 369.45) compared to lower SEMA7A expression on PNH erythrocytes (Geometric Mean: 21.4).

Figure 4.8: *P. falciparum* erythrocyte invasion is diminished in the presence anti-SEMA7A and C3 reconstituted but not C3 depleted serum. Invasion of erythrocytes by *P. falciparum* 7G8 strain was tested in the presence of 10% C3 depleted or C3 rescued serum and 40 µg/ml of anti-SEMA7A or control antibodies. Error bars represent standard deviations. P value is based on t-test.

Figure 4.9: *P. falciparum* erythrocyte invasion is unchanged in the presence anti-mTRAP and C3 reconstituted or depleted serum. Invasion of erythrocytes by *P. falciparum* 7G8 strain was tested in the presence of 10% C3 depleted or C3 rescued

serum and 100 µg/ml, 500 µg/ml of anti-mTRAP or control antibodies. Error bars represent standard deviations.

Figure 4.10: De novo complement activation is required for C3 binding to rSEMA7A. ELISA assays with rSEMA7A coated wells were incubated with A) 10% fresh, 30 min HIS, B) C3 depleted or C3 rescued serum, C) 100 µg/ml of pure C3, C3b or iC3b proteins added to 10% C3 depleted serum or D) 10% fresh, 30 min HIS, 10% C4 depleted or C4 rescue. Complement activation and binding to rSEMA7A coated wells was evaluated by probing with anti-C3 or anti-C3a primary, followed by peroxidase labeled secondary antibody. Reactions were developed with the addition of ABTS peroxidase substrate and read at OD 410 nm. Readings were calculated by averaging duplicate wells and subtracting background reading from control wells. Error bars represent standard deviations. For panels A) and B) P values are based on t-test. For panels C) P values are for one-way ANOVA with post-hoc comparisons.

Figure 4.11: Complement opsonizes microspheres in the presence of fresh but not 30 min HI serum. Microspheres (FluoSpheres) were incubated with 10% fresh (FS) or 30 min HI (HIS) serum and stained with anti-C3 antibody for complement deposition. Unstained FluoSpheres served as background control.

Figure 4.12: Anti-C3 and anti-CR1 but not anti-SEMA7A antibodies inhibit complement opsonized microspheres attachment to erythrocytes. Erythrocyte binding assays with complement coated microspheres (blue fluorescent) was performed in the presence of 40 µg/ml of anti-C3, anti-CR1, anti-SEMA7A or control antibodies. Stained erythrocytes



served as background control. Stained erythrocytes with complement coated microspheres served as 100% binding control.

Figure 4.13: The alternative pathway amplifies C3 deposition on rSEMA7A in a classical pathway independent manner. ELISA assays with rSEMA7A coated wells were incubated with 10% C1q depleted, C1q rescued, C4 depleted, C4 rescued, fB depleted, and fB rescued serum. Pure complement proteins at 100 µg/ml C3, 100 µg/ml C3b, 20 µg/ml fB, 20 µg/ml fBb, and 0.5 µg/ml fD concentration were used to generate alternative pathway convertase for complement opsonization. Complement activation and binding to rSEMA7A coated wells was evaluated by probing with anti-C3 primary antibody followed by peroxidase labeled secondary antibody. Reactions were developed with the addition of ABTS peroxidase substrate and read at OD 410 nm. Readings were calculated by averaging duplicate wells and subtracting background reading from control wells. Error bars represent standard deviations. P values are based on t-test.

Figure 4.14: Complement is activated by recombinant C1B1, RAP1A and fetuin. ELISA assays with A) rSEMA7A, rC1BP1, rRAP1A and B) fetuin coated wells were incubated with 10% C4 depleted or C4 rescued serum. Complement activation and binding to antigen coated wells was evaluated by probing with anti-C3 primary antibody followed by peroxidase labeled secondary antibody. Reactions were developed with the addition of ABTS peroxidase substrate and read at OD 410 nm. Readings were calculated by averaging duplicate wells and subtracting background reading from control wells. Error bars represent standard deviations. P values are based on t-test.

Figures:

Figure 4.1

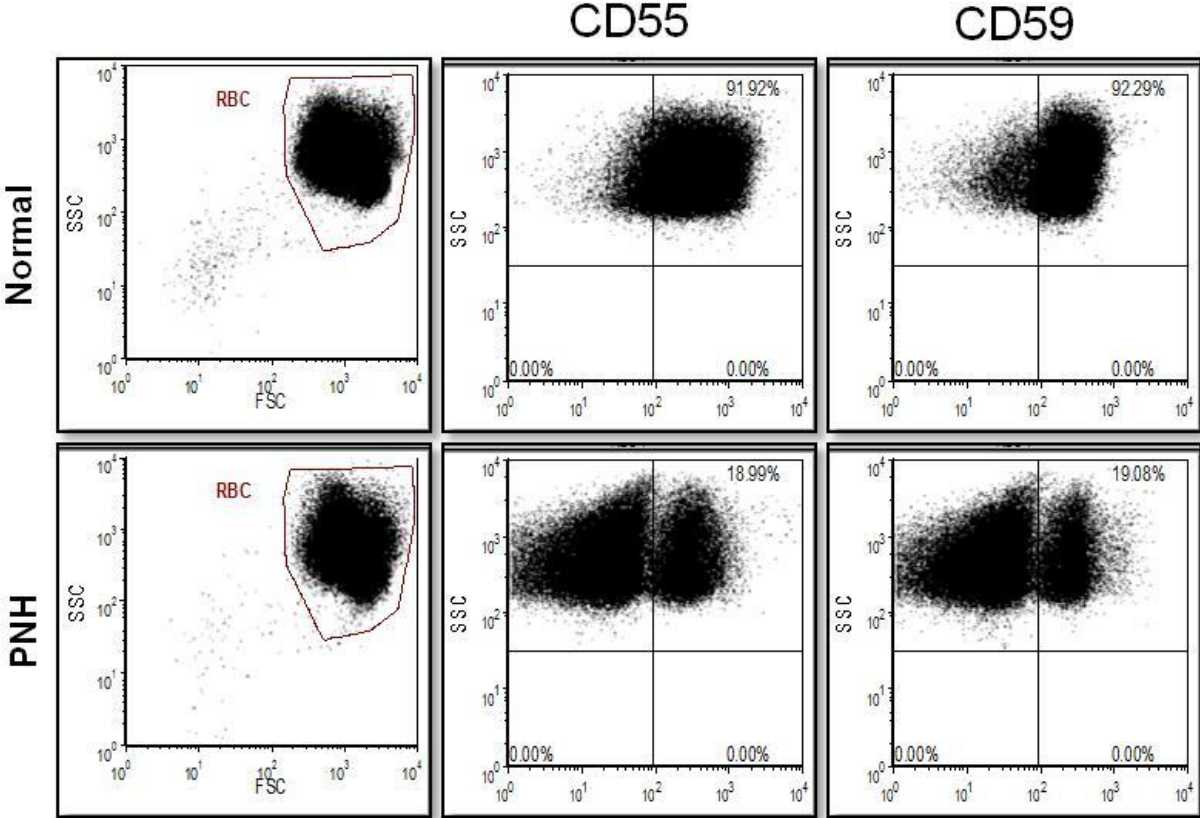
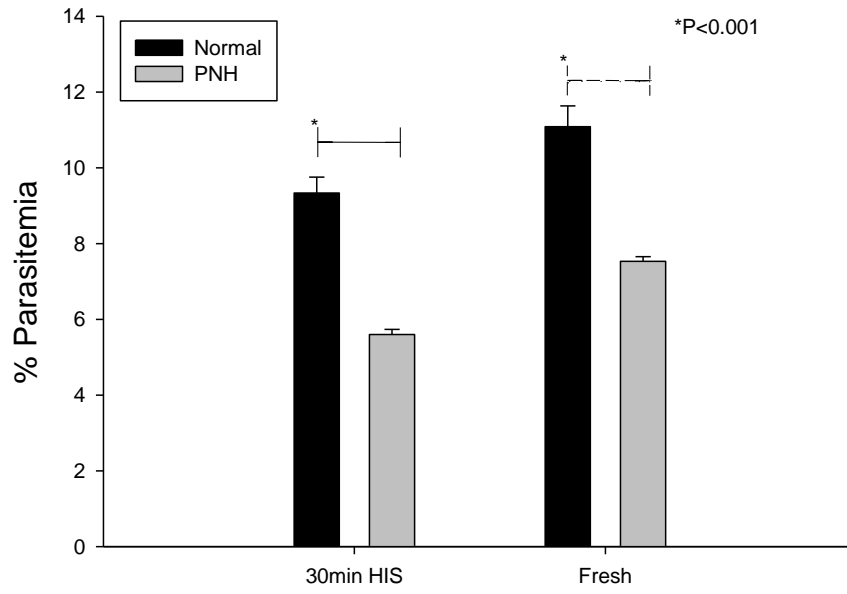


Figure 4.2

A



B

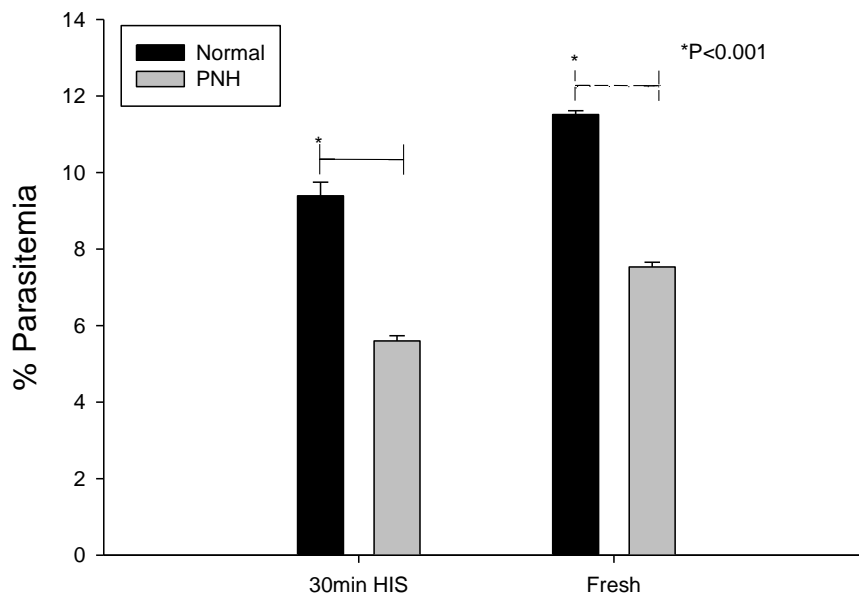
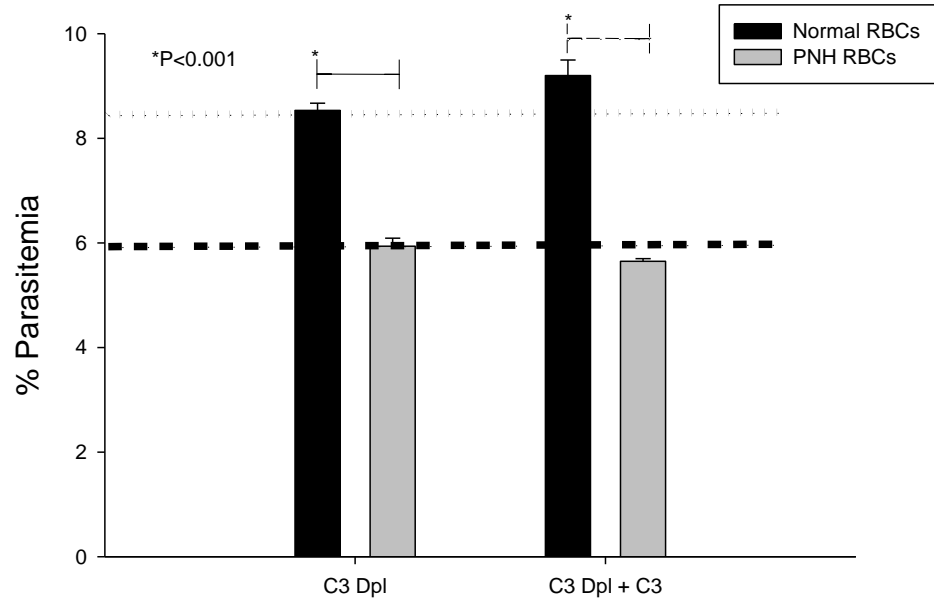


Figure 4.3

A



B

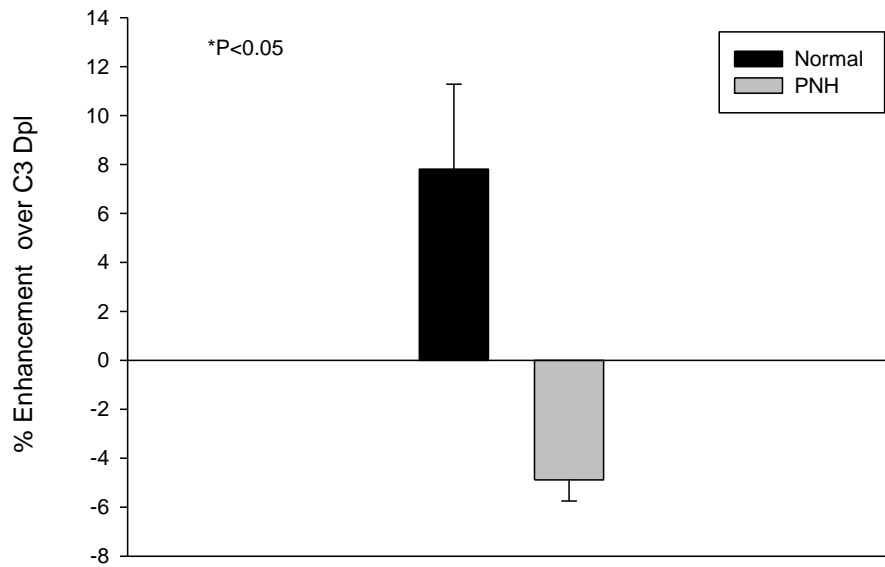
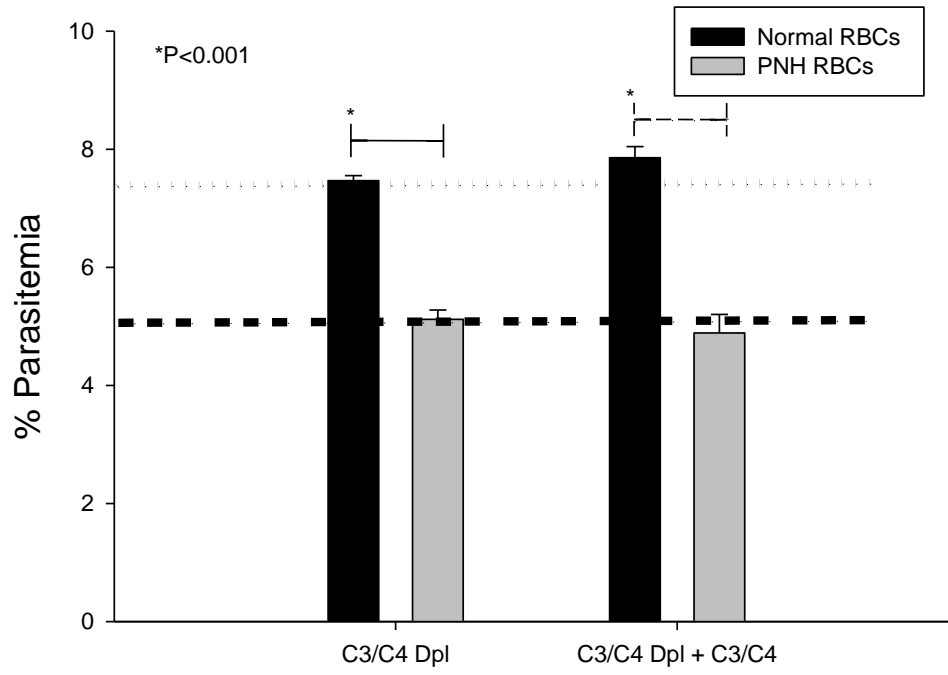


