Anti-PfGBP130 Antibodies Inhibit *P. falciparum* Growth *In vitro*

By

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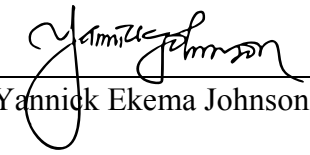

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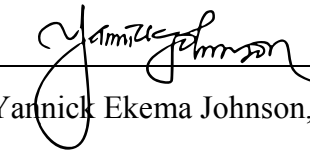

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Despite decades of effort to develop an effective vaccine against *Plasmodium falciparum*, malaria remains one of the deadliest diseases in modern times, claiming about 608,000 lives in 2022.[1] To discover new vaccine targets, we used our whole proteome differential screening method[27] to identify parasite genes that encode parasite proteins that are uniquely recognized by antibodies expressed by resistant children, but not recognized by antibodies expressed by susceptible children. We identified PfGBP130, a 130 kDa protein expressed on the surface of merozoites. To determine if antibodies to PfGBP130 inhibited parasite growth, we immunized BALB/cJ mice with lipid-encapsulated mRNA encoding PfGBP130. In *in vitro* growth inhibition assays, we determined that polyclonal anti-PfGBP130 antibodies inhibited *Plasmodium falciparum* growth by 86%. Thus, PfGBP130 is a potential vaccine target for future malaria vaccine development.

Chapter 1: Introduction

1.1 Malaria infection distribution

Malaria remains one of the deadliest diseases afflicting the modern world. It is endemic in over 90 countries, with over 300 million individuals infected worldwide and about 608,000 people dying from the infection in 2022.[1] Africa continues to bear the most burden of malaria infections. In 2022, Africa accounted for 94% of all malaria cases and 95% of deaths, with children under the age of 5 years old accounting for about 78% of all deaths due to malaria infections in Africa.[2] In the United States, approximately 2,000 individuals were diagnosed with malaria in 2017 and about 0.3% died as a result of the infection. In addition, more than 86% of patients diagnosed with malaria acquired the infection from Africa.[52] However, for the first time in 20 years, in 2023, there were 9 locally transmitted cases in the United States—seven cases in Florida and one case each in Texas and Maryland.[3] The case in Maryland is believed to be due to *Plasmodium falciparum*, while the cases in Texas and Florida are believed to be due to infection by *Plasmodium vivax*.[3]

1.2 *Plasmodium* species

Malaria is caused by protozoan parasite belonging to the genus *Plasmodium*. Six species of *Plasmodium* are known to cause malaria: *P. falciparum, P. knowlesi*, *P. vivax*, *P. malariae*, *P. ovale curtisi*, and *P. ovale wallikeri*. *P. vivax* has been documented to cause severe malaria—defined as vital organ involvement including shock, pulmonary edema, kidney impairment or anemia—predominantly in Asia and South America, including Thailand, Brazil, Indonesia and India.[4] *P. knowlesi*, *P. malariae*, *ovale curtisi*, and *P. ovale wallikeri* are less prevalent worldwide. *P. malariae*, *ovale curtisi*, and *P. ovale wallikeri* are found in Asia and West Africa and *P. knowlesi* is endemic in Southeast Asia.[5] *P. falciparum* is the deadliest of the *Plasmodium*

species and is responsible for severe disease pathology and the majority of deaths due to malaria infections in sub-Saharan Africa and Africa as a whole. Thus, *P. falciparum* has been the focus of research and therapeutic development efforts.

1.3 *Plasmodium falciparum* lifecycle

The lifecycle of *P. falciparum* is complex and is composed of mosquito and human stages (**Fig 1**). A human is infected with malaria when a female anopheles mosquito bites the person and injects *Plasmodium* sporozoite while feeding on human blood. The sporozoites migrate, via the circulatory system, to the liver, where they multiply in the hepatocytes, and merozoites are released into the bloodstream. Merozoites subsequently invade red blood cells and undergo three developmental stages—ring, trophozoite, and schizont —and then are released from the red blood cells as merozoites and invade other red blood cells. Clinical manifestations of malaria infection occur during the erythrocytic cycle of the *Plasmodium* life cycle. A small percentage of blood-stage parasites develop into sexual gametocytes (male or female) that migrates to the cutaneous microvasculature and are taken up by mosquitoes during a blood meal.[7] In the mosquito stage, female and male gametocytes are transformed into gametes, fusing and forming zygotes. Zygotes mature into ookinetes and subsequently differentiate into oocysts and lodges on the midgut's outer surface of the mosquito.[8] The oocysts have four haploid genomes from which thousands of haploid sporozoites are generated. These sporozoites invade the mosquito's salivary glands and is injected into human cutaneous microvasculature as the mosquito feeds on human blood.[9]

