Immunological Characterization of Malaria Glutamic Acid-Rich Protein (GARP)

A thesis submitted by

Maima Molvina Kaiser

In partial fulfillment of the requirements for the degree of

Master of Science

In

Pharmacology and Drug Development

Tufts University

Sackler School of Graduate Biomedical Sciences

May 2018

Adviser: Athar H. Chishti, Ph.D.

Abstract

Malaria is the most predominant parasitic infection and continues to have a significant global impact on the health and well-being of hundreds of millions of people annually [1]. *Plasmodium falciparum* Glutamic acid-rich protein(Pf-GARP) is a parasite-derived protein secreted during the blood stage of malaria. From the studies with field isolates, it appears that Pf-GARP is very immunogenic, and it is abundant in children from endemic areas [2, 3]. A key tool for the control, elimination, or even possible eradication of malaria, in addition to antimalarial drugs and vector control, is an effective vaccine [4]. My research focuses on the immunological characterization of Pf-GARP. These studies include the characterization of a new monoclonal antibody for Pf-GARP, mapping of the epitope of Pf-GARP by utilizing synthetic peptides, and initial localization of Pf-GARP using the GM7 monoclonal antibody (mAb) by immunofluorescence microscopy. Finally, to validate the proof of concept of its clinical significance, we developed an Enzyme-Linked Immunosorbent Assay (ELISA) to detect antibodies against Pf-GARP in the human malaria plasma. Our screens utilized recombinant His-Pf-GARP protein as well as defined synthetic peptides of Pf-GARP to detect and quantify antibodies in human plasma from malaria-endemic areas in Africa. In summary, we have characterized a new mAb, termed GM7, that is specific for Pf-GARP protein and determined a peptide (M1P6) that is the target of the binding of antibody (Ab) against the Pf-GARP protein. These findings are likely to have implications for screening malaria patients that are seropositive for Pf-GARP, and its functional significance in the pathogenesis of cerebral and pregnancy-associated malaria.

Dedication

This study is dedicated to my late father, Mr. Molley Goodrich Kaiser, who always guided me to seek higher education that is the key to success, and with education you can build bridges and climb mountains. Thank you for always believing in my dreams, and your continued support helped me so much during my stay away from home (Liberia).

Daddy, you have always been my role model, not only supporting us but also supporting children that were not your own with your unwavering belief that they too deserve education and always seeing more in them. Thank you for all your hard work that had led you to become the person you were always meant to be. I pray to God that he grants me the opportunity to give back to my family (Liberia), in any way possible but especially by giving less deserving children the necessary education and opportunities that will enable them to make a positive contribution to their families and community. Finally, thank you for continuing to watch over me from above, my sweet guardian angel. I love you and missed you very much.

Acknowledgement

Firstly, I would like to say thank you to my thesis adviser, Dr. Athar H. Chishti, for welcoming me into your lab, giving me the opportunity to work on various projects, and overall for being a terrific mentor. You have created a supportive team and lab environment, where students can learn and explore their ideas. I am so grateful for the opportunity, support, and guidance you have provided me during my years at Tufts.

Secondly, I would like to say thank you to Dr. Toshihiko Hanada, for being the backbone of my lab work, and for making my lab experience at Tufts very meaningful. Coming into this lab, I was nervous about what I could offer, but you have made my lab experience worthwhile. You have giving me the independence and confidence toward my lab work, and today I am leaving Tufts knowing that you have created the foundation toward my research experience.

Next, I would like to thank the Chishti lab for creating a positive and productive working environment. You all have made my lab experience so wonderful. All of you have played a key role during the course of my lab work. Thank you so much Dr. Joslyn E. Mills for sharing your lab knowledge with various protocol and always being available when I needed some advice. I would like to thank Dr. Haifa Almukadi for sharing your lab knowledge and experience during my time in the Chishti lab. I am grateful to Donna-Marie Mironchuk for keeping the lab organized and for all the technical support you have provided to me. Farha J. Mithila, thank you for always staying positive, sharing your lab knowledge along with being a good listener. Christopher Schwake, thank you for sharing

your lab knowledge, being a good lab mate, as well as always being a good listener and open minded. Daniel I. Fritz, thank you for sharing your lab knowledge and always being available to help. Finally, Shreeya S. Hegde, thank you for being a great class and lab mate, sharing your lab knowledge, and for being an amazing friend.

I would like to extend my gratitude to Dr. Miguel Stadecker for agreeing to serve as second reader of my thesis. I am very grateful, and appreciate your time, expertise, and input.

I would like to thank my classmates in the Pharmacology and Drug Development Program for being such an amazing support system during the past few years. I would not have made it without you all. I would like to acknowledge the Pharmacology and Drug Development Program in the Sackler School of Graduate Biomedical Sciences at Tufts University for giving me this opportunity and creating my research foundation.

Lastly, I would like to thank my wonderful family, for always supporting my academic goals. You all mean the world to me, and I am truly grateful to have you all as my supportive team.

Table of Contents

List of Figures

List of Table

List of Copyrighted Materials

Klein, E.Y., *Antimalarial drug resistance: a review of the biology and strategies to delay emergence and spread.* Int J Antimicrob Agents, 2013. **41**(4): p. 311-7.

List of Abbreviations

AIDS- Acquired Immunodeficiency Syndrome
CDC- Central of Disease Control and Prevention
DNA- Deoxyribonucleic Acid
ECL- Enhanced Chemiluminescent
E. *coli*- Escherichia coli
ELISA- Enzyme-Linked Immunosorbent Assay
GARP- Glutamic acid-rich protein
GARP-L- Glutamic acid-rich protein- Long
GARP-M- Glutamic acid-rich protein- Medium
GARP-S- Glutamic acid-rich protein- Short
GARPM1- Glutamic acid-rich protein Medium1
GARPM2- Glutamic acid-rich protein Medium2
GARPM7- Glutamic acid-rich protein Medium7
GM7- Glutamic acid-rich protein Medium7
GM7-1- Glutamic acid-rich protein Medium7-1
GST-GARPM- Glutathione S – transferase Glutamic acid-rich protein Medium
HCl- Hydrochloric Acid
His-GARP-Histidine - Glutamic acid-rich protein
His-GARPM- Histidine - Glutamic acid-rich protein Medium
HIV- Human Immunodeficiency Virus
HRP- Horseradish Peroxidase
IFA- Immunofluorescence Assay
IB/WB-Immuno Blot (Western Blot)
iRBC- Infected Red Blood Cells
KDa- KiloDalton
mAb- monoclonal Antibody
M1P1- Medium 1 Peptide 1
M1P2- Medium 1 Peptide 2
M1P4- Medium 1 Peptide 4
M1P6- Medium 1 Peptide 6
M1E4R- Medium 1 E- Peptide 4 Repeats
M1G4R- Medium 1 G- Peptide 4 Repeats
M2K4- Medium 2 K-Repeat 4
M2K5- Medium 2 K-Repeat 5
MSP1- *Plasmodium falciparum* Merozoite Surface Protein (MSP1)
NIH- National Institutes of Health
OD- Optimal Density
PBS- Phosphate Buffered Saline
PBST- Phosphate Buffered Saline with Tween 20
Pf-GARP- *Plasmodium falciparum* Glutamic acid-rich protein
Pf-GARPM- *Plasmodium falciparum* Glutamic acid-rich protein Medium
PfSPP- *Plasmodium falciparum* Signal Peptide Peptidase
Pf3D7 strain- *Plasmodium falciparum* 3D7 strain
Pf- *Plasmodium falciparum*
P. falciparum- *Plasmodium falciparum*

P. knowlesi- *Plasmodium knowlesi*
P. malaria- *Plasmodium malaria*
P. ovale- *Plasmodium ovale*
P. vivax- *Plasmodium vivax*
Pf-EMP1- *Plasmodium falciparum* erythrocyte membrane protein 1
RT- Room Temperature
RBC- Red Blood Cells
RH5- *Plasmodium falciparum* Reticulocyte-binding protein homologue 5 (RH5)
SDS-PAGE-GEL- Sodium Dodecyl Sulfate – Polyacrylamide gel Electrophoresis – Gel
TRX- Thioredoxin
TRX-GARPM- Thioredoxin - Glutamic acid-rich protein Medium
TRX-GARPM1- Thioredoxin - Glutamic acid-rich protein Medium1
TRX-GARPM2- Thioredoxin - Glutamic acid-rich protein Medium2
TMB- Tetramethylbenzidine
WHO- World Health Organization
X-ray- Electromagnetic Radiation
3%BSA/PBS-3% Bovine Serum Albumin in Phosphate Buffered Saline
3%BSA/PBS-3% Bovine Serum Albumin in Phosphate Buffered Saline with Tween 20

Chapter 1: Introduction

1.1 *Plasmodium falciparum*:

Malaria is caused by the single celled parasite *Plasmodium* and the lifecycle involves two animal hosts. *Plasmodium* is transmitted by the female Anopheles mosquito (vector), which transmits the parasite into human host [5]. Approximately 3.2 billion people remain at risk of malaria, and it is endemic over 106 countries [1]. Among the human malaria parasites, *Plasmodium falciparum* causes the most frequent infection in young African children who have not yet developed natural immunity, and is a major concern for non-immune travelers in malaria-endemic areas [2]. Moreover, children under the age of five, pregnant women, and people with HIV/AIDS are also most at risk for severe illness and death [6]. Malaria is the most predominant parasitic infection and continues to have a significant global impact on the health and well-being of hundreds of millions of people annually [1].

Five *Plasmodium* species are known to cause disease in humans including *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae*, and *Plasmodium knowlesi* [7]. *Plasmodium falciparum* and *P. vivax* are the most prevalent, and represent a significant global health threat however, *P. falciparum* is the most dangerous causing the highest mortality worldwide [6]. *Plasmodium falciparum* causes the highest mortality rates worldwide, but *P. vivax* has a wider geographical distribution due to its ability to infect Anopheles mosquitoes at lower temperatures. *P. vivax* remains undetectable in the blood and can stay dormant in the liver for days to years [6].

The *Plasmodium* life cycle includes the pre-erythrocyte stage, erythrocyte stage, and the sexual stage. The pre-erythrocyte stage or the liver stage is when a mosquito infected with *Plasmodium* bites a human host, the parasite migrates to the liver as a sporozoite. In the liver, sporozoites undergo multiple rounds of asexual reproduction to produce merozoites. One sporozoite can asexually reproduce to form up to 40,000 merozoites, which exit hepatocytes to invade erythrocytes or red blood cells [5]. The large parasite load often poses a serious challenge to the immune system to control and eliminate infection.

The erythrocyte stage, also known as the blood stage, begins when the parasite enters the red blood cells. A merozoite infects an erythrocyte and begins asexual reproduction thus releasing hundreds of new merozoites for reinvasion. This is the stage that causes all the disease symptoms. The periodic fever and chills originate from the bursting of infected RBCs [5].

Finally, the sexual stage begins when the mosquito takes a blood meal and ingests sexual stages of malaria parasites from the human host. Some of the merozoites in RBCs asexually differentiate into sexual forms known as male and female gametocytes. The gametocytes then sexually reproduce in the mosquito gut, developing into gametes, and finally fusing as they move up the mosquito gut wall to become an oocyst. The oocyst grows, divides, and eventually bursts and produces thousands of haploid sporozoites, which will travel to the mosquito's salivary glands to be injected into the next host during the mosquito's blood meal [5].

Cerebral malaria is the most severe neurological complication of infection caused by *Plasmodium falciparum* [8]. It is a clinical syndrome characterized by coma and

inflammation often recognized by the presence of asexual immature ring stage of the parasite in peripheral blood smears [8]. The mortality is high, and surviving patients often sustain brain injury, which manifests with long-term neuro-cognitive impairment [6]. A key tool for the control, elimination, or possible eradication of malaria, in addition to antimalarial drugs and vector control, is the development of an effective vaccine [4]. One approach to identify essential vaccine antigens is to search for malaria parasite proteins that are recognized by antibodies in the plasma of chronically exposed individuals who remain resistant to infection, and are not recognized by antibodies in the plasma of susceptible individuals [9].

