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TARGETING *PLASMODIUM* INVASION PATHWAYS IN MOSQUITOES TO BLOCK MALARIA TRANSMISSION

A DISSERTATION APPROVED FOR THE
DEPARTMENT OF CHEMISTRY AND BIOCHEMISTRY

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Abstract

Malaria remains a devastating disease. Transmission-blocking vaccines (TBVs) are being considered as a promising approach to eliminate *Plasmodium* infection. However, the challenges in developing such a vaccine are paramount, since the clinically relevant species of *Plasmodium* are transmitted by a number of different *Anopheles* mosquito vectors. Through association studies, we discovered FREP1, a mosquito midgut protein that facilitates *P. falciparum* parasite transmission. Our biochemical characterization of FREP1 discovered that this protein is secreted as a tetramer, expressed by midgut cells and located in the peritrophic matrix (PM) of mosquito midgut. Molecular analysis revealed that FREP1 anchors parasites to the mosquito PM, assisting ookinetes as a midgut receptor for *Plasmodium* migration from the blood bolus and subsequent invasion of the midgut epithelium. Since FREP1 is readily accessible to antibodies co-ingested with blood, it is a suitable antigen for targeting by TBV. Sequence comparison of orthologs showed that the fibrinogen-like (FBG) domain of FREP1 is highly conserved (>90% identical) among *Anopheles* species from different continents, suggesting that anti-FBG antibodies may block malaria transmission to all anopheline mosquitoes. Using standard membrane-feeding assays (SMFA), we showed that anti-FREP1 polyclonal antibodies significantly blocked transmission of *P. berghei* and *P. vivax* to *An. gambiae* and *An. dirus* respectively. Furthermore, *in vivo* studies of mice immunized with purified FBG showed that our experimental TBV effectively blocks *P. berghei* transmission to *An. gambiae* (>75%), without triggering immunopathology or inducing responses against mouse or human fibrinogens. Anti-FBG serum from the immunized mice also reduces *P. falciparum* infection of *An. gambiae* mosquitoes by

more than 81% during SMFA, meeting TBV criteria for clinical trials. Finally, I showed that the FBG domain directly interacts with *Plasmodium* gametocytes and ookinetes, revealing the molecular mechanisms of the transmission-blocking activity of anti-FBG antibodies. FBG also binds to peritrophic matrix, and the N-terminal region of FREP1 keeps FREP1 as tetramers. Collectively, our data support that FREP1-mediated *Plasmodium* transmission to mosquitoes is a conserved pathway, and that the targeting of the FBG domain of FREP1 will limit the transmission of multiple *Plasmodium* species to multiple *Anopheles* species. In summary, I reported here the establishment of a high-level secretion system using mosquito FREP1 signal peptide to secrete recombinant heterologous proteins. I have elucidated FREP1 molecular mechanisms as an ookinete midgut receptor that facilitates parasite invasion of the mosquito midgut, and I have determined that the highly conserved functional FBG domain of FREP1 is a broad-spectrum transmission blocking vaccine antigen.

Chapter 1: Introduction

1.1 History and Epidemiology of Malaria

Malaria is one of the major diseases affecting man, being first reported around 4,000 years ago[1]. Initially, malaria was thought to be caused by 'bad air' (from the Italian mal'aria), and its causative agent was discovered in 1880 by a French army surgeon, Charles Louis Alphonse Laveran[1]. In the late 1890s, the heamosporidan parasite of the genus *Plasmodium* was confirmed to be the etiological agent of malaria[1]. Haemosporidan parasites are apicomplexan protozoans, which are unicellular eukaryotic cells that infect the blood of vertebrates[2]. In 1897, Ronald Ross first demonstrated that patients carrying malaria parasites could infect hematophagous mosquitoes, revealing that the female *Anopheles* transmit the disease[1]. To this present date, malaria remains as one of the world's most devastating diseases with an alarming two-thirds of the world population at risk of malaria[3]. There are currently 97 countries with on-going malaria transmission and seven other countries in the prevention of introduction phase, totaling 104 countries with endemic malaria[4, 5].

According to the recent reports from the World Health Organization (WHO), malaria has decreased by 50% globally and 47% in Africa in the past ten years. This considerable decrease is largely due to the combined efforts of insecticide spraying, insecticide treated bed nets, and advancement in the early diagnostics and treatment of disease[6, 7]. Despite this progress in combating the disease, in 2013 $2.6 billion were spent on a variety of malaria control programs. Despite these efforts, 198 million malaria clinical cases and 568,000 deaths were accounted worldwide in 2013[8]. The overwhelming majority of the fatalities occur in Africa, where 87% of the deaths are

children under the age of five and pregnant women[4, 8]. The current agenda for malaria control set by several non-governmental organizations, established malaria eradication as the main goal for the next 20 years and to interrupt malaria transmission as the focus of the eradication program[9-19]. The malaria parasite has a much more complex life cycle compared to viruses and bacteria, which augment its complexity. Bacterial and viral diseases such as (e.g. smallpox, polio, and measles) not only have vaccines available, but also have been completely eradicated through vaccination programs whereas no effective vaccine is available for any parasitic disease[20, 21]. In addition to the complex biology involving both a vertebrate and an invertebrate host, *Plasmodium* parasites have genomes that are much larger than those of viruses and bacteria[22]. As a result, their genetic make-up is extremely variable across the different stages of their life cycle; this variation in the parasite expressing different proteins on the cell surface elicits different arms of the immune system to combat the disease depending on whether the parasite is inside or outside of the host cells[23, 24]. Furthermore, antibodies raised against certain stages of malaria are not effective across other stages of the infection[25, 26].

1.2 Basic Biology

There are five major *Plasmodium* species that are the etiological agents of human malaria; *P. falciparum, P. vivax, P. malariae, P. ovale* and *P. knowlesi; P. falciparum* and *P. vivax* account for 99% of the malaria cases worldwide[1, 27]. Uncomplicated malaria is more common in adolescents and adults in high transmission areas, whereas severe malaria, which mostly results from *P. falciparum* infections, is frequently observed among young children and adults who travel to areas of high malaria

transmission[28]. In extreme cases where the infected red blood cells are sequestered in the brain, cerebral malaria may occur with convulsions, followed by coma, and eventual death[1, 29]. There are approximately more than 30 species of *Anopheles* mosquitoes that transmit malaria worldwide, with a varying degree of efficiency[30]. The main malaria vector in Africa is *Anopheles gambiae;* however *A. funestus* and *A. arabiensis* are important vectors as well[30, 31]. In Asia, *A.dirus* and *A. stephensi* are responsible for the malaria cases[32]. In Europe *A. atroparvus* and in South America, *A. minimus, A.albimanus* and *A. darlingi* are the main transmission agents of the disease[14, 30, 33, 34].

To complete its complex life cycle, *Plasmodium* needs to infect a vertebrate and then a mosquito host (Figure 1). In the case of the human malaria, the vertebrate host is a human, although other mammals, reptiles and birds can be infected by their respective *Plasmodium* malaria-causing parasite. The basic biology of the *Plasmodium* parasite begins when an infected female *Anopheles* spp. mosquito takes a blood meal from human. The mosquito inoculates uninucleate sporozoites, the only form of the parasite that can infect humans, into the tissues or directly into the bloodstream. Sporozoites, as fast as 2 minutes and no more than 60 minutes, pass through the bloodstream and invade the liver where they penetrate, in the hepatic sinusoids, and could pass through the Kupffer cells and invade hepatocytes[20]. Sporozoites could enter several liver cells prior to finding the correct hepatocyte in which it can develop. The uninucleated *P. falciparum* sporozoites undergo a rapid growth yielding 10,000-40,000 liver-stage schizonts, with an average of 30,000 uninucleated merozoites, in a minimum of 5.5 days[35]. The mature liver-stage schizonts exploit the host liver cell resources leading to

its death or ultimately the rupture and release of "sacks" called merosomes containing tens of thousands of uninucleated merozoites, the infectious form of the red blood cells. In the erythrocyte, it takes approximately 48 hours for the invading merozoite to develop into a mature erythrocyte-stage schizont averaging 16 uninucleated merozoites[36]. The parasites undergo asexual multiplication inside the erythrocytes, and fully mature merozoites are released from the infected erythrocyte and invade normal erythrocytes, initiating the cycle of intraerythrocytic-stage development, rupture, and reinvasion. This process results in 10-20-fold increase in the numbers of *P. falciparum* parasites in the bloodstream every 2 days[20]. This cycle characterizes all the clinical and pathological manifestations of malaria[36]. Erythrocyte-stage parasites can, alternatively, develop into sexual-stage parasites, called gametocytes.

Plasmodium then undergoes an obligatory developmental cycle in the mosquito midgut, where gametocytes ingested by *Anopheles* spp. during a blood meal, within approximately 15 min (in the case of *P. falciparum*) egress from the erythrocytes and differentiate into gametes[37]. The male gametocytes undergo a significant transformation known as exflagellation, in which the DNA replicates to 8N and subsequent formation of eight haploid gametes (microgametocytes)[23]. Free microgametes fuse with the female gamete (macrogametocytes) forming a diploid zygote, which immediately experiences one round of DNA replication to become tetraploid[23]. This sexual multiplication of the parasite inside the mosquito gut is known as the sporogonic cycle. The zygote, 18-24 hours later, turns into a motile and elongated form, the ookinete. The motile ookinete migrates from the blood bolus to

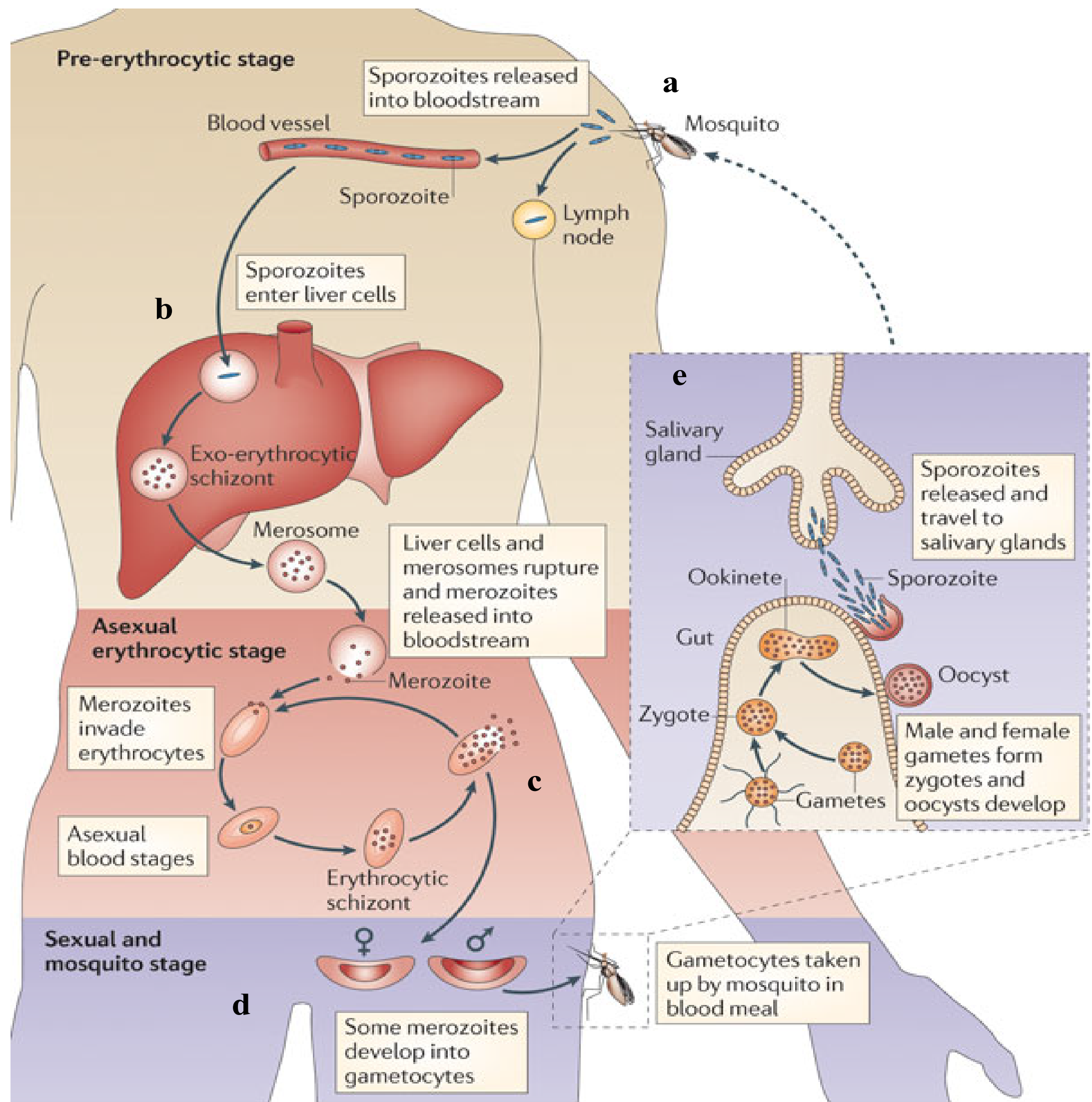

Figure 1-1. Basic features of the *Plasmodium* life cycle.
In order for *Anopheles* females to lay eggs a bloodmeal is necessary. **a**, In the process, infected females inject sporozoites, the only form of the parasite that can infect humans, into the bloodstream. **b**, Within minutes, liver cells are invaded and the sporozoites proliferate asexually. **c**, 6-7 days later, as merozoites, they invade the erythrocytes. An asexual 48-hour cycle within the erythrocytes, at which the disease clinically manifests as fever and chills, is followed by the production of male and female gametocytes. **d**, These are the only forms that can infect mosquitos during a bloodmeal, where they differentiate into male and female gametes and fuse to form ookinetes. **e**, Ookinetes penetrate the midgut epithelium and form oocysts that duly divide to create sporozoites. These migrate to the salivary glands, where the cycle of infection starts again when the mosquito takes another bite. Adapted by permission from Macmillan Publishers Ltd: [Nature Reviews Immunology] REF. 36 © (2011).

invade and traverse the midgut wall by passing through the peritrophic matrix (PM), epithelial cells and comes to rest adjacent to the basal lamina. The ookinete undergoes a series of development and multiplication and differentiates into a round oocyst. Within 10-14 days the oocyst has grown in size and undergone sporogony to produce thousands of sporozoites in the hemocoel of the mosquito. The sporozoites circulate within the haemolymph and specifically invade the salivary glands[23, 38]. When the mosquito bites another human, the sporozoites in the salivary glands are inoculated into the new host, perpetuating the malaria life cycle.

1.3 Malaria and Immunity

There are several natural barriers that the *Plasmodium* parasite encounters in both humans and invertebrate hosts. In mammals, the host defense against malaria infection relies mainly on the T-cell mediated and humoral anti-malaria response[28, 39]. In mosquitoes, there is a complete dependence of the innate immune system, which refers to the first-line host defense that serves to limit infection in the early hours after exposure to microorganisms[40].

1.3.1 Humans Immunity and Malaria

Hepatocytes, and particularly erythrocytes, have extremely low expression of both major histocompatibility complexes (MHC) on the cell surface, which represents an optimal condition for intracellular parasites like *Plasmodium*. Without the MHC class I-antigen presentation, CD8+ T cell activation does not occur, and a T cell mediated cytotoxic response to clear infected cells does not happen, granting the parasite an enormous advantage to pursue the blood stage of infection[27]. Malaria parasites are an obligate intracellular pathogen, which enables the avoidance from antibody-mediated

immune response[41]. In addition, the parasites have evolved a rapid transition from one cell to another, e.g. sporozoites take 10-15 min from skin to liver[42], and merozoites will invade a red blood cell in less than 30 s[43]. The parasite life's strategy poses difficulties for the immune system, with insufficient time for antibodies to act. Due to the reasons mentioned above, sporozoites and immature liver-stage elicits little to no inflammation[44], indicating the existence of a mechanism in the parasites that turns their pathogen associated molecular patterns (PAMPs) in the invading parasite unavailable to the pattern recognition receptors in the human host (PRR)[45]. This was demonstrated by studies that observed inflammation responses in radiation-attenuated sporozoites but not in viable *Plasmodium yoellii* infection in BALB/C mice[46, 47].

After sporozoites penetration of the skin, priming by T and B cells takes place in the lymph nodes or in the liver, where they invade hepatocytes[41, 48]. Antibodies neutralize sporozoites in the skin to prevent their invasion of liver cells[28]. Natural Killer (NK) cell and T cell derived IFN-γ is assumed to enhance phagocytosis of red blood cells by macrophages[41, 49].

Previous studies indicate that cytokines produced during *Plasmodium* infection are associated with gametocytes killing in the blood stream, due to increase of the pro-inflammatory cytokines tumor necrosis (TNF)-α and interferon (IFN)- γ produced by the host immune system[50-53]. Inflammatory cytokine's role in limiting malaria parasitemia is via activation of innate immune mechanisms of monocytes and macrophages[5]. Induction of phagocytosis and increased nitric oxide (NO) production by leukocytes are attributed to gametocyte inactivation and loss of mosquito infectivity[53, 54]. Dendritic cell (DC) encounter and recognize infected erythrocytes

via PRR and present to CD4+ cells and could cross-present antigen to CD8+ T cells[41]. Cytokines like IL-12 facilitate activation of natural killer (NK) cells, leading to IFN- γ production, which promotes the activation of adaptive and humoral immune responses via antibody subclass switching[27].

In summary, NK cells, T cells, and phagocytes mediate the main immune events following *Plasmodium* infection by restrain of the earliest phase of parasite growth. T cells may limit parasitemia and antibodies clear the residual infection[28, 49].

1.3.2 Mosquito Innate immunity and Malaria

The peritrophic matrix, known as PM, and the midgut epithelium are the physical barriers to parasite invasion in the mosquito midgut. The PM formation is stimulated by bloodmeal and its structural composition involves proteins, glycoproteins, and proteoglycans that are structurally linked by chitin[55]. The PM gradually thickens and polymerizes reaching maximal rigidity 24 h after bloodmeal; therefore it constitutes a physical barrier between bacteria and other elements present in the blood bolus and the midgut epithelium[26, 56, 57]. The motile ookinete once egressed from the PM has to traverse the midgut epithelium resulting in severe damage and eventual death of the invaded epithelial cells. As a result, there is an increase of nitric oxide synthase expression[38] along with high levels of peroxidases, which in turns leads to an increase in midgut nitration[58]. This is thought to be a critical determinant of parasite recognition by the mosquito complement-like pathway[59].

A bloodmeal containing malaria parasites, besides being sufficient to trigger *Plasmodium* immune responses prior to ookinete invasion of the midgut epithelium [60], also carries immune components from the human host (e.g. cytokines, leukocytes, NO,

and complement system) which influence parasite development inside mosquito midgut lumen[61-63]. In addition, there is evidence that the mosquito microbiota play a direct role in the activation of the mosquito immune response, due to the drastic proliferation that occurs immediately after bloodmeal[64-66]. Contributions of mosquito tissues to the anti-plasmodium immune response that limits *Plasmodium* development are multi-faceted involving multiple mosquito tissues e.g. fatbody (systemic immunity, humoral immunity via antimicrobial peptides)[67], midgut and salivary glands (local epithelial immunity, pathogen recognition and physical barrier)[60, 68], and hemocytes (cellular immunity, circulating immune components and phagocytosis)[69, 70].