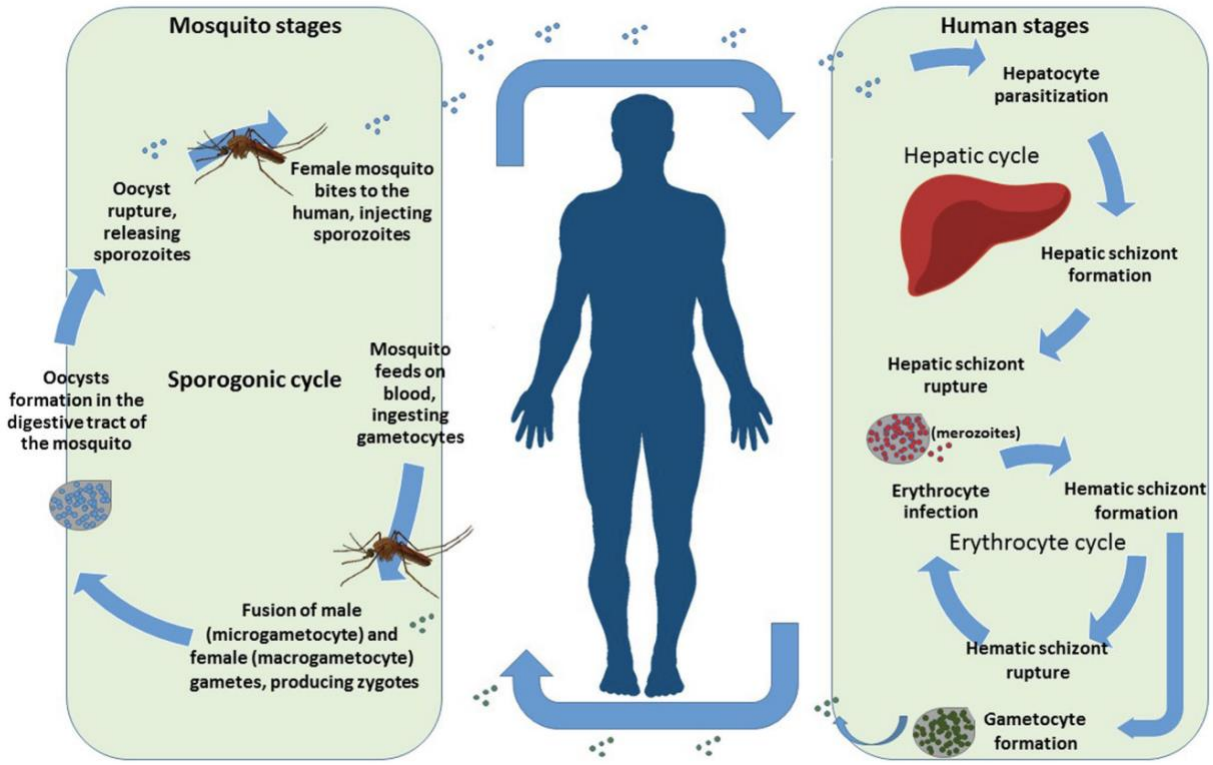

Diagram adapted from Garrido-Cardenas et al. 2019.[6]

Figure 1. *Plasmodium falciparum* Lifecycle. A female anopheles mosquito bites a human host and injects sporozoites. The sporozoites migrate to the liver where they multiply and are subsequently released into the bloodstream as merozoites which invade erythrocytes. A small percentage of blood-stage parasites develop into sexual gametocytes (male or female) that migrates to the cutaneous microvasculature and are taken up by mosquitoes during a blood meal. These gametocytes form sporozoites in mosquitoes and are injected into another human host.