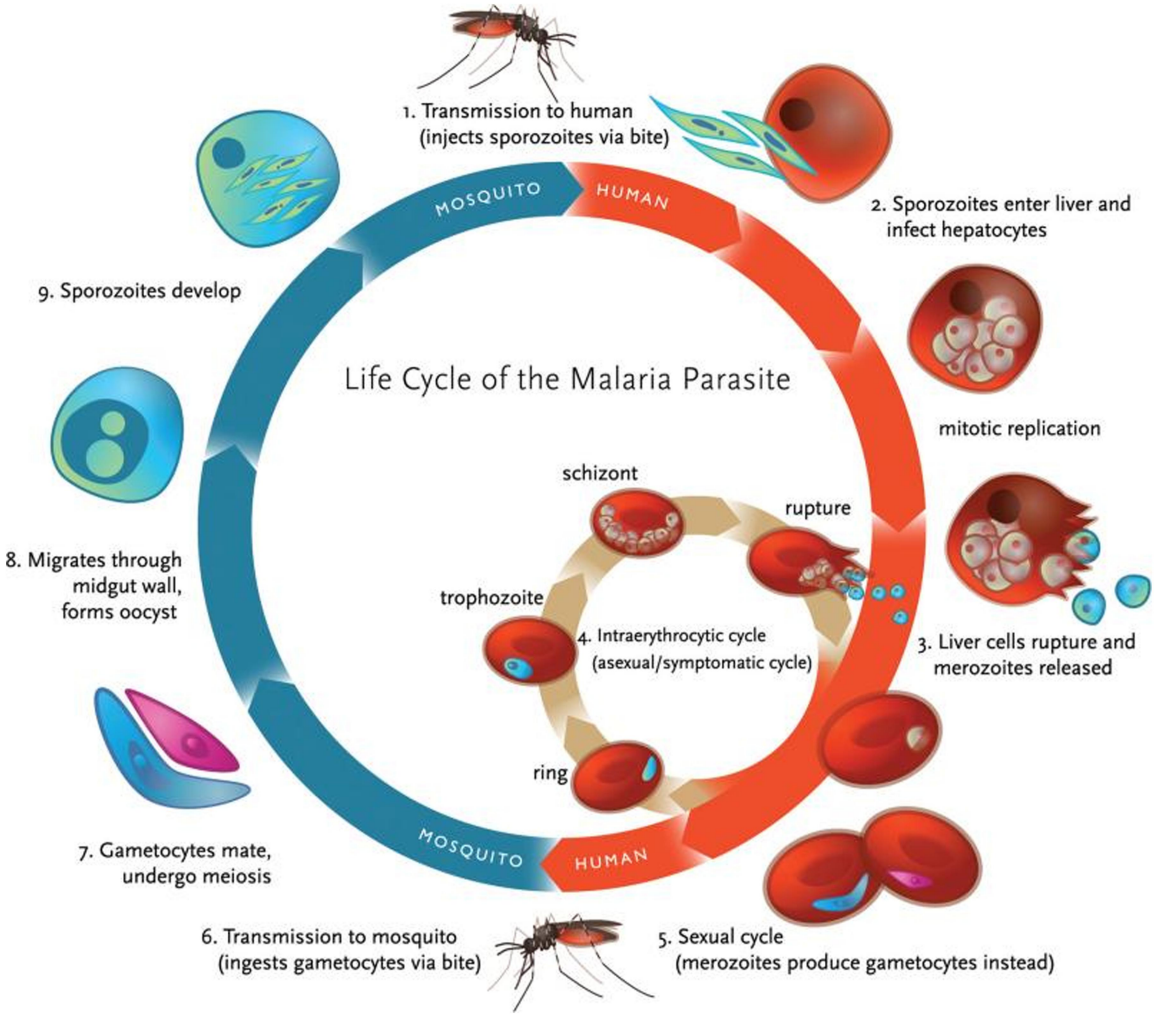

Figure 1.1 *Plasmodium falciparum* Life Cycle. Summary of the multiple stages of Malaria parasite life cycle. The three stages of *Plasmodium* life cycle are the pre-erythrocyte stage, erythrocyte stage, and the sexual stage. The pre-erythrocyte stage, which is also called the liver stage, is before the parasite infects human red blood cells. The erythrocyte stage, also is known as the blood stage, begins when the parasite infects red blood cells. The sexual stage begins when the mosquito ingests the parasite and sexual development takes place in the mosquito gut. Reprinted with permission from Klein, E.Y., *Antimalarial drug resistance: a review of the biology and strategies to delay emergence and spread.* Int J Antimicrob Agents, 2013. **41**(4): p. 311-7.

1.2 *Plasmodium falciparum* (GARP):

Plasmodium falciparum Glutamic acid-rich protein (Pf-GARP) is parasite-derived protein secreted during the blood stage of malaria. Glutamic acid-rich protein (GARP) has a predicted molecular weight of 80kDa [10] . Pf-GARP is a highly charged protein; it

contains 24% glutamic acid, 21% lysine, and 9% aspartic acid residues [3]. From the studies with field isolates, it appears that Pf-GARP is very immunogenic, and it is abundant in children from endemic areas [2, 3].

Parasite protein such as *Plasmodium falciparum* erythrocyte membrane protein (PfEMP1) facilitate adherence [11], an antigenically diverse protein family trafficked to the infected red cell surface [12-14]. An increasing number of proteins exported to the host erythrocyte in *P. falciparum* are necessary for export of *P. falciparum* specific PfEMP1 to the parasite-infected erythrocyte surface [15]. Using genetic knockdown, the authors identified exported proteins required for trafficking, display and function of the cyto-adherence protein PfEMP1, assembly of knobs and rigidification of the infected red cell, properties that are thought to be important in malaria pathogenesis [16]. Their screening revealed that disruption of Pf-GARP protein function leads to absent or greatly decreased knob structures with an abnormal distribution [16]. This finding suggests that Pf-GARP might play a key role and function of a cyto-adherence like protein.

Pf-GARP is composed of repetitive, low complexity, and intrinsically disordered sequences [3]. Pf-GARP contains three distinct lysine-rich repeat sequences presumably with a targeting function [3]. Pf-GARP contains an N-terminal signal sequence for targeting to the parasite endoplasmic reticulum, enabling the protein to be exported into the host erythrocyte and the C terminus of the protein contains an acidic stretch. [3]. Beyond the N-terminal signal sequence, Pf-GARP contains very few hydrophobic residues, suggesting that Pf-GARP doesn't contain stable folded domains [3].

Pf-GARP is effectively localized to the periphery of the infected erythrocytes [3]. Pf-GARP is highly charged due to the presence of three lysine-rich repeats, and

intrinsically disordered regions within Pf-GARP are sufficient to fold into targeting modules that localize to the erythrocyte periphery [3]. *P. falciparum* can target proteins to the periphery of the infected erythrocyte suggesting that such proteins perform key functions at the host-parasite interface [3].

The host erythrocyte undergoes drastic changes during the blood stage of the parasite life cycle [17-19]. Many proteins including Pf-GARP which is associated with erythrocyte contain tandem repeats [20, 21]. Some repeating sequences appear to be under immune selection [22], and many are highly immunogenic [23]; leading to the proposal that they may allow the parasite to evade host immune system by diverting B-cell responses toward non-protective epitopes [24]. Importantly, Pf-GARP is unique to *P. falciparum*, and is not expressed in other Plasmodium species, implying that this protein may play an important functional role in the severity of human malaria infection. Taking together, Pf-GARP is secreted during the blood stage of malaria which is essential for the parasite interaction and survival. Pf-GARP could be targeted for vaccine development.

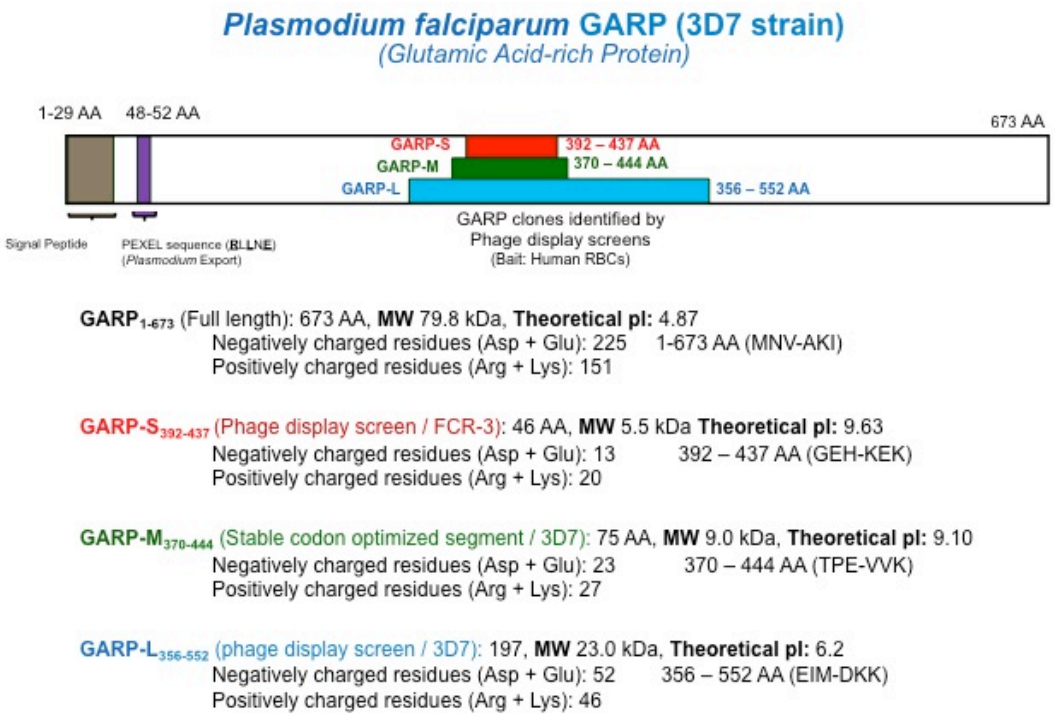

Figure 1. 2 *Plasmodium falciparum* GARP (3D7 Strain). Pf-GARP consists of 673 amino acid with a molecular weight of 80kDa. GARP-Short (GARP-S) is a 46-amino acid segment with a MW of 5.5kDa, GARP-Medium (GARP-M) is a 75-amino acid segment with a MW of 9.0kDa, and GARP-Long (GARP-L) is a 197-amino acid with a MW of 23kDa.

1.3 *Plasmodium falciparum* GARPM:

Plasmodium falciparum Glutamic acid-rich protein Medium (Pf-GARP-M) consists of 75 amino acids assembled into five amino acids repeat structure. Pf-GARP-M consists of two sets of repeats including Pf-GARP-M1 and Pf-GARP-M2. Pf-GARP-M1 and Pf-GARP-M2 were produced as recombinant proteins with a TRX (thioredoxin) tag of 12.9kDa (Figure 1.3).

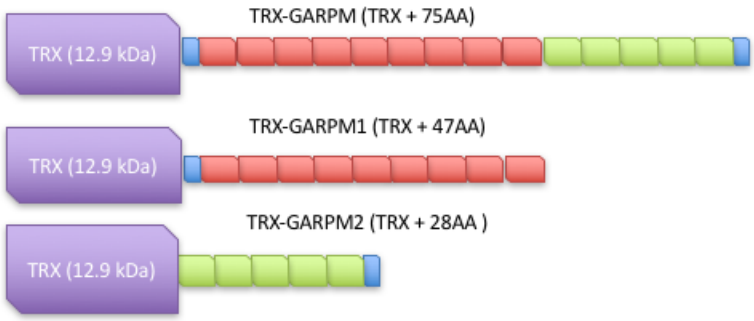

Figure 1. 3 *Plasmodium falciparum* GARP-M. Pf-GARP-M was expressed as a TRX-fusion protein where TRX tag contributes 12.9kDa and Pf-GARP-M consists of 75 amino acids. Two distinct repeats were grouped as Pf-GARP-M1 (47 amino acids, Orange) and Pf-GARP-M2 (28 amino acids, Green).

1.4 Study Aim:

The overall aim of my research was to investigate the immunological properties of Pf-GARP using a newly developed mAb and multiple synthetic peptides derived from Pf-GARP-M. The experimental focus was to map the epitope of Pf-GARP with synthetic peptides and localize Pf-GARP using GM7 mAb by immunocytochemistry. Finally, to validate the proof of concept, we developed an ELISA to screen human malaria plasma to detect antibodies against Pf-GARP in malaria patients from malaria endemic areas of Africa.

Chapter 2: Methods and Materials

2.1 Immunization of mice with GARPM

Six BALB/c female mice (8 – 12 weeks old) were immunized with recombinant Pf-GARPM protein. For the first immunization, 70μg of recombinant TRX (thioredoxin tag)-Pf-GARPM protein mixed in Freund's complete adjuvant was injected into each mouse by IP (intraperitoneal). Recombinant TRX-Pf-GARPM protein was expressed in *E. coli BL21* (DE3) and purified through Ni-beads affinity purification and Mono Q ion exchange column chromatography. To avoid excessive immune response against TRX, His-Pf-GARPM (without TRX tag) recombinant protein was used for the following boost injections. His-Pf-GARPM was expressed in *BL21(DE3)* and purified through Ni-beads and Mono-Q column. Three boost injections were given at three weeks' interval, with 50μg of His-Pf-GARPM mixed in Freund's incomplete adjuvant by IP. Test bleeds were collected 10 days after each boost, and their Ab titers were evaluated by ELISA using His-Pf-GARPM. Final (4^{th}) boost was given three weeks after the third boost to the best responding mouse, with His-Pf-GARPM in PBS, 30μg by IV and 30μg by IP. Three days after the final boost, the mouse was sacrificed, and its splenocytes were used for hybridoma production. The immunization steps were performed by Dr. Toshihiko Hanada in the Chishti lab.

2.2 Development of Monoclonal Antibody

This mAb was generated by injecting recombinant Pf-GARPM protein into BALB/c female mouse #4. One mouse (#4) showing the best immune response was selected for hybridoma fusion. Hybridoma clone (#7) was selected from 32 positive hybridoma clones. The hybridoma (#7) clone was further sub-cloned by limiting dilution.

Hybridoma clone #7 secreted an IgG3 monoclonal termed GM7-1 (for simplicity, we call it GM7 monoclonal). Final protein concentration was attained at 1.84 mg/ml in the buffer (0.02 M Potassium Phosphate, pH 7 .2 -7 .4, 0. 15 M Sodium Chloride). Since the Ab is of IgG3 class, it tends to precipitate in 4^{o}C. Warming up to 37^{o}C in water bath was necessary before use. The sub-cloning for the generation of GM7 was performed by Dr. Toshihiko Hanada in the Chishti lab.