Expressions of immune markers and mRNA levels of antimicrobial peptides e.g. defensin, a putative serine protease (ISPL5), a putative Gram-negative binding protein (GNBP), a chitinase-like domain containing protein (ICHIT), a lectin-like protein (IGALE20) and nitric oxide synthase (NOS) increase in the midgut 24 h post *Plasmodium* infection[38, 71-73]. These innate immune components are responses directed to eliminate most invading parasites. Therefore, *Plasmodium* parasites encounter major obstacles in the midgut tissue stage of mosquitoes where most parasites are cleared by the innate immune system[23, 33, 55]. In the event the ookinete successfully traverses the midgut epithelium, the mosquito host immune system has two mechanisms against the invading ookinete: an 'early-phase' and a 'late-phase' response. The early-phase response mechanism relies on the activation of complement-like system after interaction of the soluble immune proteins that circulate in the haemolymph following parasite invasion of the luminal side of the mosquito midgut[69, 74]. The 'late-phase' response attack comes upon the ookinetes that survive the 'early-phase'

immune response and it is controlled by the signal transducer and activator of transcription (STAT)-A or STAT-B. Knock down experiments showed that silencing of STAT-A or STAT-B increased oocysts survival through decreased production of NOS[75].

The Toll and Imd receptor immune signaling pathways are two conserved immune pathways in mosquitos for host defense that induce the production of effector molecules, of which antimicrobial peptides are the most prominent [76, 77]. RNAi silencing experiments are the strongest evidence of the involvement of these pathways in *Plasmodium* immunity[60, 78]. Survival of ookinetes in the midgut epithelium has been shown to depend on the action of agonists and antagonists. Recent studies have identified infection-inducible putative pattern recognition receptors, e.g. TEP1, LRIM1, APL1, and FBN9 that can mediate killing of ookinetes in the midgut epithelium[69, 79, 80]; in contrast, the c-type lectins CTL4 and CTLMA2, and Fibrinogen-like protein 1 (FREP1) can protect the ookinetes from destruction[81, 82]. The synergistic effects of the physical barriers and the immune components of the mosquito's midgut result in a bottleneck of the *Plasmodium* lifecycle, with a drastic decrease in the parasite population [23, 83]. In fact, in the majority of endemic areas only a small portion of the mosquito population carries the parasite[84-87]. Thus, the mosquito midgut stage of parasite development constitutes an optimal target for malaria interruption vaccines, namely for two reasons: the potential to enhance transmission blockade by exploiting the natural occurrence of a several fold decrease in the parasite population; and, due to the generic innate immune effector mechanism of mosquitoes, there is no highly antigen-specific immune response, which results in low polymorphism on mosquito sexual

parasite stage surface proteins, contrary to the asexual human stages[88].

1.4 Current Scenario of Malaria Control

Three main reasons could account for the current status of malaria infections around the world: first, there are no drugs available that can cure infected humans on a routine basis and on a large scale. In addition, parasites have developed drug resistance against most common anti-malarial drugs, including the recent developed family of antimalarial drug – the artemisinins, extracted from the Qinghaosu plant (*Artemisia annua*) – and quinine from the cinchona tree (*Cinchona* spp.,)[7]. This resistance is attributed to the large genetic and genomic plasticity of the parasites[89, 90]. Second, the rapid spread of insecticide resistant mosquitoes[91-94], in addition to a lack of environmentally, large-scale effective vector control programs[13]. Third, there are no commercially available vaccines that can offer protection against the protozoan *Plasmodium,* the causative agent of the disease[95]. Morphologically, malaria parasites are complex with many antigenic targets, which often result in challenges to elicit a high immunogenic response; thus an alternative approach based on subunit vaccines has been adopted[96, 97]. Subunit vaccines constitute individual recombinant parasite proteins administered either as monovalent or combined preparations incorporated with other vectors and adjuvants in order to heighten immune responses[27, 98, 99]. The WHO set the goal in 2006 to license an efficacious vaccine with >50% protection by 2015[14, 100]. However, only one candidate vaccine RTS, S/AS02A has obtained licensure. This vaccine is far from ideal, having demonstrated only 46% efficacy[101-105]. Activity of this potential vaccine is based on repeat regions and T-cell epitopes of circumsporozoite protein (CSP), and it is the primary focus of many phase 3 clinical trials among children

1.5 The call for eradication

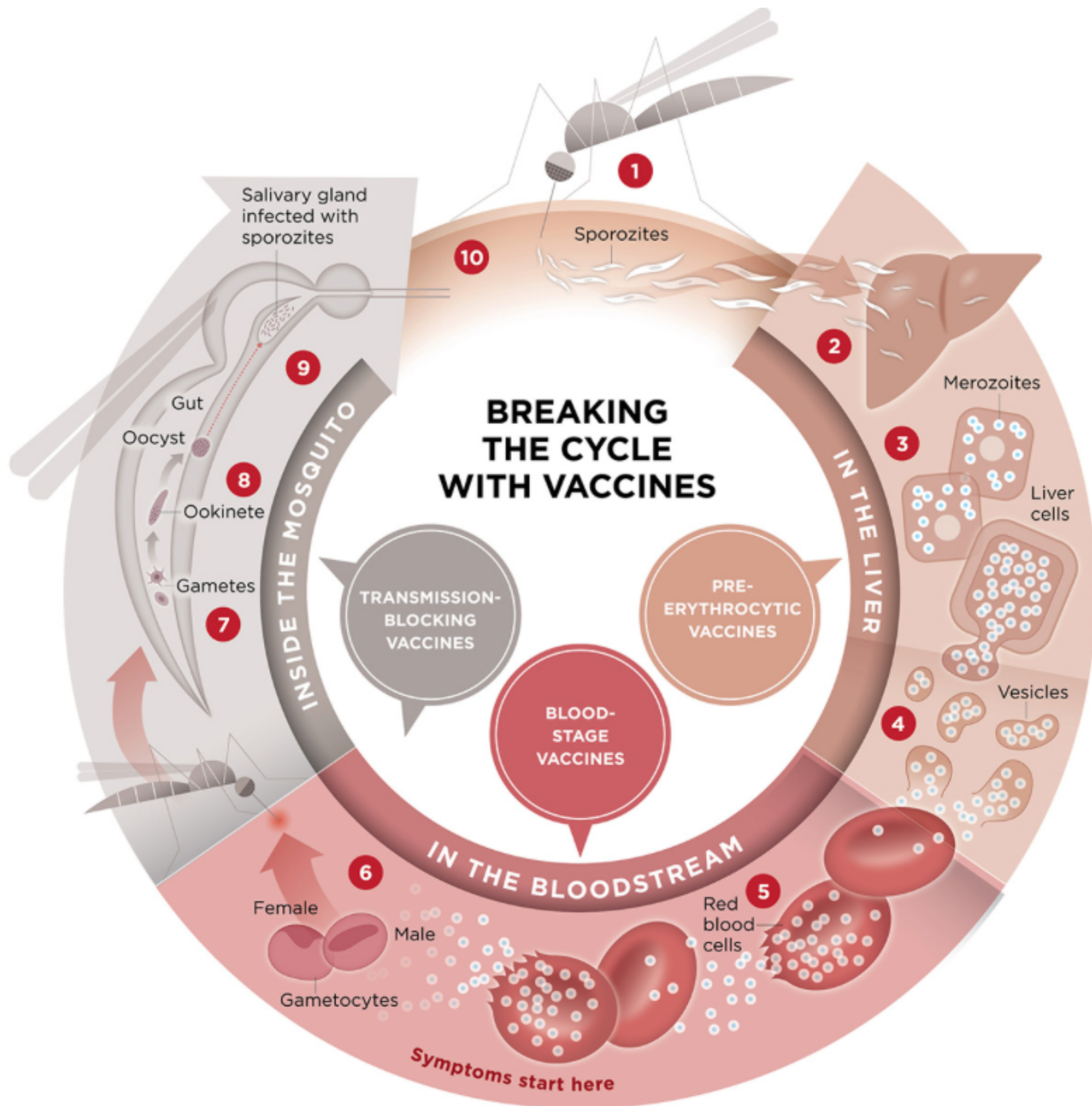

Figure 1-2. Malaria vaccines target different stages of the parasite life cycle [97]. Vaccines against the liver-stage, where following the bite **(1)** of an infected mosquito, the vaccine would prevent sporozoites from invading liver cells **(2-4).** This pre-erythrocytic vaccine is also called vaccine that interrupts malaria transmission (PE-VIMT), which interrupts transmission from mosquitoes to humans. Vaccines against the asexual blood stage, which would interfere with the disease-causing stages once the infection had entered the bloodstream **(5-6).** Last**,** vaccines targeting sexual stages of the life cycle (gametocytes **7**, gametes **8** and ookinetes **9**) or mosquito antigens to prevent the development of infectious sporozoites **(10)**. This vaccine aims at interrupting transmission from infected humans to mosquitoes and it is also called a sexual, sporogonic, or mosquito stage VIMT (SST-VIMT). *Reproduced from the PATH website at www.path.org, [October 25, 2016].*

in the sub-Saharan Africa[106, 107].

The venture to control malaria has two main approaches: a direct approach, which consists of using vaccines and drugs to cure it and the indirect approach, which focuses on vector control strategies[95]. Given the current scenario described above, there is an urgent need to develop and deploy drugs and vaccines to prevent transmission of malaria from human to mosquito, as a manner to interrupt the appearance of resistant or multiresistant *Plasmodium* genotypes[108, 109]. A transmission-blocking vaccine is seen as an essential tool in the malaria elimination and eradication efforts, including prevention of reintroduction of the disease, and when combined with other control measures, TBVs could assist a given geographic region in overcoming the threshold from control to elimination stage[12-14, 110].

Vaccines developed against the malaria parasite potentially target three stages of the development (Figure 2): vaccines against the liver-stage, where following the bite of an infected mosquito, the vaccine would prevent sporozoites from invading liver cells preventing clinical disease as well as transmission [14, 111]; those against the asexual blood stage targeting merozoites or infected red blood cells, which would interfere with the disease-causing stages once the infection had entered the blood[112, 113]; and those against the parasites sexual stages and the stages inside the mosquito midgut, aimed at preventing parasites from infecting the arthropod vector[9, 33, 114]. The latter type of vaccine is designed to reduce or block the transmission of malaria in human and mosquito populations; thus they are termed malaria transmission blocking vaccines (TBVs)[20, 33, 115]. TBVs are alternative vaccines that target sexual erythrocytic and early stage mosquito antigens, preventing the spread of the pathogen from an infected

individual to a noninfected individual. The principle of action is based on the antibodies raised against antigens expressed in the sexual stages of the *Plasmodium* parasites or antigens in the mosquito itself[116, 117]. The antibodies are presented to the mosquito in the bloodmeal, which once inside the mosquito midgut, target their antigens leading to interruption of the cascade of events that leads to the transmission cycle of the parasite[118, 119].

TBVs are commonly labeled as being altruistic vaccines since these vaccines do not offer clinical protection for the recipient but rather establish herd immunity[33]. An understanding of the biology and the epidemiology of malaria transmission is primordial for comprehending the practical employment and the potential impact of TBVs[14]. In order for malaria transmission to occur, an interaction must take place between an appropriate *Anopheles* mosquito and a human population that resides within a strictly limited distance from the aqueous breeding site of these mosquitos[114]. Thus, malaria transmission is a local feature of a given area, in which practices to reduce or exclude malaria transmission in small areas of up to 1 km of radius is attainable with measures that include drainage, residual spraying, and TBV[114]. Indeed, TBVs are intended to stop transmission in local community where their deployment would benefit members of the same household[33, 100]. In additional to their potential to reduce malaria transmission rates in human population, malaria TBVs could prolong the effective life of other malaria vaccines as they could prevent the scape of the parasites from human host. In this manner, if used in combination with liver and blood stage vaccines, TBVs could synergistically enhance the efficacy of these individual vaccines by preventing or reducing the spread of parasites that became resistant to such vaccines[14, 15, 41]. The

challenges involved in the development of TBV candidates are antigens with poor immunogenicity, epitope conformation dependence and low conservation of antigen across different species. [20, 33, 60, 114, 118, 120]. In addition, since TBV vaccines do not confer individual protection but 'herd immunity', there is a possibility that regulatory processes will be more complex, as this may influence the cost-benefit analysis made by governments and funding agencies as well as its acceptance at the community level[14, 41, 100].

Finding target molecules on parasite surface or in mosquito midguts is needed for developing vaccines against malaria.

1.6 Genomics and Vector Control

Genetic association studies are used to find candidate genes or genome regions that contribute to a specific disease by testing for a correlation between disease status and genetic variation[121]. Single nucleotide polymorphisms (SNPs) are particular base pair positions in the genome where alternative nucleotides distinguish individuals[122]. The genome of *Anopheles gambiae* is 278 million base pairs long and it is replete of nucleotide polymorphisms with an average of 1 SNP per 247 base pairs[123]. According to Aguilar *et al* (2010) the capability of the mosquito to transmit malaria to humans in a large range of ecological settings can be explained by this high density of SNPs in their genome. The authors affirmed, " …this large numbers of molecular and chromosomal polymorphisms provide a great evolutionary potential as a reservoir of genetic variability"[124]. Genetic variation in mosquito populations affects the mosquitoes' susceptibility to *P. falciparum* infection[125-127], insecticide resistance[122], and other

traits of interest. Genome wide association studies focusing on mosquito immunity have contributed to population management and disease control[128].

Through direct genome-wide association studies in wild mosquitoes from malaria-endemic regions in Africa, we recently identified a mosquito gene, fibrinogen-related protein 1 (*FREP1*) that is implicated in *Plasmodium* infection in mosquitoes[125]. Specific genetic polymorphisms in *FREP1* are significantly associated with *P. falciparum* infection intensity levels in wild *An. gambiae* populations from Kenya. The FREP1 protein is a member of the fibrinogen-related protein family (FREPs or FBNs) that contains a highly conserved C-terminal interacting fibrinogen-like (FBN) domain[80]. In vertebrates, fibrinogen molecules usually associate as hexamers and are comprised of two sets of disulfide-bridged α, β and γ chains that participate as a principal component of both cellular and fluid coagulation[129]. In invertebrates, FREPs/FBNs are common pattern recognition receptors (PRR)[130, 131] responsible mainly for initiating innate immune responses[132, 133]. For instance, tachylectin proteins in the horseshoe crab perform an effective host defense by recognizing bacterial lipopolysaccharides[134]. Since mosquitos lack an antibody-mediated response, they rely on PRRs to initiate innate immune response against pathogens[135, 136]. Previous work examining the role and function of FREP/FBN family members in *Anopheles* mosquitoes has shown that two family members, FBN9 and FBN30, appear to restrict *Plasmodium* infection of midgut epithelial cells[80]. Indeed, silencing the expression of either FBN9 or FBN30 in mosquitoes increased *Plasmodium* infection[80, 125]. Here, I report the role and function of a third FREP/FBN family member, FREP1, during *P. falciparum* infection of *Anopheles* mosquitoes. In contrast to FBN9 and FBN30 that

inhibit *Plasmodium* infection, our results[72, 114] showed that FREP1 is an important host factor that promotes infection of mosquito midguts by the major human pathogen, *P. falciparum*[82]. Altogether, our data reveal new insights into *Plasmodium-Anopheles* interactions and identify FREP1, a highly conserved mosquito antigen across malaria vectors, as a promising target for the development of vaccines that interrupt malaria transmission.

1.7 Summary of Dissertation

In my graduate studies, I investigated the biological and molecular function of our newly discovered molecule FREP1 that is related to malaria transmission in mosquitoes, and my contribution is finding the localization of endogenous FREP1 in mosquito midgut peritrophic matrix. Next, I constructed an expression vector using FREP1 signal peptide-directed secretion system to efficiently secrete heterologous proteins from insect cells. Then, I proved that the FBG is the functional domain of FREP1 having a dual role assisting *Plasmodium* invasion of mosquito midgut by directly binding to the parasite and the PM, facilitating ookinete penetration of the midgut epithelia. Finally, I demonstrated that the FBG domain could be used as a vaccine target to interrupt transmission of multiple *Plasmodium* species to different *Anopheles* mosquitoes.

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Chapter 2: *Anopheles* FREP1 Signal Peptide-Directed Secretion of Recombinant Proteins from Insect Cells

2.1 Introduction

Prokaryotic expression system, even though it has the advantage of a rapid growth rate and relatively low cost, in most circumstances recombinant proteins cannot be secreted into the extracellular medium [1]. Moreover, formation of inclusion bodies and the lack of post-translational modifications (e.g., glycosylation, phosphorylation, and disulfide bond formation) hinder the bacterial expression system for production, especially, of secreted proteins that require those modifications as well as proper folding for biological activity [2, 3]. Insect and mammalian cells have been the expression systems of choice for functional recombinant proteins for a wide range of applications[4-6]. Whilst mammalian cells cultures can be complicated, time consuming and expensive [7], baculovirus expression systems in insect cells presents a series of biological and engineering disadvantages[8, 9]. The cabbage looper ovarian cell-derived High Five cell line (BTI-TN-5B1-4), developed by the Boyce Thompson Institute for Plant Research (Ithaca, NY)[10-12] has proven to be particularly effective in producing abundant levels of biologically active recombinant proteins of different sizes and organisms[13-15]. However, it takes a considerable effort to obtain highly purified recombinant proteins expressed in the cytosol of cells. Therefore, high expression level and convenient purification of heterologous eukaryotic proteins of interest are critical for biotech industry and research labs to generate affordable active proteins.

Signal peptides have an essential role in protein export and membrane insertion of secretory and membrane proteins[16, 17]. Different signal sequences have been

observed assisting in the transport of their target proteins through common pathways and they can be interchanged between proteins, and even proteins from different origins[18, 19]. Furthermore, it has also been noted that these interchangeable signal peptides can lead to increased protein secretion[20]. Proteins secreted to extracellular media simplify the purification process, especially in serum-free insect cell cultures.

FREP1 is a blood induced secreted protein in mosquito midgut peritrophic matrix and is able to facilitate *P. falciparum* infection in *An. gambiae.* Previously, we demonstrated that FREP1 can be efficiently secreted to the culture medium. [21]. In this report, we compared FREP1 signal peptide to *Apis mellifera* melittin signal peptide [22], and *An. gambiae* FBN30 signal peptide for their ability to secrete a foreign protein in High Five insect cells. In this study, we also examined the secretion of normally non-secreted and secreted proteins using the *An*. *gambiae* FREP1 signal peptide in High Five cells[23]. The results reported here showed a substantial increase in the overall level of secretion of the recombinant proteins when using FREP1 signal peptide. Conceptually, our data support that both signal peptide and intrinsic sequences of mature proteins determine secretion efficiency.