Figure 4.3

C



D

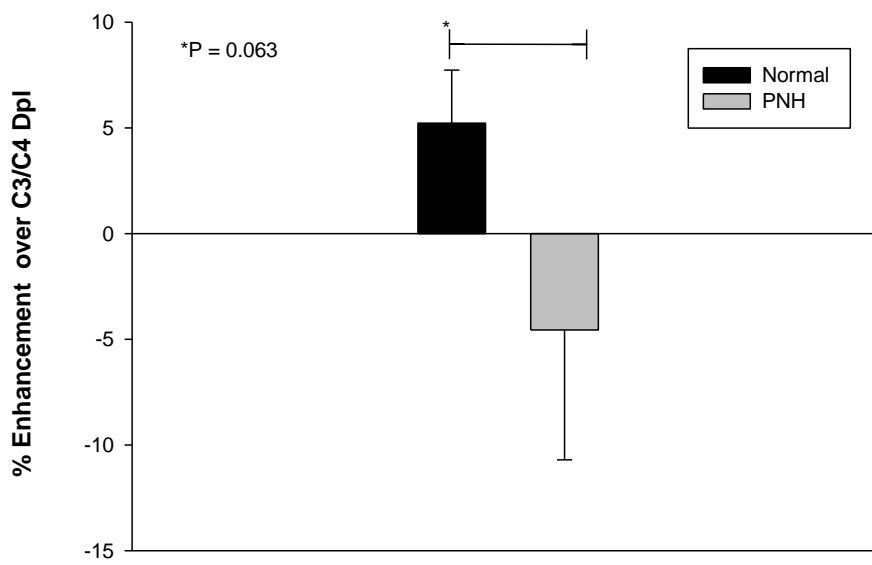


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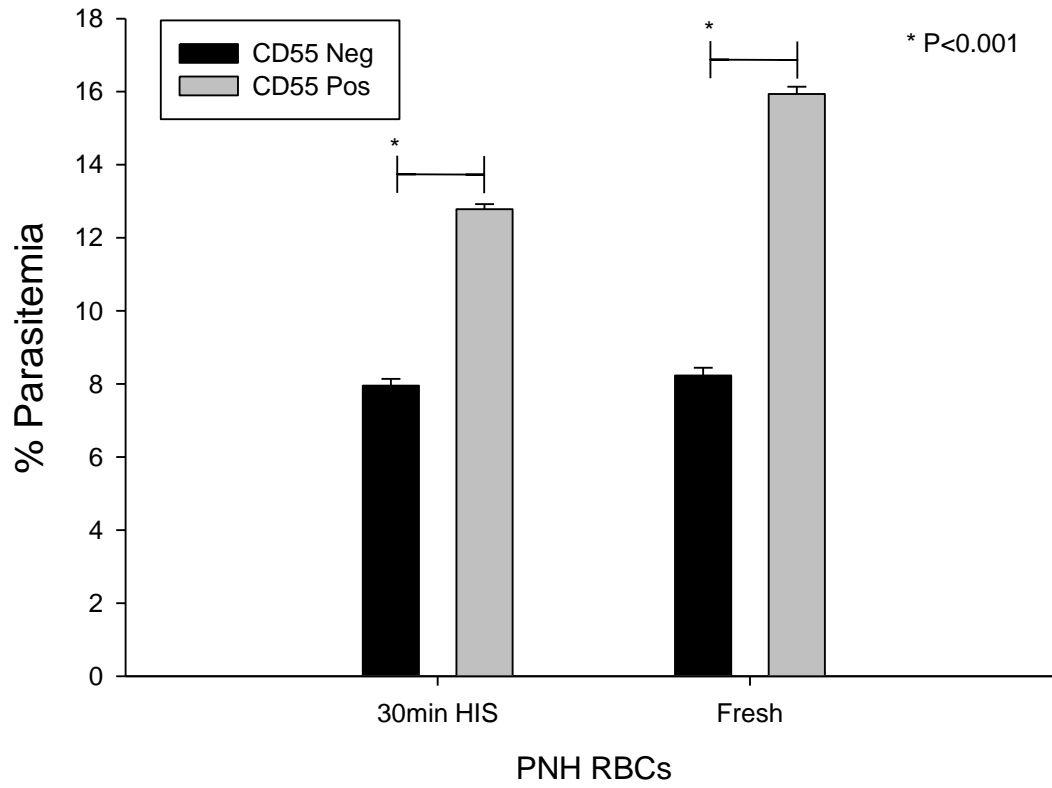
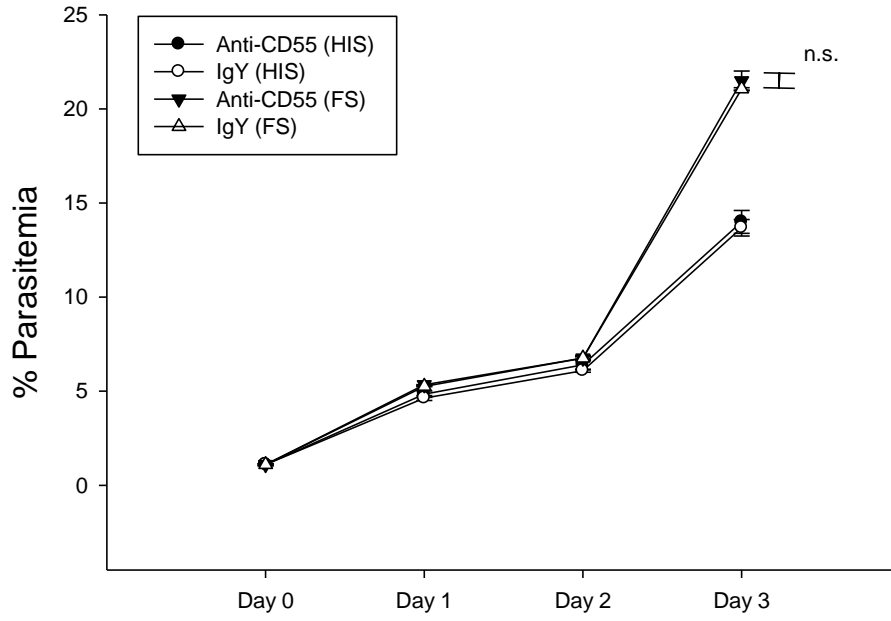


Figure 4.5

A



B

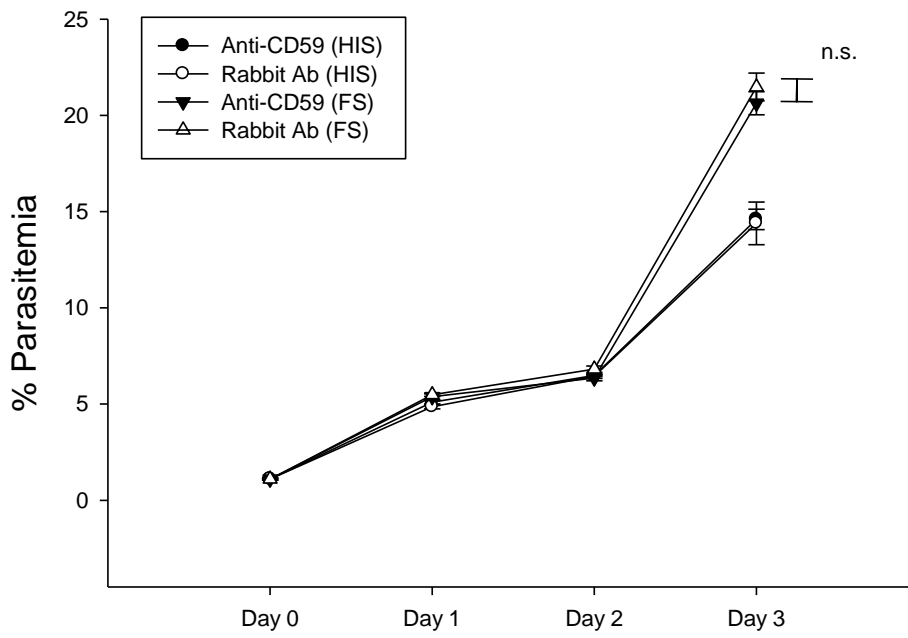


Figure 4.5

C

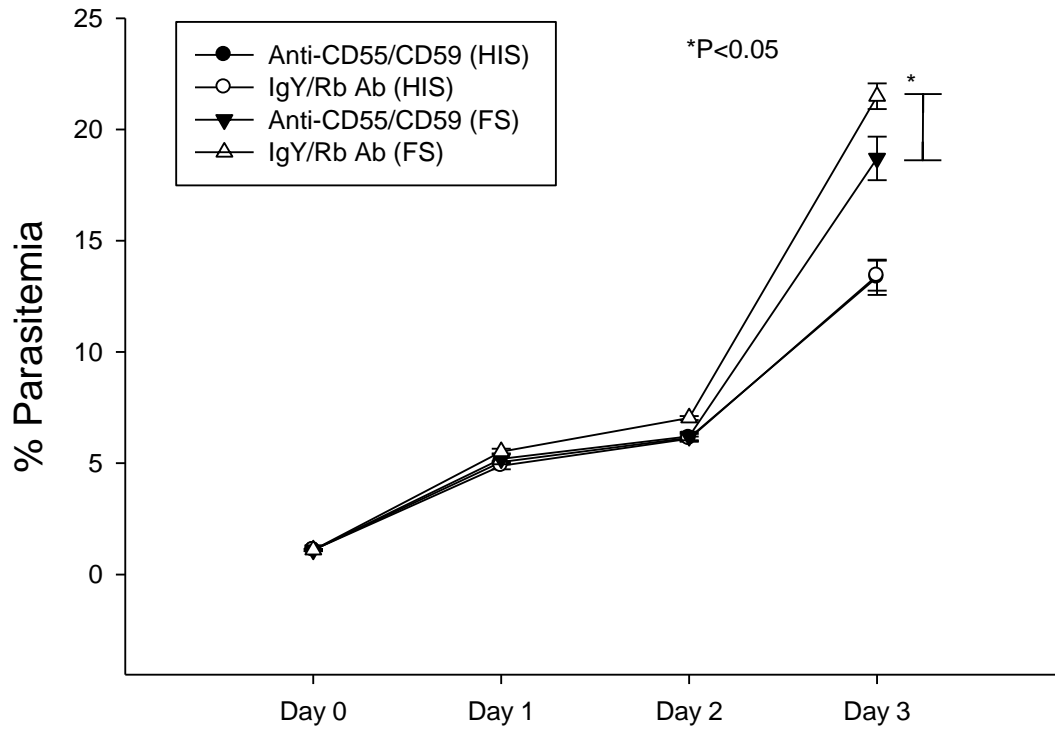
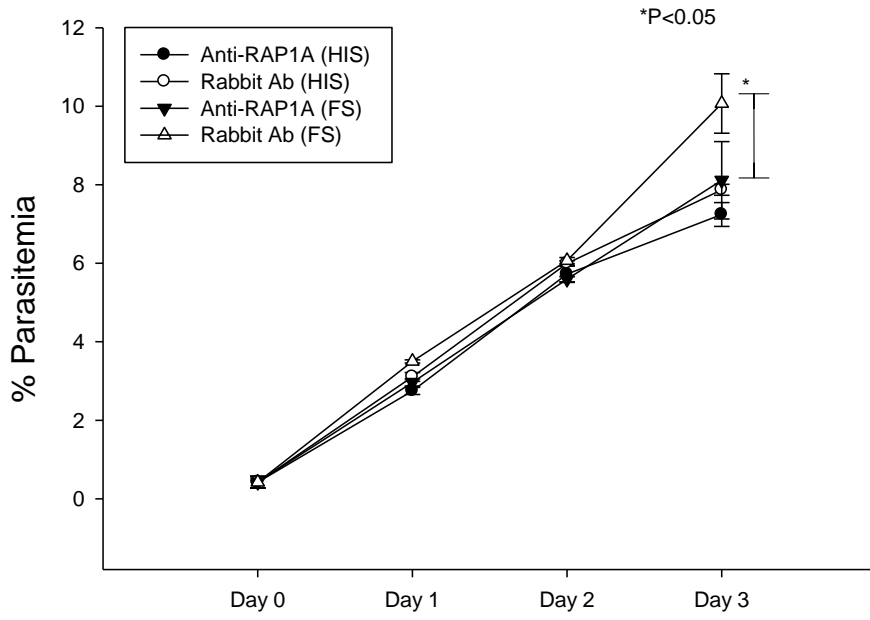




Figure 4.6

A



B

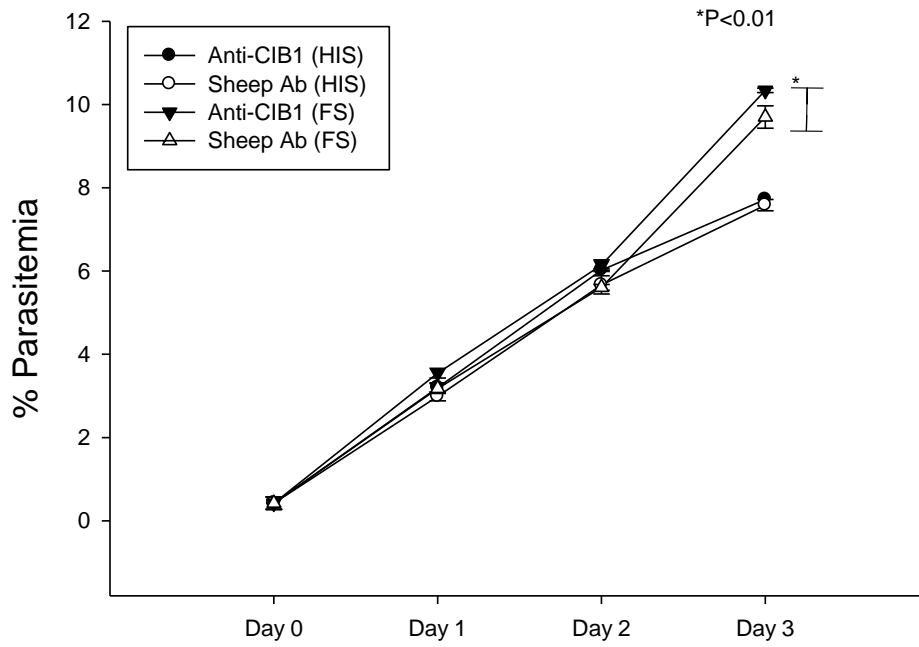
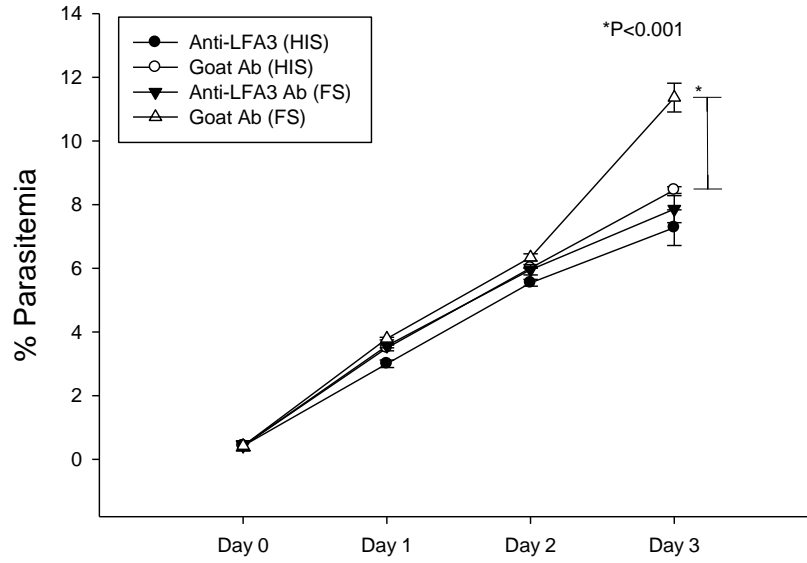