2.3 Recombinant protein expression and purification in *E. coli*

His-Pf-GARPM, GST-Pf-GARPM, and TRX-Pf-GARPM recombinant proteins were induced and expressed in *E coli BL21(DE3)*. The recombinant proteins were purified through Ni^{2+} beads, were used for affinity purification followed by Mono Q ion exchanged chromatography. The proteins were dialyzed against PBS containing 10% glycerol and stored in -80°C freezer.

2.4 (Enzyme-Linked Immunosorbent Assay) ELISA

The streptavidin-coated as well as non-coated plates were purchased from Thermo Scientific. The ELISA plates were coated either with peptide or antigen of interest and incubated overnight at 4^{o}C. The plate was then washed with 1x PBST (Phosphate buffer saline Tween 20) twice for five minutes. The plate was then blocked with 3% BSA in a 10 mL PBST and incubated for 30 minutes at room temperature. The plate was then washed with PBST twice for five minutes. Primary dilution for the human plasma was (1/400) or 1/1000 for monoclonal antibody GM7. The sample was incubated for 90 minutes at room temperature. The plate was then washed with PBST twice for five minutes. Our negative controls included Phosphate Buffer Saline (PBS) and human

Babesia plasma (609598). Secondary dilution for the anti-human Horseradish Peroxidase (HRP) was 1/10,000 or anti-mouse was (1/5,000). The sample was incubated for 60 minutes at room temperature. The plate was then washed with PBST three times for five minutes. Sigma Life Science Tetramethylbenzidine (TMB) liquid substrate system for ELISA was used. After adding the appropriate volume of TMB, the sample was then incubated for five minutes and the blue color change was observed. 1.0 M of Hydrochloric acid (HCl) was used as a stopping solution. After adding the appropriate volume of 1 M HCl, the sample was then incubated for five minutes and the yellow color change was observed. Optimal Density (OD) was quantified using VERSAmax Microplate reader.

2.5 SDS-PAGE and Western Blot analysis

After proteins were dialyzed against PBS, proteins were wash with appropriate lysate buffer, sample was further sonicated in -20^{o} C freezers to break DNA. 1X- 5X SDS-PAGE loading buffer was added to the sample, sample was then boiled at 95^{o} temperature, separated on a 10-12% Tris-glycine SDS polyacrylamide gel. Gel containing proteins were further transfer on to nitrocellulose membrane.

The nitrocellulose membrane was placed in a Phosphate buffer saline Tween 20 (PBST) for 30-minute interval at 4°C on a rotator. For the blocking buffer, 1X PBST in 5 % milk (instant non-fat dry milk) was used. Nitrocellulose membrane was placed in the blocking buffer and incubated for 30 minutes at 4°C on a rotator. After blocking the membrane, the supernatant was removed, and followed by incubation in the primary antibody of appropriate dilution. The nitrocellulose membrane was incubated for 90 minutes or overnight on a rotator. The membrane was washed with PBST in 5% milk five

times for ten minutes. Proper dilution of secondary anti-mouse was added to the membrane and incubated for 60 minutes at room temperature on a rotator. The membrane was washed with PBST five times for ten minutes. Thermo-Scientific super signal West Oico enhanced chemiluminescent (ECL) liquid substrate system for detection of HRP was used. After adding the appropriate volume of ECL, the sample was then incubated for five minutes. The membrane was developed on Fuji medical X-ray film in the dark room.

2.6 Parasite Culture

Plasmodium falciparum culture was maintained using the standard techniques [25] with slight modifications. Parasite culture media without human serum was used with the following composition; 625µL, 2 M HEPES, 50µL Gentamicin, 50µL Hypoxanthine, 2.5 ml 10% Albumax stock, in a total volume of 50 ml RPMI 1640. The parasite culture was maintained in 25 cm^2 flasks under 5% CO_2, 3% O_2, 92% N_2 at 37C. Parasite cultures were monitored every other day for growth and development by thin blood smears stained with Giemsa. Fresh complete medium was added daily, human blood was washed three times in RPMI 1640 and stored at 4^{o}C for no longer than two weeks was added, and cultures were split as necessary to maintain parasitemia below 10% and hematocrit between 3%-6%.

2.7 Immunocytochemistry using GM7 monoclonal antibody

For immunofluorescence analysis, malaria parasite culture cells were placed on a glass slide and allowed to air dry. Each slide was fixed with ice-cold methanol for 30 minutes. The slide was then rinsed with Phosphate Buffer Saline (PBS) three times for five minutes. The cells were then blocked with 3% BSA in PBS for 30-60 minutes at

room temperature (RT). The slide was then rinsed with Phosphate buffer saline Tween 20 (PBST) three times for five minutes. The slide was then incubated with a 1:2000 dilution of our mAb in PBS for 60 minutes at RT or overnight incubation at RT. The slide was then rinsed with PBS three times for five minutes. The slide was then incubated with a 1:1000 dilution of secondary goat anti-mouse Alexa Fluor 488 antibody in PBS for 60 minutes. The slide was then rinsed with PBS three times for five minutes. The coverslip was mounted with Prolong Diamond Antifade Mounting solution containing DAPI and incubated in the dark overnight. The slide was then scanned under Nikon Eclipse TE2000-E microscope using a 100X lens.

Chapter 3: Results

3.1 Production and Characterization of monoclonal antibody against Pf-GARPM

3.1.1 Characterization of mouse sera immunized with Pf-GARPM recombinant proteins

To characterize the functional role of Pf-GARP at the blood stage of malaria infection, we decided to produce mAb against Pf-GARPM. Six BALB/c mice were immunized with recombinant Pf-GARPM protein as described above. Western Blot analysis showed that five out of six serum samples were positive for reactivity against recombinant His-Pf-GARP protein at the dilution of 1:10,000 (data not shown). Non-immune mice did not show any reactivity to Pf-GARPM (data not shown). The Ponceau S stained nitrocellulose membrane showing His-Pf-GARPM at 25kDa in *E. coli* is shown (Figure 3.1). Western Blot analysis detected recombinant His-GARPM using the immunized mouse sera. The results of two mice were shown in (Figure 3.1). Both mice recognized the recombinant His-Pf-GARPM at 25kDa.

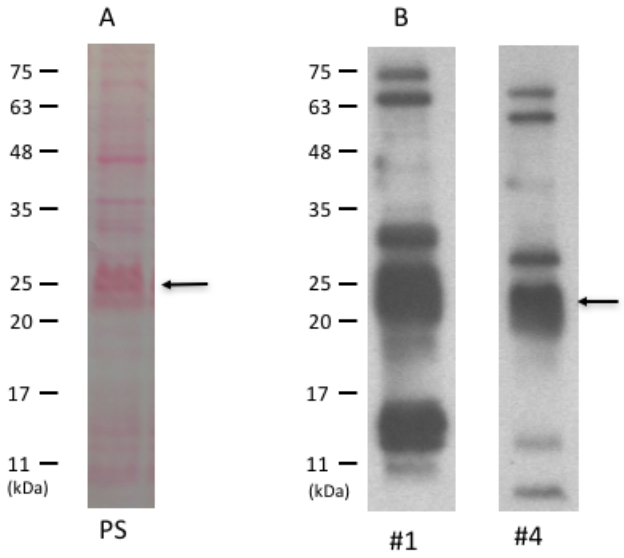

Figure 3.1 Characterization of mouse sera immunized with Pf-GARPM. A. Ponceau S (PS) stained membrane-showing His-Pf-GARPM in *E. coli* lysate. His-Pf-GARPM is shown as 25kDa band. **B. Western Blot analysis of Pf-GARPM immunized mouse sera toward recombinant His-Pf-GARPM protein.** The whole cell lysate of *E. coli* expressing His-Pf-GARPM was resolved by SDS-PAGE and transferred on nitrocellulose membrane. Out of six immunized mice (#1 - #4), two best sera (#1 and #4) are shown here. Recombinant His-Pf-GARPM (25kDa broad band) is recognized by both sera as indicated above.

3.1.2 Detection of endogenous Pf-GARP protein by sera from immunized mice

To detect the endogenous Pf-GARP protein by immune sera, I tested the mouse sera by western blot analysis. The Ponceau S staining of the membrane noted a prominent band in the *P. falciparum* infected ghosts (Figure 3.2, lane 2) at ~40kDa that does not correspond to the 48kDa detected by the antisera (Figure 3.2). The 48kDa protein is not visible in Ponceau S stained membrane. Western blot analysis showed a specific band of 48kDa in Pf infected RBC ghosts but not in uninfected ghosts by multiple sera #4 and #6 (Figure 3.2). The data for the remaining mice including sera#1, #2, #3, and #5 are not shown.

Next, I performed an antigen blocking competition experiment to test if recombinant Pf-GARPM protein reactivity is specific for Pf-GARPM at 48kDa. The sera

were pretreated with recombinant Pf-GARPM protein to compete out the specific Pf-GARP reactivity, and the 48kDa signal was blocked by Pf-GARPM treated serum (Figure 3.2), indicating that the specificity of the 48kDa band. The expected molecular weight of Pf-GARP full length coding sequence is much larger ~80kDa. Our results suggest that the 48kDa protein detected in the *P. falciparum* infected erythrocyte lysate reflects a processed form of Pf-GARP *in vivo*.

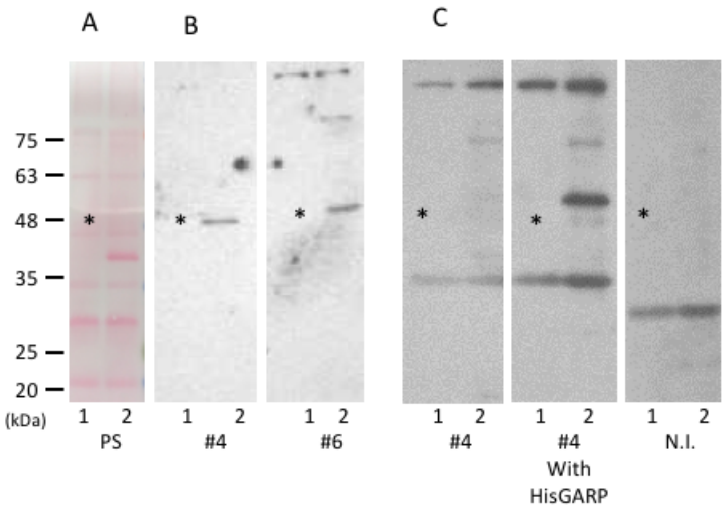

Figure 3.2 Detection of endogenous Pf-GARP protein by sera from immunized mice. A. Ponceau S (PS) staining of the membrane. Note that the prominent band in the *P. falciparum* infected ghosts (lane 2) is about 40kDa and does not correspond to the 48kDa detected by the antisera. The 48kDa protein is not visible in Ponceau S stained membrane. **B. Western Blot analysis with antisera using *P. falciparum* culture lysate.** RBC ghosts were prepared from normal human RBCs (lane 1) and from *P. falciparum*-infected RBCs (lane 2). Both mice #4 and #6 sera detected specific band of 48kDa. **C. Western blot analysis with antisera blocking competition toward *P. falciparum* culture lysate.** RBC ghosts were prepared from normal human RBCs (lane 1) and from *P. falciparum* infected RBCs (lane 2). Mouse #4 sera was pretreated with recombinant Pf-GARPM protein to compete out the specific Pf-GARP reactivity, and the 48kDa signal was blocked by Pf-GARPM treated serum, indicating that the specificity of 48kDa band. Non-immune (NI) mice did not show any reactivity to Pf-GARPM.

3.1.3 Development of a monoclonal antibody against Pf-GARP

The hybridoma culture supernatant samples were screened by ELISA (data not shown) toward His-Pf-GARPM protein. His-Pf-GARPM recombinant protein was

immobilized on 96 well ELISA plate wells. We selected 32 hybridoma clones from the 768 hybridoma culture supernatant samples. The results of 32 clones by ELISA are shown in (Figure 3.3).

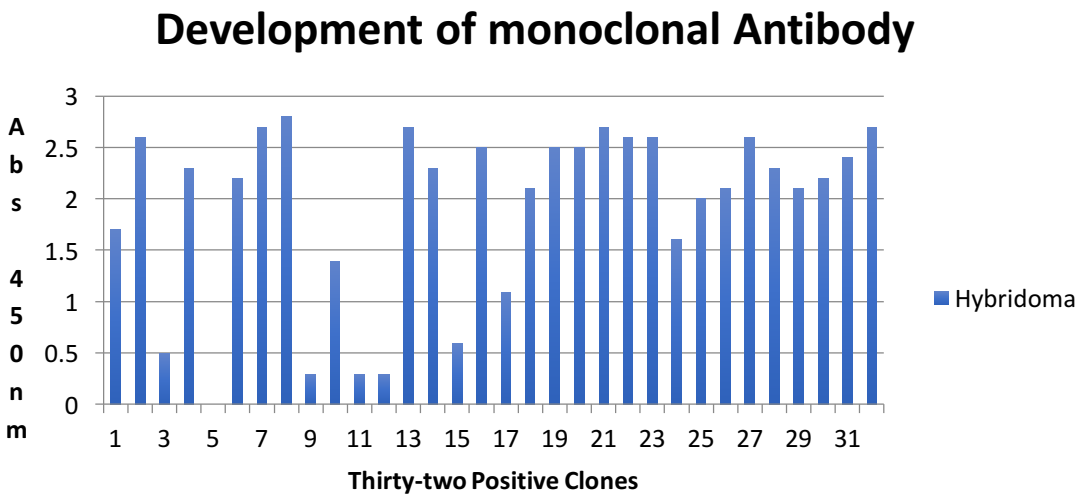

Figure 3.3 ELISA analysis of 32 hybridoma clones. His-Pf-GARPM recombinant protein was immobilized on the ELISA plate and 32 positive supernatants were selected.