2.2 Material and Methods

2.2.1 Cloning signal peptides into expression vector

The expression vector pIB/V5-His (Life Tech, Grand Island, NY) was used in order to analyze the secretion efficiency of the mosquito FREP1 signal peptide via the InsectSelect^{TM} system[23] (Invitrogen). The vector is under the control of the immediate-early promoter *OpIE2* from the baculovirus *Orgyia pseudotsugata* multicaspid nuclear polyhedrosis virus (*Op*MNPV), which, allows for constitutive

protein expression in High Five cells (Invitrogen), among other insect cell lines. Oligonucleotides containing a Kozak consensus sequence and the respective sequence coding for the *Anopheles gambiae* FREP1 signal peptide were synthesized (Live Technologies, Carlsbad, CA) (F FREP1 SigP and R FREP1 SigP 1, 2, or 3, see Table 1). The signal peptide sequence and cleavage site of FREP1 were determined with the signal peptide prediction server SignalP ver. 4.1[32]. Three different linker regions were cloned into the expression vector, with a multiple cloning site with the following endonucleases restriction sequences BamH I, Sac I, Spe I, and Xho I added to the 3' end of the signal peptide for further cloning. The signal peptide of FREP1 and its fibrinogen-like domain (FBG) were amplified using primers of F FBG Domain and R FBG Domain (Table 1) from the previously constructed pIB-FREP1/V5-His vector [21]. Oligonucleotides containing the Kozak consensus and the well-characterized honeybee melittin (HBM) signal peptide with its cleavage site[22] were synthesized with oligers of F HBM-pIB and R HBM-pIB (Table 1) through Life Technologies (Carlsbad, CA) and annealed in order to generate a dsDNA with a 5' GATC (sense strand) and a 5' TTAA (antisense strand) extension compatible with overhangs created by restriction endonuclease BamH I (NEB, Ipswich, MA) and *Eco*RI (NEB). The pIB/V5-His vector (Life Tech) was digested with the restriction endonucleases BamH I and *Eco*RI and the annealed oligonucleotides was cloned into this vector.

Table 2-1. Primers list for cloning signal peptides into pIB/V5-His.

Primer Name	Nucleotide Sequence 5'-3'
pIB/FREP1 SigP /V5-His	
F FBG pQE30	5'-ACGCATGCCACGAGTACGAGTCGATC-3'
R FBG pQE30	5'-TGCAAGCTTGTACTCGTGCTCGACCGACTC
F FREP1 SigP	5'-GCGAAGCTT*CACC*ATGGTGAATTCATT
R FREP1 SigP 1	5'-TACGCTCGAGGTCGACACTAGTGGATCCGTTA CTGCTGCTGCTGCTG-3'
R FREP1 SigP 2	5'-TACGCTCGAGGTCGACACTAGTGGATCC CCCAGACACTGCACCGTT-3'
R FREP1 SigP 3	5'-TACGCTCGAGGTCGACACTAGTGGATCCGTTC AGGGCAGT-3'
F FBG Domain	5'-GATCCACTAGTGTCGACCTCGAGGAGTCGAT CATCAAC-3'
R FBG Domain	5'-ACTCTAGAGTCGGCCGGCGGGCAATCATC ATGT-3'
F AgFBN30CDS	5'-ATGCTGCTCGCAACAGTTTC-3'
R AgFBN30CDS	5'-CTACGGTGCACTACGAAGCC-3'
F FREP1SigP/FBN30	5'-AGCGTCGACCAACTCCTTGCCGCTAGTGTT-3'
R FREP1SigP/FBN30	5'-ACGTCTAGAGTCGGTGCACTACGAAGCCGA-3'
F FBP4	5'-CGGGATCCATGAGAGAAGTAATAAGTATAC-3'
R FBP4	5'-GCTCTAGAATAGTCTGCCTCATATCCTTC-3'
pIB/HBM/V5-His	
F HBM-pIB	5'-GATCC*CACC*ATGAAATTCTTAGTCAACGTTGC CCTTGTTTTTATGGTCGTATACATTTCTTACATCT ATGCGGATCTAG-3'
R HBM-pIB	5'-AATTCTAGATCCGCATAGATGTAAGAAATGTA TACGACCATAAAAACAAGGGCAACGTTGACTAA GAATTTCATGGTGG-3'
F HBM-FBG	5'-TCAGAATTCGAGTCGATCATCAAC-3'
R HBM-FBG	5'-ACTCTAGACTCGGCCGGCGGGCAATCATCA TGT-3'

2.2.2 FBG domain cloning, expression and purification from *E. coli*

The FBG fragment was amplified with the gene specific primers of F FBG pQE30 and R FBG pQE30 (Table 1) from the full-length coding sequence of *An. gambiae* FREP1, as described previously [21]. The PCR fragment was cloned into pQE30 vector according to the manufacture's instructions (Qiagen, Hilden, Germany) and transformed into the *E. coli* strain JM109 (Promega, Madison, WI). PCR analysis and DNA sequencing further confirmed the ampicillin resistant colonies. The positive clones were then transformed into *E. coli* strain M15[pREP4] (Qiagen) competent cells and induced with 1mM isopropyl-1-thio-β-D-galactopyranoside at room temperature. The recombinant FREP1 FBG protein was purified on a Ni-nitrilotriacetic acid column (Qiagen) and the eluted fractions were resolved on 12% SDS-PAGE gel and visualized by Coomassie blue staining. All fractions containing protein were then combined and protein concentration was determined using the Bradford protein assay.

2.2.3 Cell Culture

High Five™ (BTI-TN-5B1-4) cells[10, 33] were routinely maintained at 27ºC in Express Five® Serum Free Medium (SFM) (Invitrogen) supplemented with 200mM L-glutamine (Sigma-Aldrich) in adherent culture. In order to establish stable cell lines, High Five cells were seeded into six-well culture plates (35mm diameter) at 40-60% confluent (5×10^5 cells/mL) (2.5mL/well), and transfected for 4 hours with 1mL of transfection solution containing 5µL Cellfectin® Reagent (Invitrogen) and 2.5µg of plasmid DNA in Express Five® SFM medium. Forty-eight hours after transfection, the culture medium was replaced with fresh medium containing 50µg/mL blasticidin (BSD)

(Invitrogen). Heterogeneous populations of transfected cells expressing recombinant protein were obtained by weekly sub-culturing in six-well plates in the presence of 50μg/mL BSD. Cell counts and viability were determined using trypan blue exclusion method.

2.2.4 Expression of Heterologous Proteins in Insect cells

Secretion vectors containing the FREP1 signal peptide were used for cloning different genes to test their expression levels. The FBG domain of FREP1 was cloned as described above. FBN30 was obtained by RT-PCR with gene specific primers (F AgFBN30CDS and R AgFBN30CDS, Table 1) from *An. gambiae* mosquitoes. FBN30 PCR product containing its native signal peptide was cloned into pIB/V5-His vector (Life Technology, Grand Island, NY). For comparative expression, a FBN30 without its signal sequence was amplified with primers of F FREP1SigP/FBN30 and R FREP1SigP/FBN30 (Table 1) and cloned into the expression vector pIB-FREP1 SigP-V5/His. Similarly, α-tubulin-1 (plasmodb gene ID PBANKA_0417700)[34] was amplified with gene specific primers of F FBP4 and R FBP4 (Table 1) from *Plasmodium berghei* ANKA stain by RT-PCR, and cloned into the plasmid. After being amplified in *E. coli* DH5α, the plasmids were purified using GenElute Endotoxin-free plasmid preparation kits (Sigma, USA). The purified recombinant plasmids were transfected into 40-60% confluent High Five cells. To assist plasmid transfection into cells, Cellfectin® reagent was mixed with each individual plasmids (1μL Cellfectin/μg plasmid) in 5-6 mL Express Five® SFM medium in a 25cm^2 CELLSTAR® cell culture flask (Greiner Bio-One) at 27°C. Media and cells were collected 3-5 days post transfection and analyzed for expression of recombinant protein.

2.2.5 Enzyme-Linked Immunosorbent Assay (ELISA)

After transfection, cells were assayed for expression of the heterologous protein. Transfected culture media supernatant and cell lysate (50μL) were used to coat 96-well plate (Brand, Wertheim, Germany). Following overnight incubation at 4ºC, each well was then incubated with the following solutions: 150 μL blocking buffer (2.5% BSA and 2% Normal goat serum in PBS) for 1.5 hours, 50 μL of mouse monoclonal anti-His antibody (1:1,000 dilution with blocking buffer) for 1 hr at RT and 50 μL of alkaline phosphatase-conjugated goat anti-mouse IgG (1:20, 000 diluted in blocking buffer) for 45 min at RT. The wells were washed with PBST (PBS with 0.2% Tween-20) three times between incubations. Plates were developed with 50 μL of pNPP solution (Sigma-Aldrich, St. Luis, MO) and the absorbance at OD_{405} was measured using an Epoch Microplate reader (Biotek).

2.3 Results

2.3.1 Expression of recombinant FBG protein in *E. coli* and insect cells

FREP1 containing 738 amino acids mediates *Plasmodium* invasion in mosquitoes, and fibrinogen-like domain (FBG) is a functional domain, which is at the FREP1 C-terminal spanning from amino acids 463-688. We cloned the FBG domain in pQE-30 plasmid and expressed it in *E. coli*. After breaking the cells by sonication, results showed that the expressed recombinant FBG protein was insoluble in PBS (Fig 2-1A). To obtain soluble functional protein, the FBG fragment was cloned into pIB/V5-His plasmid and expressed in High Five cells. SDS-PAGE showed that FBG was expressed in the cytoplasm at very low levels (<0.1μg/mL, Fig 2-1B). Due to low expression and high contamination, it is an arduous task to purify FBG.

2.3.2 Evaluation of secretion efficiency of two signal peptides of insect origin in insect cells

To increase the production and facilitate the purification, we modified pIB/V5-His by adding a HBM signal peptide sequence at the beginning of the multiple cloning site- linker region, to generate pIB-HBMSigP/V5-His (Fig 2-1C). The high expression levels of the HBM signal peptide has been demonstrated previously [24]. The coding region for HBM was generated through total synthesis (F HBM-pIB and R HBM-pIB, Table 2.1), and inserted into polyclonal sites (Fig 2-1C). Then the FBG fragment was obtained through PCR using the F HBM-FBG and R HBM-FBG primers (Table 2.1). After digestion by restriction enzymes, FBG was inserted into this modified plasmid adjacent to the HMB signal peptide (Fig 2-1C). The plasmid was transfected into High Five cells, and recombinant FBG was successfully secreted into the culture medium. The transient expression levels were kept stable after 48 hours and were consistently expressed with the stable cell lines as well (Fig 2-1D).

We then substituted HBM signal peptide with FREP1 signal peptide to secrete recombinant FBG fragment (Fig 2-1C) from High Five. This plasmid was also transfected into High Five cells. After incubation for two days, 10 μL supernatant were loaded on SDS-PAGE. The Coomassie blue stained-gel exhibited a denser band from FREP1 signal peptide guided secretion than that from HBM signal peptide (Fig 2-1D and 2-1E). Furthermore, the secreted recombinant FBG protein was quantified by ELISA using anti-FBG polyclonal antibodies, and the results revealed that FREP1 signal

peptide secreted more than 10-fold of FBG protein in comparison to HMB signal peptide (Fig 2-1F).

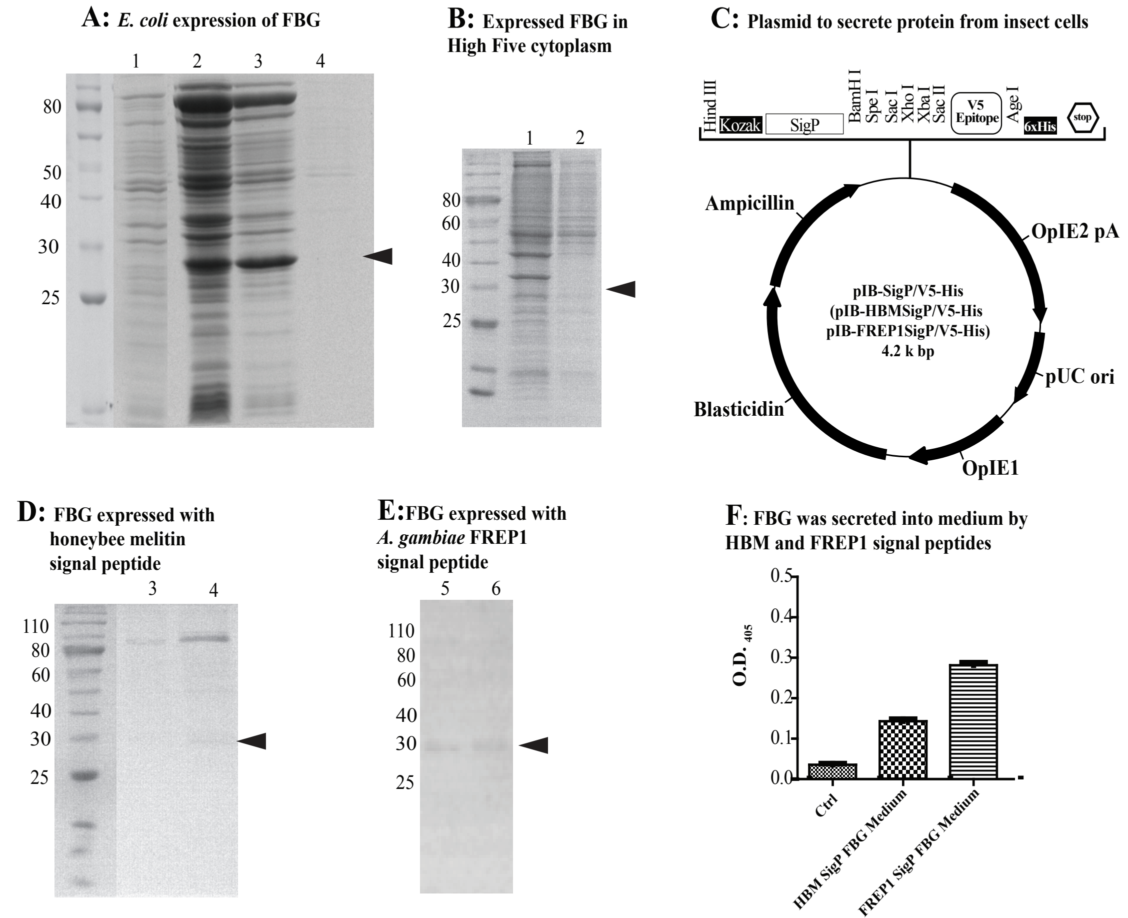

Figure 2-1. Expression of heterologous *An. gambiae* FREP1 FBG domain in *E. coli* and insect cells.

(A) FBG is insoluble after induced expression in *E.coli* showing on Coomassie-stained 12% SDS-PAGE gel. Lanes: 1: cells before induction; 2: cells induced; 3: cell lysate pellet; 4: cell lysate supernatant. **(B)** Recombinant FBG expressed in High Five cells. Lanes: 1: cell lysate; 2: Ni-NTA purified FBG (28.2kDa) from cell lysate. **(C)** DNA plasmid map of the pIB-FREP1 SigP/V5-His. (**D**) FBG was secreted from High Five via HBM signal peptide. (**E**) FBG was secreted from High Five via FREP1 signal peptide. Lanes: 3,5: culture supernatant; 4,6: 10X concentrated culture supernatant. (**F**) ELISA assay shows higher recombinant FBG secretion was achieved through FREP1 signal peptide compared to HBM's signal peptide.

2.3.3 The length of linker region does not affect FBG expression

The signal peptidase cleavage site of FREP1 signal peptide was predicated to be located at the position 22 (Serine) and 23 (Threonine). We engineered three different lengths of amino acids (5, 9 and 22) at the linker region between the signal peptide and the FBG domain (Fig. 2-2A). After expression in High Five cells, all constructs resulted in secreted FBG proteins into the culture medium. No significant difference in expression levels of the recombinant FBG protein among constructs was observed as the linker region varied from 5 amino acids to 22 amino acids (Fig. 2-2B), which indicates that the size of linker regions has no detectable effect on the expression of the FBG recombinant protein. We also expressed the full length FREP1 under the same condition (Fig. 2-2A), and expression level of full-length FREP1 is much higher than the others (Fig. 2-2B). This indicates that intrinsic mature proteins as a whole influences the protein secretion efficiency.

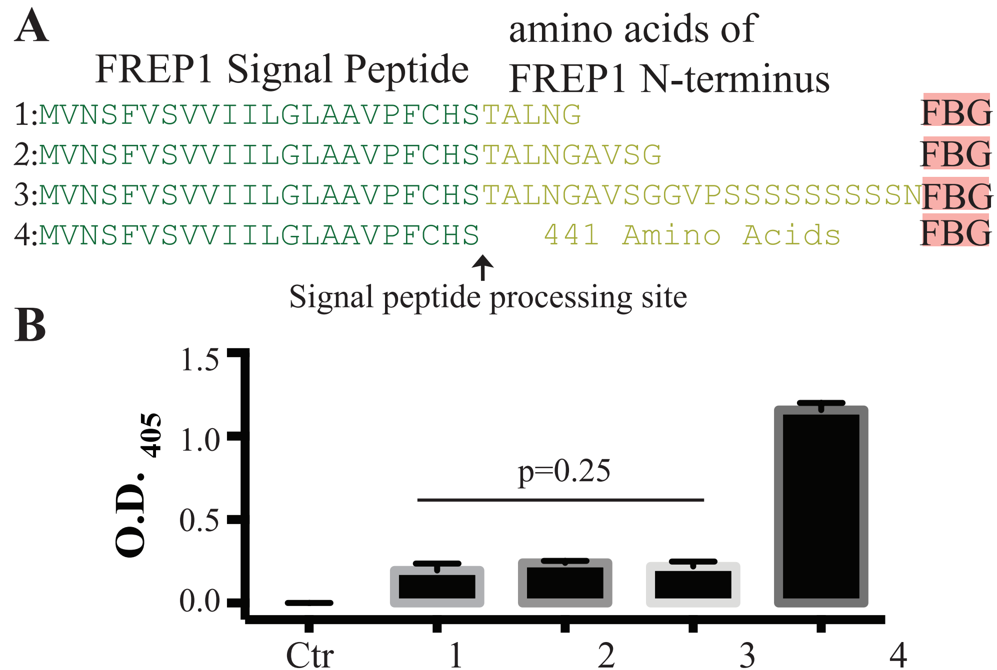

Figure 2-2. Various length of linker between signal peptide and mature FBG does not influence the secretion efficiency of FBG.
(A) Four different lengths of amino acid sequences from FREP1 N-terminus were added between signal sequence and FBG. **(B)** Secreted FBG in culture medium was quantified by ELISA. Lanes: Ctr: cells with empty vector; 1, 2, 3: cells containing vector with five, nine, and 22 extra amino acids at cleavage site respectively. Lane 4: full-length FREP1.