1.4 Anti-malarial medication and vaccines

Uncomplicated *P. falciparum* malaria is treated with artemisinin-based combination therapy (ACT).[10] This replaced the failing chloroquine and sulfadoxine-pyrimethamine monotherapy.[11] ACT is a combination of a fast-acting artemisinin derivative with a longer-acting partner drug. The fast-acting artemisinin component rapidly eliminates malaria parasites in the blood, both in the asexual and sexual stages of the parasite lifecycle. Whereas the longer-acting partner drug provides a period of post-treatment prophylaxis, preventing the development of resistance to the artemisinin derivative and clearing the remaining parasite in the blood.[10] Patients with severe malaria are recommended to be administered with intravenous or intramuscular artesunate for at least 24 hours and until they can tolerate oral medication.[12] Additional important antimalarial medications are primaquine and tafenoquine. Primaquine is recommended to be taken daily for 14 days and tafenoquine is taken as a single-dose gametocytocide, thus preventing malaria transmission to mosquitoes.[11] However, they are underused because of concerns over haemolytic toxicity in glucose-6-phosphate dehydrogenase (G6PD) deficient patients.[13]

After four decades of research and clinic trials, the malaria vaccine RTS, S was recommended by the WHO for use among children living in malaria endemic areas.[14] RTS, S vaccine is a hepatitis B virus-like particle that contains the genes of the repeat domain and C-terminal region of the *P. falciparum* circumsporozoite protein (CSP)—a sporozoite-specific antigen that is expressed on the surface of sporozoites of different *Plasmodium* species—fused with hepatitis B surface antigen (HBsAg) and additional unfused HBsAg.[15,16,19] Vaccination with RTS, S induces the production of antibodies against CSP expressed on sporozoites which immobilize the sporozoites, thus preventing hepatocyte infection and therefore interfering with one of the key

steps in *Plasmodium falciparum* infection in the human body. From an extensive phase 3 study conducted in various *Plasmodium falciparum* endemic regions in sub-Saharan Africa, RTS, S was effective against clinical malaria in children aged 5 to 17 years, reducing severe disease by 75%, 18% and 9% a few weeks after immunization, 1 year and 5 years, respectively. The participants were vaccinated at months 0, 1, 2 and a booster dose at month 20.[17,18,40] In addition, in children aged 6 to 12 weeks, the efficacy was estimated to start at 63% and diminished to 11% and 3% after 1 and 5 years, respectively.[14] However, RTS, S has no effect on the infectivity of gametocytes and thus mosquitoes can be infected by individuals carrying the parasite, and transmission remains unchanged in malaria endemic areas.

In April 2023, the Ghanaian Food and Drug Administration licensed a second malaria vaccine, R21/Matrix M, for use in 5-36 months old children.[20] And in October 2023, the WHO officially recommended R21/Matrix M for use in high transmission areas.[21] Similar to RTS, S, R21/Matrix M contains a fusion of hepatitis B surface antigen (HBsAg) and CSP genes in a hepatitis B virus-like particle but without the additional unfused HBsAg (as in RTS, S). A preliminary review of a phase 3 trial of R21/Matrix M suggested that R21/Matrix M was effective against clinical malaria through 18 months post-dose in areas of highly seasonal transmission and through 12 months in areas with low/moderate transmission.[24]

There has been considerable effort invested in developing a vaccine effective in halting *Plasmodium falciparum* transmission in endemic areas. The major focus has been on pre-erythrocyte vaccines as they block *Plasmodium falciparum* lifecycle when the parasites are fewest in the body and prevent major illness associated with malaria infections, with CSP being

the major target as it is known to elicit protective B and T-cell responses. Vaccines containing attenuated whole-sporozoite capable of invading hepatocytes but do not progress to the blood stage infection are currently being evaluated in Mali and Kenya.[22, 23] In addition, vaccine candidates based on merozoite surface protein 1 (MSP1) constructs and formulations have been studied in monkeys and are currently being evaluated in volunteers undergoing experimental vaccination.[25]

1.5 PfGB130 discovery and lipid-encapsulated mRNA delivery platform

In the fight against *Plasmodium falciparum*, it is believed that a vaccine capable of inducing protective immunity against all stages of malaria infection is necessary to have the highest impact on morbidity and transmission.[14] Therefore, there is definitely a need for new vaccine targets that can be used in tandem with current malaria vaccines on the market. In order to identify novel vaccine candidates for *Plasmodium falciparum* infection, a longitudinal cohort study in which 785 children were monitored for *P. falciparum* infections from birth to 3.5 years of age as part of the Mother Offspring Malaria Studies (MOMS) project was conducted.[41] From age 1.5 years to 3.5 years, routine blood samples were collected every 6 months. *P. falciparum* parasitemia was determined from Giemsa staining of blood smears from participating children; venous blood was collected, centrifuged and plasma stored at -80 ^{o}C. In order to determine the acquired differences in antibody repertoire that drive parasitemia levels, the participants were ordered by their mean parasite densities and individuals in the low and high extremes of the distribution were designated as "resistant" (n =12) and "susceptible" (n =14), respectively.[27] Subsequently, the plasma of participants who were highly resistant or highly susceptible to malaria infection were pooled and differential biopanning using a *P. falciparum* blood-stage