Generation of the monoclonal antibody involves harvesting of B cells from the spleen of an animal that has been challenged with an antigen, and subsequently fusion with an immortal myeloma tumor cell line (Figure 3.4). A tumor of the fused cells is called a hybridoma, and these cells secrete mAb specific for the antigen. The B cells confer Ab production capability, while the myeloma cells enable hybridoma to divide [26]. Hybridomas secrete only one Ab type, effectively ensuring a long-term supply of antibodies selective for a single epitope, which are also known as monoclonal (Figure 3.4).

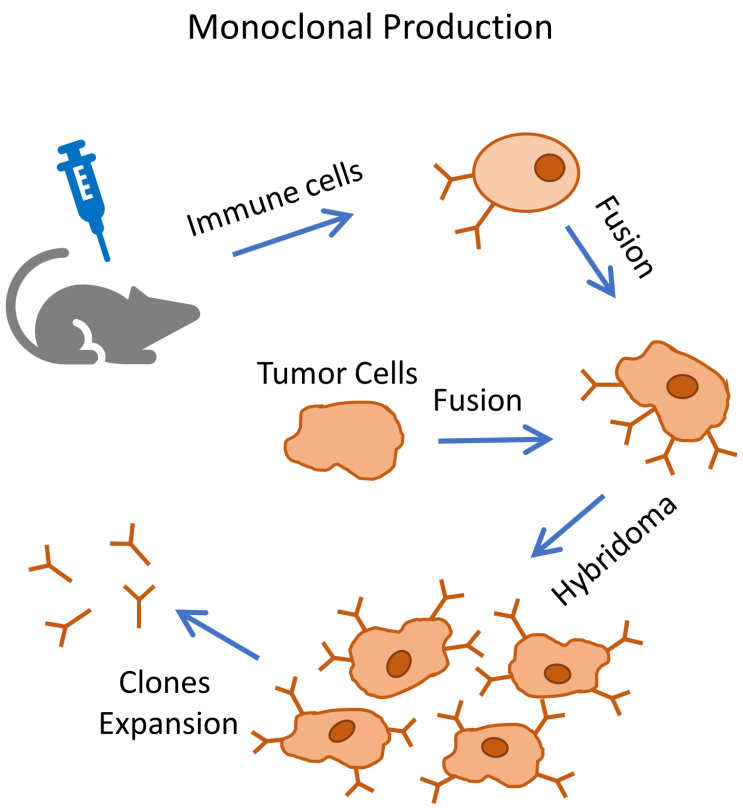

Figure 3.4 Summary of monoclonal antibody production. Producing monoclonal antibody involves immunization of an animal, usually a mouse; obtaining immune cells from its spleen; and fusing the cells with a myeloma cell line to make them immortal so that they can grow and divide indefinitely.

The 32 clones of hybridoma we identified were expanded further and the culture supernatants were obtained. Many of the clones retained strong reactivity toward His-Pf-GARPM by ELISA, whereas some hybridoma clones lost the reactivity. These supernatants were further characterized by western blotting and the results are summarized in (Table 3.1).

32 Hybridoma Clones Tested against Malaria Plasmodium falciparum Lysate				
Hybridoma	ELISA	IB Dilution	WB Quality	WB Detection of Pf Lysate
1	1.7	neat	good	no band
2	2.6	1/5	good	48kDa
3	0.5	neat	no band	ND
4	2.3	neat	good	no band
5	0	ND	ND	ND
6	2.2	1/25	good	high background
7	2.7	1/25	good	48kDa
8	2.8	1/25	good	48kDa
9	0.3	neat	good	no band
10	1.4	neat	good	48kDa
11	0.3	neat	no band	ND
12	0.3	neat	no band	ND
13	2.7	neat	good	48kDa
14	2.3	neat	good	no band
15	0.6	neat	no band	ND
16	2.5	neat	good	no band
17	1.1	neat	good	no band
18	2.1	neat	good	nonspecific 70kDa
19	2.5	neat	good	48kDa
20	2.5	1/5	good	48kDa
21	2.7	1/5	good	48kDa
22	2.6	1/5	good	48kDa
23	2.6	neat	good	48kDa
24	1.6	neat	no band	ND
25	2	neat	good	48kDa
26	2.1	neat	poor	48kDa
27	2.6	neat	good	48kDa
28	2.3	1/25	good	48kDa
29	2.1	1/5	good	48kDa
30	2.2	1/5	good	48kDa
31	2.4	neat	good	no band
32	2.7	1/5	good	48kDa

Table 3.1 Summary of the hybridoma reactivity. To select the best monoclonal antibody produced hybridoma clones, all 32 clones were analyzed by both ELISA and Western blotting to determine the clones that recognize the 48kDa protein of Pf-GARP. Table 1 outlines the summary of all 32 clones analyzed by ELISA and western blotting.

3.1.4 Final selection of positive clones

After completing the identification of 32 positive clones, the next step was to select five or six clones that meet all the criteria as listed above. These characteristics were based on ELISA absorbance, Western Blot (IB) dilution, Western Blot quality, and the Western Blot detection of Pf-GARPM in the Pf lysate. Final screen included hybridomas # 2, 7, 19, 21, 26, and 28. Each hybridoma was tested against lysate from uninfected RBCs (lane 1) and Pf infected RBCs (lane 2). All clones recognized the 48kDa antigen in *P. falciparum* lysate by western blot analysis (Figure 3.5). Based on these data, we selected hybridoma #7 as the best clones and named it GARPM7 or GM7. The GM7 hybridoma was selected for further sub-cloning and expansion. The GM7 hybridoma cells were clonally purified by limiting dilution, and final clone, GM7-1, was used for large scale mAb production.

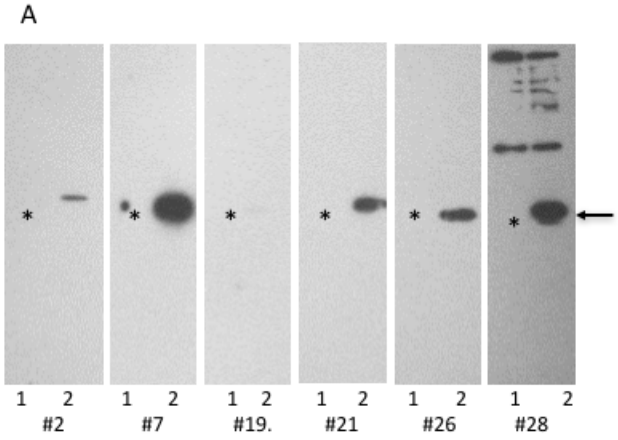

Figure 3.5 Final selection of clones by Western blotting. Six best hybridoma supernatants were identified using Pf culture lysate. RBC ghosts were prepared from uninfected human RBCs (lane 1) and from Pf infected RBCs (lane 2). All clones recognized 48kDa antigen in *P. falciparum* lysate. The hybridoma clone#7 was selected as the best supernatant.

3.1.5 Mapping of GM7 epitope within Pf-GARPM.

The mAb was purified by Protein G Sepharose column. Isotype of the mAb was determined to be IgG3, and the purified GM7 mAb was used to map the epitope within GARPM (Figure 3.6). First, we were interested in mapping the epitope of GM7 within Pf-GARPM. One reason was to identify two independent monoclonal antibodies that recognize two distinct non-overlapping parts of Pf-GARPM. The rationale was to develop a sandwich type ELISA system to detect Pf-GARP protein in the human malaria plasma samples.

Pf-GARPM was divided into two segments termed Pf-GARPM1 and Pf-GARPM2. These proteins were expressed and purified as TRX proteins as described earlier (Figure 3.6). Using western blotting, the GM7 mAb recognized TRX-Pf-GARPM protein and TRX-GARPM1 protein (Figure 3.6). However, GM7 did not recognize TRX-Pf-GARPM2 or the negative control TRX (Figure 3.6). Therefore, we screened the remaining 31 hybridoma clones for the reactivity toward Pf-GARPM2. Again, none of the clones reacted with TRX-Pf-GARPM2 (data not shown). Based on these observations, it was not feasible to develop a sandwich type ELISA for Pf-GARP at this stage.

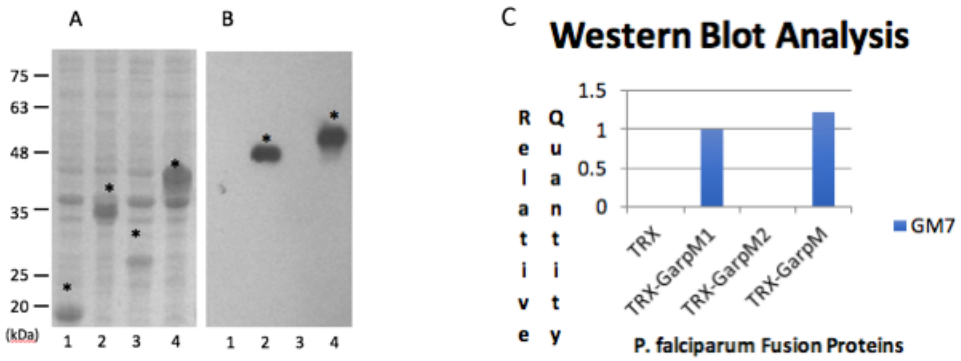

Figure 3.6 Characterization of the purified GM7 mAb by Western Blotting. A. Coomassie stained SDS-PAGE gel of TRX-proteins. Cell lysate of *E. coli* expressing TRX-GARPM proteins was resolved on 12% gel. Lanes show TRX as a negative control (lane 1), TRX-Pf-GARPM1 (lane 2), TRX-Pf-GARPM2 (lane 3), and TRX-pf-GARPM (lane 4). The position of respective recombinant protein is shown by the arrowheads. **B. Western blot data of purified GM7 mAb.** Trx-GARPM1 (lane 2) and TRX-GARPM (lane 4) are recognized by GM7 strongly and specifically. **C. Quantification of the Western Blot data.**

3.1.6 Localization of Pf-GARP using GM7 monoclonal antibody by Immunofluorescence

The generation of GM7 mAb enabled us to determine the localization of Pf-GARP in *P. falciparum* infected RBCs. This approach allowed to us to confirm the principle that proteins from *Plasmodium falciparum* contain lysine-rich tandemly repeating sequences that confer their peripheral localization in infected RBCs [3]. Therefore, I decided to examine the localization of Pf-GARP in a Pf-infected RBCS by immunocytochemistry using GM7 mAb. The *P. falciparum* 3D7 stain was used for immunofluorescence analysis by GM7 mAb. The GM7 detected Pf-GARP on the surface of infected RBCs indicating that *P. falciparum* exports Pf-GARP to surface compartments of infected erythrocytes (Figure 3.7). The GM7 did not detect any signal in uninfected RBCs, further demonstrating the specificity of our antibody for Pf-GARP (Figure 3.7).

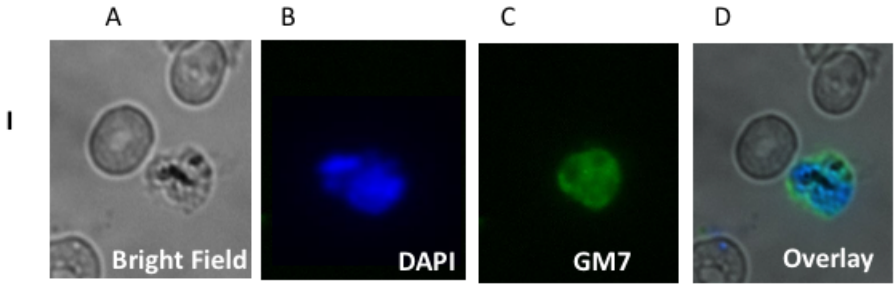

Figure 3.7 Immunofluorescence analysis of Pf-GARP using GM7 monoclonal antibody. I. Pf infected human RBCs were fixed and stained with GM7 mAb. **A.** Bright field image of Pf infected RBC. **B.** DAPI stain. **C.** Pf-GARP staining with GM7. **D.** Overlay.

3.1.7 Mapping of the GM7 epitope within Pf-GARPM using synthetic peptides

Since all 32 hybridoma clones secreting monoclonal antibodies reacted against Pf-GARPM1 and none reacted against Pf-GARPM2, we wanted to test whether there are any hybridoma clones that recognize different epitopes within Pf-GARPM1. If successful, these two monoclonal antibodies would allow us to develop a sandwich ELISA as outlined before. To accomplish this goal, we elected to use synthetic peptides of defined specificity.

As shown in (Figure 3.8), Pf-GARPM is composed of characteristic repeat structures. GARPM1 consists of nine repeats of five amino acid motifs represented by the consensus xEHKx. Moreover, Pf-GARPM1 can be further divided into two groups; first six repeats consist of highly conserved EEHKE or GEHKE repeats and following three repeats consists of more diverse EEHKK-EEHKS-KEHKS sequence elements.