Figure 4.6

C



D

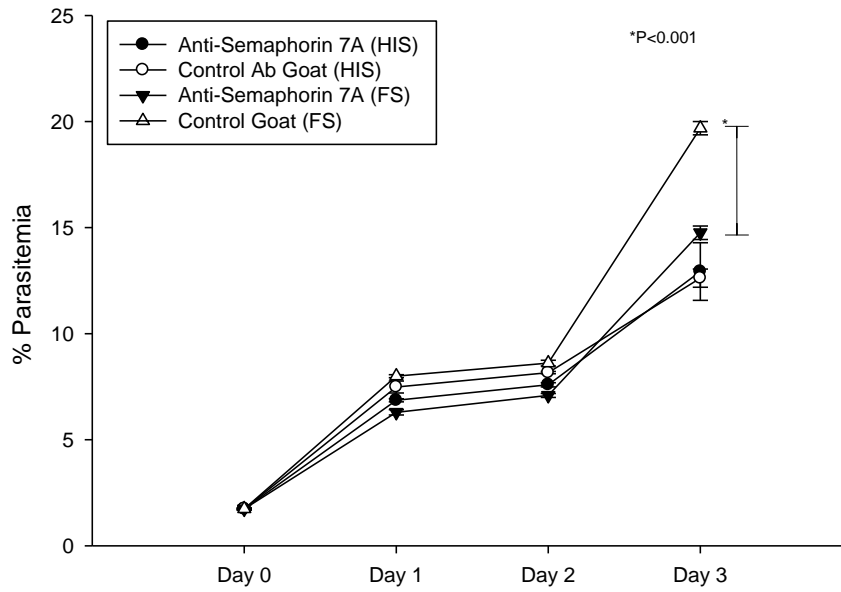
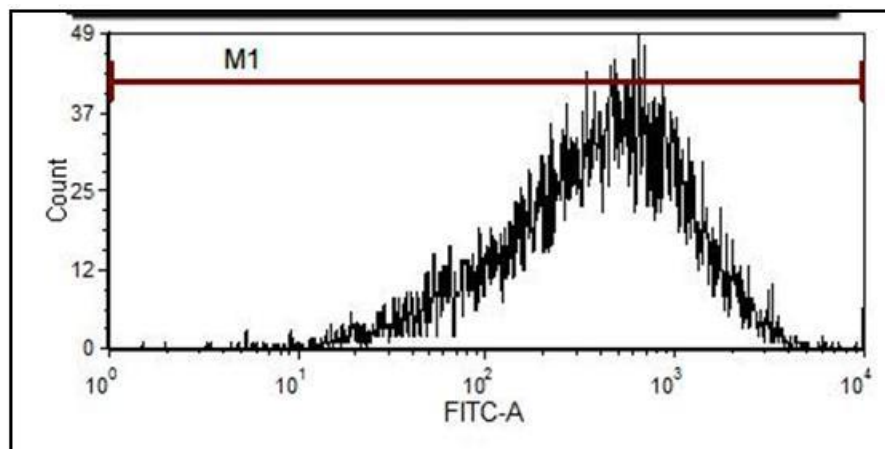


Figure 4.7

Normal



PNH

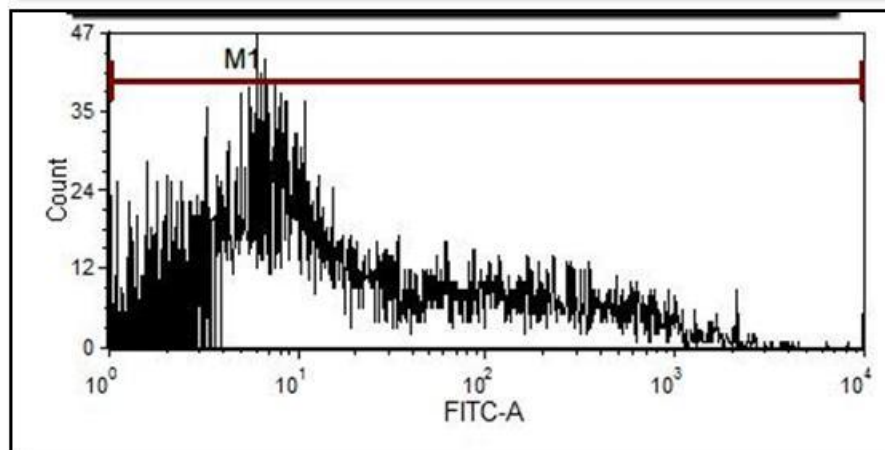


Figure 4.8

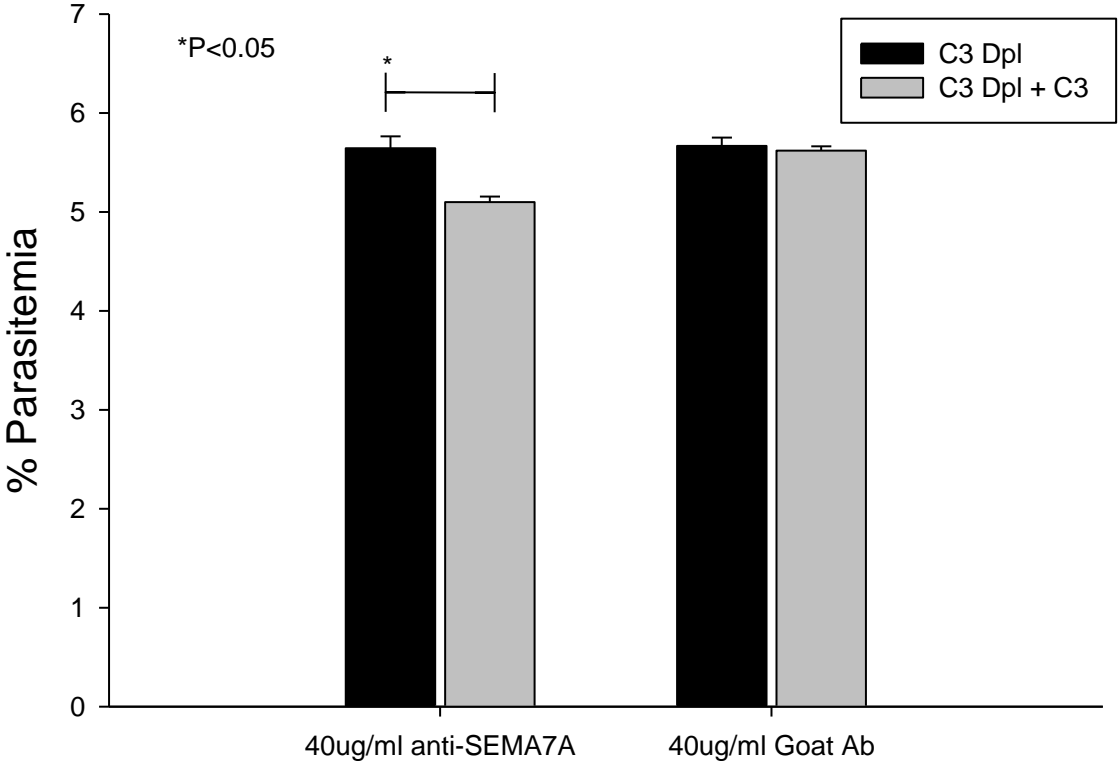


Figure 4.9

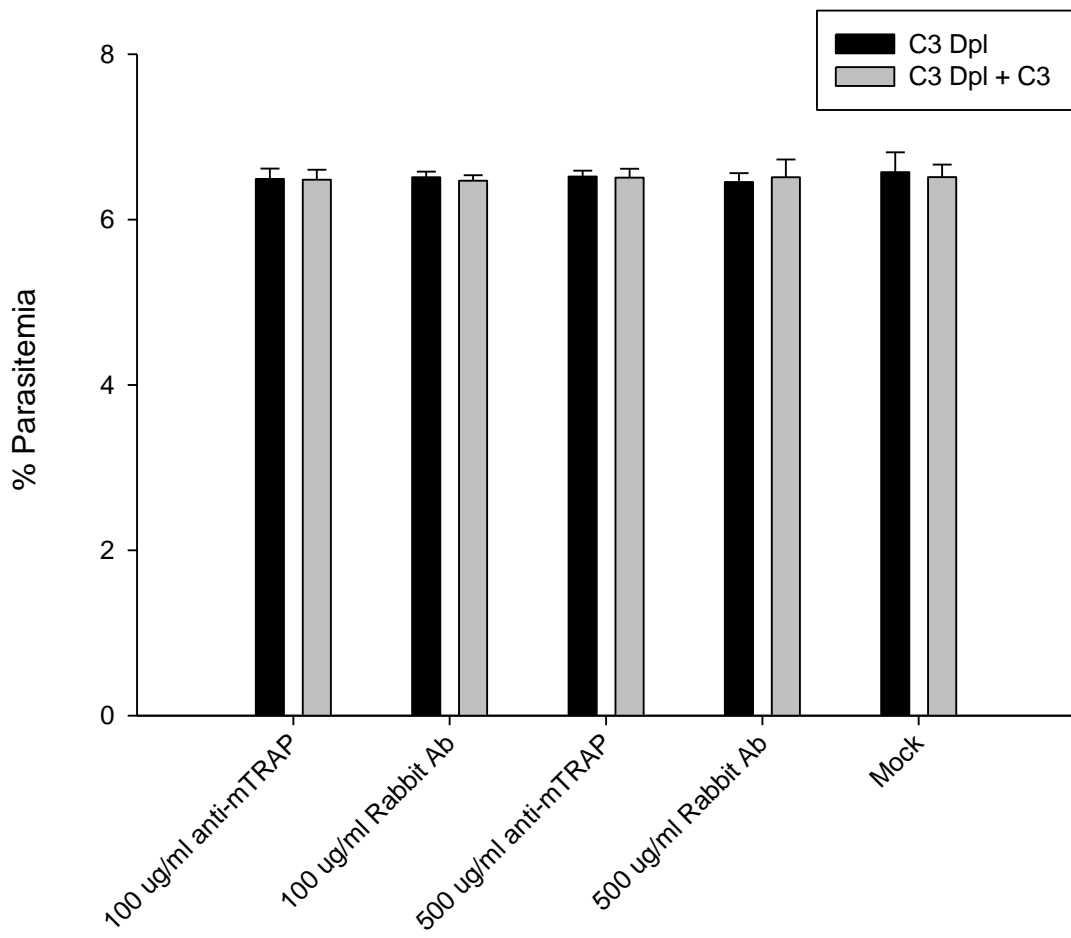
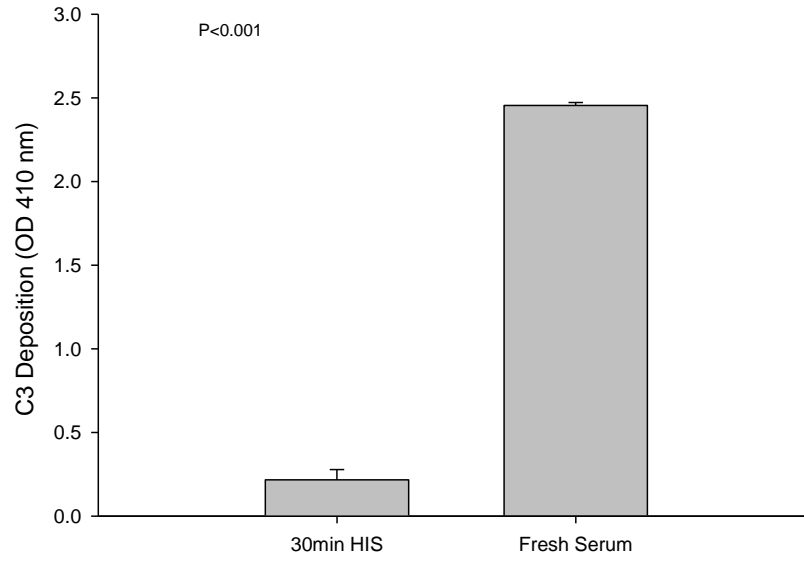
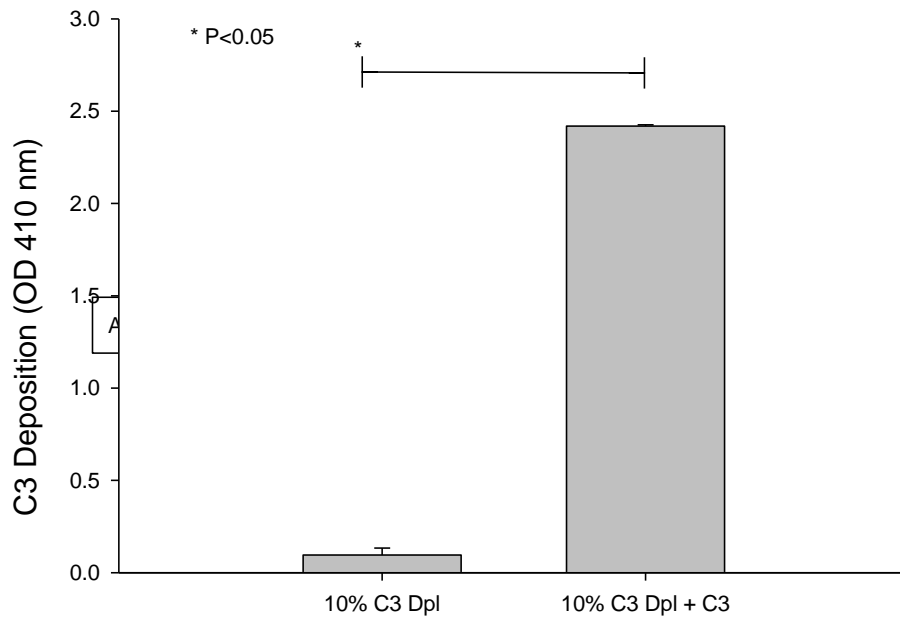