cDNA library constructed in the T7 bacteriophage was conducted.[27] Following three rounds of differential biopanning, PfGBP130 was identified as one of the targets of antibodies present in the plasma of children highly resistant to *P. falciparum* infection but absent in the plasma of children highly susceptible to *P. falciparum* infection. PfGBP130 encodes an ectodomain (PfGBP130-ecto, aa 89-824) which contains a charged 137 aa domain followed by 12 copies of a 50 aa repeat (**Fig 2**). From the differential screening, a clone of PfGBP130 was identified, PfGBP130-A (aa 111-374) which contains the majority of the charged N-terminal domain and three copies of the 50 aa repeat.

PfGBP130 has been proposed to have a role in merozoite invasion of erythrocytes[29, 30] and thus probing the ability of anti-PfGBP130 antibodies to limit *P. falciparum* growth *in vitro* was the next logical step. Based on the recent success of the mRNA delivery platform in SARS-CoV2 vaccines [28], we constructed lipid nanoparticle encapsulated mRNA encoding PfGBP130-A and PfGBP130-ecto and vaccinated BALB/cJ mice with these constructs. The goal was to investigate if mice could express polyclonal anti-PfGBP130 antibodies and if these antibodies could inhibit *P. falciparum* growth *in vitro*.

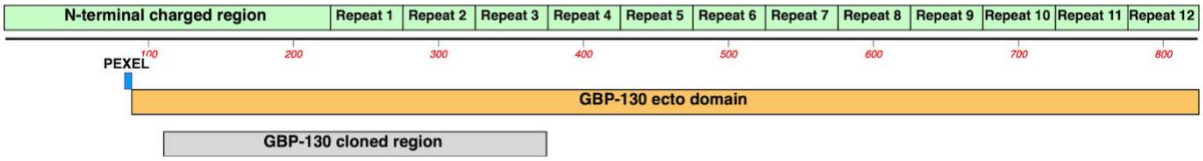

Figure 2. Domain structure of PfGBP130. PfGBP130 is a merozoite surface protein that is composed of a charged 137 aa domain followed by 12 copies of a 50 aa repeat (PfGBP130-ecto, aa 89-824). PfGBP130-A (aa 111-374)—GBP130 cloned region—contains the majority of the charged N-terminal domain and three copies of the 50 aa repeat.

Chapter 2: Methods

2.1 Parasite strain and culture

Plasmodium falciparum strain, 3D7, was purchased from MR4. The parasites were cultured *in vitro* using the method outlined by Trager and Jensen, with minor modifications.[31] 3D7 parasites were cultured in RPMI 1640 medium containing 5% human O+ red blood cells, 5% Albumax II (Invitrogen), 25 mm HEPES, 24 mm sodium bicarbonate and 10 μg/ml gentamycin at 37 ^{o}C with 5% CO_2, 1 % O_2 and 94% N_2.

2.2 Production of GBP130-A mRNA

mRNAs were produced using T7 RNA polymerase (Megascript, Ambion) on a linearized plasmid encoding codon-optimized PfGBP130-A or PfGBP130-ecto,[47,48] with each mRNA transcribed to contain 101 nucleotide-long ply(A) tails. Modified nucleoside-containing mRNA was generated using 1-methylpseudouridine-5-triphosphate (TriLink). The RNAs were capped using the m7G capping kit with 2'-O-methyltransferase (ScriptCap, CellScript) and purified by Fast Protein Liquid Chromatography (FPLC) (Akta Purifier, GE Healthcare).[49] All mRNAs were analyzed by denaturing or native agarose gel electrophoresis and frozen at -20 ^{o}C.