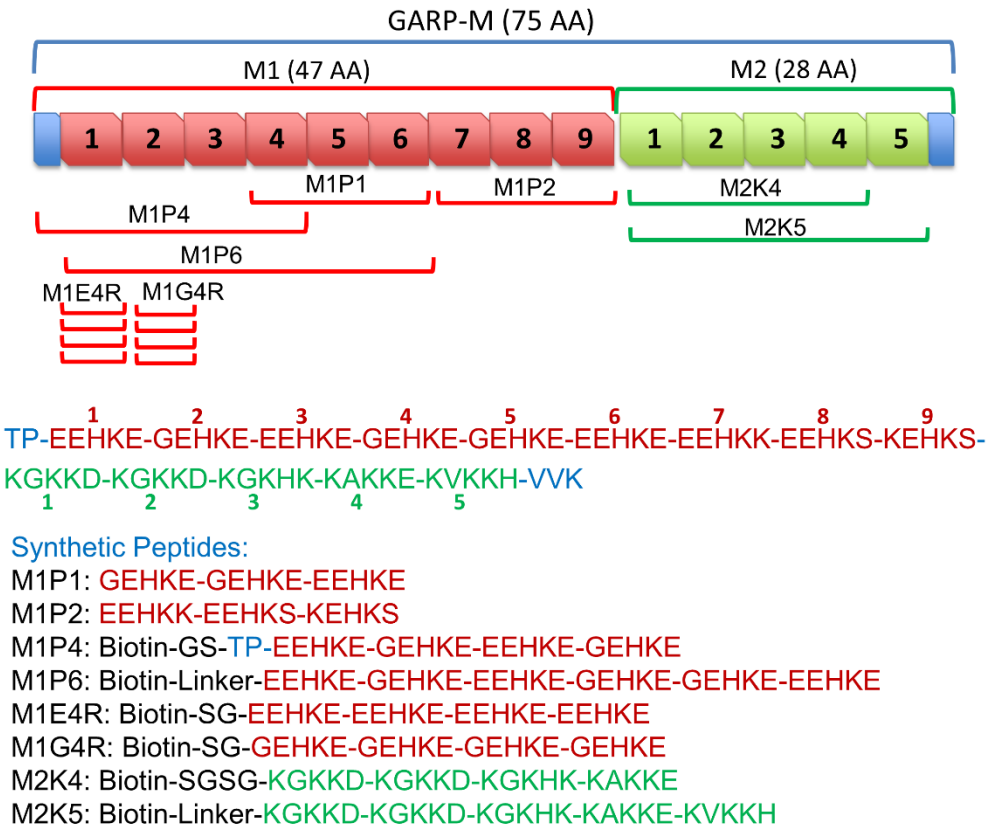

Figure 3.8 Summary *Plasmodium falciparum* GARPM synthetic peptides. The Pf-GARPM protein was divided into several segments based on its known repeat structure. These segments were produced as synthetic peptides. M1P1 peptide contains two repeats of GEHKE and one repeat of EEHKE. M1P2 contains one repeat of EEHKK, EEHKS and KEHKS. M1P4 contains a biotin–GS-TP linker- and the first four repeats. M1E4R peptide contains four single repeats of EEHKE. M1G4R peptide contains four single repeats of GEHKE. Both peptides contain the N-terminal biotin SG–linker tag. M1P6 peptide contains six repeats (three E repeats and three G repeats) linked to flexible linker.M2K4 and M2K5 peptides contain four and five repeats of the Pf-GARPM2, respectively.

M1P1 is a synthetic peptide composed of two repeats of GEHKE and one repeat of EEHKE. M1P2 peptide is composed of one repeat of EEHKK, EEHKS, and KEHKS sequence. M1P4 peptide is 20-amino acid long consisting of a biotin–GS-TP linker conjugated to two repeats of EEHKE and two repeats of GEHKE. Similarly, the M1E4R peptide consists of four repeats of single EEHKE. M1G4R peptide consists of four single repeats of GEHKE. Both M1E4R and M1G4R peptides were assembled to

determine whether multiples of a single repeat can provide a more optimal surface for reactivity against monoclonal antibodies. Finally, the M1P6 peptide was designed with a flexible linker conjugated to three repeats of each EEHKE and GEHKE.

Since Pf-GARPM2 consists of five repeats of highly basic five amino acids defined by the consensus sequence KxKKx, we elected to synthesize two peptides covering the entire Pf-GARPM2. M2K4 peptide contains four repeats whereas M2K5 peptides five repeats. Both peptides contained an SGSG linker to provide flexibility to Biotin. The composition and location of each synthetic peptide is shown in (Figure 3.8) where the Pf-GARPM1 and Pf-GARPM2 segments are highlighted by red and green color.

I first tested which of these repeats is recognized by the GM7 mAb. The peptide M1P1 containing repeats four-six and M1P2 containing repeats seven-nine were tested first (Figure 3.8). ELISA showed that GM7 recognized M1P1 but not M1P2 (Figure 3.9) indicating that the GM7 epitope is located within these repeats. I then tested the remaining 31 hybridoma clones to see if any of these clones will react with M1P2 by ELISA. No reactivity was detected with M1P2 (data not shown). As expected, no reactivity was detected with M2K4 and M2K5 peptides. Together, these results demonstrated that GM7 and all other hybridomas recognize only Pf-GARPM1 within an epitope assembled by both EEHKE-GEHKE repeats. This epitope is highly immunogenic, and essentially all the positive clones we identified are directed toward that same epitope.

A summary of GM7 mAb reactivity to various synthetic peptides is given below:

M1P1 and M1P2 peptides ELISA using GM7 demonstrated that the reactive epitope resides within the repeats contained within M1P1. The absorbance signal (~0.2 OD) from the M1P2 peptide was comparable to negative controls (Figure 3.9). Based on these data, peptide sequence contained within M1P2 was not further pursued.

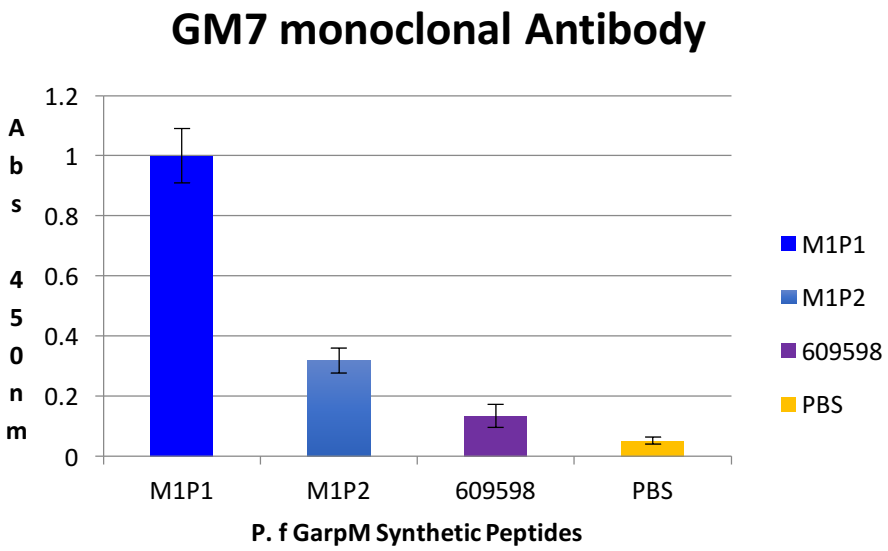

Figure 3.9 *Plasmodium falciparum* GARPM synthetic peptides M1P1 and M1P2. Data show that GM7 recognized M1P1 but not M1P2. This result suggests that Pf-GARPM1 epitope is likely to be encoded by EEHKE-GEHKE repeats since essentially all positive clones identified the same epitope.

M1P4 This 20-amino acid peptide contains two repeats of EEHKE and two repeats of GEHKE. ELISA indicated the GM7 mAb detects M1P4 (Figure 3.10). It is to be noted that the binding affinity for M1P4 appears to be slightly better than M1P1 presumably due to the presence of an additional repeat in M1P4 (Figure 3.10). Another possibility could be the presence of a biotin molecule linked to four repeats via a flexible linker.

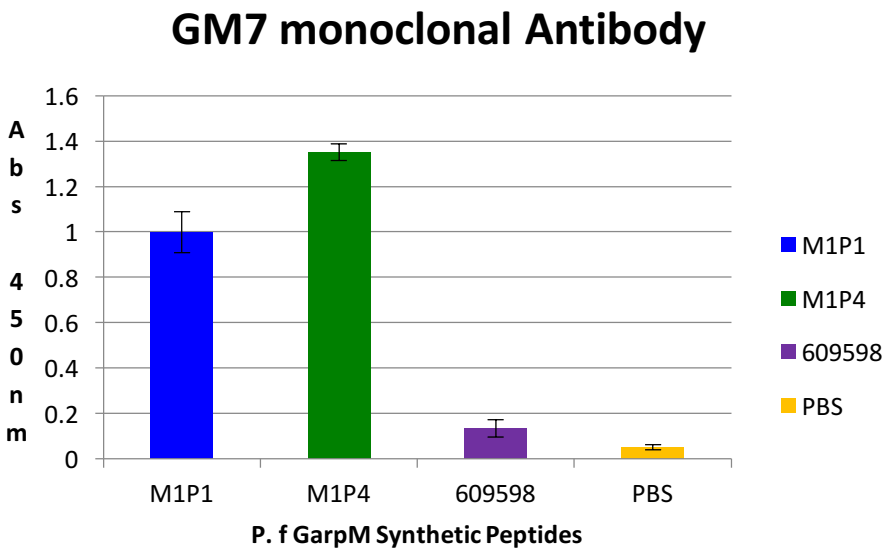

Figure 3.10 *Plasmodium falciparum* GARPM synthetic Peptides M1P1 and M1P4. Results show that GM7 recognized both M1P1 and M1P4 indicating that GM7 epitope is located within EEHKE-GEHKE repeats.

M1E4R and M1G4R peptides - These peptides were designed to determine the relative reactivity of EEHKE and GEHKE repeats by GM7. Each peptide contains four repeats.

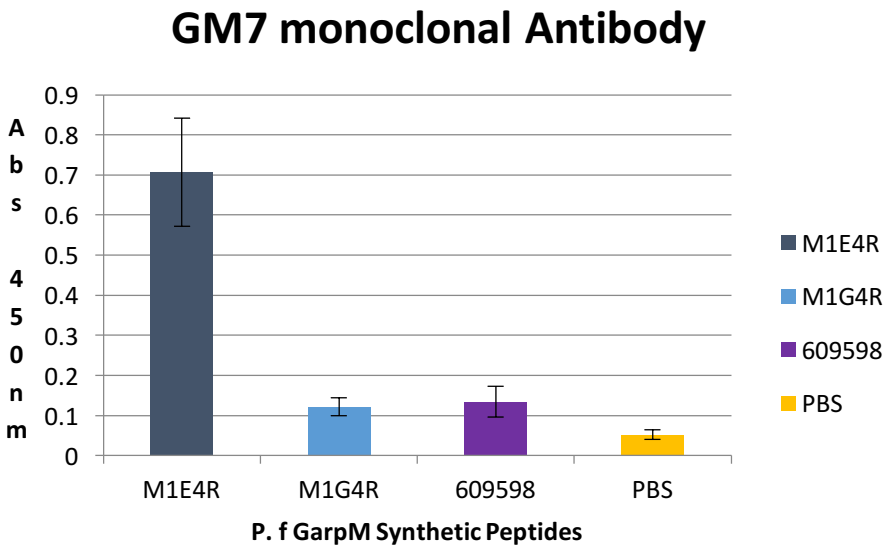

Figure 3.11 *Plasmodium falciparum* GARPM synthetic peptides M1E4R and M1G4R. Data show that GM7 recognized M1E4R but not M1G4R. This result suggests that Pf-GARPM1 epitope is likely to be encoded by EEHKE repeats since essentially all positive clones identified the same epitope.

GM7 recognized M1E4R peptides but didn't M1G4R peptide (Figure 3.11). This result suggests that GARPM1 epitope particularly EEHKE motif is highly immunogenic.

M1P6 –This peptide contains three EEHKE repeats and three GEHKE repeats. The six repeats were conjugated to biotin via a flexible linker. ELISA data demonstrated that GM7 reacted with relatively higher affinity to M1P6 peptide as compared to M1P4 and M1P1 peptides (Figure 3.12). Based on these data, it was concluded that M1P6 peptide encodes an optimal immunogenic epitope recognized by GM7 mAb. The M1P6 peptide was selected for the development of an ELISA-based screen to test human plasma samples from patients from malaria endemic areas of Africa.