2.3.4 FREP1 signal peptide outperforms a mosquito FBN30 native signal peptide in recombinant expression of FBN30

It has been indicated previously the dependence of a protein on its original signal peptide for its proper synthesis and expression [25]. To demonstrate that the high secretion levels mediated by FREP1 signal peptide are not restricted to *FBG* fragment, we cloned a mosquito gene *FBN30* (*AGAP006914*) into the plasmid pIB-FREP1SigP/V5-His (Fig 2-1C). FBN30 was previously found to be related to malaria infection in mosquitoes [26], and it is a secreted protein endogenously. Therefore, we constructed two pIB/V5-His expression vectors to secrete recombinant FBN30 protein:

one used FBN30 native signal peptide and the other used FREP1 signal peptide. The two plasmids were used to express FBN30 in High Five cells under the same expression conditions. Ten μL concentrated from ~0.25 mL cell culture medium supernatant was loaded to SDS-PAGE followed by staining or western-blotting, detected by anti-FBN30 polyclonal antibodies. Results from Coomassie blue-stained SDS-PAGE gel and Western blot show that both systems secreted recombinant FBN30 protein. FREP1 signal peptide secreted higher amount of FBN30 protein than FBN30 native signal peptide. The recombinant FBN30 expressed with its native signal peptide is barely detectable (Fig. 2.3A) whereas recombinant FBN30 expressed with the FREP1 signal peptide is promptly detectable (Fig. 2-3B). Less than 0.1 μg/mL of recombinant FBN30 was recovered from expression with its native signal peptide and could only be detected in the Western blot (Fig. 2-3A), whereas recombinant FBN30 expressed with FREP1 signal peptide was about 10μg/mL (Fig. 2-3B). This result supports our hypothesis that a signal peptide of an endogenous protein must play a role to regulate a protein expression.

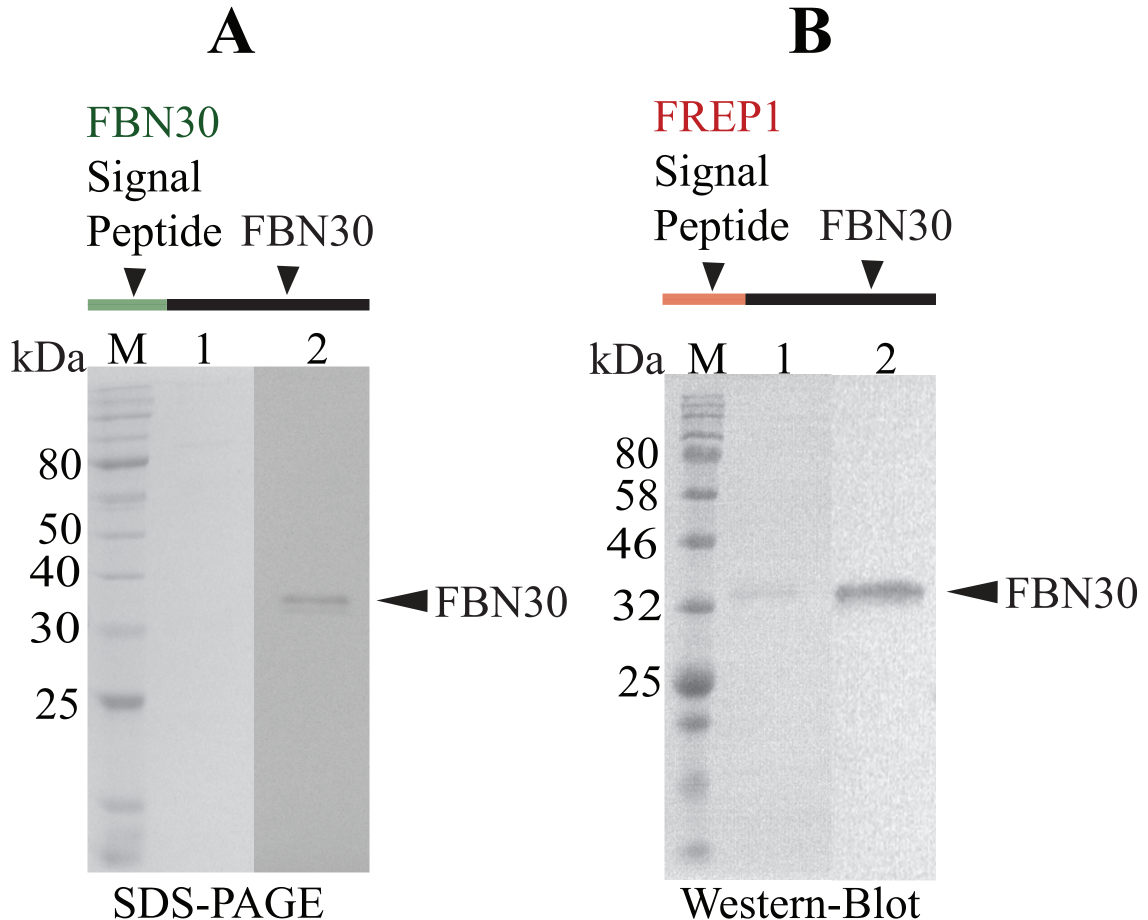

Figure 2-3. Secretion of recombinant FBN30 protein using FREP1 signal peptide is higher compared to its original signal peptide. (A): Recombinant FBN30 was secreted into culture medium using the native signal peptide. Lane 1: Coomassie-stained SDS-PAGE gel of 10µL sample of Ni-NTA purified FBN30 from 25X concentrated supernatant culture media; lane 2 Immunoblotting of gel shown in lane 1. (B): Recombinant FBN30 was secreted in culture medium through FREP1 signal peptide. Lane 1: 10µL sample of Ni-NTA purified FBG from ~0.25mL culture supernatant; lane 2 Immunoblotting of gel shown in lane 1.

2.3.5 Evaluation of FREP1 signal peptide expressing a non-insect cytoplasmic protein

It is important for the applicability of the signal peptide to be compatible with a broad range of heterologous proteins. In order to determine the potential of the mosquito FREP1 signal peptide to secrete a cytosolic protein into the extracellular medium, we

transfected High Five cells with the secretion vector fused with the *Plasmodium berghei* protein α-tubulin-1 (PBANKA_0417700). The recombinant α-tubulin-1 protein expression was analyzed two days post transfection by ELISA. As shown in Fig. 2-4, the FREP1 signal peptide resulted in the extracellular secretion of a non-secreted *Plasmodium* protein. The expressed proteins were completely secreted since we did not detect any trace amount of the protein. This observation supports the ability of FREP1 signal peptide to target foreign non-secreted proteins for secretion in High Five cells expression system.

2.4 Discussion

High production and easy purification are two important criteria to express heterologous proteins in eukaryotic cells. Therefore, establishing a serum-free insect cell secretion system that can secrete a protein of interest has values. It has been previously demonstrated that protein secretion can be improved by alternative signal peptides [27, 28]. Initially, we attempted to secrete FBG protein using well-characterized HBM signal peptides. Although the recombinant protein was detected in culture medium, but the expression level is very low. To resolve the problem, we constructed FREP1 signal peptide-directed protein secretion plasmid, and successfully secreted 6μg/mL of active FBG recombinant protein in a serum-free adherent culture in two days, which is more than 10-fold higher than HBM signal peptide.

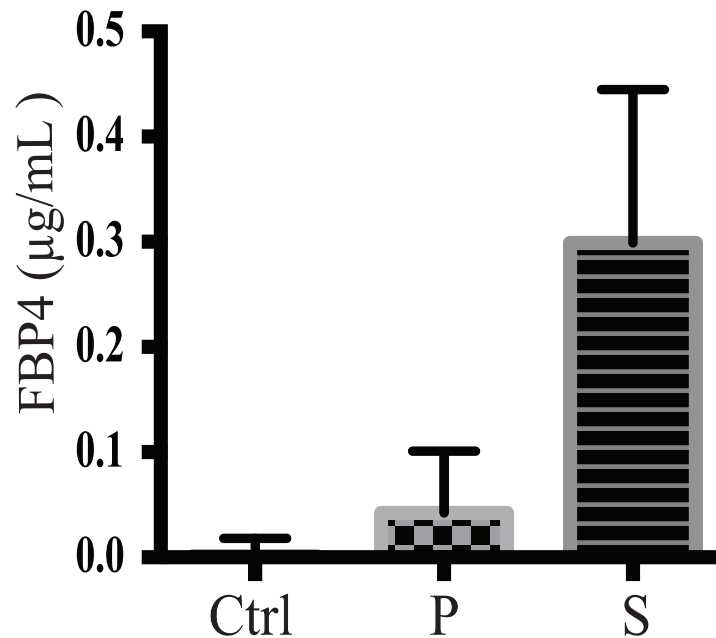

Figure 2.4. Secretion of *P. berghei* tubulin, a cytoplasmic protein using this system. Lanes: Ctr: culture medium with empty vector; P: cell pellet with expression vector; S: culture medium with expression vector. ELISA was used to detect expression of α-tubulin-1 and its product concentration was determined via standard curve.

Furthermore, we studied the secretion efficiency related to different signal peptides, varying length of the linker regions, and different sources of proteins. In this study, signal peptides from three different genes (HBM, FREP1 and FBN30) were studied. For the same recombinant protein, a protein led by FREP1 signal peptide resulted in secretion levels about 10-fold higher than HBM signal peptide or 100-fold greater than FBN30 signal peptide. Thus, signal peptides have an immense impact to the protein production. Second, previous studies have suggested that the secretion efficiency of signal peptides could be affected by the amino acid sequence downstream of signal peptide, particularly at cleavage sites [29, 30]. By keeping the same cleavage site, our results indicate that the length of amino acids from 5 to 22 amino acids at linker regions

does not affect FBG protein secretion. However, the secretion of full length of FREP1 (441 amino acids between signal peptide and FBG domain) is much higher than the FBG alone. This result indicates that a whole mature protein instead of a domain also determines secretion efficiency.

It is notable that protein secretion of FBN30 is lower under its own signal peptide than FREP1 signal peptide. Therefore, the signal peptide must be one of regulatory points for expression of endogenous proteins inside organisms, as a means to respond to the environment. Mutations within signal peptides could thus affect secretion levels of a given functional protein and consequently the organism trait. This is consistent to our previous findings of a naturally occurring mutant of FBN30 signal peptide, resulting in low expression of FBN30 and rendering mosquitos susceptible to *Plasmodium falciparum* infection, contrary to the refractoriness observed in the higher expressed FBN30 in mosquitoes harboring non mutated copies of the gene [26].

In summary, we reported here *An. gambiae* FREP1 signal peptide-directed secretion system that is able to efficiently secrete heterologous proteins from insect cells. The signal peptides and whole intrinsic proteins have an immense impact on protein secretion efficiency.

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Chapter 3: Secreted FREP1 Localizes in the Peritrophic Matrix of *Anopheles gambiae* Mosquitoes and Interacts with sexual stage *Plasmodium* Parasites[1]

3.1 Introduction

Malaria remains a worldwide public health crisis, and *Anopheline* mosquitoes transmit malaria pathogens, of which *P. falciparum* is the most dangerous species(1). Female mosquitoes require bloodmeal for egg production (2). Feeding on *Plasmodium*-infected blood can result in the ingestion of male and female haploid gametocytes that fuse to form diploid ookinetes: a process that initiates *Plasmodium* infection of the mosquito vector. Ookinetes start invading mosquito midgut epithelial cells between 12 to 24 hours after a bloodmeal (3). Un-fused gametocytes or ookinetes located near the periphery of the blood bolus in the mosquito midgut are susceptible to the attack by diverse digestive proteases and bacteria (4-6), while gametocytes and ookinetes inside the blood bolus are protected by blood. However, mature ookinetes must cross and exit the blood bolus in order to initiate invasion of epithelia. Mosquito blood feeding regulates the gene expression (7,8) and also stimulates the formation of the peritrophic matrix (PM) inside the midgut (9). The newly formed PM completely surrounds the ingested blood, separating the blood bolus from secretory midgut epithelia, providing a second physical barrier that limits the infection by pathogens ingested during blood feeding (10). The PM is composed of 3-13% chitin microfibrils and is embedded with many identified (3) and un-identified proteins (11). Notably, when the ookinetes are mature 12 hours after the bloodmeal (9), the PM also becomes visible in the midgut

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lumen. In order to infect mosquitoes, the motile, banana-shaped ookinetes must sequentially attach to and penetrate the PM and the midgut epithelium (12). At present, the detailed molecular mechanisms involved in ookinete penetration of the PM are unclear.

We recently identified a mosquito gene, fibrinogen-related protein 1 (*FREP1*), that is implicated in *Plasmodium* infection in mosquitoes (13). Genetic polymorphisms in *FREP1* are significantly associated with *P. falciparum* infection intensity levels in wild *An. gambiae* populations from Kenya. The FREP1 protein is a member of the fibrinogen-related protein family (FREPs or FBNs) that contains a highly conserved C-terminal interacting fibrinogen-like (FBN) domain. In vertebrates, fibrinogen molecules usually associate as hexamers and are comprised of two sets of disulfide-bridged α, β and γ chains that participate as a principal component of both cellular and fluid coagulation (14). In invertebrates, FREPs/FBNs are common pattern recognition receptors (PRR) (15,16) responsible mainly for initiating innate immune responses (17). For instance, tachylectin proteins in the horseshoe crab perform an effective host defense by recognizing bacterial lipopolysaccharides (18). Previous work examining the role and function of FREP/FBN family members in *Anopheles* mosquitoes has shown that two family members, FBN9 and FBN30, appear to restrict *Plasmodium* infection of midgut epithelial cells. Indeed, silencing the expression of either FBN9 or FBN30 in mosquitoes increased *Plasmodium* infection (13,19). Here, we report the role and function of a third FREP/FBN family member, FREP1, during *P. falciparum* infection of *Anopheles* mosquitoes. Our genetic and biochemical assays reveal that FREP1 functions as a critical molecular anchor in the PM that facilitates *Plasmodium* invasion

and infection of mosquito midguts. Furthermore, our results demonstrate that the highly conserved FBG is a functional domain of FREP1 that is responsible for the interaction between FREP1 protein and *Plasmodium* parasites. Collectively, our data reveal new insight into *Plasmodium-Anopheles* interactions and identify FREP1 as a promising transmission-blocking target.

3.2 Material and Methods

3.2.1 Rearing *An. gambiae* mosquitoes

An. gambiae G3 strain was maintained at 27°C, 80% humidity with a 12-hour day-night cycle. Larvae were reared on ground KOI fish food supplements (0.1 mg per larvae per day). Adult mosquitoes were maintained with 8% sucrose and fed with mouse blood (mice were anesthetized with ketamine/xylazine) for egg production.

3.2.2 Preparation of *P. falciparum* gametocytes and ookinetes

P. falciparum parasites (NF54 strain from MR4) were added into O^+ fresh human blood (4% red blood cells (RBC), 0.25-0.5% parasitemia). Cultures were maintained in 6-well plates (Corning Incorporated Costar) with 5.0 ml complete RPMI-1640 medium supplemented with 10% heat-inactivated human AB-type serum (Interstate blood bank, Memphis) and 12.5 µg/ml hypoxanthine. The plates were maintained under 37 °C in a candle jar (20), and the medium was replaced daily until day 15-17. The parasitemia or gametocytemia was checked every other day by Giemsa staining of thin blood smears. To prepare ookinetes, the cultured *P. falciparum* cultures harboring stage V gametocytes were diluted 10-fold in complete RPMI-1640 (no sodium bicarbonate). The cultures were incubated at room temperature (RT) for 24 hours to simulate the formation of

zygotes and ookinetes (21). The culturing and infection experiments of *P. falciparum* were conducted in the biosafety level 2 lab at the University of Oklahoma.

3.2.3 Generating anti-FREP1 polyclonal antibody

FREP1 was cloned using PCR with primers shown in Table 2 from *An. gambiae* mosquito cDNA library that was generating by reversely transcribing from total RNA. The PCR product and pQE30 plasmid were digested with restriction enzymes Xma I and Hind III. After ligation and transformation into *E. coli* JM109, the positive plasmid verified by PCR was transformed into *E. coli* M15 strain. 1mM IPTG was used to induce gene expression in *E. coli* M15 strain. The expressed cells were lysed in buffer B (8 M Urea, 100mM NaH_2PO_4, 10mM Tris·Cl, pH 8.0). Since there was a 6xHis tag at the N-terminal of expressed protein, the recombinant FREP1 protein was purified by Ni-NTA column using a standard protocol (22). SDS-PAGE and Coomassie Brilliant Blue R-250 staining confirmed the purity of recombinant FREP1 protein. The purified recombinant FREP1 was then used as an antigen to generate customized polyclonal antibody against FREP1 in rabbits (Thermo Fisher Scientific, Rockford, IL, USA). Rabbits were boosted three times in 2-week intervals, after which anti-FREP1 antibody in anti-serum was purified with the affinity chromatograph protein A-agarose and suspended in PBS.

3.2.4 Expressing recombinant full FREP1, FBG and N-FREP1 protein in insect cells

FREP1 complete coding sequence was obtained by PCR with primers shown in Table 3.1 from adult *An. gambiae* cDNA library. Primers specific to the N terminal FREP1 (amino acids 1-461) were used to amplify this fragment (see primers in Table

3.1). FBG domain of FREP1 (amino acids 462-677) was cloned as described in chapter 2. Full length FREP1, N- FREP1 and FBG domain were cloned into plasmid pIB/V5-His (Life Tech, Grand Island, NY) to generate pIB-FREP1, N-FREP1, or FBG-His (encoding the respective protein with a 6xHis-tag), respectively. After amplified in *E. coli* DH5a, plasmids were purified with endotoxin-free plasmid preparation kits (Sigma-Aldrich, St. Louis, MO).

The cabbage looper ovarian cell-derived High Five cells(23) was used to express all three recombinant proteins according to the manufacturers instructions (24). In brief, endotoxin-free recombinant pIB-FREP1 plasmids were mixed with Cellfectin® Reagent (1µl Cellfectin/µg plasmids, Invitrogen) in 5-6ml Express Five® SFM medium (Invitrogen, Grand Island, NY). The cells were cultured in 25cm^2 cell culture flask (Greiner Bio-One, Monroe, NC) for 48 hours at 27°C. Medium and cells were separated by centrifugation at 300 g for 5 minutes. The proteins in medium were concentrated using Amicon® ULTRA-4 Centrifugal Filter Devices (Milipore, Billerica, MA) by centrifugation at 5,000 g for 10 minutes. All recombinatnt FREP1 proteins with 6xHis-tag were purified using Ni-NTA column using a standard protocol (25).

3.2.5 Gel Filtration Chromatography to determine the FREP1 protein size

Similar to the previous description (23), about 0.03-1mg purified high five-expressed recombinant full FREP1 and N FREP1 protein in 0.1ml PBS was applied onto Äkta™ Pure FPLC chromatography system (GE Healthcare) with superdex G-200 increase column (60 cm in length, 0.5 cm in diameter) with flow rate controlled to 0.2 ml/min. Superdex G-200 increase can separate proteins ranging from 5kDa to 600kDa. Fractions of approximately 0.1 ml were collected in an automated fraction collector.

UV280 absorbance was detected constantly, and ELISA was used to detect recombinant FREP1 protein in each fraction. Standard curve of molecule weight for the gel filtration column was obtained using a set of proteins with known molecular weights.