2.3 Lipid nanoparticles encapsulation of mRNA

Using a self-assembly process in which an aqueous solution of mRNA at pH=4 is rapidly mixed with a solution of lipids dissolved in ethanol, FPLC-purified m1ψ-containing mRNA were encapsulated in LNPs.[42] The LNPs in this study contained ionizable cationic lipid (proprietary of Acuitas)/phosphatidylcholine/cholesterol/PEG-lipid (50:10:38.5:1.5 mol/mol) and were

encapsulated at an RNA to total lipid ratio of ~0.05 (wt/wt), with a diameter of ~80 nm. The lipid encapsulated mRNA formulations were stored at -80 °C and at a concentration of ~1 μg/μL.

2.4 Anti-PfGBP130 antisera

BALB/cJ mice were immunized intradermally with 10 μg lipid encapsulated mRNA encoding either GBP130-A or GBP130-ecto every three weeks, for a total of three doses. After immunization, blood was collected by cardiac puncture and centrifuged, with the resulting serum heat-inactivated (56 °C for 30 minutes) and stored at -80 °C.

2.5 Growth inhibition assays

Growth inhibition assays[32] were conducted with control mice sera and sera obtained from mice vaccinated with either GBP130-A or GBP130-ecto. *P. falciparum* parasites were maintained between 0.1% to 3% parasitemia and 2% hematocrit. The parasites were synchronized to the ring stage using 5% sorbitol[33] over three successive cycles before incubation with mice sera. Parasites were incubated with mouse sera at 2% hematocrit and 0.2%-0.5% parasitemia up to a final volume of 100 μL in 96-well plates. Each experimental condition was conducted in triplicates, and the incubation period lasted 72 hours; the parasite culture was eventually taken down for parasitemia analysis via flow cytometry.

2.6 Flow cytometry analysis

Parasite culture was stained with 4 nM Hoechst dye—10 μL of Hoechst dye for every 1 μL of parasite culture— and incubated at room temperature for 30 minutes, washed three times with PBS to remove unbound reagent and analyzed on the flow cytometer (CytoFLEX, Beckman

Coulter). The parasitemia of the control and treatment groups were obtained and graphed using GraphPad PRISM.

2.7 Anti-PfGB130 antibody assays

Anti-PfGBP130 antibody levels were measured using previously published methods.[34] 100 μg of rPfGB130-A, rPfGB130-ecto or rGARP-ecto (negative control) was conjugated to 1.25 x 10^7 microspheres (Luminex). From this, two sets of beads were generated: the first set containing only rPfGB130-A, and the second set containing rPfGB130-ecto and rGARP-ecto. The beads were reconstituted with deionized water and Assay Buffer E (0.1% BSA, 0.05% Tween-20, 0.05% sodium azide and PBS pH 7.4)—ABE. Reconstituted beads were incubated at room temperature for one hour with mouse serum beginning at 1:1000 dilution and followed by a two-fold serial dilution series with ABE in 96-well filter bottom plates. The beads were washed three times with ABE, vacuum filtrated and incubated at room temperature for one hour with anti-mouse IGg (BD Biosciences) conjugated to phycoerythrin diluted 1:500 in ABE. The beads were washed three more times with ABE, vacuum filtrated, resuspended in ABE and analyzed on BioPlex 200 multi-analyte analyzer. The titer was calculated as reciprocal of last dilution that generated a fluorescence value greater than 1000 units.

Chapter 3: Results

Due to the recent success of lipid encapsulated (LNP) mRNA as a vaccine delivery platform in the Pfizer-BioNTech COVID-19 vaccine, we hypothesized that vaccination with PfGBP130 mRNA would lead to the generation for anti-PfGBP130 antibodies in our model organism. Thus, we vaccinated mice with lipid encapsulated PfGBP130-A or PfGBP130-ecto mRNA and collected their serum for analysis. Using a bead-based antibody detection assay previously developed[38], the lipid encapsulated PfGBP130 mRNA constructs were found to be highly immunogenic in mice, with both generating a titer value of 1:512,000 (**Fig 3 (**A) and (B)). Because anti-PfGBP130 antibodies were identified in children who developed resistance to severe malaria[27], we speculated that PfGBP130 will be implicated in inhibiting malaria parasite growth in-vitro. To assess this, we conducted growth inhibition assays with both PfGBP130-A and PfGBP130-ecto mRNA sera. Anti-PfGBP130 antibodies generated by LNP PfGBP130-A mRNA and LNP PfGBP130-ecto mRNA inhibited parasite growth by 86% (**Fig 3** (C)).