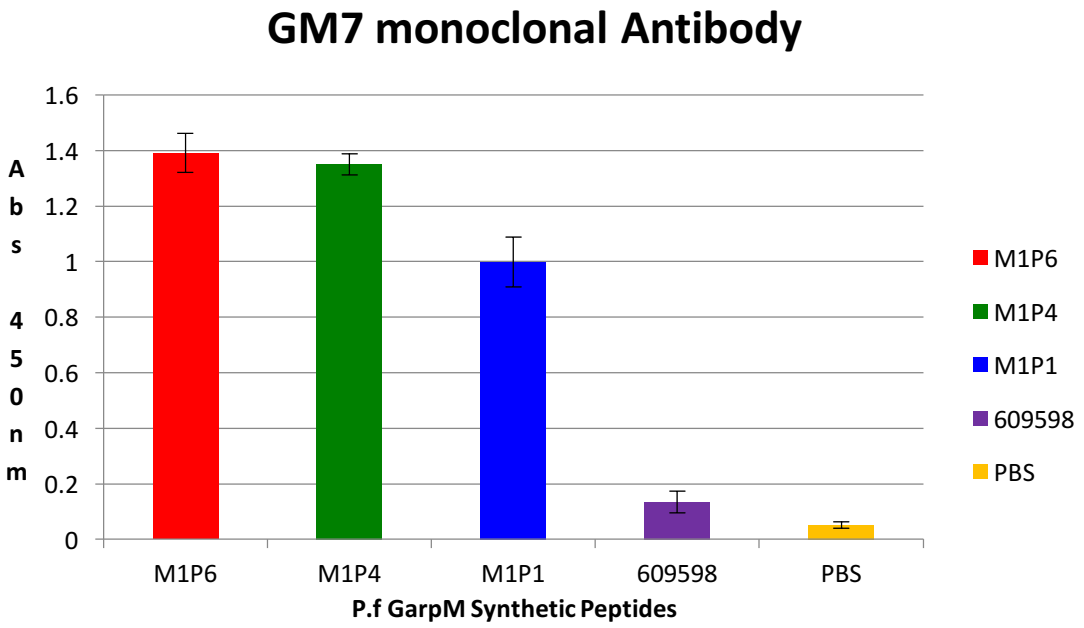

Figure 3.12 *Plasmodium falciparum* GARPM synthetic peptides M1P6, M1P4, and M1P1. Data indicate that M1P6 peptide encodes the most reactive antigenic epitope recognized by GM7 indicating that the epitope is located within EEHKE-GEHKE repeats.

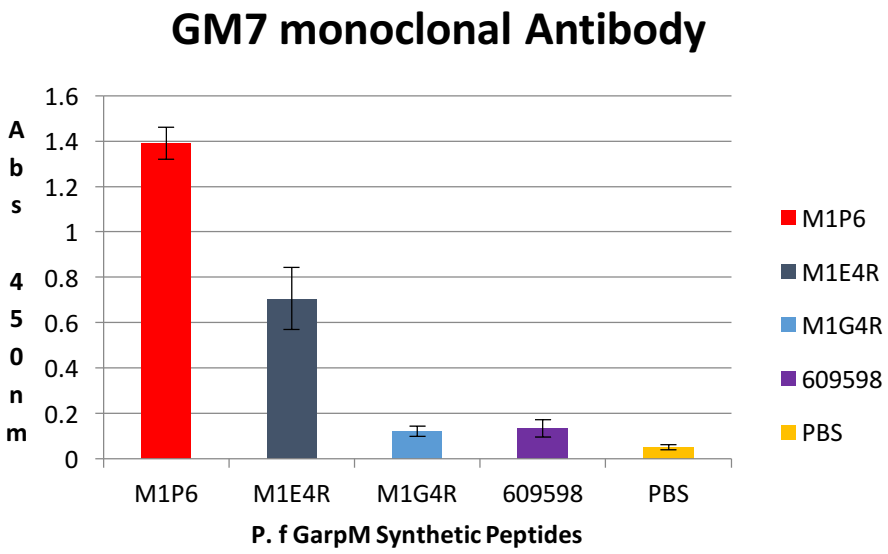

Figure 3.13 *Plasmodium falciparum* GARPM synthetic peptides M1P6, M1E4R and M1G4R. Data indicate that M1P6 peptide encodes the most reactive antigenic epitope recognized by GM7 indicating that the epitope is located within EEHKE-GEHKE repeats.

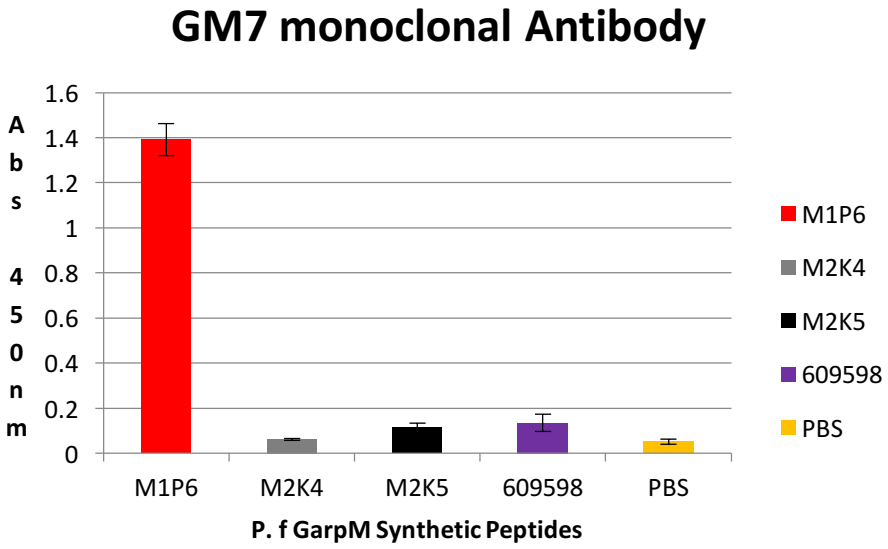

Figure 3.14 *Plasmodium falciparum* GARPM synthetic peptides M1P6, M2K4 and M2K5. Data indicate that M1P6 peptide encodes the most reactive antigenic epitope recognized by GM7 indicating that the epitope is located within EEHKE-GEHKE repeats.

3.2 Clinical application of Pf-GARP ELISA: Proof of Concept

3.2.1 Screening of patient plasma against Pf-GARPM

Previously, we tested 10 plasma samples from malaria endemic areas in Africa for reactivity against *Plasmodium falciparum* Signal Peptide Peptidase (PfSPP) [27]. These samples were originally obtained from the National Institutes of Health (NIH) as part of a screen to search for potential biomarkers of malaria infection in endemic areas. These plasma samples were obtained from 10 healthy female adults living in the rural village of Kambila, Mali where transmission of *P. falciparum* is seasonal and intense [28].

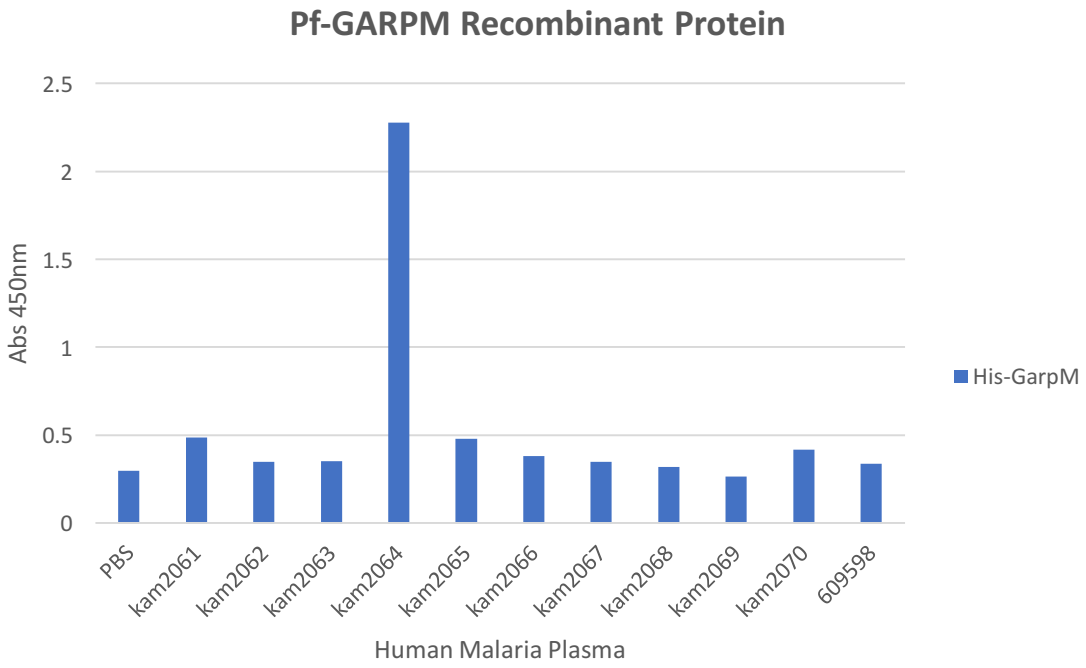

Figure 3.15 Screening of patient plasma against *Plasmodium falciparum* GARPM. His-GARPM was used to detect antibody response against Pf-GARP in 10 malaria patients living in malaria endemic areas. The screening revealed high plasma reactivity against His-GARPM in one of the ten samples. ELISA screen also showed relatively low plasma sero-reactivity against His-GARPM in some of other 9 plasma samples.

To validate our findings with Pf-GARP, we used the same 10 samples to screen for antibodies against Pf-GARPM using His-Pf-GARPM as a bait using our ELISA

protocol (Figure 3.15). Since we detected very high seropositive response from Kam 2064 sample, and low signal from other samples (Figure 3.15), we decided to screen additional plasma samples from the same region in Africa. The NIH laboratory provided us an additional 370 plasma samples from the same malaria endemic area as described above. These plasma samples came from subjects representing both sexes as well as young children exposed to malaria.

Using recombinant His-Pf-GARPM as bait, we screened 370 malaria subjects by ELISA. Again, our ELISA screening identified several samples of high seropositive response. Some of the representative samples of this screen are shown in (Figure 3.16). The seropositive response from the remaining plasma samples is shown in (Table 3.2 and Table 3.3).

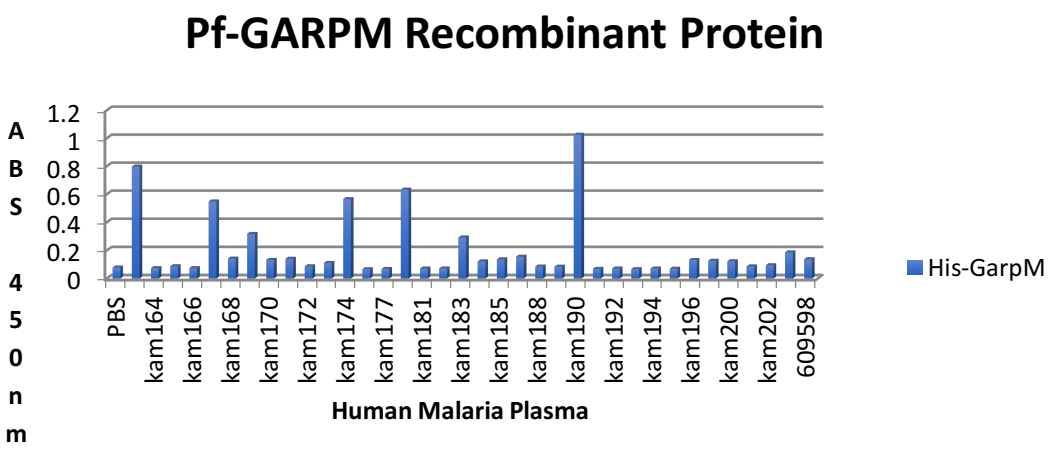

Figure 3.16 Screening of patient plasma against *Plasmodium falciparum* GARPM. The ELISA screening of the 370 malaria samples revealed high plasma reactivity against His-GARPM in some samples. This histogram shows sero-positivity in some representative samples. Data from the remaining plasma samples are shown in Table 3.2 and Table 3.3.