3.2.6 Immunohistochemical (IHC) assay to determine protein distribution in mosquito tissues

Midguts from 3-5 day-old naïve and bloodfed female mosquitoes were dissected in PBS with protease inhibitor (Thermo Scientific, Rockford, IL, USA). Tissues were embedded completely in optimal cutting temperature (OCT) compound and immediately frozen in liquid nitrogen. Frozen midguts were sectioned (8-10 μm) with a cryostat. The sections were mounted on super frost plus slides (positively charged), air dried for 30 minutes at room temperature and fixed in 4% paraformaldehyde-PBS for 20 minutes and stored at -20ºC until use. Prior to staining, sections were re-hydrated in Tris-buffered saline (50 mM Tris, 150 mM NaCl, pH 7.6) with 0.05% tween-20 (TBST) for 10 minutes. Sections were blocked with 1-3 drops of blocking solution (5% dry milk in TBST) for 30 minutes, and then incubated with 2.5μg/ml purified rabbit anti-FREP1 antibodies in blocking solution for two hours. Control sections were incubated with blocking solution containing pre-immune rabbit antibodies. Samples were washed 3 times for 5 minutes with TBST. Next 100 μl of 1:20,000 diluted goat anti-rabbit antibody conjugated with alkaline phosphatase (Sigma-Aldrich, St. Louis, MO) in blocking solution was incubated with each sample for 30 minutes at room temperature. Slides were then washed 3 times in TBST. Finally, the sections were developed with 100 μl of BCIP/NBT Chromogenic Solution (Sigma-Aldrich; St. Louis, MO) for 10-20 minutes, rinsed with water, and examined under a microscope at 4x and 40x

magnification. The Photoshop software (version CC 2014) was used to measure the gray values of selected areas. The darkness values were obtained by subtracting the measured gray values of a targeting area from background gray values. It is worth noting that we have validated this method of measuring darkness values by using different exposing time and using other software (such as Microsoft PowerPoint) to change the brightness and/or contrast of a photo. After the calculation, we always obtained similar values for a certain area.

3.2.7 indirect immunofluorescence assays (IFA) to examine the binding between parasites and insect cell-expressed recombinant Full FREP1, FBG and N Terminal proteins.

Standard IFA was performed as described previously (26). In brief, *P. falciparum* cultures were deposited on premium cover glass slip slides (Fisher Scientific) to make blood smears. Dry smears were fixed in 4% paraformaldehyde in PBS at RT for 30 minutes, and then sequentially incubated with 100 mM glycine in PBS for 20 minutes, 0.2% bovine serum albumin (BSA) in PBS for 90 minutes, high five cell-expressed FREP1 (10μg/ml) in 0.2% BSA-PBS for two hours, enhancer (Alexa Fluor ® 594 Goat Anti-Mouse SFX kit, Invitrogen) for 30 minutes, 5μg/ml anti-FREP1 antibody in PBS containing 0.2% BSA for one hour, and 2μg/ml secondary antibody (Alexa Fluor ® 594 Goat Anti-rabbit Antibody, in PBS containg 0.2% BSA, Life Technologies Inc.) for 30 minutes. Between each incubation, the smears were washed 3 times for 3 minutes in PBS containing 0.2% BSA. Cover slips were rinsed in distilled water for 20 seconds, and 50 µl vectashield mounting media (Vector Laboratories, Burlingame, CA) was added onto the cover slip and the cover slip was covered onto

slides. After incubating for at least 2 hours in dark, the cells were examined using Nikon Eclipse Ti-S fluorescence microscope. The fluoresence intensity was measured by the Photoshop software (version CC 2014). The actual fluoresence intensity of a target was calculated by substracting the mean background fluoresence intensity (gray values) from the mean target fluoresence intensity (gray values). At least three replicates were measured to calculate the mean values and standard deviations.

3.2.8 Binding assays between FBG and PM.

3-5-day old G3 *An. gambiae* mosquitoes were fed with mouse blood. The engorged mosquitoes were maintained in insectary (27^0C, 80% humidity) for 18 hrs. Ice-anesthetized mosquitoes were then dissected to obtain midguts, and the blood bolus inside a midgut was removed by puncturing it. Blood fed-mosquito midguts and naïve mosquito midguts were incubated with 200µL of 0.1mg/ml BSA, full-length FREP1, N-FREP1, and FBG separately for 2 hrs at RT. Ten midguts were used in each treatment. Three independent experimental replicates were performed. After incubation, midguts were homogenized with pestles in 300µL of 0.5% Tween-20 in PBS. Midgut lysate supernatants (50µL) were used to coat 96-well plates overnight at 4ºC. Since recombinant proteins of FREP1, FBG, N-FREP1 contain His-tag, the bound protein was probed with 50µL of mouse anti-His monoclonal antibody and developed as we mentioned above.

3.3. Results

3.3.1 Generating anti-FREP1 polyclonal antibody

To understand the basic biochemical characteristics of FREP1 protein, we first examined its functional domains. According to our previous genome annotation (27), the

full length of FREP1 protein has 738 amino acids, including a 22-amino acid signal peptide at the N-terminal, three coiled-coil regions, and a conserved ~200-amino acid FBN domain at the C-terminal (Figure 3.1A). All six cysteine amino acid residuals are within FBN domain.

To generate anti-FREP1 antibodies as an analytical tool, *FREP1* coding region excluding the signal peptide region was cloned and expressed in *E. coli* M15 strain, and the expressed protein was purified by Ni-NTA column. The result indicated that the purity of *E. coli*-expressed recombinant FREP1 is >95% (Figure 3.1B, lane 3).

Table 3-1. PCR primers.

Primer name	Primer sequence
F FREP1 pQE30	5'-A*CCCGGGCAC*TGCCCTGAACGGTGCAG-3'
R FREP1 pQE30	5'-GGC*AAGCTT*CGCGAACGTCGGCACAGTC-3'
F FREP1 pIB/V5-His	5'-TCA*AAGCTT***CACC**ATGGTGAATTCATTCGTGTCG-3'
R FREP1 pIB/V5-His	5'-AC*TCTAGA*GCGAACGTCGGCACAGTCGTG-3'
F FBG FREP1 pIB/V5-His	5'-TCAGAATTC**CACC**ATGGAGTCGATCATCAAC-3'
R FBG FREP1 pIB/V5-His	5'-ACTCTAGACTCGGCCGGCGGGCAATCATCATGT-3'
F N FREP1 pIB/V5-His	5'-TCAAGCTT**CACC**ATGGTGAATTCATTCGTGTCGGTA-3'
R N FREP1 pIB/V5-His	5'-ACTCTAGACTGTACTCGTGCTCGACCGACTC-3'

Italic and underlined sequences denote restriction recognition sites. The bold sequence is Kozak consensus sequences. The primers were synthesized through Integrated DNA Technologies Inc.

Injecting the purified FREP1 protein into rabbits generated the anti-FREP1 anti-serum. The antibodies were purified using affinity chromatography protein A/G-agarose. Western blot result showed that the purified polyclonal rabbit anti-FREP1 antibody can

recognize recombinant FREP1 specifically (Figure 3.1C). The purified anti-FREP1 antibodies were used in this study.

3.3.2 Recombinant FREP1 protein is secreted from insect cells into culture medium, and form tetramers

We examined cellular FREP1 expression patterns *in vitro.* We cloned the full-length *FREP1* into the plasmid pIB/V5-His under the baculovirus *OplE2* promoter and expressed its product in High Five insect cells. The results showed that FREP1 protein was exclusively detected in the cell culture supernatant, and no FREP1 protein was detected in the cell pellet (Fig. 3.1D), indicating FREP1 is a secreted protein. Notably, only one band was detected by anti-FREP1 antibody in the western blot membrane.

Next, we determined whether FREP1 protein assumes distinct quaternary structures. On SDS-PAGE gel, the purified insect cell-expressed recombinant FREP1 protein showed the same molecular weight under both reducing (with 2-mercaptoethanol) and non-reducing conditions (Fig. 3.1E), indicating that insect cell-expressed recombinant FREP1 protein exists as either monomers or multimers that associate via non- covalent bonds.

The molecular weight of insect-expressed recombinant FREP1 protein (~95kDa, Fig. 3-1E) is greater than the calculated molecular weight (83.5kDa), suggesting post-translational modification of secreted FREP1 protein. Size exclusion chromatography was then utilized to separate recombinant FREP1 protein and protein complexes. Based on the gel-filtration standard curve, the major recombinant FREP1 peak appeared to be between 308kDa and 409kDa (Fig. 3-1F). Because the unit molecular weight of recombinant FREP1 is about 95kDa, our data support that the majority of FREP1 protein

exists as tetramers (~380kDa). In addition monomers, dimers and trimers also exist (Fig. 3-1F).

3.3.3 The FREP1 N-terminal domain forms oligomers

Sequence alignments revealed that the orthologs of N-terminal of FREP1 between amino acids 23 and 462 are highly variable. To understand the function of this region, we cloned this fragment into pIB/V5-His and expressed it in High Five cells. This recombinant protein (named N-FREP1 in this paper, 52.9kD) was secreted into medium and purified with Ni-NTA column. SDS-PAGE showed one enriched band, indicating the purified recombinant N-FREP1 did not contain any major contaminations (Fig. 3.2A). Furthermore, a size exclusion chromatography using Superdex G200 increase column was applied to determine molecular weight of native N-FREP1. Results (Fig 3.2B) indicate that the majority of N-FREP1 recombinant protein forms tetramers, which is consistent to our published molecular model of FREP1 as a tetramer.

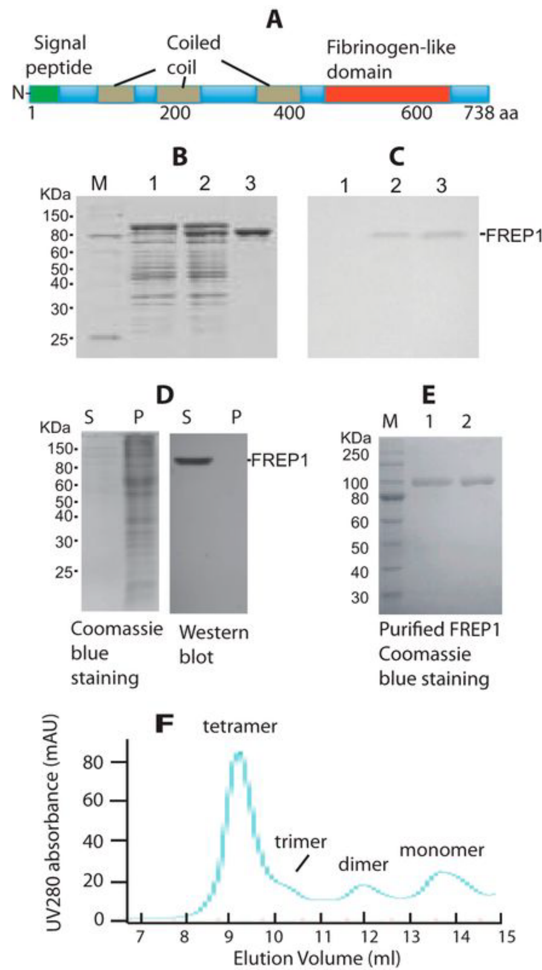

Figure 3-1. FREP1 is secreted from insect cells and forms tetramers.

FREP1 is secreted from insect cells and forms tetramers. **A:** FREP1 protein has a N-terminal signal peptide, three coiled coils, and a C-terminal FBN domain. **B:** The *FREP1* gene was cloned and expressed in *E. coli* M15 strain. On 12% SDS-PAGE stained with Coomassie Brilliant Blue, lane 1: before induced; lane 2: after induced by IPTG); lane 3: the expressed recombinant FREP1 was purified with Ni-NTA column. **C:** Western blot using anti-FREP1 antibody to detect specifically recombinant FREP1 protein. **D:** SDS-PAGE (left) and western blot (right) showing that the recombinant FREP1 protein is secreted from High Five cells into culture medium. “S” and “P” represent supernatant and cell pellet respectively. Only one band was detected by anti-FREP1 antibody. **E:** The purified recombinant FREP1 expressed in High Five insect cells on reducing (lane 1) and non-reducing (lane 2) 12% SDS-PAGE, stained with Coomassie Brilliant Blue. **F:** The UV280 absorbance profile of the purified insect cell-expressed recombinant FREP1 in PBS using Superdex G-200 gel filtration chromatography.

3.3.4 FREP1 protein localizes to the peritrophic matrix in mosquito midguts after blood feeding

Published microarray-based gene expression data (28,29) support that FREP1 has higher expression in mosquito midguts, compared to other tissues, and FREP1 is up regulated by bloodmeal. We then, used the generated polyclonal anti-FREP1 antibodies in IHC assays to determine the localization of endogenous FREP1 in mosquito midguts.

To determine whether anti-FREP1 can specifically recognize FREP1 in mosquitoes, we dissected midguts from bloodfed mosquitoes (18 hours after bloodmeal) and naïve mosquitoes. The blood in bloodfed mosquito midguts was manually removed.

On SDS-PAGE, the protein composition of bloodfed mosquito midguts was distinct from naïve mosquitoes (Figure 3-3A), which is consistent to previous reports of blood-regulation of mosquito gene expression (13). The western blot detected only one band in homogenized bloodfed mosquito midguts, and no band was detected in naïve mosquito midguts (Figure 3-3B). These results are consistent to microarray data showing that blood up-regulates *FREP1* expression. These data also further confirm the specificity of rabbit polyclonal anti-FREP1 antibody for endogenous FREP1 protein expressed in mosquitoes.

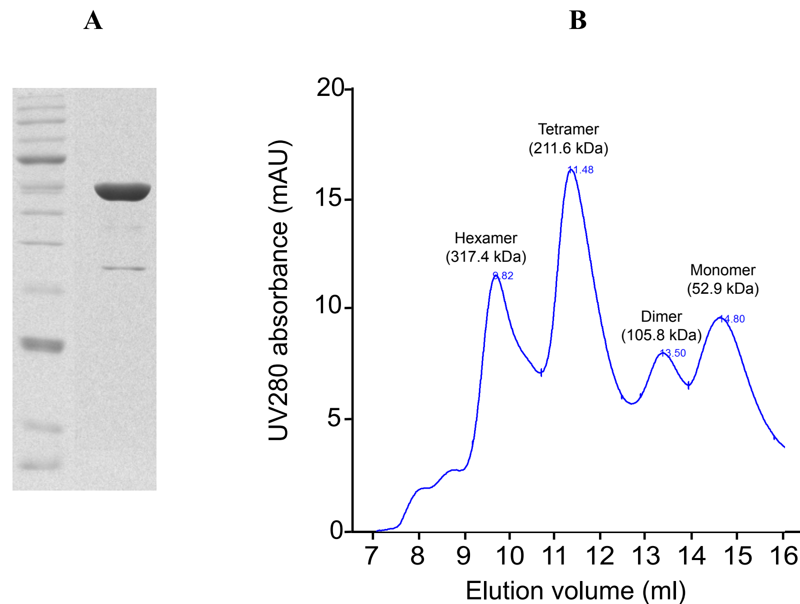

Figure 3-2. The most abundant form of native N-FREP1 is tetramer.
A: SDS-PAGE of purified recombinant N-FREP1 (10µL). **B**: Size exclusion chromatography (SEC) of insect cell expressed recombinant N-FREP1purified by Ni-NTA affinity chromatography. Approximately 8 nmoles of the sample was injected into a 500µL loading loop and then run over a Superdex 200 increase column using 50mM NaH_2PO_4 pH 8.0, 300mM NaCl at a flow rate of 0.5ml/min. A fixed wavelength UV monitor monitored the elutions. Elution time corresponding to the oligomers size was estimated from N-FREP1 molecular weight (52.9 kDa).

IHC assays were performed to localize FREP1 proteins in mosquitoes. Comparisons between the negative control IHC sections (Figure 3-3C, pre-immune antibody) and the experimentally stained sections (Figure 3-3D, anti-FREP1 antibody) of naïve mosquito midguts did not show any significance (Figure 3-3E). However, 12 hours post bloodmeal, the FREP1 signal (purple color) in experimental sections (Figure 3.2G) is significantly more intense ($P<0.01$, Figure 3-3H) than that in control sections (Figure 3-3F), indicating that the FREP1 protein was up-expressed in mosquito midguts after bloodmeal. These data are also consistent with the western blot result (Figure 3-3B)

and published microarray-based expression data showing up-regulation of *FREP1* mRNA expression in mosquitoes three hours after bloodmeal feeding (13). Furthermore, two portions of stained midgut sections (rectangles in Figure 3-3F and G) were magnified 40X to show a more detailed structure (Figure. 3-3I and Figure. 3-3J, respectively). Strikingly, we found that the majority of FREP1 protein was localized in the mosquito PM that resides within the midgut lumen. Consistent staining patterns and FREP1 localization were observed in more than 3 independent experiments, and the statistical analysis of darkness values between control and experiment groups confirms that significantly more FREP1 protein was detected after bloodmeal (Figure 3-3H and 3-3K). Together, the data from the microarray analyses and our new western blot and IHC studies consistently show that *FREP1* gene is up-regulated after blood feeding and that FREP1 protein is secreted into mosquito midgut lumen and associated with the PM.