Figure 4.10

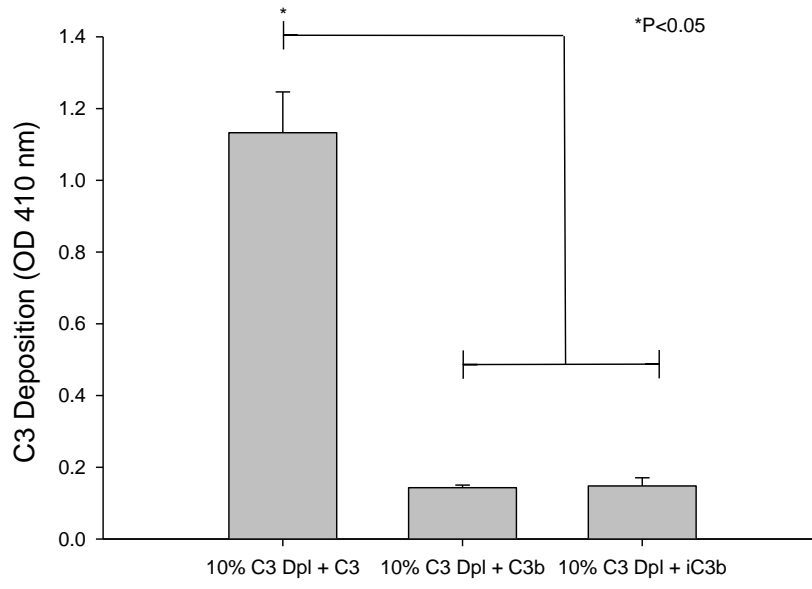
A



B



C



D

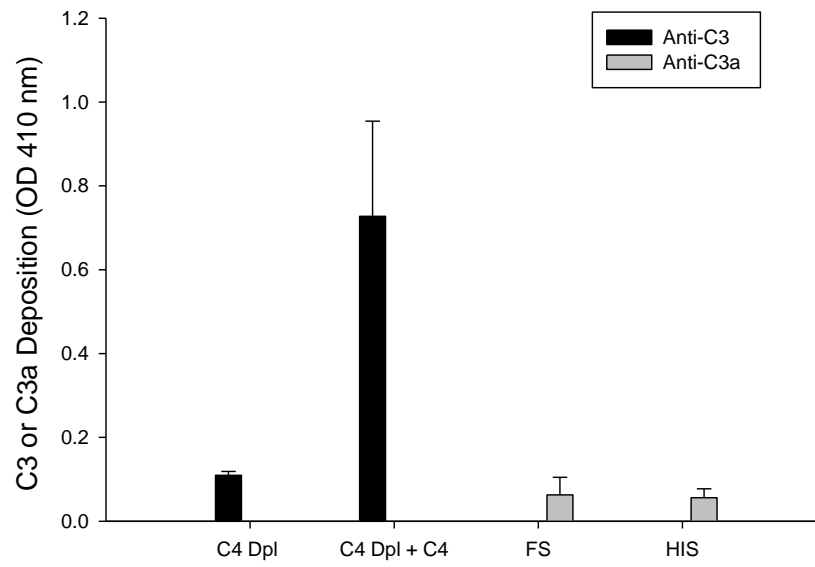


Figure 4.11

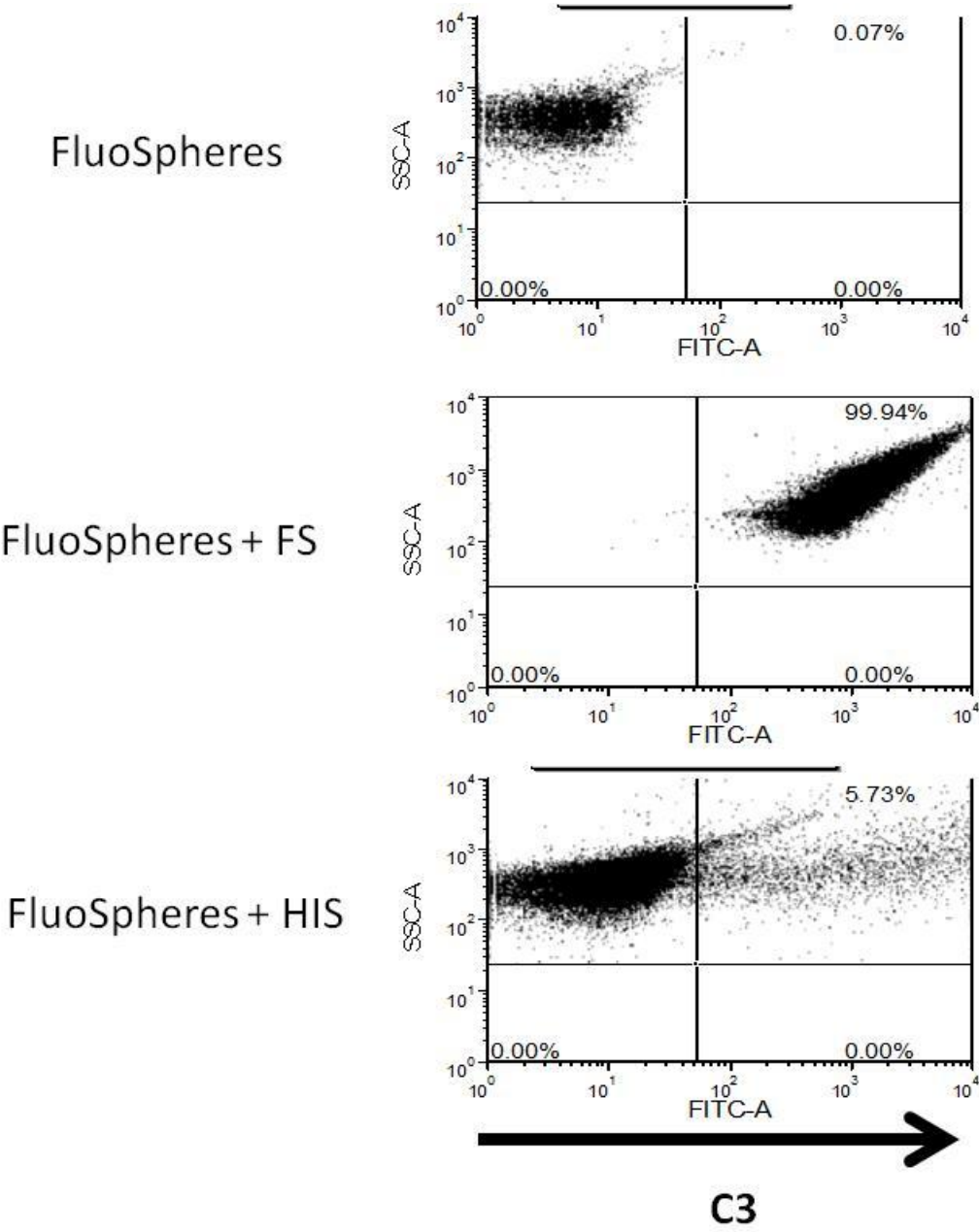




Figure 4.12

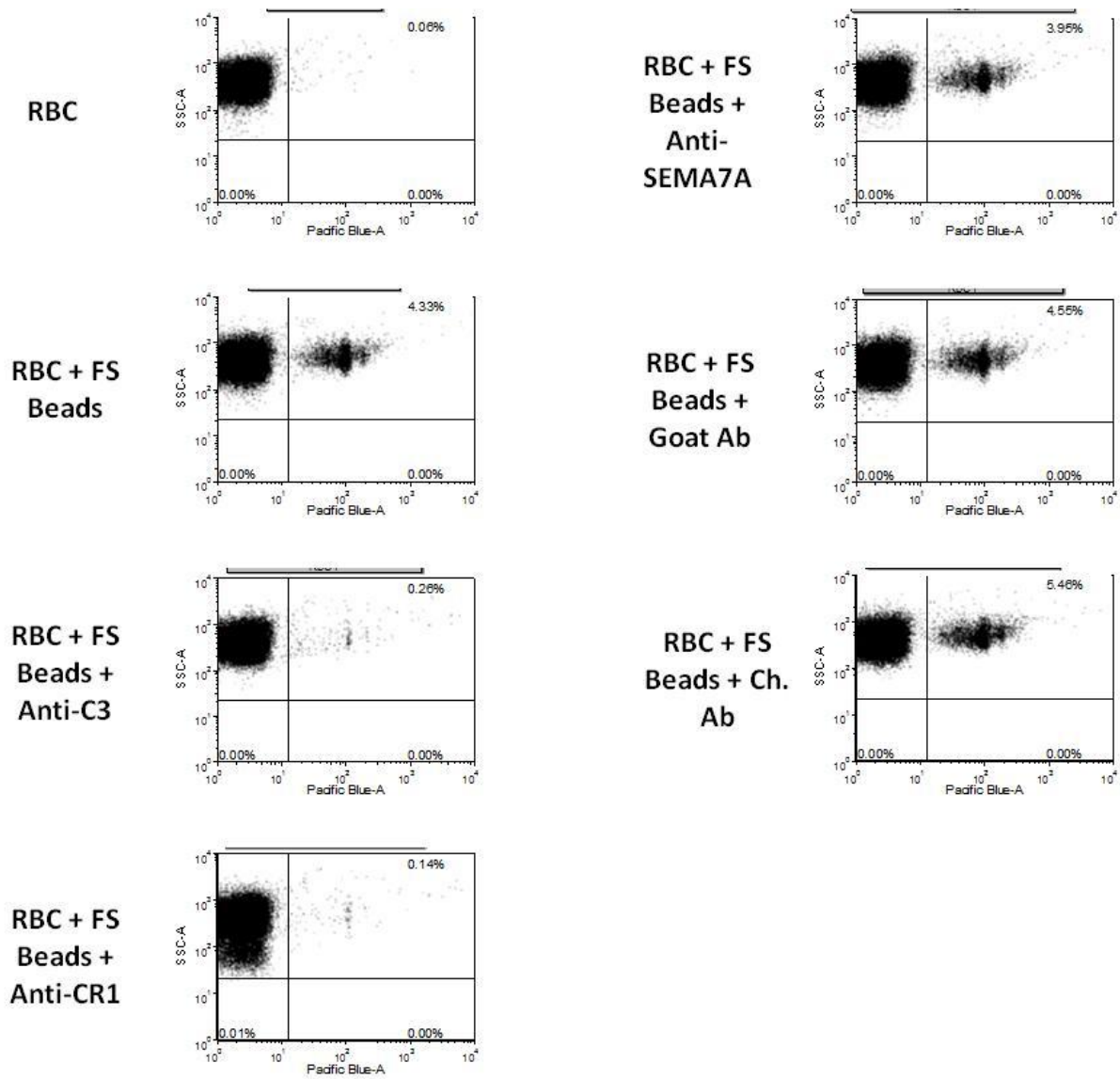


Figure 4.13

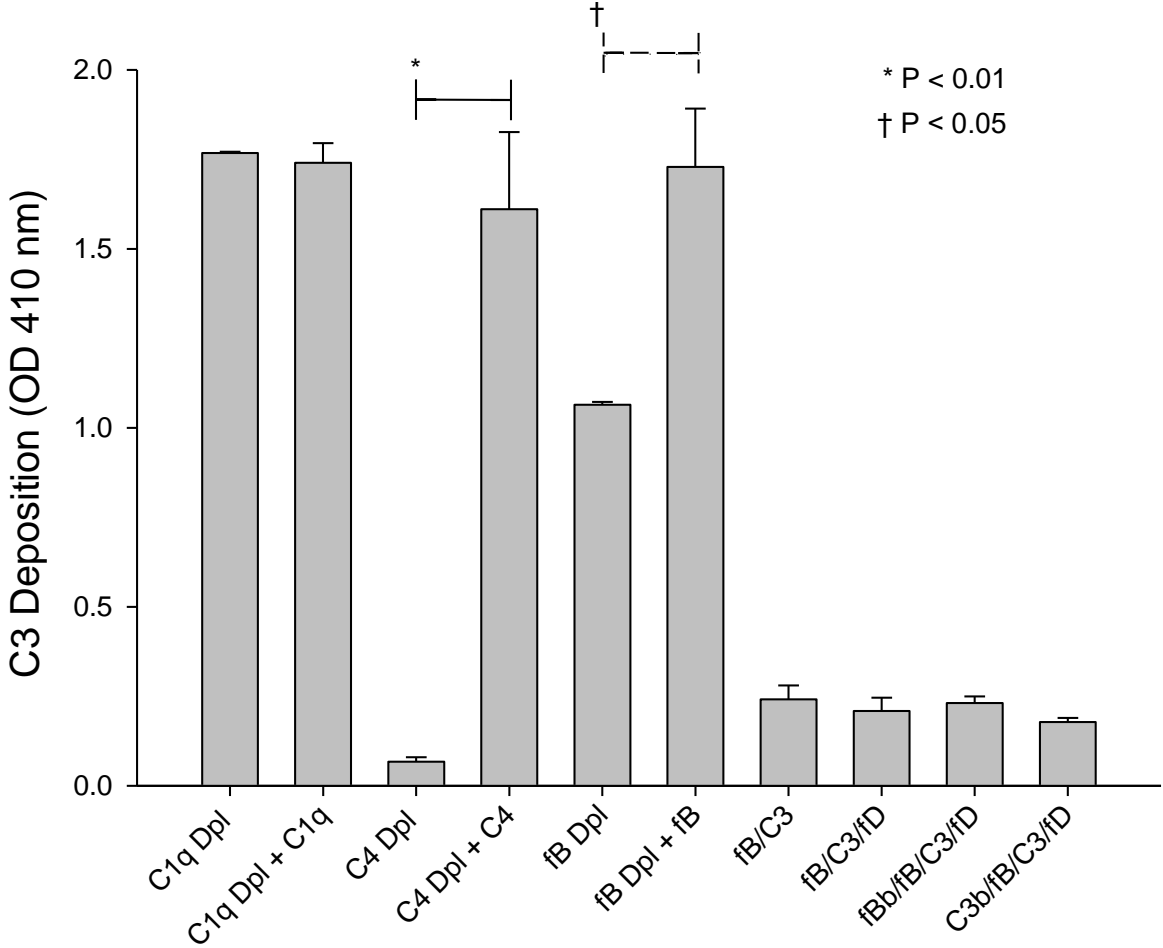
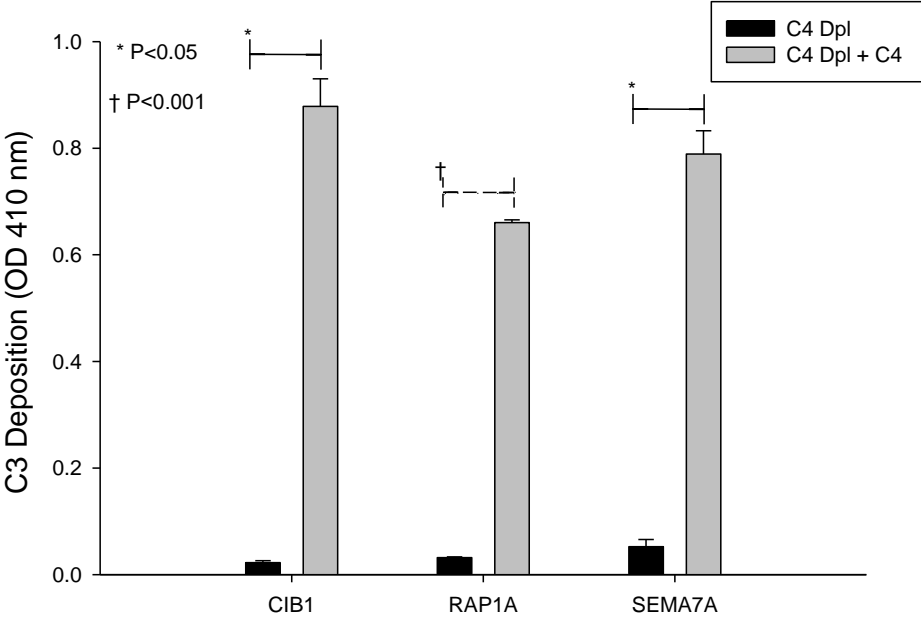
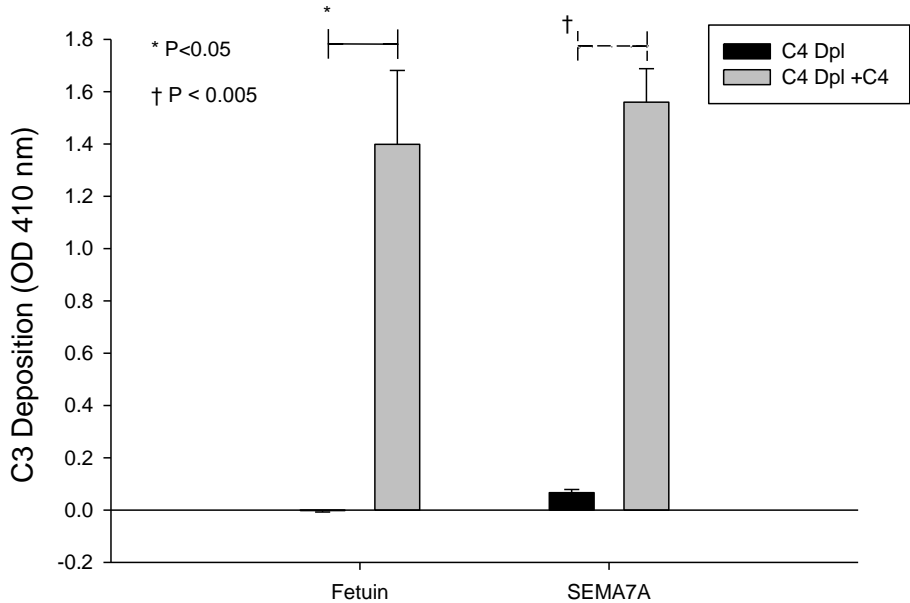


Figure 4.14

A



B



## Chapter V: Summary

### Overview of the Data Chapters:

Our research focuses on the important role that complement plays in the invasion of erythrocytes by *P. falciparum*. We discovered a novel complement mediated erythrocyte invasion pathway that is in part independent of sialic acid-dependent and -independent pathways utilized by the merozoites for invasion of erythrocytes (Figure 5.1). Here we show that complement and antibody intrinsic functions of host protection are hijacked for the benefit of the parasite. Consequently this has serious implications for current vaccine development as a result future vaccine formulations should strive to promote less complement fixing antibody isotypes.