		Screening of Patient Plasma against Plasmodium falciparum His-GARPM							
Sample	OD	Sample	OD	Sample	OD	Sample	OD	Sample	OD
PBS	0.0703	kam092	0.6565	kam146	0.2231	kam124	0.2476	kam134	0.6639
kam006	0.1119	kam091	0.6601	kam034	0.301	kam111	0.345	kam103	0.4791
kam004	0.3603	kam094	0.9323	kam059	0.3061	kam051	0.4666	kam060	0.2671
kam001	0.3061	kam093	0.519	kam049	0.0973	kam050	0.3688	kam108	0.3074
kam008	0.1807	kam095	0.5868	kam053	0.2758	kam129	0.0967	kam142	0.0578
kam015	0.4608	kam077	0.8771	kam060	0.4519	kam063	0.6497	kam150	0.5132
kam010	0.3147	kam020	1.1477	kam105	0.3694	kam055	0.2706	kam126	0.1727
kam003	0.2484	kam021	1.1091	kam123	0.1717	kam032	0.4661	kam124	0.7414
kam012	0.49	kam018	0.4634	kam054	0.2405	kam140	0.6783	kam099	0.5987
kam005	0.1893	kam024	0.1856	kam061	0.5958	kam1134	0.4261	kam038	0.3186
kam076	1.1239	kam025	0.8742	kam107	0.9067	kam110	0.2856	kam059	0.6729
kam072	0.7553	kam149	0.4221	kam114	0.3026	kam121	0.4538	kam110	0.5629
kam070	0.348	kam150	0.6792	kam127	0.2737	kam062	0.7329	kam119	0.2405
kam007	0.2203	kam088	0.7762	kam108	0.4743	kam120	0.317	kam120	0.4315
kam014	0.3072	kam087	0.5514	kam109	0.4989	kam098	0.2128	kam127	0.4144
kam011	0.4351	kam023	0.4881	kam153	0.3982	kam033	0.5249	kam128	0.2719
kam009	0.5136	kam047	0.1895	kam038	0.2165	kam145	0.5749	kam036	0.375
kam013	0.4622	kam042	0.4632	kam037	0.2131	kam126	0.3647	kam105	0.4274
kam002	0.5946	kam043	0.6134	kam119	0.1057	kam148	0.411	kam121	0.1187
kam082	0.3074	kam044	0.431	kam135	0.2505	kam125	0.2908	kam129	0.0517
kam085	0.8907	kam045	0.6095	kam117	0.1868	kam096	0.4816	kam111	0.4436
kam073	0.243	kam040	0.3761	kam130	0.3594	kam141	0.4454	kam039	0.3722
kam069	0.3676	kam041	0.9278	kam122	0.5734	kam036	0.5221	kam114	0.4568
kam071	1.0902	kam046	0.6196	kam035	0.0406	kam142	0.0966	kam140	0.5025
kam067	0.4254	kam028	0.7835	kam057	0.3171	kam156	0.2973	kam097	0.4618
kam065	0.2787	kam030	0.9281	kam131	0.0932	kam110	0.5139	kam048	0.3946
kam078	0.5617	kam029	0.7058	kam158	0.2785	kam102	0.2501	kam051	0.2135
kam074	0.5051	kam016	1.1135	kam104	0.455	kam052	0.0929	kam125	0.0627
kam068	0.7403	kam051	0.7548	kam115	0.2605	kam099	0.2402	kam153	0.4665
kam079	0.541	kam026	0.9275	kam112	0.3858	kam106	0.3936	kam130	0.0556
kam075	0.575	kam027	1.2688	kam039	0.2145	kam133	0.424	kam055	0.7014
kam066	0.6272	kam022	0.7467	kam097	0.471	kam157	0.5608	kam135	0.0492
kam064	0.4552	kam031	1.1478	kam155	0.0987	kam118	0.2692	kam143	0.0468
kam081	1.0467	kam017	0.8383	kam100	0.6322	kam143	0.5484	kam112	0.4831
kam080	0.6828	kam108	1.1088	kam103	0.6011	kam144	0.1934	kam032	0.3957
kam084	1.0876	kam058	0.4582	kam048	0.8322	kam154	0.2308	kam050	0.4119
kam083	0.5358	kam151	0.2794	kam128	0.1808	kam136	0.2355	kam089	1.0212
kam086	0.4979	kam137	0.1824	kam152	0.3994	kam090	0.5829	kam147	0.2392
kam132	0.1981	609598	0.3297						

Table 3.2 Summary of seropositive response from the remaining plasma samples
The ELISA screening of the 370 malaria samples revealed high plasma reactivity against His-GARPM in tabulate samples. This data shows sero-positivity in some representative samples.

		Screening of Patient Plasma against Plasmodium falciparum His-GARPM							
Sample	OD	Sample	OD	Sample	OD	Sample	OD	Sample	OD
PBS	0.0619	kam159	1.0636	kam157	0.3409	kam056	0.097	kam049	0.2099
kam061	0.5611	kam163	0.797	kam204	0.5388	kam008	0.9764	kam078	0.9825
kam037	0.322	kam164	0.0741	kam205	0.9326	kam001	1.1284	kam069	1.0191
kam141	0.4306	kam165	0.0872	kam206	1.2909	kam013	0.059	kam076	1.0915
kam054	0.6008	kam166	0.0741	kam207	0.5019	kam006	0.3832	kam094	1.4649
kam034	0.5412	kam167	0.548	kam208	1.1512	kam015	1.312	kam095	1.0573
kam115	0.3648	kam168	0.141	kam209	0.5371	kam004	0.5647	kam092	0.8002
kam136	0.0482	kam169	0.3173	kam211	0.6318	kam087	0.6486	kam093	0.4166
kam156	0.5492	kam170	0.1323	kam212	0.7211	kam011	0.9189	kam090	0.6773
kam155	0.7584	kam171	0.1403	kam213	0.7318	kam002	0.3943	kam091	0.3733
kam131	0.0454	kam172	0.0866	kam214	0.5993	kam009	0.5195	kam088	0.3505
kam053	0.2319	kam173	0.1108	kam215	0.6036	kam005	0.4141	kam089	0.3073
kam132	0.6133	kam174	0.5649	kam216	0.7048	kam014	0.7006	kam086	0.2876
kam098	0.4138	kam176	0.0659	kam217	1.0766	kam007	0.7003	kam023	0.1542
kam057	0.2492	kam177	0.0679	kam218	0.5041	kam003	0.8275	kam100	0.8748
kam122	0.4259	kam180	0.6322	kam219	0.5396	kam010	0.8996	kam051	0.5509
kam139	0.159	kam181	0.0706	kam220	0.5446	kam012	0.9241	kam016	0.9022
kam058	0.2464	kam182	0.0713	kam221	0.519	kam075	1.1185	kam025	0.7415
kam052	0.2041	kam183	0.2919	kam222	1.1563	kam073	0.5617	kam026	1.5913
kam118	0.7108	kam184	0.1217	kam223	0.8816	kam077	0.7027	kam108	0.9553
kam123	0.4862	kam185	0.1359	kam224	0.8285	kam064	0.3911	kam040	0.0786
kam137	0.3611	kam186	0.1551	kam094	0.7164	kam066	0.3365	kam044	0.2829
kam102	0.5041	kam188	0.0847	kam138	0.5859	kam079	0.3577	kam042	0.4449
kam104	0.6274	kam189	0.0838	kam145	0.7236	kam068	0.5245	kam041	0.2472
kam101	0.6889	kam190	1.0249	kam160	0.5689	kam072	0.8098	kam046	0.3991
kam154	0.3747	kam191	0.0683	kam161	1.0661	kam070	1.0435	kam029	0.7218
kam096	0.3873	kam192	0.0705	kam162	1.1769	kam074	0.9982	kam022	1.0283
kam147	0.3829	kam193	0.067	kam028	0.0979	kam065	1.0035	kam024	0.7258
kam062	0.1677	kam194	0.0704	kam033	0.0936	kam067	1.4407	kam018	0.6072
kam148	0.2786	kam195	0.0693	kam035	0.1005	kam076	1.1934	kam020	1.1665
kam117	0.5724	kam196	0.132	kam063	0.7308	kam084	0.5916	kam021	0.2855
kam107	0.8033	kam198	0.1259	kam134	0.3604	kam085	0.8284	kam030	0.5293
kam146	0.3298	kam200	0.1225	kam133	0.4468	kam082	0.7315	kam031	0.4604
kam144	0.29	kam201	0.0858	kam151	0.0448	kam083	0.8852	kam028	0.3233
kam158	0.4684	kam202	0.0944	kam109	0.2281	kam080	0.3704	kam027	0.3321
kam149	0.8619	kam203	0.1858	kam106	0.2953	kam081	0.3478	kam0198	0.2197
kam017	0.5523	kam2064	1.5576	609598	0.1372				

Table 3.3 Summary of seropositive response from the remaining plasma samples
The ELISA screening of the 370 malaria samples revealed high plasma reactivity against His-GARPM in tabulate samples. This data shows sero-positivity in some representative samples.

To further validate our model that subjects living in the malaria endemic areas could be screened for antibodies against Pf-GARP by our ELISA screens, we used the Pf-GARPM derived synthetic peptide M1P6. We selected 20 positive and negative plasma samples from our His-GARPM based ELISA screens of 380 human plasma samples (Figure 3.17 and Table 3.2 and Table 3.3). Consistent with the results of His-GARPM screens, the ELISA screening using M1P6 peptide also revealed high plasma reactivity

against Pf-GARP in some samples as shown in (Figure 3.17). Also, to compare the data from (Figure 3.17), I selected the same 20 positive and negative plasma samples from our His-GARPM based ELISA screens of 380 human plasma samples. Consistent with the results, the ELISA screening using recombinant His-GARPM also revealed high plasma reactivity against Pf-GARP in some samples as shown in (Figure 3.18). Together, these results demonstrate that information from the peptide mapping, His-GARPM fusion protein, and M1P6 synthetic peptide allowed us to develop an ELISA screening strategy to detect antibody reactivity in subjects exposed to malaria infection in the endemic areas of Africa.

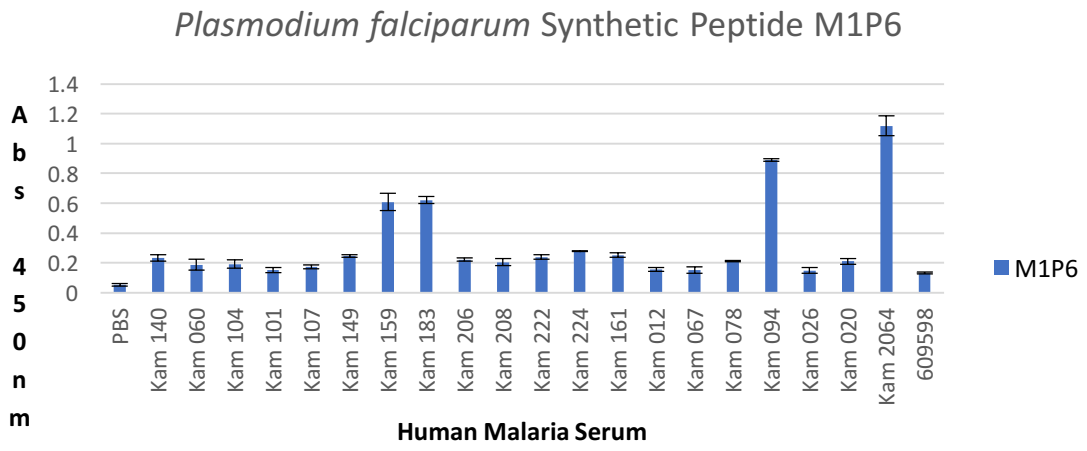

Figure 3.17 Screening of patient plasma against *Plasmodium falciparum* GARP synthetic peptide M1P6. The ELISA screening of 20 out of the 380 malaria samples revealed high plasma reactivity against the synthetic peptide M1P6 in some samples. This histogram shows sero-positivity in some representative samples.

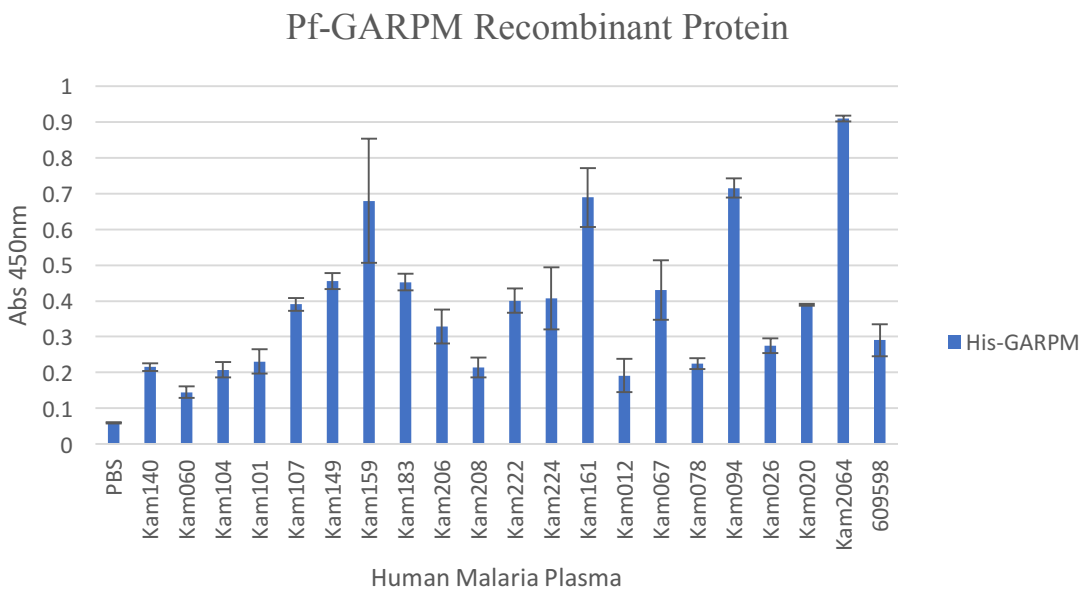

Figure 3.18 Screening of patient plasma against *Plasmodium falciparum* GARPM. The ELISA screening of 20 out of the 380 malaria samples revealed high plasma reactivity against the synthetic peptide M1P6 in some samples. This histogram shows sero-positivity in some representative samples.

Chapter 4: Discussion

Plasmodium falciparum malaria remains a leading cause of mortality, and vaccines are urgently needed to attenuate this public health threat [9]. Severe malaria caused by *P. falciparum*, due to multiple organ failures which is associated with increased adhesiveness of parasite-infected erythrocytes contributing to the blockage of micro-capillaries [29]. Malaria disease is influenced by the ability of parasites to express specific subsets of adhesive proteins, such as those expressed in parasites causing cerebral and pregnancy associated malaria. Therefore, there is an urgent need to identify proteins whose expression is indicative of disease severity. This information could guide intervention strategies aimed at lessening the symptoms of malaria in both non-immune and partially immune individuals [2].

Plasmodium falciparum is known to encode a Glutamic Rich-Acid Protein termed Pf-GARP that is expressed in various parasite isolates that were selected for their ability to bind particular host receptors [2, 3]. From the studies with field isolates, it appears that Pf-GARP is very immunogenic, and it is abundant in children from endemic areas [2, 3]. Importantly, Pf-GARP is unique to *P. falciparum*, and is not expressed in other *Plasmodium* species, implying that this protein may play an important functional role in the severity of human malaria infection. These features suggest that Pf-GARP could be critical for the virulence of human malaria pathogenesis [2].