In other studies, mice were vaccinated with *E. coli*-expressed PfGBP130-A formulated in TiterMax® adjuvant or as a codon optimized DNA vaccine construct.[43] The recombinant protein and DNA-based vaccine constructs were found to be immunogenic, generating a titer value of 1:512,000 and 1:8,000, respectively. However, in parasite growth inhibition assays, anti-sera from mice immunized with PfGBP130 recombinant protein had no impact on parasite growth for 3 strains (3D7, INDO and NIH 710), and anti-sera from mice immunized with the DNA construct inhibited parasite growth by 79-89%. The inability of GBP130-A protein vaccinated mice anti-sera to inhibit parasite growth may be due to misfolded *E. coli*-expressed protein. In addition, GBP130-A has not been successfully expressed in mammalian expression system.[43]

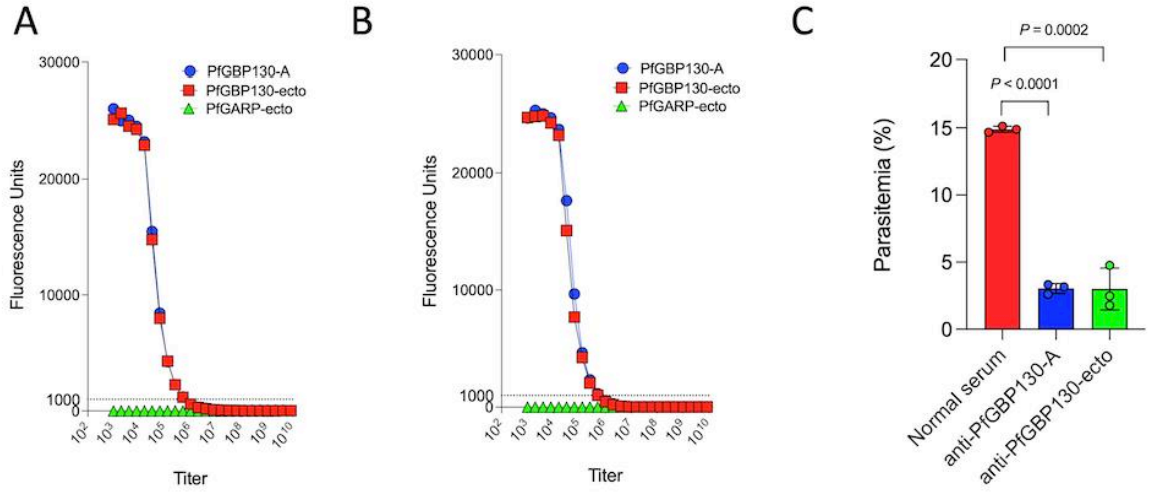

Figure 3: Vaccination with lipid-encapsulated PfGBP130 mRNA generated polyclonal antibodies that inhibit parasite growth. A) Vaccination with lipid encapsulated mRNA encoding PfGBP130-A or (**B**) PfGBP130-ecto generates high titer (1:512,000) antibodies against PfGBP130-A and PfGBP130-ecto coated beads with no reactivity against PfGARP-ecto (negative control) coated beads. **C)** Anti-PfGBP130-A and anti-PfGBP130-ecto antibodies inhibit parasite growth by 86% *in vitro*. Ring-stage parasite were incubated with either anti-PfGBP130-A or anti-PfGBP130-ecto mouse sera at 1:10 dilution. Negative control was pre-immune mouse sera. For **A** and **B,** the circles and squares represent the mean of triplicates**.** For **C,** the bars represent the mean and the error bars represent SEM. Also, results in **C** represent three independent experiments.

Chapter 4: Discussion and Conclusion

P. falciparum exists intracellular for most of its life cycle in human hosts—except as sporozoites or merozoites in which the parasite exists extracellular for a short time. Sporozoites invade the liver and merozoites invade erythrocytes. In their intracellular form, malaria parasites are generally shielded from the host immune system. Thus, developing vaccines or therapeutics that target sporozoites and/or merozoites is paramount in mounting an effective defense against malaria parasite growth in human hosts.