Numerous studies, in both humans as well as animal models, support our finding of complement activation during malaria infection (96-102). As the merozoites egress from the protective environment of the host erythrocyte they are exposed to antibody and complement mediated opsonization, as the host immune system struggles to neutralize the merozoites during their transient migration into a new host cell. Overall our data supports complement activation on the surface of the merozoites with the deposition of C3b fragments. Recently, another group also confirmed complement deposition on purified merozoites *in vitro* with deposition of C1q and C3b (59). Yet others have shown no evidence of complement activation on the merozoite surface (92,93). Discrepancy in these studies may stem for drastically different merozoite isolation methods in addition to the different parasite strains that were used.

We propose that complement activation on the parasite surface and the opsonization of the merozoites leads to enhancement of erythrocyte invasion. We consistently demonstrated *in vitro* enhancement of erythrocyte invasion by *P. falciparum* in the presence of fresh or complement rescued sera relative to sera deficient in complement activity after heat inactivation or selective complement depletions. In theory, any complement pathway may recognize the merozoites and become activated on the merozoite surface. Overall our *in vitro* data demonstrated that the classical pathway predominates, particularly in the presence of anti-merozoite antibodies, resulting in enhancement of opsonization and erythrocyte invasion by merozoites. This antibody-mediated complement enhancement of erythrocyte invasion was also supported by parasite growth in our *in vivo* passive transfer experiments in *P. berghei* mouse models. In the presence of anti-*P. berghei* antibodies the end point parasitemia was enhanced in wild type mice, presumably through the activation of complement. Also, due to lack on complement opsonization in C3 knockout mice the end point parasitemia was much lower than in wild type mice. There was an inverse correlation between passively transferred anti-*P. berghei* antibody dose and the level of parasitemia, such that the lower anti-*P. berghei* antibody doses led to significant enhancement of parasitemia in mice. This *in vivo* enhancement in parasitemia diminished with increasing doses of anti-*P. berghei* antibodies. Previous animal studies that administered high doses of passively transferred antibodies, from immune rodents, also inhibited parasite growth in infected naïve animals (239-243).

Contrary to our findings, Dasari *et al.*, proposed that as the infected erythrocytes rupture, the expelled DV activate, become opsonized by complement, and serve as

decoys that allow merozoites to escape complement attack (92,93). Conflicting results by Boyle *et al.*, demonstrated that the classical pathway mediates deposition of complement on merozoites but instead of enhancement of erythrocyte invasion, complement opsonization leads to merozoite destruction (59). Considering these differences, it is possible that the stoichiometric quantities of the anti-parasitic antibodies, complement, and parasite concentrations govern whether the parasitemia will be inhibited, enhanced or unaltered, specifically during primary acute infection. At a low parasite load, during the initial phase of the primary infection, partial complement removal by the DV may be beneficial to the limited number of egressed merozoites in the vicinity of erythrocyte. The DVs may reduce excessive complement deposition and potential lysis of merozoites by MAC before the merozoites have time to invade the subsequent erythrocyte. As the infection progresses, anti-merozoite antibody titers increase and so does the potential for excessive classical pathway driven complement activation on the merozoite surface. This excessive opsonization may block critical receptor-ligand interactions necessary for invasion of erythrocytes. DV, acting as decoys for complement activation, may reduce excessive complement deposition on merozoites thereby prolonging the chronic phase of the infection for the benefit of the parasite.

As with most biological systems, intermittent synchronous erythrocyte lysis would release a highly heterogeneous merozoite population into the bloodstream with a fraction of merozoites being less fit. In the presence of anti-merozoite antibodies and complement, these less robust merozoites may be at a disadvantage and be more susceptible to complement damage (59). Additional variability in the results may arise

from availability of complement components (59,92,93). Our experiments were performed in the presence of 10% complement sufficient or deficient serum, a concentration that is standard for performing invasion and growth inhibition assays. Others carried out their experiments at serum concentrations of up to 50% which may partially account for their inhibitory activity (59). Nevertheless, both our human *in vitro* and animal *in vivo* data demonstrated that, for the majority of the merozoites, complement opsonization would promote merozoite invasion of erythrocytes.

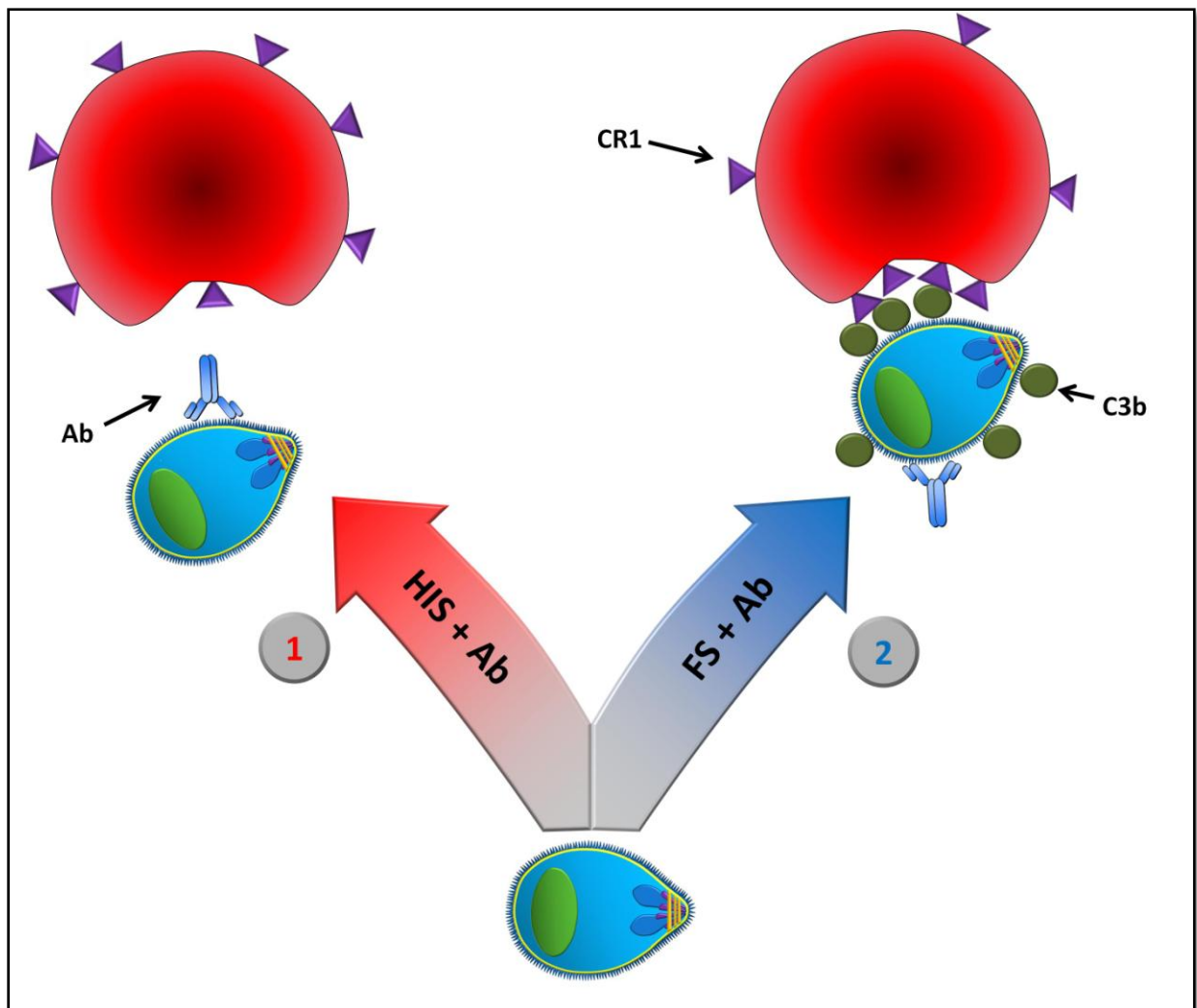


Figure 5.1: A model of antibody mediated complement enhancement of erythrocyte invasion by Plasmodium merozoites. **1)** In the absence of complement, such as via heat inactivation of serum (HIS), the merozoites depend on interactions between the erythrocyte receptors and the parasite ligands. Antibody (Ab) directed against merozoites may have inhibitory effect, and thereby block erythrocyte invasion. **2)** In the presence of complement, such as in fresh serum (FS), the merozoites may utilize not only specific receptor ligand interactions, as described in **1**, but also an alternative pathway of erythrocyte invasion becomes available which may be mediated by complement opsonins. Complement opsonins (C3b) on the merozoite surface may act as an alternative, host derived, parasite ligand that the merozoite can utilize to bind to the erythrocyte receptors, such as CR1, and counteract some of the invasion blocking effects of anti-merozoite antibodies.

Our interest in complement mediated enhancement of erythrocyte invasion stems from seminal studies describing the role of complement receptor and regulator on the erythrocytes, CR1, in the invasion by merozoites (15,37). Blocking CR1 caused invasion inhibition of sialic acid-independent *P. falciparum* strains especially in the absence of functional glycoporphin receptors on erythrocytes. The inhibition was exacerbated with sCR1 when it was used as a competitor in the presence of fresh serum. This was potentially due to steric hindrance from aggregates of sCR1-complement complexes that obstructed critical merozoite ligand-erythrocyte receptor invasion interactions as well as destabilized convertases and broke down complement opsonins. Surprisingly, sialic acid-dependent *P. falciparum* strains that are unable to utilize CR1 for invasion also benefit from complement presence. This may be due to CR1 playing a dual role during the invasion process. CR1 not only acts as an invasion receptor for sialic acid-independent strains but also as a receptor for complement opsonins on the merozoites that can be exploited by both sialic acid-dependent and -independent strains. Of course other receptors, specifically GPI anchored proteins such



as DAF and SEMA7A, on the erythrocyte surface may also interact with complement bound merozoites and enhance invasion (198,276).

Merozoites interaction with complement is hard to dispute, as is their relative resistance to complement mediated lysis, at least in the physiologically relevant time frame between their transitions from one host cell to the next. How merozoites resist MAC mediated lysis prior to erythrocyte invasion is unknown. Although different stages in the *Plasmodium* lifecycle, specifically the emerging gametes and young zygotes, through gamete surface protein PfGAP50, are able to bind to complement regulator factor H and hijack its functionality and inhibit complement cascade progression on the surface of the parasitic sex stages within the mosquito midgut (277). Furthermore, a novel protein was identified named the apical Sushi protein, which contains a domain present in many complement regulatory proteins called the Sushi domains (110). Apical Sushi protein may also serve a complement modulatory role for the benefit of the parasite. In our attempt to explain the merozoite's relative resistance to complement lysis, our preliminary results indicated potential host derived CR1 localization on the surface of merozoites which may limit MAC formation. It is conceivable that the parasite internalizes host CR1 and incorporates it into merozoite's membranes, since both the transmembrane protein Duffy and the GPI-anchored CD59 have been found to be in the apicomplexan vacuole (191). Incorporated CR1 on the merozoite surface may serve as an additional ligand for parasites to invade host cells through CR1-CR1 interactions by way of a complement opsonin bridge. Host subverted and modified or parasite de novo encoded complement regulatory proteins may also be present on infected erythrocytes. This is supported with *P. berghei* infected erythrocytes being more resistant to

complement mediated lysis than uninfected erythrocytes (173). Furthermore *P. berghei* infected erythrocytes, based on immunoblots of CD55 and CD59, displayed differences in molecular masses, and CD59 glycosylation pattern changed compared to uninfected erythrocytes (173).

One of the main questions our research aimed to address was why anti-merozoite antibodies generated from potential vaccine targets block erythrocyte invasion *in vitro*, but are inefficacious *in vivo*. The potential explanation is, antibodies generated from vaccine targets, are tested *in vitro* for their erythrocyte invasion inhibitory efficacy in the absence of complement (80,82,219-225). The antibody-mediated complement enhancement of erythrocyte invasion by merozoites would be missed under normal testing conditions. When we purified total antibodies from human MSP1<sub>42</sub> vaccine recipients, the antibodies inhibitory activity was reduced in the presence of complement. Even if invasion inhibitory antibodies are generated from vaccines, activation of the classical pathway would negate some inhibitory activity since the parasite is able to manipulate complement for its own benefit. Hence historically studies struggled to find a correlation between anti-merozoite *in vitro* erythrocyte invasion blocking and *in vivo* erythrocyte invasion permissive antibody results. Another possibility is direct activation and opsonization of merozoites by complement, although to a lesser extent, in the absence of anti-merozoite antibodies especially if merozoites are able to modulate complement's lytic terminal pathway. Opsonization of merozoites by complement fragments may create a complement coat thereby disguising the parasite by masking essential highly conserved surface antigens from immune pattern recognition receptors.

Finally, by focusing on the effects of complement on erythrocyte invasion by merozoites and using PNH erythrocytes, cells deficient in GPI anchored receptors, we were able to gather preliminary data on potential novel erythrocyte receptors utilized by merozoites in the invasion of erythrocytes in a complement dependent manner. We reduced invasion enhancement of *P. falciparum* only in the presence of complement by blocking SEMA7A, RAP1A and LFA3 on the surface of erythrocytes. The reduction in parasitemia subsequent to blocking these receptors was primarily observed after two rounds of erythrocyte invasion in growth assays. It is easy to dismiss the results from a growth assay as just an amplification in parasitemia from a secondary round of invasion, but during initial invasion factors, such as antibodies and complement, bound to the merozoites may potentially be brought into the erythrocyte (63,278,279). These discrete merozoite bound factors may influence the intracellular parasite growth, development, and fitness, which would impact the egress of merozoites out of infected host cell and the secondary round of erythrocyte invasion. Bergmann-Leitner *et. al* demonstrated that anti-merozoite antibodies not only inhibit erythrocyte invasion but are transported with the merozoite into the infected erythrocyte where they induce stage specific growth retardation of late stage trophozoite, and in some cases even inhibited schizont rupture which was parasite strain specific (278). Also, the infected erythrocytes become leaky especially prior to rupture enabling macromolecules, such as beads with a diameter of up to 70 nm, to enter the cells (280,281). It's feasible for complement components, just like it is for antibodies, to enter the infected erythrocytes throughout the duration of the parasites intracellular growth and influence the overall parasite development, in addition to the invasion enhancement we currently observe.