To investigate the functional role of Pf-GARP, we decided to produce a mAb and develop a quantitative assay to measure Ab response against Pf-GARP in human subjects exposed to *P. falciparum* malaria. Based on our recent findings, we identified a core domain of Pf-GARP that binds to human erythrocytes/RBCs using phage display cDNA

technology (unpublished data). This core domain of Pf-GARP was designated as Pf-GARPM. Since Pf-GARPM binds to RBCs, we cloned and expressed a TRX fusion protein of Pf-GARPM and injected purified protein into six BALB/c mice to induce an immune response. After several boosts with a His-Pf-GARPM fusion protein, mice were tested for immune response by Western Blotting. Among the six mice injected, five mice produced the most robust response against the 25kDa His-Pf-GARPM protein, two mice western blotting analysis is shown (Figure 3.1).

Before proceeding with the generation of a mAb, we first decided to test for the reactivity of mouse serum against endogenous Pf-GARP protein by Western blotting. The mouse serum detected a single 48kDa band in *P. falciparum* infected RBCs but not in uninfected RBCs (Figure 3.2). Although a positive signal was detected by sera from all immunized mice, two mice showed the best response (Figure 3.2).

The specificity of 48kDa was further confirmed by the antigen blocking competition experiments where recombinant His-GARPM protein blocked the reactivity of mouse sera against 48kDa band of endogenous Pf-GARP in parasite cell lysates (Figure 3.2). Since the predicted molecular weight of Pf-GARP is ~80kDa, we concluded that the 48kDa band reflects a processed form of Pf-GARP *in vivo*. A similar processing of malaria antigens including *Plasmodium falciparum* Merozoite Surface Protein (MSP1), and *Plasmodium falciparum* Reticulocyte-binding protein homologue 5 (RH5) has been demonstrated for multiple proteins detected during the blood stage of malaria pathogenesis[30-32]. Together, these observations suggest that the core RBC-binding domain of Pf-GARP encodes a highly immunogenic sequence that appears to be suitable for the generation of monoclonal antibodies.

To generate monoclonal antibodies against Pf-GARP, we screened 32 hybridomas and selected the GM7 as the best mAb as described in the Results section (Figure 3.3).

First, we were interested in mapping the epitope of GM7 within Pf-GARPM. The GM7 recognized TRX-GARPM and TRX-GARPM1 but not TRX-GARPM2 or the negative control TRX. We tested 31 additional hybridoma clones (data not shown) for their reactivity toward Pf-GARPM2. We did not find any clone with reactivity for Pf-GARPM2. Based on these data, it was not feasible to develop a sandwich-type ELISA suitable for the detection of endogenous Pf-GARP in future studies.

We then tested GM7 mAb for its ability to detect endogenous Pf-GARP in *P. falciparum* infected RBCs by immunofluorescence microscopy. GM7 successfully detected Pf-GARP on the surface of infected RBC (Figure 3.7). These observations suggest that the native Pf-GARP is transported to the cell surface consistent with the presence lysine-rich tandemly repeating sequences that confer a peripheral localization in the infected RBCs. Pf-GARP contains three distinct lysine-rich repeat sequences with a potential targeting function [3]. Moreover, successful localization of Pf-GARP in infected RBCs indicates that GM7 is a suitable mAb to investigate both denatured and native Pf-GARP in future studies.

The next step was to map the GM7 recognition epitope within Pf-GARPM1 using synthetic peptides. The rationale for this approach was based on the assumption that if GM7 recognizes a dominant immunogenic epitope in Pf-GARP, then identification of this epitope may allow us to develop an ELISA to detect GM7 like Ab response in subjects from malaria endemic areas. As shown in (Figure 3.8), Pf-GARP is composed of

characteristic repeat structures. Pf-GARPM1 consists of nine repeats of five amino acid motifs represented as xEHKx, and GARPM1 can be also be divided into two groups; first six repeats consist of highly conserved EEHKE or GEHKE repeats, followed by three repeats that are relatively more diverse EEHKK-EEHKS-KEHKS.

We designed several synthetic peptides encoding a combination of various repeats (Figure 3.8.). By performing a series of ELISA screens, a core sequence containing the EEHKE repeat was identified showing the highest reactivity for GM7 (Figure 3.11). Although the GEHKE repeat alone did not show much reactivity, its presence was required to confer optimal response from the peptide containing EEHKE repeats. Based on these observations, we designed a peptide containing three EEHKE and three GEHKE repeats. This peptide termed M1P6 was linked to biotin via a flexible linker and served as the primary bait peptide to develop an ELISA for subsequent antibody screening of patient plasma samples.

As described in the Results section above, we obtained 380 plasma samples from subjects living in malaria endemic areas of Mali. These samples were originally acquired by the NIH for their malaria research program. We have previously published the Ab reactivity of 10 plasma samples for another malaria antigen termed PfSPP [27]. We first screened all 380 plasma samples using His-Pf-GARPM fusion protein as bait in our ELISA screens. The rationale for using the His-Pf-GARPM protein was to capture all potential Ab positive samples that might be otherwise missed by a single peptide-based ELISA screens. Using this approach, we identified a number of Ab positive samples for Pf-GARPM (Figure 3.16). From these screens, we selected 20 samples that included both positive and negative hits using the His-Pf-GARPM as bait. These 20 samples were then

analyzed by the M1P6 peptide-based ELISA screen as well as His-GARPM recombinant protein. Consistent with the screen performed with the His-Pf-GARPM protein, several samples that were positive with the fusion protein also tested positive by the peptide-based screen (Figure 3.17 and Figure 3.18). These observations suggest that our newly developed peptide-based ELISA may serve as a suitable diagnostic tool to screen for Ab reactivity against Pf-GARP in subjects from malaria endemic areas.

4.1 Conclusion

In summary, we have developed a new mAb that is specific for *Plasmodium falciparum* GARP protein. The GM7 mAb can detect both native and recombinant Pf-GARP using western blotting and immunofluorescence assays. We have identified a key peptide repeat of Pf-GARP recognized by GM7 mAb. Based on this information, we developed a quantitative ELISA that can detect Ab reactivity against Pf-GARP in human subjects from malaria endemic areas. Our anticipation is that the relatively higher Ab response against Pf-GARP may correlate with the protection of severe malaria disease, particularly the symptoms observed in cerebral and pregnancy-associated *P. falciparum* malaria. These findings may lay the foundation for the discovery of Pf-GARP as a biomarker for the disease progression in severe malaria.

4.2 Future Direction

The feasibility of our ELISA based screen for testing the presence of Pf-GARP antibodies would require screening of human blood samples from malaria endemic areas of Africa and around the world. If these samples establish a correlation between Pf-GARP Ab response in severe malaria, our findings may provide the basis of a novel diagnostic tool for future studies.

The GM7 mAb against Pf-GARP will allow further characterization of the function of Pf-GARP using immunoprecipitation and mass spectrometry-based approaches.

Chapter 5: Bibliography

1. Prevention, C.f.D.C.a. *Malaria Facts*. Malaria [Web Site] 2017 [cited 2017 12/10]; Available from: https://www.cdc.gov/malaria/about/facts.html.
2. Vignali, M., et al., *NSR-seq transcriptional profiling enables identification of a gene signature of Plasmodium falciparum parasites infecting children.* J Clin Invest, 2011. **121**(3): p. 1119-29.
3. Davies, H.M., K. Thalassinos, and A.R. Osborne, *Expansion of Lysine-rich Repeats in Plasmodium Proteins Generates Novel Localization Sequences That Target the Periphery of the Host Erythrocyte.* J Biol Chem, 2016. **291**(50): p. 26188-26207.
4. Crompton, P.D., S.K. Pierce, and L.H. Miller, *Advances and challenges in malaria vaccine development.* J Clin Invest, 2010. **120**(12): p. 4168-78.
5. Philadelphia, T.C.o.P.o. *Malaria and Malaria Vaccine Candidates*. History of Vaccines [Web Site] 2017 [cited 2017 09/21]; Available from: https://www.historyofvaccines.org/content/articles/malaria-and-malaria-vaccine-candidates.
6. Organization, W.H. *Fact Sheet: World Malaria Report 2016*. Malaria [Web Site] 2016 [cited 2017 12/10]; Available from: http://www.who.int/malaria/media/world-malaria-report-2016/en/.
7. Singh, B., et al., *A large focus of naturally acquired Plasmodium knowlesi infections in human beings.* Lancet, 2004. **363**(9414): p. 1017-24.
8. Idro, R., et al., *Cerebral malaria: mechanisms of brain injury and strategies for improved neurocognitive outcome.* Pediatr Res, 2010. **68**(4): p. 267-74.
9. Raj, D.K., et al., *Antibodies to PfSEA-1 block parasite egress from RBCs and protect against malaria infection.* Science, 2014. **344**(6186): p. 871-7.
10. Triglia, T., et al., *Structure of a Plasmodium falciparum gene that encodes a glutamic acid-rich protein (GARP).* Mol Biochem Parasitol, 1988. **31**(2): p. 199-201.
11. Leech, J.H., et al., *Identification of a strain-specific malarial antigen exposed on the surface of Plasmodium falciparum-infected erythrocytes.* J Exp Med, 1984. **159**(6): p. 1567-75.
12. Baruch, D.I., et al., *Cloning the P. falciparum gene encoding PfEMP1, a malarial variant antigen and adherence receptor on the surface of parasitized human erythrocytes.* Cell, 1995. **82**(1): p. 77-87.
13. Smith, J.D., et al., *Switches in expression of Plasmodium falciparum var genes correlate with changes in antigenic and cytoadherent phenotypes of infected erythrocytes.* Cell, 1995. **82**(1): p. 101-10.
14. Su, X.Z., et al., *The large diverse gene family var encodes proteins involved in cytoadherence and antigenic variation of Plasmodium falciparum-infected erythrocytes.* Cell, 1995. **82**(1): p. 89-100.
15. Marti, M., et al., *Signal-mediated export of proteins from the malaria parasite to the host erythrocyte.* J Cell Biol, 2005. **171**(4): p. 587-92.
16. Maier, A.G., et al., *Exported proteins required for virulence and rigidity of Plasmodium falciparum-infected human erythrocytes.* Cell, 2008. **134**(1): p. 48-61.

17. Marti, M. and T. Spielmann, *Protein export in malaria parasites: many membranes to cross.* Curr Opin Microbiol, 2013. **16**(4): p. 445-51.
18. Spillman, N.J., J.R. Beck, and D.E. Goldberg, *Protein export into malaria parasite-infected erythrocytes: mechanisms and functional consequences.* Annu Rev Biochem, 2015. **84**: p. 813-41.
19. Boddey, J.A. and A.F. Cowman, *Plasmodium nesting: remaking the erythrocyte from the inside out.* Annu Rev Microbiol, 2013. **67**: p. 243-69.
20. Glenister, F.K., et al., *Functional alteration of red blood cells by a megadalton protein of Plasmodium falciparum.* Blood, 2009. **113**(4): p. 919-28.
21. Baruch, D.I., et al., *Identification of a region of PfEMP1 that mediates adherence of Plasmodium falciparum infected erythrocytes to CD36: conserved function with variant sequence.* Blood, 1997. **90**(9): p. 3766-75.
22. Conway, D.J., et al., *A principal target of human immunity to malaria identified by molecular population genetic and immunological analyses.* Nat Med, 2000. **6**(6): p. 689-92.
23. Kaur, P., et al., *Synthetic, immunological and structural studies on repeat unit peptides of Plasmodium falciparum antigens.* Int J Pept Protein Res, 1990. **36**(6): p. 515-21.
24. Kemp, D.J., R.L. Coppel, and R.F. Anders, *Repetitive proteins and genes of malaria.* Annu Rev Microbiol, 1987. **41**: p. 181-208.
25. Trager, W. and J.B. Jensen, *Human malaria parasites in continuous culture.* Science, 1976. **193**(4254): p. 673-5.
26. Sciences, N.A.o. *Monoclonal Antibody Production*. Committee on Methods of Producing Monoclonal Antibodies [Web Site] 1999 [cited 2017 09/21]; Available from: https://grants.nih.gov/grants/policy/antibodies.pdf.
27. Li, X., et al., *Plasmodium falciparum signal peptide peptidase is a promising drug target against blood stage malaria.* Biochem Biophys Res Commun, 2009. **380**(3): p. 454-9.
28. Crompton, P.D., et al., *Sickle cell trait is associated with a delayed onset of malaria: implications for time-to-event analysis in clinical studies of malaria.* J Infect Dis, 2008. **198**(9): p. 1265-75.
29. Nash, G.B., et al., *Abnormalities in the mechanical properties of red blood cells caused by Plasmodium falciparum.* Blood, 1989. **74**(2): p. 855-61.
30. Ord, R.L., M. Rodriguez, and C.A. Lobo, *Malaria invasion ligand RH5 and its prime candidacy in blood-stage malaria vaccine design.* Hum Vaccin Immunother, 2015. **11**(6): p. 1465-73.
31. Das, S., et al., *Processing of Plasmodium falciparum Merozoite Surface Protein MSP1 Activates a Spectrin-Binding Function Enabling Parasite Egress from RBCs.* Cell Host Microbe, 2015. **18**(4): p. 433-44.
32. Galaway, F., et al., *P113 is a merozoite surface protein that binds the N terminus of Plasmodium falciparum RH5.* Nat Commun, 2017. **8**: p. 14333.