3.3.5 FBG binds mosquito midgut peritrophic matrix

To verify FREP1 protein association with the PM, we analyzed the interactions between mosquito midguts and recombinant full-length FREP1, FBG and N-FREP1 proteins to determine which domain is responsible for this interaction. Since PM forms only after a bloodmeal, blood fed-mosquito midguts and naïve mosquito midguts were incubated with BSA, recombinant FREP1, N-FREP1, and FBG separately. Ten midguts were used in each treatment. Three independent experimental replicates were performed. BSA and FREP1 are negative and positive control respectively. Since naïve mosquito midguts do not contain PM, they are used as negative controls as well. After

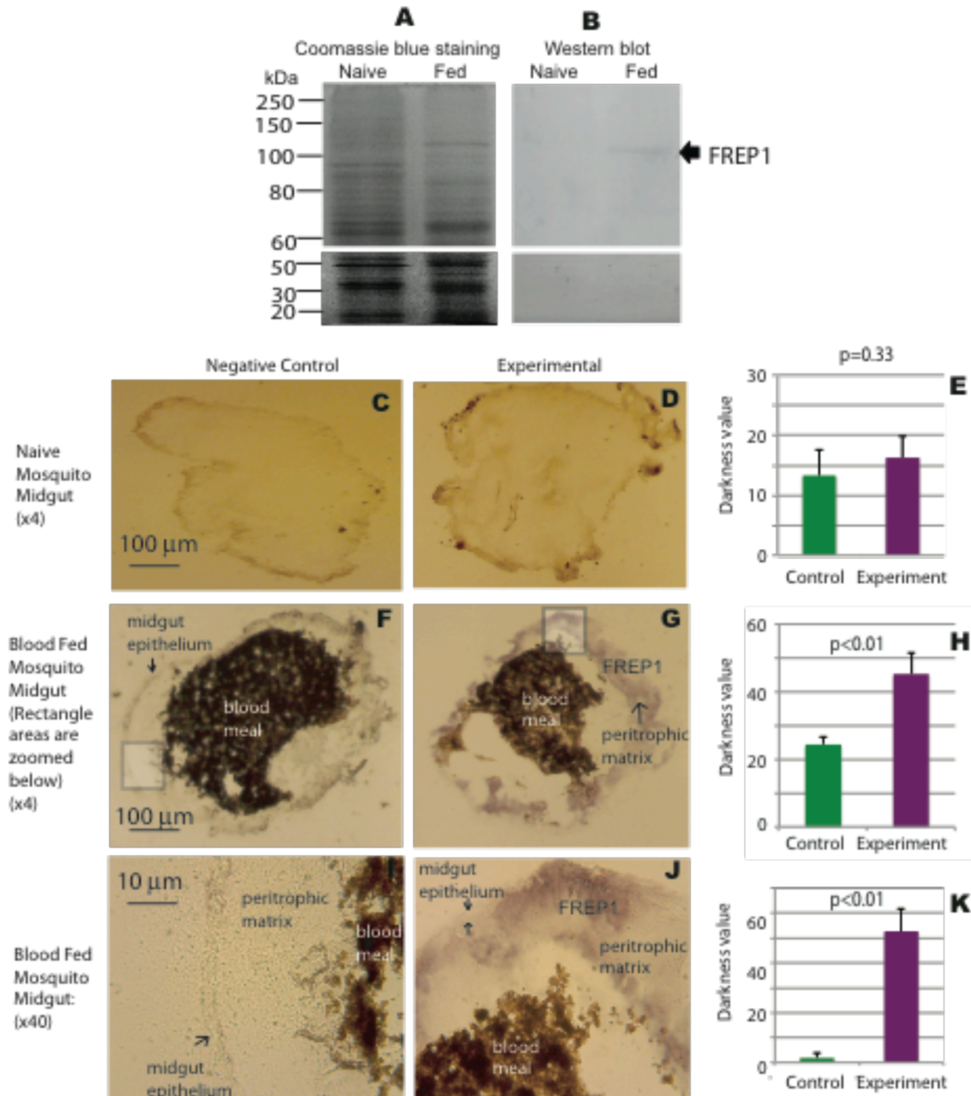

Figure 3-3. FREP1 localizes in the mosquito midgut peritrophic matrix.
FREP1 protein localizes to the mosquito midgut peritrophic matrix**. A:** Midgut proteins from bloodfed and naïve mosquitoes were fractionated on 10% SDS-PAGE and stained with Coomassie Brilliant Blue. **B:** Midgut proteins of bloodfed and naïve mosquitoes were fractionated on 10% SDS-PAGE, transferred to membranes and probed with anti-FREP1 antibody. Western blot data show anti-FREP1 antibody specifically recognized FREP1 in bloodfed mosquito midguts. **C, F,** and **I:** Negative control of naïve and blood fed *An. gambiae* midgut in which purified pre-immune rabbit antibody was used to detect FREP1 protein. **D, G,** and **J:** Experimental groups of naïve and blood fed *An. gambiae* midguts in which anti-FREP1 rabbit antibody was used to detect FREP1 protein; **I** and **J** are the magnification of areas on **F** and **G** (highlighted by rectangles) respectively. Locations of the midgut epithelium, the peritrophic matrix, the FREP1 protein and the blood bolus are annotated on the images. Summary data in E, H, and K show the statistical difference of darkness values between negative controls and experimental groups in three experimental replicates.

incubation, midguts and their binding proteins were homogenized, and extracts were used to coat an ELISA plate. The bound recombinant FREP1, N-FREP1, and FBG were probed with anti-His monoclonal antibody. After development with ELISA reagents, FREP1- and FBG- incubated with blood-fed mosquito midguts exhibited significantly ($p<0.005$) higher signal than BSA-incubated midguts. There is no significant ($p>0.4$) difference between N-FREP1 incubated midguts and negative controls (Fig 3.4). These results support FBG binding to PM.

3.3.6 FBG binds sexual stage ookinetes

We determined the molecular relationship between FBG and parasites. We previously showed that FREP1 binds to *P. falciparum* (NF54) sexual stage gametocytes and ookinetes [30]. In this study, we examined the capacity for the purified FBG domain of FREP1 to bind *P. falciparum* parasites, gametocytes and ookinetes in particular.

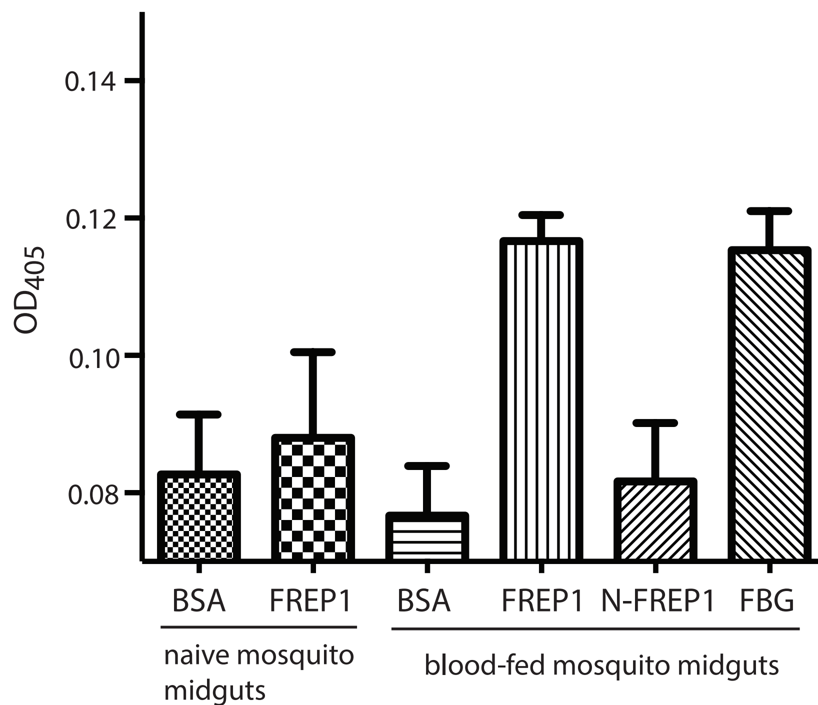

Figure 3-4. FBG binds to mosquito peritrophic membrane.

Non-infected human red blood cells (RBC), *P. falciparum* gametocytes, and ookinetes were fixed on cover slips (non-permeabilized). After incubation with insect cell-expressed FBG recombinant protein, we detected parasite bound FBG protein by indirect immunofluorescence assays. The results showed that FREP1 FBG bound gametocytes (Fig. 3-5, row B), early stage ookinetes (just merged with a sharp tail, Fig. 3-5, row C) and ookinetes (Fig. 3-5, row D). The binding signals were not observed when non-infected human RBC were assayed (Fig. 3-5, row A), confirming that FBG does not bind healthy human RBCs. Additional negative controls included incubation of *P. falciparum* gametocytes with an irrelevant protein (chloramphenicol acetyltransferase, CAT) expressed in the same system. As shown in Fig. 3-5, row E, there were no binding signals in control groups. Therefore, FBG fragment is a functional domain of FREP1 that is responsible for the interaction between FREP1 protein and *Plasmodium* parasites.

3.4 Discussion

We previously reported that the polymorphisms within *FREP1* were strongly associated with reduced *P. falciparum* infection intensity (13). Our current work aimed to investigate the molecular mechanisms and functions of FREP1 protein during infection of mosquitoes by the human malaria pathogen *P. falciparum*. First, we demonstrated that FREP1 protein is expressed in the mosquito midgut PM and exerts its effects on *Plasmodium* infection in the PM. It is well known that PM formation occurs ubiquitously in the midguts of hematophagous insects and serves as an important physical barrier to resist or prevent invasion by pathogens in blood(11). Previous studies showed that the *FREP1* gene expression is up-regulated by bloodmeal (29).

Here we showed that FREP1 protein is secreted into the mosquito midgut lumen after a bloodmeal and is associated with the PM. It is reported that FBN domains in FREPs can recognize N- acetylglucosamine of chitin (31,32), other carbohydrates and their derivatives (17,32), and our data confirmed that FREP1 protein binds PM. We speculate that the FREP1 protein is a structural and functional protein in PM that interacts with chitin and other carbohydrates via the FBN domain of FREP1. This interaction keeps FREP1 proteins closely associated with the PM following bloodmeal feeding.

Second, our data supports the quaternary structure of FREP1 to be tetramers. Invertebrate FREP/FBN family members tend to multimerize in order to exert their physiological functions (33). For example, the *An. gambiae* mosquito FBN9 protein forms dimers that interact with Gram-positive and Gram-negative bacteria (19). Moreover, the functional forms of horseshoe crab TL-5A and TL-5B proteins form propeller-like structures with each blade corresponding to a disulfide-linked dimer (34). Similarly, our results showed that FREP1 forms tetramers through hydrophobic forces instead of disulfide-bonds since non-reducing SDS-PAGE showed that insect cell-expressed FREP1 are a monomer and size exclusion chromatography showed that FREP1 forms a tetramer. Coiled-coil motifs in proteins have been reported to mediate protein homodimer complexes (35) and tetramer complex formation (36). Consistent with this, the elution profile of N-FREP1 accounts for the tetrameric form of FREP1.

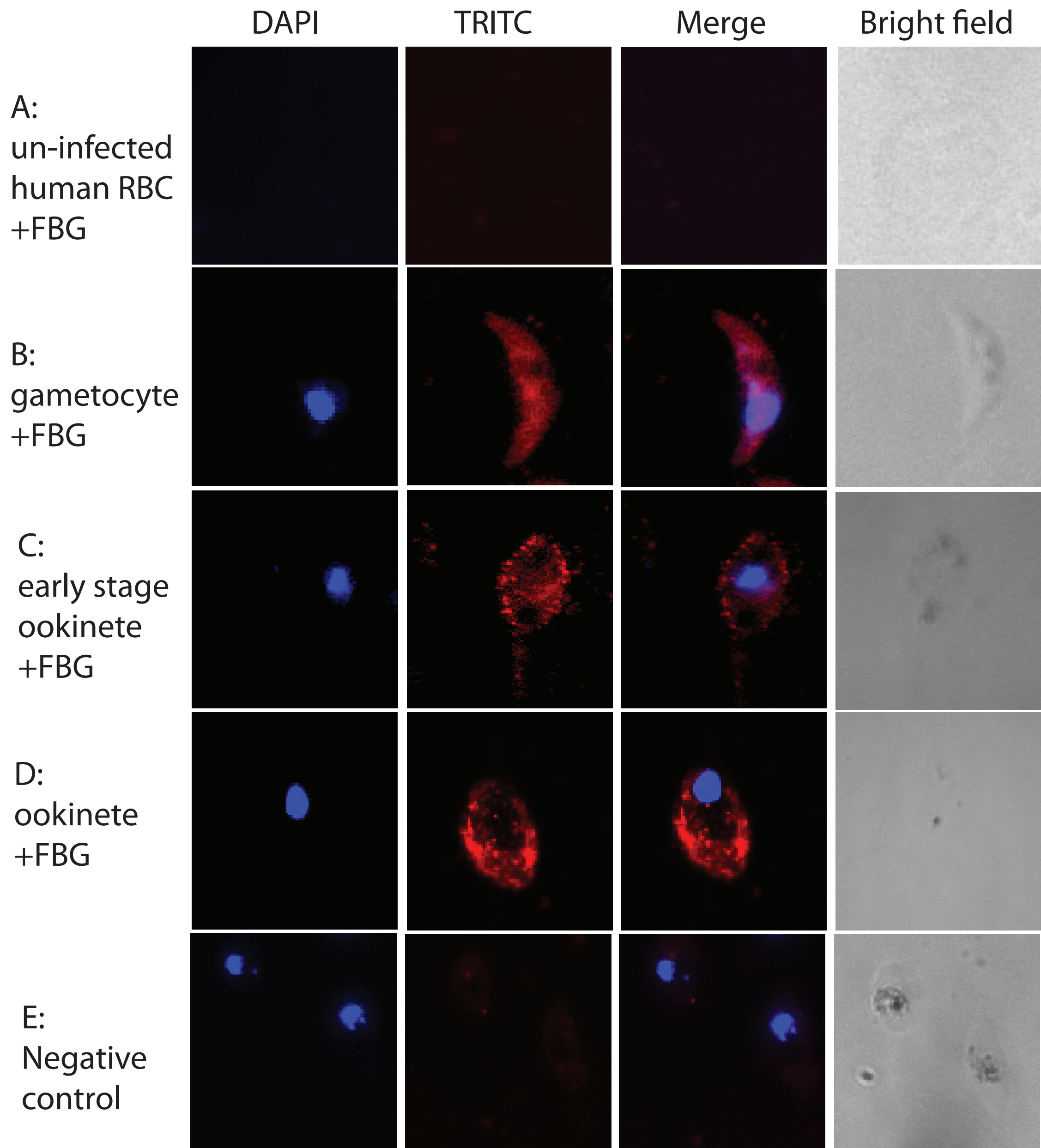

Figure 3-5. Insect cell-expressed recombinant FREP1 FBG protein interaction with *Plasmodium falciparum* was detected by indirect immunofluorescence assay (IFA). The first and second columns depict cell nuclei stained with DAPI and binding FBG proteins, respectively. Merging column one and two generated the third column, showing the co-localization of *P. falciparum* (nuclei) and FBG protein binding. The 4th column shows the bright views of the cells. Row A: FBG does not bind to un-infected RBC. Row B: FBG binds to *P. falciparum* gametocytes. Row C, D: FBG binds to *P. falciparum* ookinetes. Row E: An irrelevant control protein (CAT) does not bind *P. falciparum* parasites.

Third, IFA assays demonstrated that the FREP1 protein interacts with sexual stage (gametocytes) and mosquito midgut invasion stage (ookinetes) *P. falciparum* parasites. The putative parasite-expressed FREP1-binding partners are expected to localize on the cell surface of the parasites (including asexual stages, sexual stages, and ookinetes), because non-permeabilized approaches were used in IFA to detect the interaction between FREP1 protein and parasites. We discovered that FBG domain in FREP1 binds *Plasmodium* gametocytes and ookinetes. Since FREPs were proposed to act as pattern recognition molecules (19), the C-terminal FBN domain within FREP1 is likely responsible for mediating interactions between FREP1 proteins and *Plasmodium* parasites. Since FREP1 is localized in the mosquito midgut PM, our data support our prediction that FBG domain interacts with PM as well.

In summary, we discovered that *Anopheles gambiae* FREP1 is a secreted protein that forms oligomers (tetramer) via the coiled-coil regions in the N terminal, and it localizes within the peritrophic matrix of mosquitoes. We propose that the functional FBG domain of FREP1 has dual role in assisting *Plasmodium* invasion of mosquito midgut by directly binding to parasite and the PM, facilitating ookinete penetration of the midgut epithelia. Thus, FREP1 constitutes an ideal mosquito antigen target for malaria transmission-blocking vaccines.

3.5 Acknowledgment

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Chapter 4: Fibrinogen domain of FREP1 is a broad spectrum malaria transmission-blocking vaccine antigen

4.1 Introduction

Malaria death rates have dropped by 47% between 2000 and 2013 globally, and by 54% in Africa due to applications of several anti-malaria strategies including anti-malaria drugs, insecticide-treated nets, and indoor insecticide spraying. Despite these efforts, more than 587,000 still died and 90% of these deaths occurred in Sub-Saharan Africa in 2014 [1]. The rapid spread of drug-resistant malaria parasites and insecticide-resistant mosquitoes along with the absence of efficient vaccines against malaria present major challenges for malaria control. Therefore, new approaches are urgently needed. Transmission blocking vaccines (TBVs) have been recently considered as a promising measure to combat malaria. TBVs are designed to block parasite development in the mosquito midgut upon ingestion with the human antibodies against antigens from either parasites or mosquitoes.

Human malaria is caused by *Plasmodium falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*, of which *P. falciparum* and *P. vivax* are responsible for ~99% of malaria cases. Since only gametocytes can infect mosquitoes, antigens on the surface of gametocytes and/or ookinetes such as Pfs25, Pfs48/45 and Pfs230 have been evaluated as TBV candidates in preclinical studies [2-4]. Among them, Pfs25 and its ortholog Pvs25 from *P. vivax* are the only candidates to progress to clinical trials. Pfs25 is a 25-kDa sexual stage specific protein expressed on the surface of the parasite during several sexual developmental stages including gamete, zygote and ookinete [5]. Clinical

trials of Pfs25 only showed moderate levels of transmission-blocking activity [6], underscoring the need to identify additional and novel antigens for TBV development.

About 30 anopheline mosquito species transmit malaria[7]. The major malaria vectors in Africa are *Anopheles gambiae, An. arabiensis* and *An. funestus*. In Asia, the most important species are *An. stephensi* and *An. dirus.* In South America, *An. minimus*, *An. albimanus* and *An. darlingi* are responsible for malaria transmission [2, 8, 9]. To successfully transmit malaria, *Plasmodium* parasites must complete a complex developmental cycle in both human and mosquito hosts. Thus, mosquito midgut molecules that facilitate ookinete invasion are likely to serve as ideal targets for TBVs. Previous studies showed that polyclonal antibodies against mosquito alanyl aminopeptidase 1 (APN1) or carboxypeptidases B (CPB) [2, 10] inhibited 73% and 51% parasite development in mosquito midguts respectively using *P. berghei*-mouse infection system.

Since human malaria is caused by several *Plasmodium* species and transmitted by numerous *Anopheles* species, and many endemic areas have both *P. falciparum* and *P. vivax* malaria cases and transmitted by several different *Anopheles* species, an ideal TBV antigen would effectively block malaria transmission of multiple parasite species to multiple mosquito species. We recently reported that FREP1 plays a pivotal role in ookinete invasion of the mosquito midgut [11]. FREP1 is a tetramer that localizes within the peritrophic matrix, and facilitates *Plasmodium* invasion through direct binding to gametocytes and ookinetes. In this study, we demonstrate that a highly conserved FBG domain within FREP1 is a broad-spectrum TBV antigen that blocks transmission of multiple *Plasmodium* species to multiple *Anopheles* species, which supports FREP1-

mediated *Plasmodium* invasion to mosquitoes as a conserved pathway. In particular, *in vivo* mouse model demonstrates FBG as a vaccine that blocks >75% transmission of *P. berghei*, better than reported mosquito TBV antigens (APN1, CBP) [2, 10]. It is worth noting that only three mosquito proteins have been identified suitable for malaria TBV antigens. Membrane feeding assays showed anti-FBG serum blocked >81% transmission of *P. falciparum*, which meets the target product profile (TPP) set as guideline for malaria TBVs for clinical trials (>80%) [12].

4.2 Material and Methods

4.2.1 Ethics Statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH). Mice were used according to approved protocols (R15-012) by the University of Oklahoma Institutional Animal Care and Use Committee (A3240-01). The *P. vivax* infected patients blood was used to examine the efficacy of antibodies. We followed the NIH Human Subjects Policies and Guidance. The Ethical Review Committee of the Faculty of Tropical Medicine, Mahidol University approved *P. vivax* infection protocols (MUTM 2011-040-05).

4.2.2 Mosquito and *P. falciparum* maintenance

An. gambiae G3 strain and *An. dirus* were reared in an insectary room maintained at 27°C, 80% humidity with a 12-hr day/night cycle. Larvae were fed with ground fish food (KOI, 0.1mg per larvae per day). Adult mosquitoes were maintained on 8% (w/v) sucrose and fed with mouse blood for egg production. *P. falciparum* parasites (NF54 strain from MR4, Manassas, VA) were maintained in RPMI-1640 medium (Life

Tech, Grand Island, NY) supplemented with 10% heat-inactivated (56°C for 45 min) human AB+ serum (Interstate blood bank, Memphis, TN), 12.5 μg/mL hypoxanthine and 4% haematocrit (O+ human blood) in a candle jar at 37°C as described previously [11].