## **Advantages and Limitations of Employed Methods and Alternative Approaches:**

In the majority of our experiments we used fresh erythrocytes, that were less than a week old, and complement sufficient serum isolated from whole blood of a single donor. This reduced any potential artifacts in our assays that may stem from blood group incompatibilities since, only 2, ABO and Rh, out of 33 currently recognized blood groups are verified for compatibility for malaria culture (282). Use of a single blood donor also prevented any variability that would result from variations in expression levels of blood group antigens on erythrocytes between individuals. The level of enhancement in erythrocyte invasion we observed in the presence of fresh serum, from the same donor, compared to heat inactivated serum, even with only 3 min of heat treatment, were usually greater than from the pooled serum for numerous different donors depleted of specific complement components. This may be due to less interference in invasion from blood group specific antibodies from the homologous serum donor as the erythrocytes. Use of fresh erythrocytes, also limited the level of auto- or natural antibody binding to senescent cell antigens generated due to changes associated with erythrocyte anion exchange protein (band 3) which becomes more numerous as cells age or are stored *ex vivo* (283). During erythrocyte storage, there is also the loss of cell membrane due to vesiculation which produces changes in the erythrocyte shape from normal, reversible and biconcave discocyte to reversible but deformed echinocyte to irreversibly deformed spherocochinocyte and finally to erythrocyte ghost (284-287) . Prolonged erythrocyte storage also reduces cell surface

receptor such as CD35, CD55 and CD59 making erythrocytes more susceptible to complement mediated lysis and refractory to invasion by merozoites (286,288). Furthermore, lipid content in the erythrocyte membrane changes based on medications, such as statins, and diet, both of which may affect membrane fluidity, receptor distribution and number, that would then translate to inconsistency in merozoite invasion between different blood donors (289-291). Variation in blood donor's diet may also attribute to some inherent variability between serum batches potentially from something as trivial as the quantities of common dietary plant flavonoids that have known antiplasmodial activity (292). Serum complement levels may fluctuate based on the intensity, muscle damage and inflammatory responses sustained from day to day exercise activity (293-295). Thereby using reagents from a single healthy blood donor offers the advantages of controlling the potential impact that these extraneous factors may have on erythrocyte invasion and growth by *Plasmodium*. This comes at the cost of reduced robustness of the data and limited representation of the whole population. In the future more donors will need to be recruited for both serum and erythrocyte donations from diverse ethnic and/or racial backgrounds. Information from high-throughput SNP based blood group genotyping of erythrocyte donors may be useful in assessing effects of less prominent blood groups on long term *in vitro* culturing of *Plasmodium* (296). This may potentially lead to discovery of novel invasion receptors as well as understanding which blood group combinations synergize in enhancing or inhibiting erythrocyte invasion and intracellular growth.

Our current work only utilized one mouse monoclonal antibody, mAb5.2, directed against MSP1 on the surface of the merozoites. Although mAb5.2 was considered to

have no invasion inhibitory activity we observed minor erythrocyte invasion inhibition in the presence of heat inactivated serum (297). Future work would benefit from an expanded antibody repertoire to other merozoite surface antigens as well as intracellular stage specific targets in both invasion and growth inhibition assays. It is possible that unlike mAb5.2, other antibodies, against different epitopes on MSP1 or other *Plasmodium* targets, would be refractory to induction of antibody mediated complement enhancement and offer more protection for the host. Our data on the reduction in erythrocyte invasion by *P. falciparum* from purified total IgG isolated from human MSP1<sub>42</sub> vaccinees in the presence of complement would benefit from isotyping the antibody response in these individuals as well as look at the pattern of glycosylation. A potential correlation may exist between high complement fixing antibody response that is skewed towards IgG3 and IgG1 in vaccinees and reduction in antibodies ability to inhibit invasion. Other complement fixing antibodies such as IgM, directed against sporozoites, have been correlated with protection against sporozoite transmission; this same level of protection may translate to parasite blood stages (298). Conversely, in malaria patients high titers of IgM, four times the level of IgG, directed against erythrocytes along with complement consumption were associated with reduction in the Hct leading to anemia (299). Further involvement of IgM in exacerbation of blood stage infections comes from the study of placental malaria. The PfEMP1 protein VAR2CSA expressed on infected erythrocytes bound to natural IgM and protected the parasites from FcγR-dependent phagocytosis, masked PfEMP1-specific IgG epitopes without obstructing CSA binding and prevented C1q deposition (300,301). It's possible that unlike the exoerythrocytic stage that direct the antibody mediated immune response

against the free sporozoites, the involvement of intracellular parasite forms and manipulation of the host erythrocyte during blood stage infection may inadvertently create an autoimmune like environment. This may potentially cause more collateral damage to the uninfected erythrocytes while partially concealing the infected erythrocytes from antibody responses. Additional studies on specific IgM responses against the merozoites and the infected erythrocytes in the context of complement should prove useful in understanding not only this early antibody response but also the role this marginalized yet critical antibody class plays in infection.

Furthermore, our focus *in vitro* has been limited to a model for an acute primary infection; additional work would need to be done on the role of complement in the presence of a polyclonal antibody response from individuals that had numerous exposures to the parasite. An assessment of the antibody repertoires that are affinity purified for specific antigenic recognition isolated from clinically immune individuals from endemic areas and compared to antibodies from vaccinees may define which parasite targets induce inhibition independent of complement fixation or are exclusively dependent on specific antibody isotype. Affinity screening, titrating and isotyping of antibodies, and evaluating complement levels and functionality from chronically infected individuals that have not been exposed to recurring parasite infections yet are still sporadically symptomatic may also prove effective when compared to clinically immune individuals (302).

Our mouse *in vivo* results seem to demonstrate that complement mediated enhancement is also highly dependent on antibody titers and/or concentrations. Our method of producing mouse antibodies induced high titered polyclonal responses that

are partially reminiscent of multiple consecutive natural infections. Parasitemia enhancement in mice was seen only in the presence of low passively transferred doses (250 µg/mouse) of anti- *P. berghei* antibodies while high antibody doses reduced parasitemia. It's possible that the immunodominant merozoite epitopes stimulated the production of non-inhibitory complement fixing antibodies that enhanced invasion. Whereas, the immunorecessive epitopes induced invasion inhibitory antibodies but at a much lower titer or concentration and hence their invasion inhibitory activity was only apparent at a high antibody dose per mouse. Also, complement driven invasion enhancement with non-neutralizing immunodominant epitope specific antibodies may be present in acute primary infection but evolve into complement inhibition with inhibitory immunorecessive epitope specific antibodies during reinfection. This was observed with impaired control of parasitemia in C1q deficient mice reinfected with *P. chabaudi* (124). Additionally, mice injected with control antibodies usually exhibited reduced parasitemia relative to untreated control. This may have been due to intravenous immunoglobulin (IVIG) like therapeutic activity of control antibodies, with modulation of excessive inflammatory responses, that are observed during malaria infection, by binding to complement fragments, such as C3b and C4b, and to anaphylatoxins such as C3a and C5a (132,147,303,304). There was apparent variability in the level of *Plasmodium* growth inhibition in mice treated with control antibodies which was batch dependent. It's possible that this control antibody batch variability is due to the difference in age of animals as well as primary housing location that would dictate pathogen exposure milieu; culminate in the inconsistent diversity and exposure level to different pathogens by mice prior to serum collection for control



antibody purification (305). Future work may test different control antibodies batches in passive-transfer experiments with *P. berghei* infected mice, comparing variables such as age, sex, and housing location on the level of *Plasmodium* growth inhibition. Yet, even in the absence of passively transferred antibodies, parasitemia in C3 deficient mice was significantly lower than in wild type counterparts. Future experiments in the presence of passively transferred anti-*Plasmodium* antibodies, with additional knockouts mice that are deficient in specific complement pathways, such as C1q, C2, or fB knockouts, may add further insight as to how the parasite escapes and subverts complement for its own benefit. Creation of a mouse model suitable for *P. falciparum* growth and *in vivo* experimentation would prove invaluable, yet to date; most attempts have proven unsuccessful or unreliable. Differences between murine and human hemoglobin may partly be the cause for poor growth sustainability of *P. falciparum* in mouse erythrocytes. One possible approach may be to cross breed recently developed mice expressing human hemoglobin knock-in genes (B6;129-*Hba*<sup>tm1(HBA)Tow</sup> *Hbb*<sup>tm2(HBG1,HBB\*)Tow</sup>/*Hbb*<sup>tm3(HBG1,HBB)Tow</sup>/J, The Jackson Laboratory) with human CR1 transgenic mice.

Prospective work should focus on identifying how merozoites are able to manipulate complement for their own benefit and escape MAC lysis. Complement manipulation by merozoites may involve parasite encoded complement regulator binding proteins, similar to PfGAP50, which may bind to host derived complement regulators and stop further progression of the complement cascades (277). Of course it's possible that the merozoites are able to encode their own complement regulators, such as novel Sushi domain-containing proteins, without the need to rely on the host

regulatory factors (110). Based on our data, the merozoite surface may potentially be decorated with host complement regulators, such as CR1, that was internalized and transferred from the erythrocyte surface and incorporated into the merozoite membrane (191). Further discovery of novel ligand receptor interaction and/or complement regulators utilized by the parasite may employ the co-immunoprecipitation assays using complement opsonins as the primary targets or the pull down assays with labeled complement opsonins used as bait for the prey ligands that are of parasite, modified host or native host origin. Protein complexes contained in eluted samples could be visualized by the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or the native PAGE. Finally identification of novel complement binding proteins would necessitate protein band isolation from the polyacrylamide gel followed by enzymatic treatment of the isolated proteins, such as by tryptic digestion, and mass spectrometric identification of digested peptides. Alternatively for less stable receptor ligand interactions, the far-western blot analysis can be performed with samples of the merozoite or the infected erythrocyte lysates containing the unknown prey proteins separated by the SDS-PAGE or the native PAGE followed by membrane transfer. Post transfer, the blocked membranes can then be probed with a known bait proteins, such as complement components prior to further mass spectrometric analysis.

The totality of this research may need to be extended to all other stages of the malaria lifecycle as well as how the parasite and host are affected during co-infections with other pathogens. Recently, elevated levels of IgM antibodies directed against Gal $\alpha$ 1-3Gal $\beta$ 1-4GlcNAc-R ( $\alpha$ -gal) have been reported to be protective against malaria transmission in individuals from malaria endemic regions (298). Yilmaz et al. reported

that the human gut pathobiont  $\alpha$ -gal expressing *E. coli* O86:B7 induced anti- $\alpha$ -gal Abs in mice which blocked sporozoite migration from the skin to the liver. Furthermore, the  $\alpha$ -gal vaccination conferred sterile immunity in mice against subsequent natural infection (298). Even though the bacterial driven immune responses are potentially protective to the sporozoite stages, children with *Plasmodium falciparum* malaria are at higher risk of invasive bacterial infections and increased mortality (306). Surprisingly, individuals may acquire immunity faster to schistosomiasis by producing more anti-schistosome IgE and IgG3 if they are infected with malaria (307). A negative interaction between the two parasites was observed when infection with schistosomiasis offered greater protection against acquisition of *Plasmodium* infection (308). On the other hand, infection with HIV increases malaria susceptibility, while malaria infection increased the risk of HIV disease progression (309). Additional clinical and animal co-infection studies should focus not just on the interplay between the pathogens but the cumulative effects on the host's immune response.

### **Potential Applications:**

A comprehensive understanding of complement activation and exploitation of erythrocyte invasion and growth by the parasite for its own benefit will pave the way for more efficacious anti-malarial treatments. Complement inhibitors such as the C3 inhibitor Compstatin and/or the monoclonal anti-C5 antibody Eculizumab may play a significant role as supplemental treatments in combating severe malaria. Reduction in availability of C3 could partially limit erythrocyte invasion and hence curb kinetics of

parasitemia during acute infections, potentially providing enough time for antimalarials to take effect. Specific mitigation, by these therapies, of excessive C5a activation would limit the systemic inflammation, which is possibly a prominent contributing factor in malaria specific mortality. We must be mindful of the critical role complement plays in the host defense against pathogens, in the modulation of the adaptive immune system and in the clearance of apoptotic cells. Extreme caution is needed in reduction of complement activity especially since complement depletion increases the susceptibility to infections by bacteria such as *Haemophilus influenzae* and *Neisseria* (310,311). Future vaccine designs may benefit from adjuvant formulations that influence specificity, glycosylation and isotype of antibody responses, and potentially limiting the generation of complement fixing isotypes like IgG3 in the immune responses (312). Antibody responses induced by swine-origin influenza virus H1N1 vaccine containing oil-in-water adjuvant MF59 were increased in both avidity and the diversity of antibody epitope repertoire compared to unadjuvanted vaccine, as determined by surface plasmon resonance and genome fragment phage display libraries (313). In individuals vaccinated with influenza A anti-vaccine IgG demonstrated increased levels of galactosylation and sialylation upon active immunization while there was no change in the glycosylation of total IgG (314). Furthermore, level of IgG Fc sialylation increased during the vaccination time course (314). Antibody effector function, due to sialylation, may differ depending on when the pathogen encounters the antibodies post vaccination. Antibody isotype response in animals immunized with copolymer L141 generated a predominantly non-complement fixing IgG1 antibody response and smaller quantities of complement fixing IgG2a and IgG2b antibodies (315). In contrast, immunization with

LPS as an adjuvant favored the production of IgG2a and IgG2b isotypes (315). Also, alum preferentially augments a T helper 2 response that consecutively drives B cells to produce IgG1 and IgE antibodies (316-318). The diminished transmission of sporozoites, as a result of IgM mediated complement inhibition, may also be applicable to the reduction of blood stage parasitemia due to individual's specific microbiota (298). The modulation of intestinal microbiota through dietary intake of probiotics may prove beneficial in combating drug resistant parasite strains.

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## **Appendix A: Cobra Venom Factor Invasion Enhancement and Inhibition of Erythrocytes by *Plasmodium falciparum***

### **Introduction:**

Cobra venom factor (CVF) is a complement-activating, structural, and functional C3 analog found in *Naja naja* venom (319,320). Similar to C3b, CVF binds to factor B, forming a CVF:fB complex which is subsequently cleaved by factor D, producing fluid phase CVF:fBb convertase, capable of cleaving C3 and C5, that is partially analogous to host derived C3b:fBb alternative complement pathway convertase (321,322). The C3b:fBb convertase exhibits a spontaneous decay dissociation into its subunits with a half-life of 1.5 min, while the CVF:fBb convertase has a half-life of 7 hours (323,324). Unlike C3b:fBb, CVF:fBb is resistant to factor H mediated convertase decay and factor I proteolytic inactivation (325-328). Without regulatory restraint, and greater stability, CVF:fBb convertase is able to deplete complement through continuous activation of C3 and C5. Use of CVF is a common safe method to deplete laboratory animals (329,330).