PfGBP130 is a 130kDa merozoite surface protein that binds to glycophorins on erythrocytes[36] and is implicated in the invasion of RBC by merozoites. Previous studies have shown that [^{125}I] glycophorins, when added to isolated merozoites, bind to saturated sites on the merozoite surface.[45] In addition, purified glycophorin A, when added to isolated merozoites, block erythrocyte invasion, and erythrocytes treated with the Fab' fragment of anti- Glycophorin A antibodies are resistant to merozoite invasion.[44] Also, human erythrocytes deficient in glycophorin A or B have been shown to be resistant to merozoite invasion.[37, 39] This body of evidence suggests the existence of merozoite surface proteins that mediate attachment to glycophorins on erythrocytes. Furthermore, PfGBP130 (130 kDa) and PfGBP155 (155 KDa), both proteins on merozoite surface coat, have been shown to bind specifically to glycophorin coupled to an acrylamide matrix.[36] Also, antibodies to PfGBP130 significantly inhibited the binding of PfGBP130 to the glycophorin-matrix, thus suggesting that PfGBP130 interaction with glycophorins on erythrocytes is important for merozoite binding and invasion of erythrocytes. In addition, because PfGBP130 binding to glycophorins on erythrocytes is important for the parasite's survival in human hosts, it follows that PfGBP130 would be conserved. The primary

sequence of a cDNA clone has shown that PfGBP130 was composed of highly conserved 50 aa repeats which were retained in different geographical isolates of *P. falciparum*.[46]

Squirrel monkeys (*Saimiri sciureus*) immunized with protein fractions (100 KDa and 70 KDa) obtained from crude extract prepared from mature parasite schizont stages (cultured on human erythrocytes) conferred significant protection against *P. falciparum* challenge infection.[50] The sequence identity of the antigens was not determined and cannot be definitively interpreted as being PfGBP130. In addition, *Aotus* monkeys were immunized with a GBP130 recombinant protein containing three of 50 aa repeats—GBP130 gene encodes 12 tandemly repeated 50 aa repeat units— expressed in *E. coli*. Sera from the immunized monkeys did not inhibit parasite growth *in vitro*, and no protection was observed after intravenous challenge with *P. falciparum*-infected erythrocytes.[51] This result suggests that potential protective epitopes in PfGBP130 are not located in the repeated aa region but in the N-terminal charged domain (aa 1-225) which overlaps with the region identified by our whole proteome differential screening (aa 111-374)—denoted as GBP130-A.[27]

A whole proteome differential screening (WPDS) method was previously conducted to identify the subset of parasite antigens recognized by antibodies expressed by children resistant to severe malaria but not expressed by children susceptible to severe malaria.[27] PfGBP130-A (aa 111-374), a partial sequence of PfGBP130-ecto (aa 89-824), was one of the antigens identified by this study. Anti-PfGBP130 antibodies could have contributed to conferring protection against severe malaria by blocking merozoite invasion of erythrocytes and preventing the blood stage parasite life cycle which is responsible for the clinical manifestation of severe malaria. Thus, by

inhibiting the blood stage parasite lifecycle, the host is less likely to suffer from severe malaria. In our study, we vaccinated mice with lipid encapsulated PfGBP130 mRNA. Our bead-based antibody detection assay showed that mice significantly expressed anti-PfGBP130 antibodies, and via Growth Inhibition Assay, we showed that PfGBP130 anti-serum inhibited parasite growth by 86%. From these results, it can be deduced that anti-PfGBP130 antibodies generated in mice inhibit malaria parasite growth *in vitro*. This is in line with the reasoning that anti-PfGBP130 antibodies bind and block PfGBP130, a merozoite surface protein, from binding to glycophorins on erythrocytes and, in the process, prevent merozoite invasion and inhibit parasite growth.

Malaria infections remains one of the world deadliest diseases, especially for children in sub-Saharan Africa. In addition, resistance to current medication continues to grow and spread across Africa and Asia. Thus, there is a dire need for the development of novel antimalarials and vaccines. PfGBP130 is a promising vaccine target candidate that can mediate the blocking of erythrocytes invasion by merozoites and inhibit malaria parasite growth. In conjunction with other vaccines that target other aspects of the parasite's lifecycle, vaccines that target PfGBP130 have the potential to confer protection against malaria.

Based on the promising results that show that anti-PfGBP130 antibodies inhibit parasite growth *in vitro*, the next step will be to investigate if anti-PfGBP130 antibodies confer protection in non-human primates. In addition, future research efforts can be concentrated on investigating and developing a vaccine construct based on PfGBP130 in tandem with other vaccine targets with the goal of developing a vaccine that produce robust protection against *P. falciparum* infections.

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