4.2.3 *In vitro* transmission-blocking assay of *P. berghei* infection in *An. gambiae* with anti-FREP1 antibodies

P. berghei (ANKA GFPcon strain) infected mouse blood was precipitated by centrifugation (2000xg for 3 minutes). The blood pellet was then mixed with an equal volume of anti-serum. The standard membrane-feeding assay (SMFA) [15] was conducted using 3-days old female naïve mosquitoes. After feeding for 20 minutes, the engorged mosquitoes were maintained on 8% sugar (w/v) at 19°C. Seven days after infection, mosquitoes were dissected and the midguts were stained with 0.2% mercurochrome and examined using light microscopy to count the number of oocysts. Data were analyzed with nonparametric Wilcoxon test implemented in software R-project.

4.2.4 *In vitro* transmission-blocking assay of *P. vivax* infection in *An. dirus* mosquitoes with anti-FREP1 antibodies

Field isolates of *P. vivax* were collected from patients attending malaria clinics in Ubonratchanthani province, Thailand. Within 10 hours after collecting blood, aliquots of 350 μl of infected blood were prepared. The infected blood was centrifuged at 1,500 xg for 5 min and plasma was removed. Packed blood was washed once with RPMI-1640 incomplete medium. The antiserum was mixed with *P. vivax* infected packed blood at 1:1 ratio (v/v). The suspension was incubated at room temperature for 15 minutes before

being fed to 100 female *An. dirus* (age 5-7 days) per treatment for 30 minutes using membrane-feeding device. The packed infected blood mixed with naïve human AB serum at 1:1 ratio (v/v) was used as a control. Engorged mosquitoes were kept on 10% sugar solution. The number of oocysts in mosquito midguts was determined under a microscope at day 7-post bloodmeal.

4.2.5 Gene cloning, protein expression, and purification

The full length coding sequence of *An. gambiae* FREP1 was amplified as described previously [11]. The FREP1 FBG fragment was amplified with the gene specific primers (5'-ACGCATGCCACGAGTACGAGTCGATC-3', 5'-TGCAAGCTTGTACTCGTGCTCGACCGACTC-3'), PCR fragment was cloned into pQE30 vector and expressed *E. coli* strain M15[pREP4] (Qiagen, Valencia, CA) with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside at room temperature. The recombinant protein was purified on a Ni-nitrilotriacetic acid column (Qiagen) and eluted with Buffer D (20 mM Sodium Phosphate, 500 mM NaCl, 8M Urea, PH4.0). The eluted fractions were analyzed on 12% SDS-PAGE gel followed by Coomassie blue staining to examine the purity. All fractions containing protein were then combined and protein concentration was determined using the Bradford protein assay [16].

4.2.6 Expression of recombinant FREP1 protein in insect cell High Five cells

As described previously [11], **t**he full-length FREP1 was cloned into plasmid pIB/V5-His (Life Technologies, Carlsbad, CA). After being amplified in *E. coli* DH5α, plasmids were purified using GenElute Endotoxin-free plasmid preparation kits (Sigma-Aldrich, St. Lois, MO). The purified recombinant plasmids pIB-FREP1 and pIB-chloramphenicol acetyltransferase (CAT) (as a control) were transformed into 40-60%

confluent cabbage looper ovarian cell-derived High Five cells. Cellfectin® Reagent (Invitrogen, Carlsbad, CA) was mixed with each individual plasmids (1μl Cellfectin/μg plasmids) in 5-6 ml Express Five® SFM medium (Invitrogen) to transfect into cells. After 5 generations of dilution, the cells that stably expressed the recombinant protein were cultured in 25cm^2 CELLSTAR® cell culture flask (Greiner Bio-One, Monroe, NC) for 48 hrs at 27°C. The secreted FREP1 protein in medium was purified using Ni-NTA column and dissolved in PBS for use.

4.2.7 Immunizations of mice with FREP1 and FBG

Five Hsd:ND4 female (20-25g; 7 to 8 weeks old) mice were primed via subcutaneous (s.c.) injection and boosted two times at 3-week intervals via (s.c.) injection with 20μg of FREP1 per mouse in 200 μL phosphate buffer saline (PBS) adsorbed (1:1) in Alhydrogel adjuvant. The control mice were primed or boosted with PBS only and emulsified (1:1) with Alhydrogel. Similarly, five mice were injected (s.c.) with FBG protein under the same regime for optimal-prime boosting with 20μg of the purified recombinant protein in 0.5X buffer D, with control group comprised of five mice injected with 0.5X buffer D emulsified in alhydrogel instead. Serum was collected from each mouse prior to each priming and boosting immunization. Animals were sacrificed following *in vivo* feeding assays, and blood was collected via orbital sinus [17]. Blood samples were centrifuged at 2,000x g, for 20 minutes at 4ºC for serum separation.

4.2.8 *In vivo* transmission-blocking assay with the immunized mice

Mice were immunized with the same regime for optimal-prime boosting as described above. Ten days after second boost, naïve and optimally prime-boosted

Hsd:ND4 mice were infected via i.p. route with *P. berghei*–infected blood. When parasitemia reached <6% mice were injected with phenylhydrazine hydrochloride (60 mg/Kg) (Santa Cruz Biotechnology, Dallas, Texas) to stimulate gametocytemia. Mice harboring circulating gametocytes (confirmed by Giemsa staining) were anesthetized and used to feed 100 3-day old *An. gambiae* female mosquitoes for 20 minutes. After feeding, un-engorged mosquitoes were removed and engorged mosquitoes transferred to a cage and kept at 19°C and 8% (w/v) sucrose. Seven days after infection, mosquitoes were dissected and the midguts were stained with 0.2% mercurochrome and examined using light microscopy to count the number of oocysts. Two independent experiments were performed for each treatment. Transmission blocking activity was calculated as the $Transmission\ Reduction\ Activity = 1 - \left(\frac{mean\ \#\ oocysts\ experimental\ group}{mean\ \#\ oocysts\ in\ control\ group}\right) x100.$

4.2.9 Determining antibody titer with ELISA assays

Mice sera were collected and assayed for the presence of FREP1 specific IgG antibodies using ELISA. In brief, recombinant affinity-purified FREP1 was diluted to 0.5μg/ml in 0.1M sodium phosphate (Na_2HPO_4), pH 9.0 binding buffer and used to coat 96-well plate MediSorp^{TM} plates (NUNC –Denmark) overnight at 4 ^{o}C. Wells were washed three times with 0.2% Tween 20 in PBS (PBST) and blocked with 2.5% bovine serum albumin (BSA) and 1% normal goat serum in PBS for 2 hrs at room temperature. Serum samples were prepared by 1:5 serial dilutions starting at 1:100 to 1:8x10^6, 50 μl were added into each wells and incubated at room temperature for 1 hr. Bound IgG antibodies were detected using AP-conjugated goat anti-mouse IgG (Sigma-Aldrich) at 1:800 in blocking solution for 2 hrs at room temperature and visualized using p-Nitrophenylphosphate (Sigma-Aldrich, St. Louis, MO) as the substrate. Absorbance at

450nm was measured using Epoch microplate spectrophotometer (Biotek, Winooski, VT). Antibody titer was expressed as endpoint titer, the highest dilution of serum that gives a reading above the cutoff (2x standard deviation of the signal generated from pre-immune serum).

4.2.10 Cross-reactivity of mouse FREP1 serum to human plasma fibrinogen

Human blood (AB+) was purchased from Oklahoma Blood Institute. Whole blood collected from donors were transferred into BD Vacutainer® blood collection tubes (Becton and Dickinson Company, Franklin Lakes, NJ) coated with ethylenediaminetetraacidic acid dipotassium salt dihydrate (K_2EDTA) to prevent coagulation. Blood sample was centrifuged at 3,000x g for 5 minutes at 4ºC to separate plasma. 50µL of human plasma samples without dilution (1) or diluted 10-fold in PBS (2) were coated overnight at 4 ºC in a 96-well plate (Brand, Wertheim, Germany). 50µL insect cell-expressed recombinant FREP1 (0.5µg/ml) and BSA (10mg/ml) were coated as positive and negative controls respectively. Samples were diluted in 0.1M Na_2HPO_4 (pH 9.0). Each well was then incubated with the following solutions at RT: 150 µl blocking buffer (2.5% BSA and 1% normal goat serum in PBS) for 2 hrs, 50 µl of anti-FREP1 mouse serum (1:1,000), control mouse serum (1:1,000) and anti-FREP1 rabbit serum (1:2,000) diluted in blocking buffer for 1 hr, 50µL of alkaline phosphatase-conjugated goat anti-mouse IgG (1:800) and goat anti-rabbit IgG (1:20,000) for 1.5 hrs. Wells were washed for 5 minutes with PBST three times between incubations. At the end, the samples were developed with 50 µl of p-Nitrophenylphosphate solution (Sigma-Aldrich).

4.2.11 Immunofluorescence assays (IFA)

Nonpermeabilized immunofluorescence assays were performed as described previously [11]. In brief, *P. falciparum* cultures, containing cells and parasites, were smeared on coverslips (Fisher Scientific, Waltham, MA) and fixed in 4% paraformaldehyde in PBS at room temperature for 30 minutes. After quenching with 0.1M glycine in PBS, coverslips were blocked overnight at 4ºC in 2.5 % BSA and 1% normal goat serum in PBS (blocking solution). Cells were incubated for 2 hours at room temperature with High Five cell-expressed FBG (100µg/ml) in blocking solution. Sequentially, cells were incubated for 1 hour with mouse monoclonal anti-His antibody (1:1, 000) and Goat anti-mouse (1:1, 000) secondary antibody (Alexa Fluor® 555, Life Technologies, Inc., Carlsbad, CA) for 45 mins, both diluted in blocking solution. Cells were washed between each step three times for 5 mins in PBST. Cells in the control group were incubated with High Five expressed 100 µg/ml chloramphemicol acetyltransferase (CAT). Coverslips were mounted on glass slides using 20µL vectashield mounting media (Vector Laboratories, Burlingame, CA) and visualized using a Nikon Eclipse Ti fluorescence microscope.

4.2.12 Alignment of FREP1 sequences from multiple species of anopheline mosquitoes

The orthologs of FREP1 in various anopheline species were obtained from the Vector-Base genome server [18]. The multiple sequence alignment of *An. gambiae* FREP1 with its orthologs was built using ClustalO program ver. 1.2.1[19] and visualized with Jalview [20].

4.2.13 Using ELISA approach to demonstrate that anti-FBG serum inhibits the interaction between FREP1 and parasites

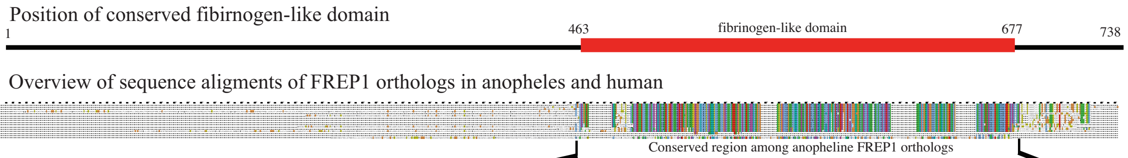
15-day cultured *P. falciparum* NF54 cells were lysed in PBST. About 50µL of supernatant (2.0mg/ml) per well was used to coat a 96-well plate. Purified recombinant full-length FREP1 was mixed with pre-immune mouse serum (1:150) or anti-FBG serum (1:150, final titer: $1x10^4$). BSA (2.5 mg/mL) was used as a negative control. The bound FREP1 in wells was probed with anti-N-FREP1 mouse serum (1:1000 dilution), followed by incubation with alkaline phosphatase-conjugated goat anti-mouse IgG (1:20,000). After developed with pNPP (Sigma), OD_{405} was measured using Epoch microplate reader. Three replicates were conducted.

4.3 Results

4.3.1 The FREP1 fibrinogen-like domain is highly conserved in anopheline mosquitoes

We examined the FREP1 orthologs among anopheline mosquitoes to find conserved regions. We obtained 13 orthologs from major malaria vectors in Africa (*An. gambiae*, *An. funestus, An. arabiensis, An. coluzzii*, *An. merus,* and *An. albumnus*), South America (*An. darlingi*), Asia (*An. sinesis, An. stephensi, An. minimus,* and *An. epiroticus*), and Europe (*An. atroparvus*). The results from multi-sequence alignment revealed a highly conserved region between amino acids 463 and 677 of *An. gambiae* FREP1 (Fig. 4-1A).

A: Sequence aligments of FREP1 orthologs in anopheles and human



B: Alignment of conserved region in detail

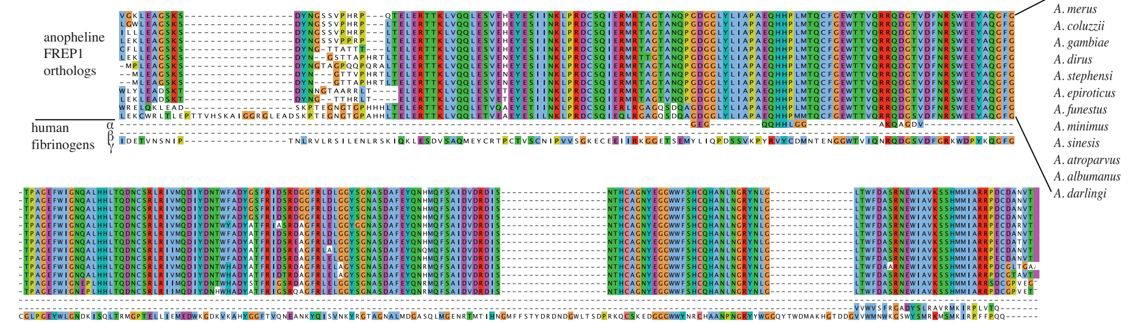


Figure 4-1. Multiple sequence alignment of FREP1 from *An. gambiae* and other major malaria vectors.
A: The overview of sequence alignments of FREP1 orthologs in *Anopheles* mosquitoes and human fibrinogens. **B**: Detailed alignment of conserved FBG domains. Dots and dashes are insertions or deletions. Colored letters depict conserved amino acids.

Detailed analyses of this conserved region found that more than 90% of the protein sequences are identical among all 13 anopheline species (Fig. 4-1B), suggesting that antibodies raised against this domain might be able to block that transmission of multiple *Plasmodium* species to multiple *Anopheles* mosquitoes. Since this conserved region is a FBG domain, we also compared FREP1 with human fibrinogens α, β, and γ chains. Multiple sequence alignment found less than 10% identical sequences between the mosquito conserved FBG domain and human fibrinogens, supporting that vaccination with recombinant mosquito FREP1 or the FBG domain protein would be unlikely to trigger autoimmune reactions.

4.3.2 Rabbit Anti-FREP1 antibodies inhibit malaria transmission in *P. berghei* and *P. vivax* in *Anopheles gambiae* and *Anopheles dirus* respectively

Previously, we reported that anti-FREP1 rabbit polyclonal antibodies effectively blocked *P. falciparum* in a major malaria vector, *An. gambiae* [11]. Since there is a highly conserved FBG domain among anopheline orthologs, we determined whether anti-FREP1 antibody would also inhibit transmission of other *Plasmodium* species to additional *Anopheles* species. To address this question, *An. gambiae* mosquitoes were fed with *P. berghei* infected blood mixed with rabbit anti-FREP1 serum (1:1 dilution, final titer 5×10^4), and subsequently examined the number of developing oocysts in mosquito midguts. Rabbit pre-immune serum was used as a negative control. The results showed that anti-FREP1 serum significantly reduced the number of *P. berghei* oocysts, compared with the control group that substituted anti-FREP1 serum with pre-immune serum (Fig. 4-2A). The average number of *P. berghei* oocysts per midgut significantly decreased from 10 in the control group to 3 in the experimental group ($p<0.0001$). The results were consistent in two biological replicates.

Figure 4-2. Rabbit anti-FREP1 antiserum inhibits *P. berghei* and *P. vivax* infection of *An. gambiae* and *An. dirus* mosquitoes, repectively.
Control: Pre-immune rabbit serum was used. **Experiment (Exp)**: anti-FREP1 rabbit serum was used.

Next we tested whether anti-FREP1 antibody could inhibit the transmission of another major human malaria pathogen, *P. vivax*, to another major malaria vector in Asia, *An. dirus*. *P. vivax*-infected blood (2 field isolates) was mixed with rabbit anti-FREP1 serum (1:1 dilution, final titer $5x10^4$) and fed to *An. dirus*. The results showed that the anti-FREP1 antibody significantly reduced the number of oocysts per midgut more than two folds compared to the control serum (Fig. 4-2B). Statistical analysis showed that the inhibitory effect of anti-FREP1 antibodies against *P. vivax* infection in *An. dirus* is significant ($p < 0.005$). Together, these data support that anti-FREP1 antibodies can block the transmission of multiple species of malaria parasites to multiple mosquito species.

4.3.3 Experimental immunization of mice with FREP1 does not trigger toxicity or elicit antibodies that cross-react with human fibrinogen

Our sequence alignment displayed a minor degree of homology between mosquito FREP1 and mammalian fibrinogens. Despite this, we also investigated whether immunization of mice with FREP1 causes any autoimmune response. We expressed the recombinant FREP1 protein in *E. coli* and insect cells and purified the recombinant proteins using Ni-NTA affinity columns [11]. For both *E. coli* and insect cell expressed recombinant FREP1, mice were immunized with a series of FREP1 doses (0.2, 2, or 20µg) and boosted with the same dose twice at three-week intervals. Pre-immune human plasma coated ELISA assays were performed to assess the cross reactivity between anti-FREP1 polyclonal antibodies generated in mice and human blood plasma (human fibrinogens).

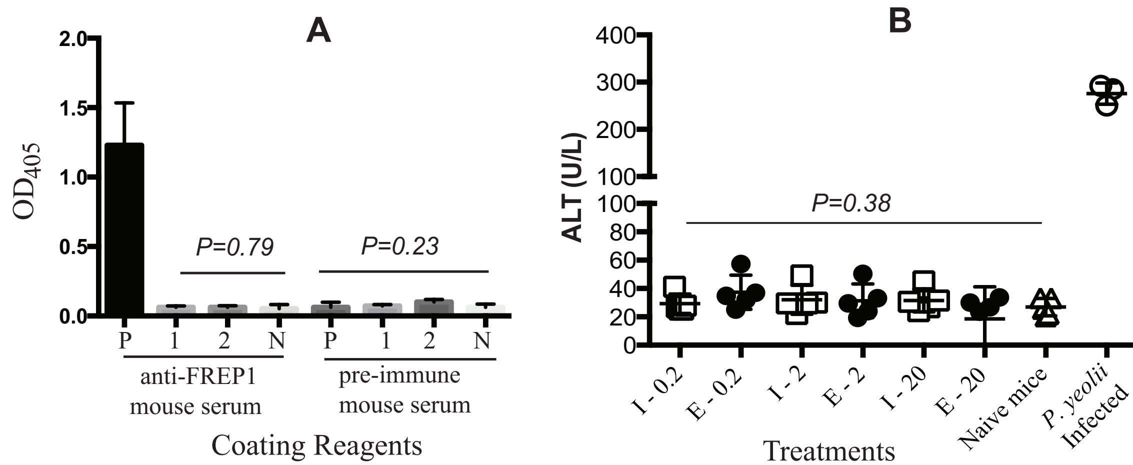


Figure 3-3. FREP1 immunizations do not trigger autoimmune reactions against mammalian or human fibrinogens and are nontoxic to mice.
A: Mouse anti-FREP1 antiserum does not cross-react with human fibrinogens, and no autoimmunity was induced either. P: coated with insect cell-expressed recombinant FREP1; 1: coated with human plasma; 2: coated with 10-fold diluted human plasma; N: wells coated with BSA. **B:** The serum ALT activity does not change significantly between the FREP1-immunized mice and control mice. I-: insect cell expressed recombinant FREP1. E-: *E. coli* expressed recombinant FREP1.