Previous studies examining the role of complement in controlling parasitemia produced mixed results. Initial findings detected no difference in parasitemia in rhesus monkeys upon depletion of the alternative complement pathway and complement factors downstream of C3 with CVF in both pre-immune and immune animals (122). These results were substantiated in CVF-treated and untreated *P. chabaudi* infected mice that were similarly protected by passive immunization with hyperimmune serum (239). Alternatively, infection progressed more rapidly, resulted in higher parasitemia

and increased mortality rate, in *P. berghei* infected rats treated with CVF (125). We initially observed, *in vitro*, antibody mediated complement enhancement of erythrocyte invasion by *P.falciparum* (see figure 3.1-3.4). Our previous results also demonstrate antibody mediated enhancement of parasitemia in *P. berghei* infected mice that were passively immunized with purified total antibodies from anti-*P. berghei* hyperimmune serum (see figure 3.10-3.11). We thus postulated that complement depletion of mice with CVF would reduce complement mediated enhancement.

## **Results and Discussion:**

Our initial aim was to determine the effects of complement depletion with CVF on endpoint parasitemia in *P. berghei* infected mice. Two different CVF treatment regimens were tested to determine the duration of complement depletion. Mice were injected IP with 3 µg per mouse daily of CVF (Complement Technology, Inc., Tyler, Texas) on day -3, -2 prior to and on the day of infection (CVF Pre) or injected with CVF on the day prior to infection and every other day post infection (CVF EOD). Endpoint parasitemia was assessed on day 6 post infection. Elevated parasitemia was present in mice from both CVF injection regimens (Figure A.1). In addition, we intended to determine the effects of complement depletion with CVF on anti-*P. berghei* antibody mediated enhancement in *P. berghei* infected mice. To our surprise, 750 µg of anti-*P. berghei* antibody per mouse in the presence of CVF treatments synergistically enhanced endpoint parasitemia (Figure A.2). This may have been due to the inherent properties of complement depletion thought complement activation by CVF. Whereby, facilitation of complement activation in the presence of anti-*P.berghei* antibodies promoted optimal

opsonization of merozoites for invasion of erythrocytes leading to elevated parasitemia in mice.

To establish if enhancement of *P.berghei* parasitemia in mice is translatable to *P. falciparum* invasion enhancement of erythrocytes *in vitro*, parasite invasion and growth were monitored in the presence of varying concentrations of CVF in the presence or absence of complement activity. CVF inhibited erythrocyte invasion and growth in a dose dependent and complement independent manner (Figure A.3.A). There was a noticeable change in the morphology of the erythrocyte population by FSC/SSC dot plot in CVF treated compared to untreated erythrocytes (Figure A.3.B). Giemsa stained thin blood smears revealed changes in the erythrocyte shape from normal, biconcave discocyte to echinocyte in CVF treated erythrocytes (data not shown).

We postulated that the inhibitory effects of CVF on *P. falciparum* invasion of erythrocytes was due to the morphological changes in the erythrocyte membranes thereby reducing merozoites ability to bind to and/or invade CVF treated erythrocytes. Erythrocytes were incubated in FS or HIS and in the presence or the absence of 5 µg/ml of CVF overnight prior to growth assay setup in FS or HIS serum to determine if CVF damages erythrocytes thereby obstructing *P. falciparum* invasion. Surprisingly, erythrocytes pretreated with CVF were preferentially invaded and were receptive to parasite growth (Figure A.4.A and A.4.B). Giemsa stained thin blood smears of overnight CVF treated erythrocytes revealed changes in the erythrocyte morphology from the normal biconcave discocyte to the reversible but deformed echinocyte the following day. The erythrocyte morphology returned back to the normal, biconcave discocyte form after removal of CVF prior to growth assay setup (data not shown).



These results indicate that CVF exerts dually opposing functions on the erythrocyte invasion and/or growth by *P. falciparum*. Firstly, in the presence of CVF parasite invasion was inhibited, potentially due to the damage to the erythrocytes and/or merozoites by CVF binding to the cell membranes. Based on the morphological changes in the erythrocyte membrane, from a discocyte to a echinocyte forms, the damage may be lipid membrane specific hence in the presence of CVF the reduction in erythrocyte invasion by *P. falciparum* was not due to the alterations in the erythrocyte membrane but due to the disruption of the merozoite membrane. Also CVF may function as a calcium ionophore elevating intracellular calcium at the expense of both potassium and hydrogen ion loss (331). Both calcium and potassium play a major role as signals in merozoites for the secretion of microneme and rhoptry proteins during the invasion of erythrocytes (33). Or CVF may act as an anionic amphipath which tend to produce echinocytes (332). Future experiments will involve addition of CVF at different stages of the parasites intracellular development for different durations of time and test the effect on invasion. It is possible that CVF may also have inhibitory activity against the ring and/or the late trophozoite stages and that would translate into drastic reduction in erythrocyte invasion by merozoites. Merozoite egress and attachment assays may also be performed in the presence of CVF and cytochalasin D to determine if CVF blocks merozoite attachment to erythrocytes.

Secondly, it is puzzling why CVF treated erythrocytes were preferentially invaded by merozoites. It is possible that CVF treatment of the erythrocytes imparts some age specific phenotype on the erythrocytes, such as increasing the level of deformability that is reminiscent of the younger erythrocytes (333). While *P. falciparum* is able to invade

erythrocytes of all ages, some species of *Plasmodium*, such as *P. vivax* and *P. ovale* preferentially invade the reticulocytes. Further understanding of these differences may hint at the mechanism of CVF action on the erythrocyte physiology (334). Erythrocyte invasion is in part controlled by signaling through the erythrocyte  $\beta$ 2-adrenergic receptors which stimulated the production of cyclic adenosine 3',5'-monophosphate (cAMP) which consecutively increased the erythrocyte membrane deformability (335,336). Agonists that stimulate cAMP production in the erythrocytes led to an increase in malarial infection (335). In addition, as the merozoite facilitates the invasion process into the erythrocyte, the erythrocyte undergoes a rapid echinocytosis, this has been postulated to be due to the erythrocytic cytoskeletal changes attributable to the merozoite contact induced localized influx of calcium ions (337). Increased surface area as a result of deformation of the erythrocyte membrane may assist binding and reorientation of the merozoites (9,337). Future research may focus on the potential interaction of CVF with the erythrocyte  $\beta$ 2-adrenergic receptors. This potential novel role of CVF as a  $\beta$ 2-adrenergic receptor agonist should be of no surprise considering that much like adrenalins ability to raise blood flow, CVF may facilitate the dissemination of venom through out the host (338).

## Figure Legends:

Figure A.1: Cobra venom factor enhances endpoint parasitemia. Mice were injected IP with 3  $\mu\text{g}$  (per 200  $\mu\text{l}$  injection) daily of CVF on day -3, -2 prior to and on the day of infection (CVF Pre) or injected with CVF on the day prior to infection and every other day post infection (CVF EOD). Mock control mice were injected with 200  $\mu\text{l}$  PBS. Endpoint parasitemia was assessed by tail vein blood sample six days post infection, stained with Hoechst 33342 for flow cytometric acquisition. Error bars represent standard deviations.

Figure A.2: Cobra venom factor and anti-*P. berghei* antibodies synergistically enhance endpoint parasitemia. Mice were injected IP with 3  $\mu\text{g}$  (per 200  $\mu\text{l}$  injection) of CVF on the day prior to infection and every other day post infection. Wild-type (C57BL/6) mice were infected with *P.berghei* ANKA ( $1.5 \times 10^7$  pRBC/100  $\mu\text{l}$ ) and injected with anti-*P. berghei* antibodies or control antibodies at 750  $\mu\text{g}$  (per 100  $\mu\text{l}$  injection). Control mice were injected with 200  $\mu\text{l}$  or 300  $\mu\text{l}$  PBS depending on the control group. Tail vein blood sample six days post infection was stained with Hoechst 33342 for flow cytometric acquisition. Error bars represent standard deviations.

Figure A.3: Cobra venom factor inhibits endpoint parasitemia in a dose-dependent manner. A) Invasion and growth of *P. falciparum* strain 7G8 was tested in the presence of 1, 5, 10,  $\mu\text{g/ml}$  CVF or PBS (Mock) in the presence of 10% fresh serum or 30 min HIS. Error bars represent standard deviations. B) Flow cytometry plots show the total gated erythrocyte population, identified by SSC vs FSC, with perturbation of morphology

due to 1, 5, 10,  $\mu\text{g/ml}$  CVF treatment compared to normal (Mock) CVF untreated erythrocytes.

Figure A.4: Cobra venom factor treated erythrocytes are preferentially invaded. Erythrocytes were pretreated with 5  $\mu\text{g/ml}$  CVF or PBS (Mock) overnight in the presence of A) 10% fresh serum or B) 10% 30 min HIS. Prior to assay setup erythrocytes were washed to remove CVF. Invasion and growth of *P. falciparum* strain 7G8 was tested in the presence or absence of 10% fresh serum or 30 min HIS. Error bars represent standard deviations

## Figures:

Figure A.1

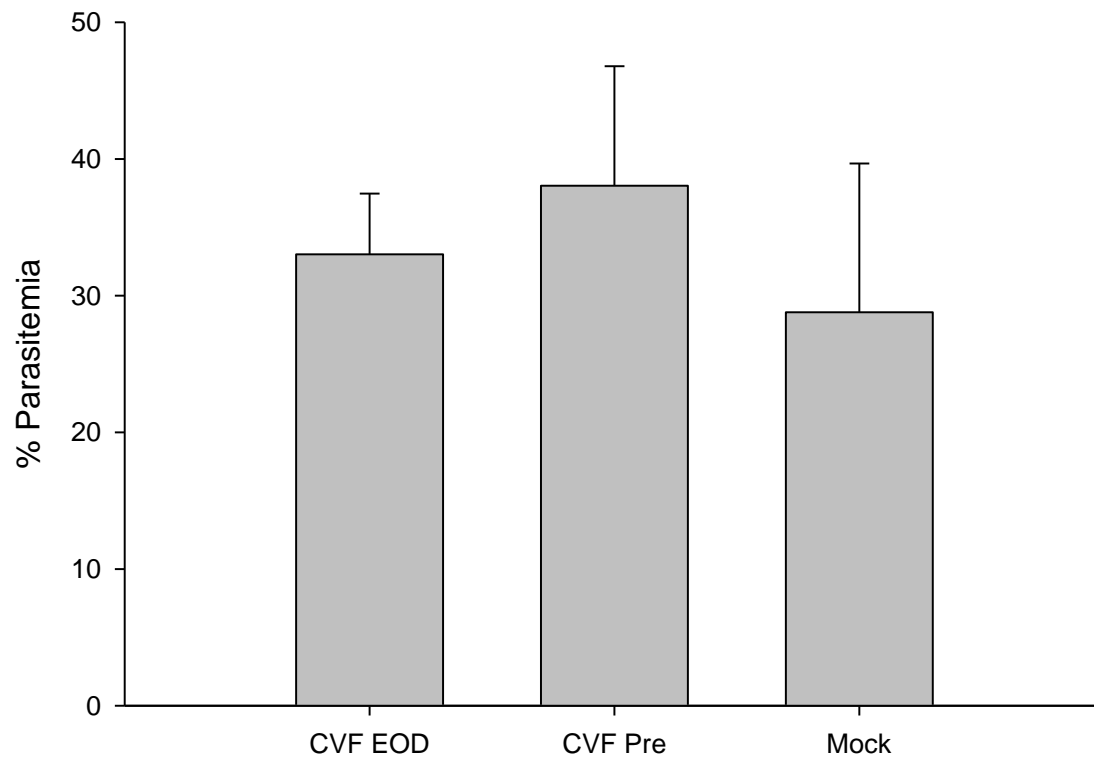


Figure A.2

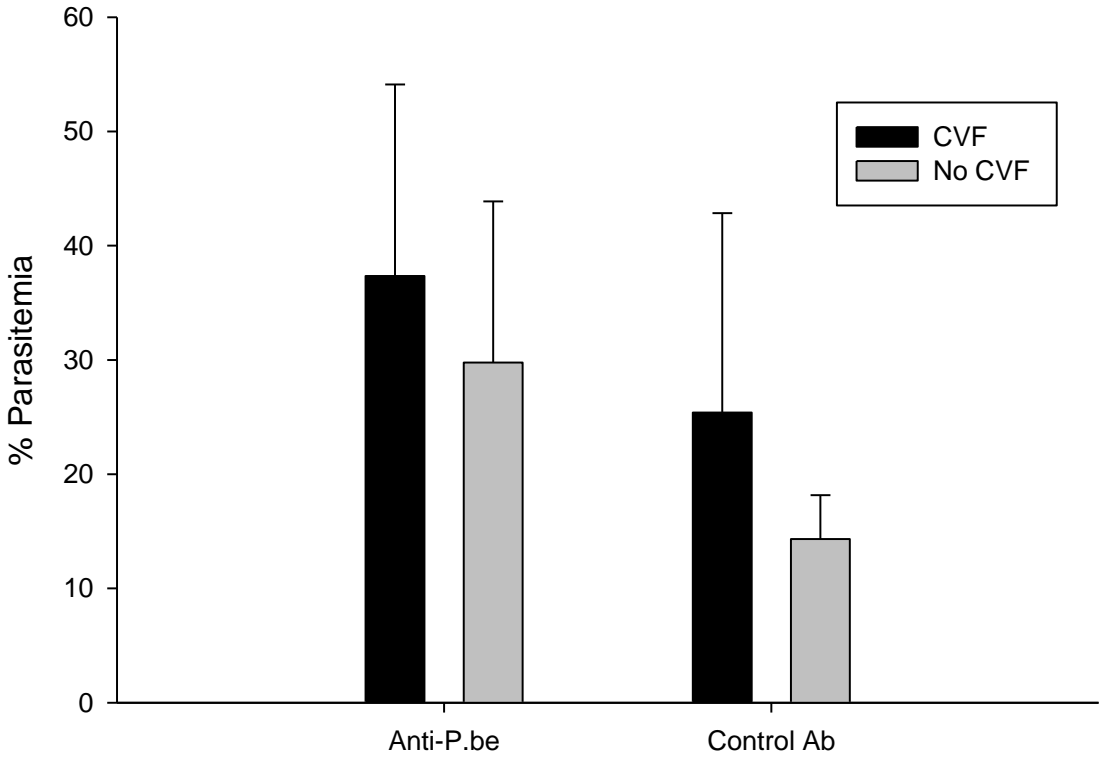
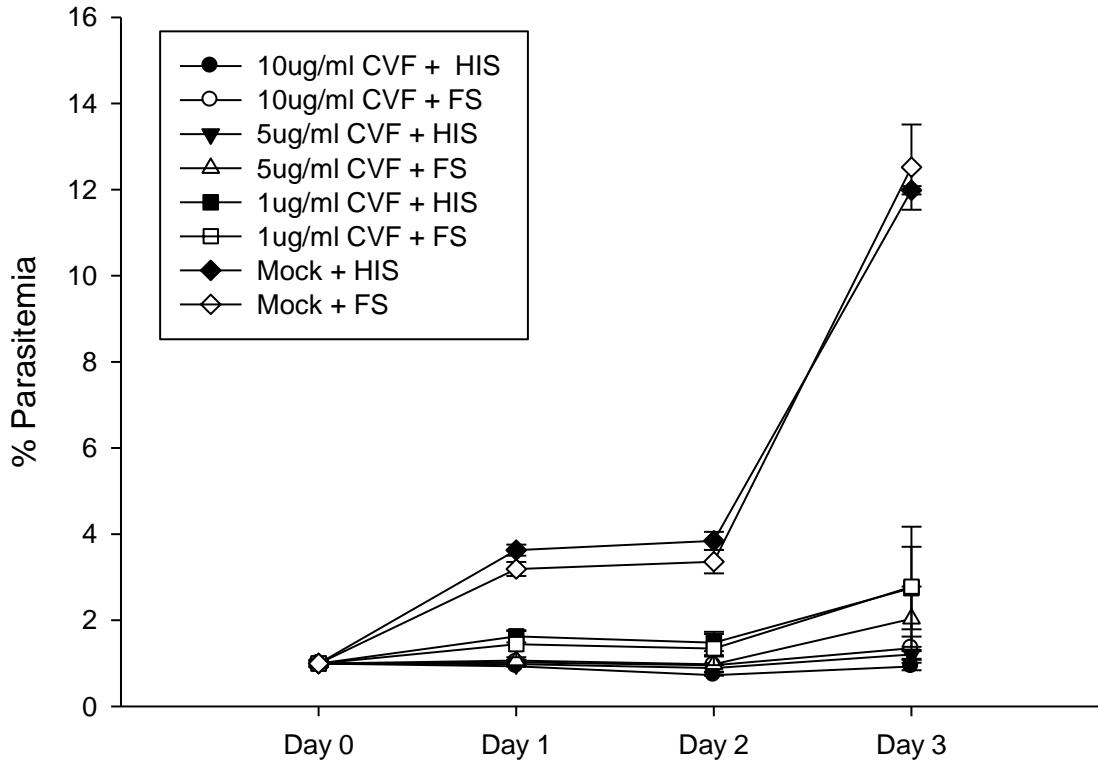


Figure A.3

A



B

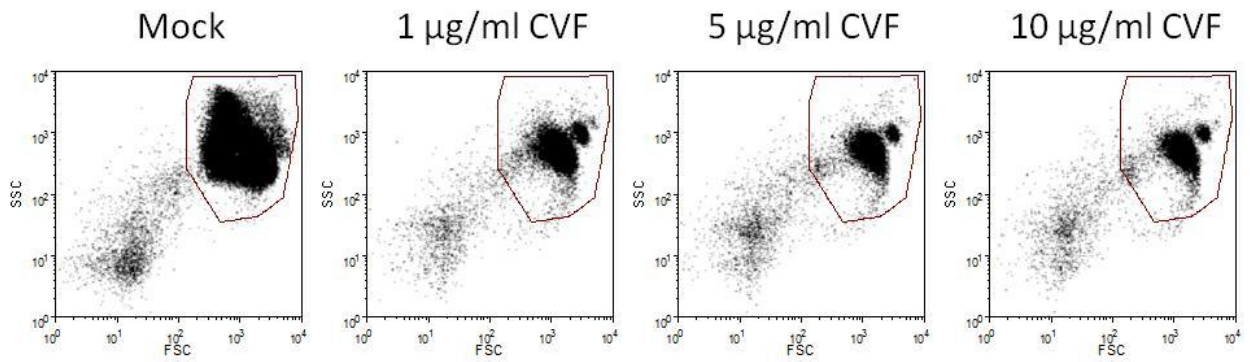
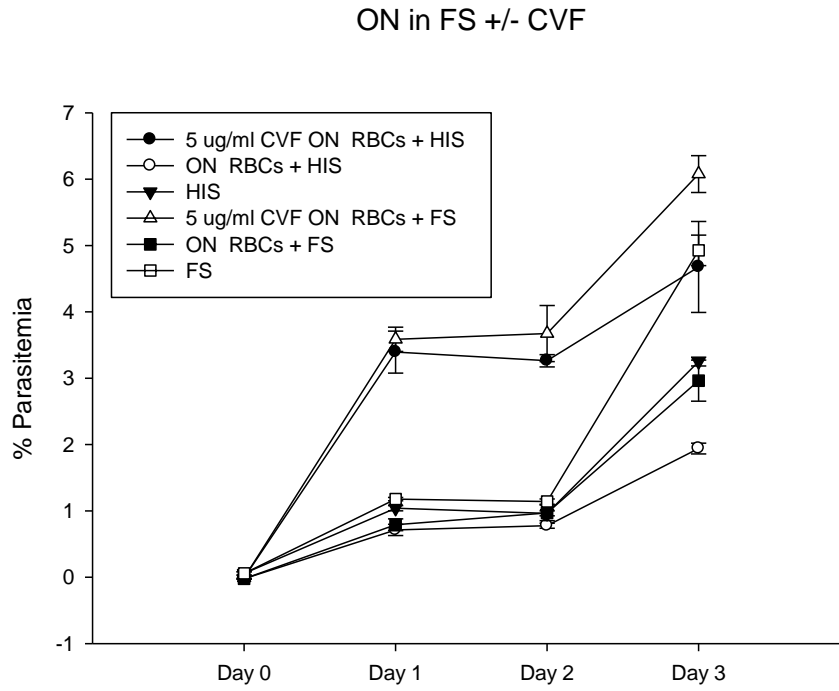
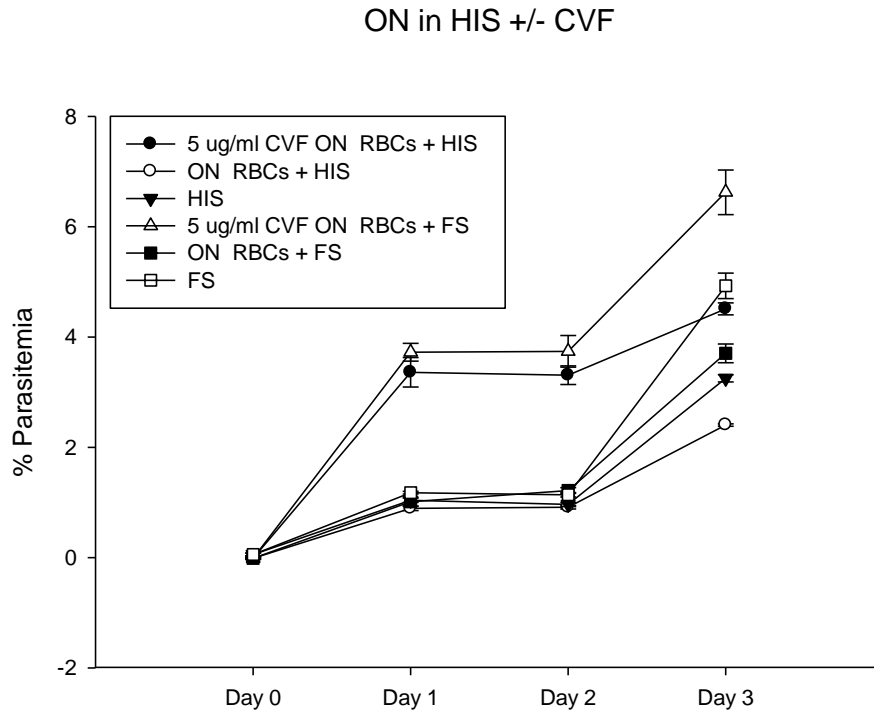


Figure A.4

A



B





## **Appendix B: The Enhancing and The Inhibitory Effects of Purified Antibodies From Individuals Living in Malaria Endemic Regions**

### **Introduction:**

Development of naturally acquired immunity to *P. falciparum* is lengthy and is govern by transmission intensity. In areas of high malaria transmission infants and young children are at a greater risk of sever malaria but acquire protective immunity faster, and by early childhood and into adulthood suffer less severe disease (153,339). In areas of low malaria transmission, immunity develops slowly and all age groups are susceptible to severe disease (340,341). Antibody mediated natural immune response to *P. falciparum* merozoites has an inhibitory role on erythrocyte invasion and intraerythrocytic growth. Prospective longitudinal studies identified an increase in prevalence and concentration of antibodies with age against merozoite targets (59-61). Purified total gamma immunoglobulin from immune adults passively transferred to malaria infected children resulted in drastic reduction in parasitemia and amelioration of clinical symptoms (43). The role of complement in the presence of naturally acquired anti-merozoite antibodies has not been extensively studied.

Our initial findings expose a potential anti-merozoite antibody mediated complement enhancement of erythrocyte invasion (see Chapter 3). A recent study, contrary to our results, observed an age dependent antibody mediated complement inhibition of erythrocyte invasion in the presence of 50% serum with total purified antibodies from individuals living in endemic areas (59). Incongruity of our results with Boyle *et al.* may be due to their use of significantly higher antibody concentrations,

which were not disclosed, as well as their use of 50% serum concentrations as the source of complement. In addition, alternative erythrocyte invasion strategies, with potential alteration of merozoite invasion fitness due to the parasite separation protocols may have also contributed to conflicting results between our studies. Our goal was to determine the role complement plays in the presence of purified antibodies from children living in malaria endemic areas with different yearly transmission intensities.

## **Results and Discussion:**

To test the potential impact of complement on the erythrocyte invasion by *P. falciparum* in the presence of naturally acquired antibodies, total IgG was purified from serum samples obtained from children living in two different malaria endemic areas of Ghana. Malaria transmission is all year round in Kintampo, located in the central forest-savannah transitional zone of Ghana (342). Seasonal transmission predominates in Navrongo, which is located in the savannah belt near the northern border with Burkina Faso (343). Antibodies from malaria exposed or non-exposed individuals, depending on transmission intensity, were evaluated in the presence of 10% C3/C4 inactivated or rescued serum. No erythrocyte invasion inhibition was observed for the majority of the antibody samples independent of transmission intensity which was similar to control antibodies from malaria naïve donors. Rescue of complement activity produced a negligible, statistically non-significant, increase in inhibition in all groups. A few antibody samples possessed complement independent invasion enhancing and inhibitory activity, specifically in the Kintampo cohort. Antibody samples with enhancing

properties may have been due to autoagglutination because of blood group incompatibilities.

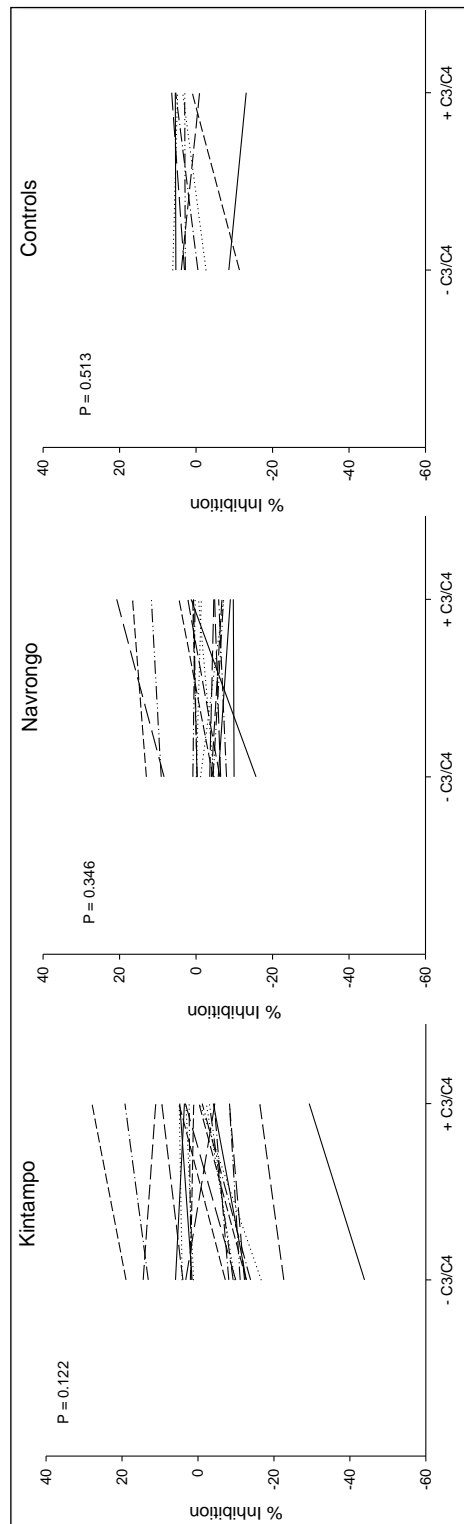
Further research may involve assessment of the antibody's effects on erythrocyte invasion in dose response assays in the context of complement which may generate similar results that were obtained from our mouse studies (see Figure 3.10). Antibody samples, from Kintampo and Navrongo, with significant invasion enhancing and inhibitory activity will be tested by *P. falciparum* protein microarray (*P. falciparum* Reactive Antigen Microarrays, Antigen Discovery Inc., Irvine, California) antibody profiling in combination with isotyping to determine if correlation exists between antibodies antigenic specificity, isotype prevalence and effect on erythrocyte invasion. Regular serum sampling of these individuals to adulthood would enable longitudinal studies of the antibody repertoire acquisition and isotype fluctuation as well as the determination of the invasion/growth inhibition of these antibodies in the presence of complement.

## Figure Legends:

Figure B.1: Antibodies from children living in malaria endemic areas lack erythrocyte invasion inhibitory activity independent of complement presence. Invasion of *P. falciparum* strain 3D7 was tested in the presence of purified total IgG (2.5 mg/ml) from individuals from Kintampo ( N = 24), Navrongo (N = 19), or malaria naïve controls (N = 9) in the presence of C3/C4-inactivated or C3/C4-reconstituted serum. P is by paired t-test.

# Figures:

Figure B.1



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## EDUCATION

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## PUBLICATIONS

- **Biryukov S.**, & Stoute, J. A. (2014). Complement activation in malaria: friend or foe?. *Trends in molecular medicine*, 20(5), 293-301.
- Vinayaka, A. C., Sadashiva, M. P., Wu, X., **Biryukov, S. S.**, Stoute, J. A., Rangappa, K. S., & Gowda, D. C. (2014). Facile synthesis of antimalarial 1, 2-disubstituted 4-quinolones from 1, 3-bisaryl-monothio-1, 3-diketones. *Organic & biomolecular chemistry*, 12(42), 8555-8561.
- **Biryukov S**, Angov E, Landmesser ME, Spring MD, Ockenhouse C, and Stoute J. Complement and Antibody-mediated Enhancement of Red Cell Invasion and Growth of *Plasmodium falciparum*. *Submitted*

## PRESENTATIONS

- **Biryukov S.** "Inactivation of Myogenesis Using COX2 Inhibitors through the Analysis of MyoD and MEF2 Expression." Research Seminar Series (Master's Thesis Presentation), University of New Haven. November 2006.
- **Biryukov S**, and Stoute J. "Complement and Antibody Enhancement of Red Cell Invasion by *Plasmodium falciparum*." Molecular Parasitology Meeting XXII. Woods Hall, MA. September 2011. Poster Presentation.
- **Biryukov S**, and Stoute J. "Antibody and complement-mediated enhancement of red cell invasion by *Plasmodium falciparum*." Graduate Student Research forum 2014. March 2014. Poster Presentation.
- **Biryukov S**, and Stoute J. "Antibody and complement-mediated enhancement of red cell invasion by *Plasmodium falciparum*." The American Association of Immunologists Annual Meeting. Pittsburgh, PA. May 2014. Poster Presentation.
- **Biryukov S**, and Stoute J. "Antibody and complement-mediated enhancement of red cell invasion by *Plasmodium falciparum*." Departments of Medicine and Public Health Sciences Research Day. May 2014. Poster Presentation.
- **Biryukov S**, Angov E, Landmesser ME, Spring MD, Ockenhouse C, and Stoute J. "Complement and Antibody-mediated Enhancement of Red Blood Cell Invasion by *Plasmodium falciparum*." American Society of Tropical Medicine and Hygiene 64<sup>th</sup>. Philadelphia, PA. October 2015. Oral Presentation.