Wells coated with bovine serum albumin or insect cell-expressed recombinant FREP1 were used as negative and positive controls, respectively. The results showed no cross-specific recognition between the anti-FREP1 antibodies generated by *E. coli* or insect cell expressed FREP1 to human plasma fibrinogen (Fig. 4-3A).

Complementarily, we examined whether immunization with recombinant FREP1 triggered toxicity or caused inflammatory immunopathology in mammals by examining the activities of alanine aminotransferase (ALT) in the immunized mice. The positive control was the blood from *P. yoelii*-infected mice, which exhibited high levels of ALT, characteristic of inflammation and liver damage (Fig. 4-3B). Notably, the levels of ALT activity in the mice injected with *E. coli*- or insect cell-expressed FREP1 protein was

similar to the baseline activity observed in naïve mouse serum (p>0.38), supporting that immunization of FREP1 does not cause inflammatory autoimmune or toxicity responses.

4.3.4 Immunizing mice with FBG inhibits the transmission of *P. berghei* to mosquitoes *in vivo* using direct feeding on mice

Although anti-FREP1 antibodies block the transmission of multiple *Plasmodium* species to multiple *Anopheles* species, only FBG domains are conserved among anopheline mosquitoes. Therefore, we next determined whether the highly conserved FREP1 FBG domain alone was an effective TBV antigen *in vivo* and *in vitro*. We cloned the FREP1 FBG domain (from 463 to 729 aa) and expressed it in *E. coli*. Mice were immunized with purified FBG protein mixed with Alhydrogel adjuvant using an optimal prime-boost regimen. High-titer antibody levels ($3x10^6$ on average) against FBG were achieved in anti-serum (Fig. 4-4A). Mice in the control group were immunized with buffer mixed with Alhydrogel under the same regimen. Ten days after second boost, mice were infected with *P. berghei*, and were further used to infect *An. gambiae* directly. Results showed that *An*. *gambiae* mosquitoes fed on the FBG-immunized mice had 4.2 and 3.5 oocysts per midgut on average in two experiments, which was significantly ($p<0.0004$) fewer than mosquitoes fed on mock-immunized mice that had 16.7 and 19.8 oocysts on average in two experiments (Fig. 4-4B). Anti-FBG antibodies reduced the number of oocysts by 75% and 82% in these two independent replicates. We collected the mouse sera and measured titers of anti-FBG antibodies by ELISA endpoint titer assays.

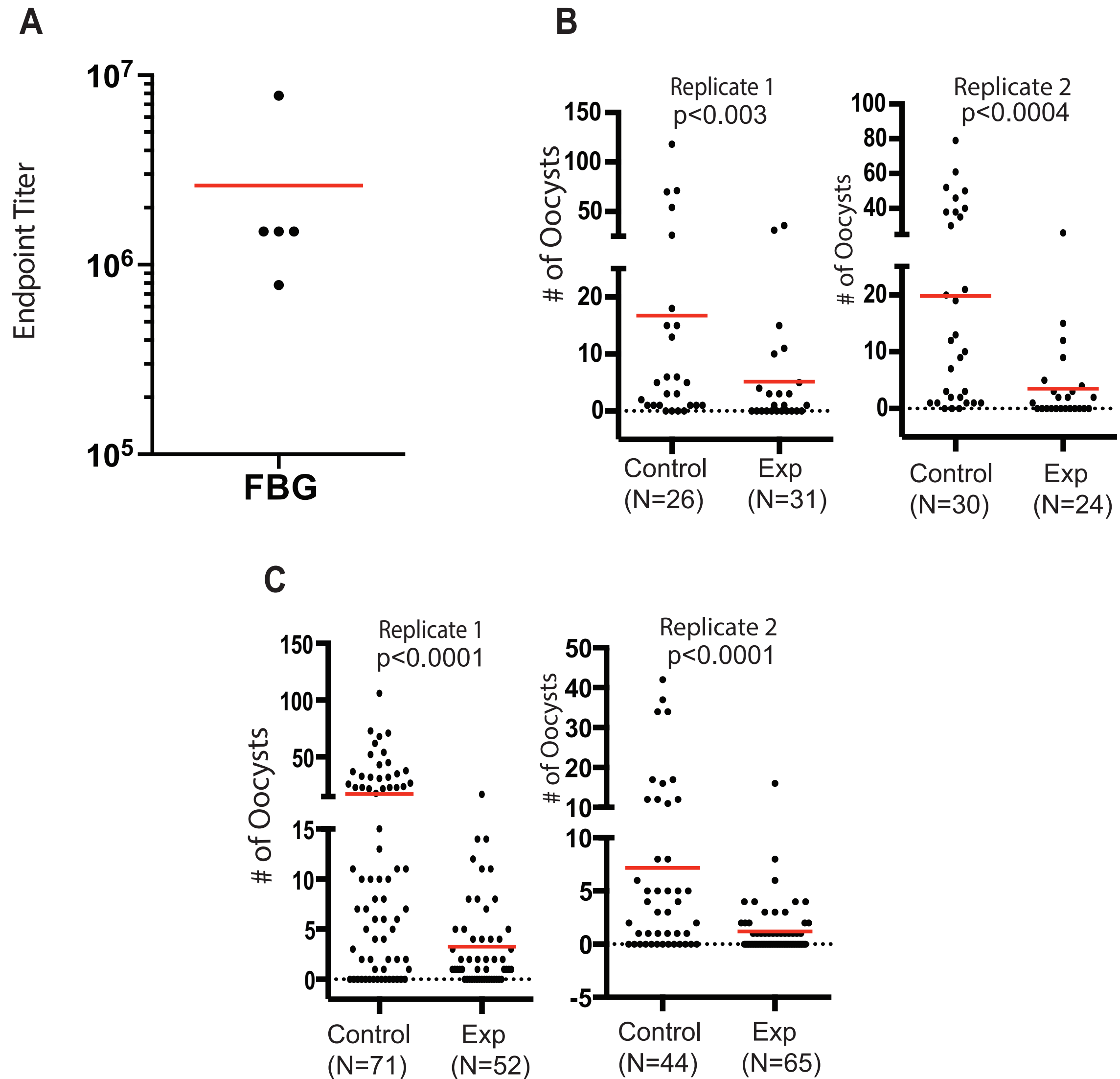


Figure 4-4. Immunization of mice with purified FBG formulated with Alhydrogel inhibits *P. berghei* transmission to *An. gambiae* in *in vivo* studies and *P. falciparum* transmission to *An. gambiae in vitro* with SMFA.

A: Endpoint-titer shows recombinant FBG is highly immunogenic **B:** *in vivo* studies results of direct feeding assays using two FBG-immune mice and two control mice showed the transmission-blocking activity of *in vivo* generated anti-FBG antibody response against *P. berghei* to *An. gambiae* mosquitoes. **C:** In *in vitro* studies, serum collected from FBG-immune mice also blocks *P. falciparum* (NF54 strain) infection to *An. gambiae* mosquitoes. Control or anti-FREP1 immunized mouse sera were mixed with human serum (1:4 ratio) and mixed with *P. falciparum*-infected packed human blood prior to its use in SMFA. The final anti-FBG antibody titer was $4x10^5$.

4.3.5 Anti-FBG anti-serum inhibits the transmission of *P. falciparum* to mosquitoes in membrane-feeding assays

To test whether mouse anti-FBG serum also exhibits transmission-blocking activity against *P. falciparum*, we added immune serum from immunized mice to *P. falciparum*-infected blood containing gametocytes and fed to mosquitoes using SMFA. The average number of oocyst per midgut was reduced from 17.4 and 7.2 in control groups to 3.3 and 1.2 in experimental groups, respectively, in two replicates (Fig. 4-4C). The anti-FBG serum significantly ($p<0.0001$) reduced the number of oocysts of *P. falciparum* by more than 81%. These *in vivo* and *in vitro* experiments demonstrate that the conserved FBG alone is a potent universal TBV antigen.

Since anti-FBG anti-serum blocks *Plasmodium* transmission and FBG domain of FREP1 binds *Plasmodium* parasites, anti-FBG anti-serum should inhibit the interaction between FREP1 and parasites. We used ELISA and IFA to test this hypothesis. For ELISA, we coated a plate with the lysate of sexual stage parasites. The purified FREP1 and anti-FBG serum were mixed and added to coated wells.

4.3.6 Anti-FBG antibodies prevent FREP1 from binding to *Plasmodium* parasites

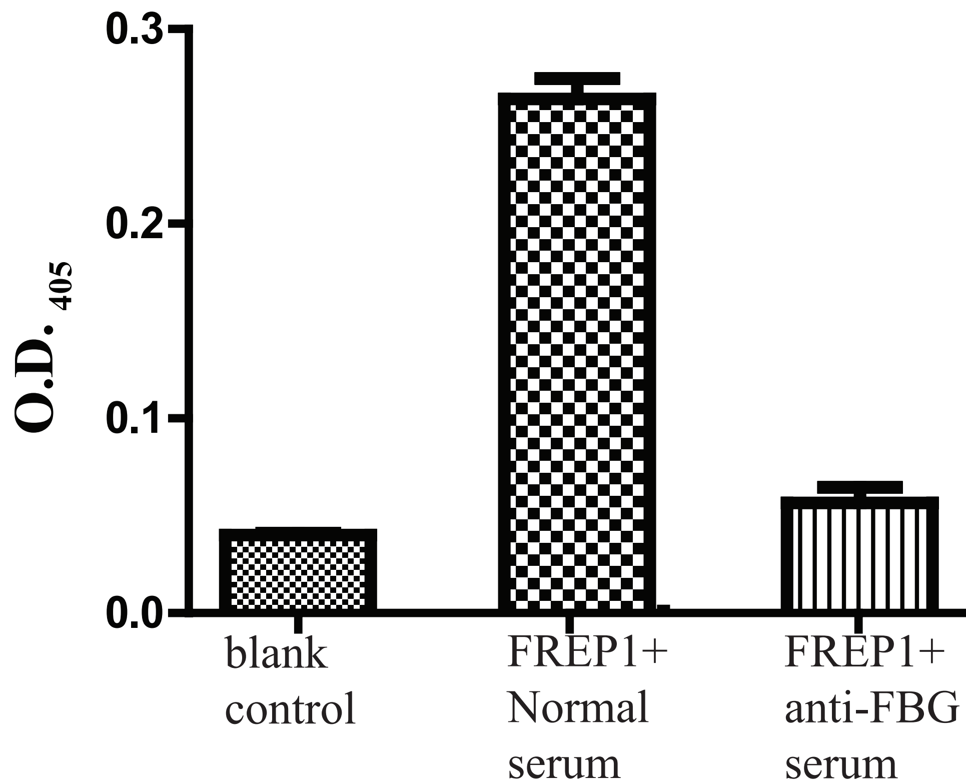


Figure 4-5. ELISA assays demonstrate that anti-FBG mouse serum prevents FREP1 from binding to *Plasmodium* parasites.

The normal mouse serum replacing anti-FBG anti-serum was used as a negative control (no blocking activity). Incubation of coated plate without FREP1 was used as a positive control (completely blocked). Antibodies against N-FREP1 protein quantified the parasite-bound FREP1. Results indicated that anti-FBG anti-serum significantly reduced binding signals between FREP1 and parasites (Fig. 4-5). In order to confirm that anti-FBG anti-serum blocks the interaction between FREP1 and sexual stage parasites, IFA assays were conducted. As shown in Fig. 4-6, *P. falciparum* gametocytes and ookinetes incubated with FREP1 mixed with anti-FBG serum have much weaker signals than those mixed with pre-immune mouse serum.

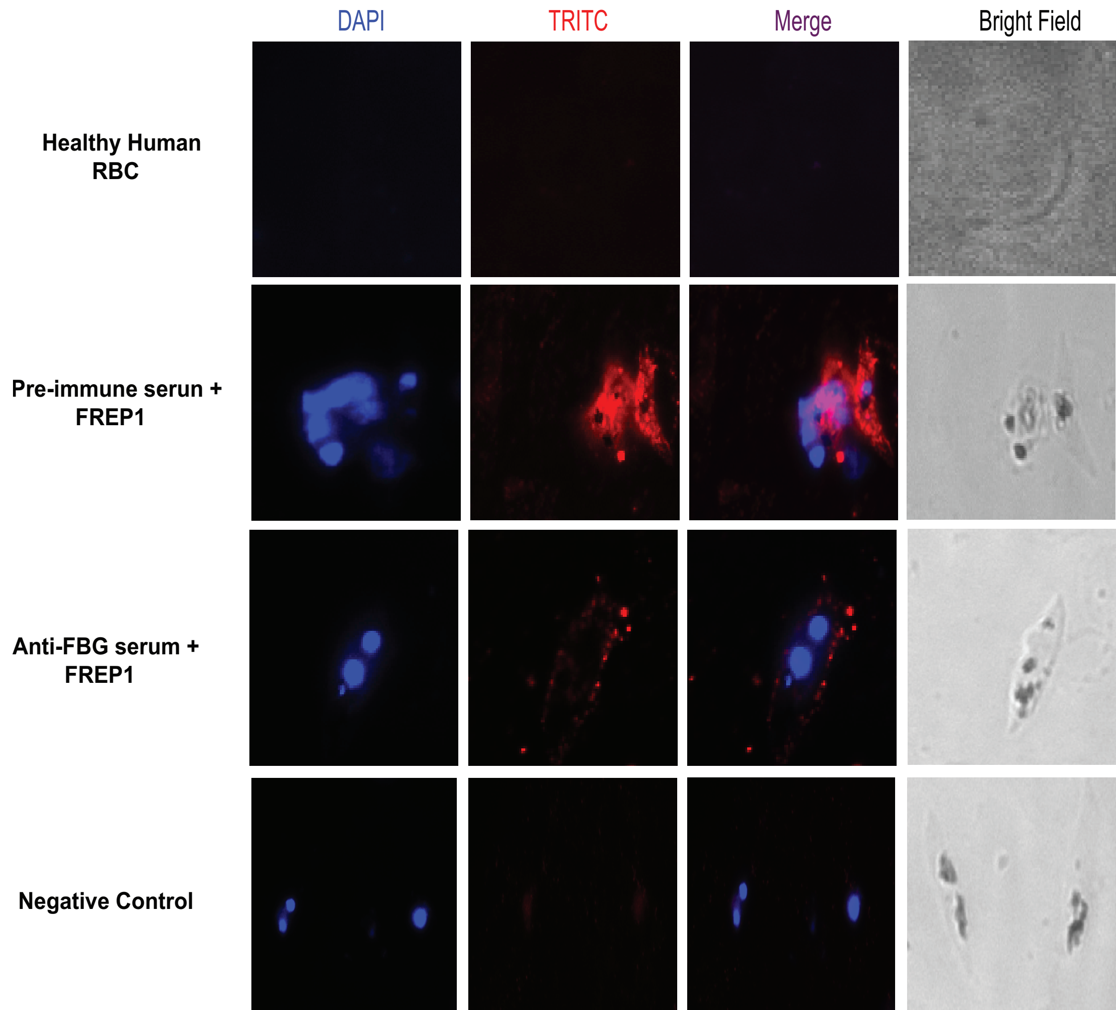


Figure 4-6. IFA assays found that anti-FBG serum prevents FREP1 from binding to parasites.

4.4 Discussion

Malaria TBVs are novel approaches for malaria control. However, there are few efficacious antigen targets that have been described. We identified a novel *Plasmodium* invasion pathway, e.g. FREP1 mediated *Plasmodium* invasion through direct binding to *P. falciparum* gametocytes and ookinetes [11]. Since FREP1 localizes in the mosquito midgut peritrophic matrix, it is readily accessible to antibodies in co-ingested blood. Therefore, mosquito FREP1 is an ideal target for a TBV.

One of the scientific hurdles in developing TBVs is that malaria is caused by multiple species of parasites transmitted by multiple species of mosquito vectors. Identification of an antigen with the potential to limit the transmission of multiple major parasite species by multiple major vectors is critical for developing a successful TBV. Previously, we demonstrated that anti-FREP1 antibody effectively inhibited the infection of *An. gambiae,* a major malaria vector in Africa, by *P. falciparum,* a major human malaria pathogen [11]. Here, we show that anti-FREP1 antibody also significantly reduced the transmission of *P. vivax,* another important human malaria pathogen by *An. dirus,* another major mosquito malaria vector in Asia. Moreover, our data show that anti-FREP1 antibodies also inhibit transmission of *P. berghei* to mosquitoes. *P. berghei* is a distantly related parasite causing rodent malaria. Together, we propose that FREP1 is an excellent candidate as a universal TBV antigen.

We examined the orthologs of FREP1 in anopheline mosquitoes. The length of FREP1 orthologs is various, from 311 amino acids in *An. dirus* to 738 amino acids in *An. gambiae*. However, they share a highly conserved FBG domain that is about 210 amino acids in length. Notably, antibodies against FREP1 FBG domain effectively blocked the transmission of *P. berghei* and *P. falciparum* to mosquitoes. Because the *E. coli*-expressed FREP1 FBG domain used for immunization was purified under a denaturing condition and the transmission-blocking activity of anti-FBG antibody was functional a fully folded FBG is not required as a vaccine antigen. This is an advantage for this highly conserved mosquito midgut antigen [3, 13, 14]. In chapter 3, we discovered that FBG domain in FREP1 binds *Plasmodium* gametocytes and ookinetes.

Here, we verified that anti-FBG serum prevents parasites from binding to FREP1, which explains the molecular mechanisms of FREP1 FBG as a universal TBV antigen.

We also investigated the immunogenicity, toxicity and autoimmunity following FREP1 and FBG immunization of mice. Immunization with either *E. coli* or insect cell expressed FREP1 proteins elicited high titers of antibodies, supporting that recombinant FREP1 and FBG is highly immunogenic. High-titer functional antibodies are required for TBVs. The principle mode of action of this approach depends on high levels of antibodies circulating in the blood of the human host at the time that the malaria mosquito vector takes a bite, in order for the antibodies to prevent parasites from invading mosquito guts. Importantly, FBG immunization of mice with a clinically relevant adjuvant (Alhydrogel) did not induce autoimmune reactions, immunopathology, or elicited cross-reactive antibodies against endogenous or human fibrinogens.

Our data collectively demonstrate that FREP1-mediated *Plasmodium* invasion pathway is highly conserved in *Plasmodium* parasites and *Anopheles* mosquitoes. Indeed, a cocktail of vaccine antigens that includes both FBG and the parasite expressed FREP1-binding partners would be predicted to synergistically increase transmission-blocking efficacy and potentially enable the vaccines to completely inhibit malaria transmission.

4.5 References

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