Determinants of enhanced immunogenicity of *Plasmodium falciparum* transmission blocking DNA vaccines

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Abstract

Malaria is responsible for significant disruption of human lives through huge economic losses, high morbidity and mortality. It is a vector-borne disease transmitted by female *Anopheles* mosquito that is caused by protozoan parasites of the genus *Plasmodium.* Four species of *Plasmodium* are specific for human infections, of which *P. falciparum* is the deadliest form, resulting in over a million deaths each year in children under the age of five. Current control measures rely heavily on treatment and vector control, which, while beneficial, are unlikely to stop transmission and help with malaria elimination. In order to realize World Health Organizations (WHO) short-term objective of disease elimination with the eventual goal of eradication, additional tools that can interrupt transmission of the parasite within communities are urgently required. In order to achieve this, we propose the use of **transmission-blocking vaccines (TBV)** that can target parasite developmental stages in the vector, thereby preventing any further transmission of malaria between hosts, and both infected and uninfected host. Two *P. falciparum* TBV candidate antigens, Pfs25 and Pfs48/45, were tested for functional antibody responses in rodent and nonhuman primate models. In addition, keeping in mind the economic limitations of target population for such a vaccine, we evaluated a DNA vaccine platform for its known benefits as a cost-effective and easy to develop, store and transport platform. However, due to low immunogenicity outcomes often seen with DNA vaccines, we also tested various DNA vaccine enhancement mechanisms such as the use of codon optimization of DNA encoding antigen, in vivo

electroporation and studying the impact of N-linked glycosylation post-translational modification on the immune responses generated against target antigens. The findings reported here demonstrate potent functional transmission reduction with Pfs25 and Pfs48/45 DNA vaccines in rodents and nonhuman primates. In addition, we report the benefits of using codon optimization and in vivo electroporation in enhancing the antibody and functional immunogenicity responses of DNA vaccines. Furthermore, we found that mutating putative N-glycosylation did not significantly impact the immunogenicity outcome of Pfs25 and Pfs48/45 DNA vaccines, suggesting that leaving glycosylation sites intact would be most beneficial for these antigens, and also making a case for determining the impact of N-linked glycosylation of *Plasmodium* proteins on a case by case basis as the outcomes are likely to vary from antigen to antigen. Overall, our findings support the requirement for further investigation into transmission-blocking vaccines using the DNA vaccine platform with the eventual goal of developing a clinical grade vaccine.

This dissertation is dedicated to the most important facets of my life. Ma, baba, didum, dada, bhabhi and Ada.

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Chapter 1: Introduction

1.1 Burden of disease:

Despite the changing patterns of global disease burden, with non-communicable diseases gaining momentum, malaria continues to play a significant role in the disruption of human lives through huge economic losses, morbidity and mortality. As of 2013, there were still 104 countries where malaria is considered endemic with an estimated 3.4 billion people at risk of contracting disease (WHO, 2013). It is estimated that the economic burden of malaria in the African continent alone is around $12 billion per annum [1,2]. In addition, of the 7 million all cause deaths each year of children under the age of 5 years, over 99% are in low-income settings and a significantly large portion of these can be attributed to *Plasmodium falciparum* alone, the causative agent of the most pathogenic form of malaria (WHO, 2011). As a matter of fact, every 30 seconds, a child dies of malaria, resulting in a daily loss of over 3000 children, making it the largest killer of children worldwide (Unicef.org). It is worth noting that between 2000 and 2012, there has been a drop of mortality rates by 42% globally and 49% in the African region and a decline in incidence rates as well (25% globally and 31% in the African region). However, the absolute numbers of cases and deaths have not seen any significant reduction (WHO, 2013). Furthermore, the initial rise in funding seen until 2010 has since plateaued between 2010-2012 [1-3] and a decline in funding and coverage of key interventions that has contributed to the progress made so far, is all but inevitable. Studies by the malaria eradication research agenda (malERA) have found that

aggressively scaling up current control measures with all the available tools, while beneficial, will still fall short of global eradication. It suggests that new tools are needed to complement current strategies that are mainly geared toward reducing morbidity and mortality such that the basic reproduction rate (R_0, i.e. the number of secondary cases arising from a single case) is reduced to less than 1 with the ultimate goal of eradicating malaria from human populations [4,5]. It is also important to keep in mind that the genetic plasticity of malaria parasites dictates that antimalarial drug resistance to even the most recently discovered drugs, is all but guaranteed [6,7]. Taken together, these findings suggest that new preventive tools such as vaccines that could reduce disease transmission within an at-risk community in combination with current control strategies might be crucial to achieving the short-term goal of malaria elimination (Roll Back Malaria Partnership, 2008), while ultimately aiming for global eradication [5,8].

1.2 Lifecycle, immune response and pathogenesis:

Malaria is a parasitic disease caused by Apicomplexan pathogens belonging to the Genus *Plasmodium* under Kingdom Protozoa [9,10]. *Plasmodium* parasites have a wide range of vertebrate hosts but human infection is limited to 4 species: *P. falciparum*, which is the deadliest form, *P. vivax*, *P. malariae* and *P. ovale* (WHO, 2012). A 5th species, *P. knowlesi*, has recently been shown to cause disease via zoonosis [11,12]. The complex lifecycle (Fig 1) of these parasites involves sexual maturation and transmission via female *Anopheles* mosquitoes, the primary host and vector; and asexual development in a secondary, vertebrate host. Human

infection begins with injection of sporozoites in the skin, which then migrate via blood vessels and lymphatics within a few minutes, to infect hepatocytes and begin pre-erythrocytic schizogony (liver stage infection), which lasts for 5-8 days. One *P. falciparum* sporozoite has the ability to develop into as many as 40,000 merozoites per infected liver cell [10,13]. Merozoites rupture from hepatocytes and commence erythrocytic asexual lifecycle stage of the parasite[14,15]. During asexual cycling over a 44-48 hour period, newly formed merozoites infect naïve erythrocytes and either continue asexual development or commit to terminal sexual stage gametocyte formation. Erythrocytic male and female gametocytes are then taken up by female *Anopheles* mosquitoes in a blood meal, leading to the formation of male and female gametes, which fuse to form zygotes. Zygotes transform into motile ookinetes that traverse the epithelium of midgut and develop into oocysts that attach to the basal laminar surface of the midgut. Sporozoites released from mature oocysts then find their way to the salivary glands, ready to transmit malaria to the next human host [16,17].

The human immune system is capable of mounting a wide range of stage-specific responses against the complex and variable lifecycle stages employed by the parasite (Fig. 2). Immune responses to the parasite begin with sporozoites injected into skin, some of which drain to lymph nodes and prime B and T cells. Antibodies are also able to trap some sporozoites in the skin and prevent invasion of hepatocytes. Sporozoites that are able to evade these immune responses go on to infect hepatocytes, once within hepatocytes however, IFNγ producing CD4+ and CD8+ T cells are able to prevent some parasites development into merozoites,

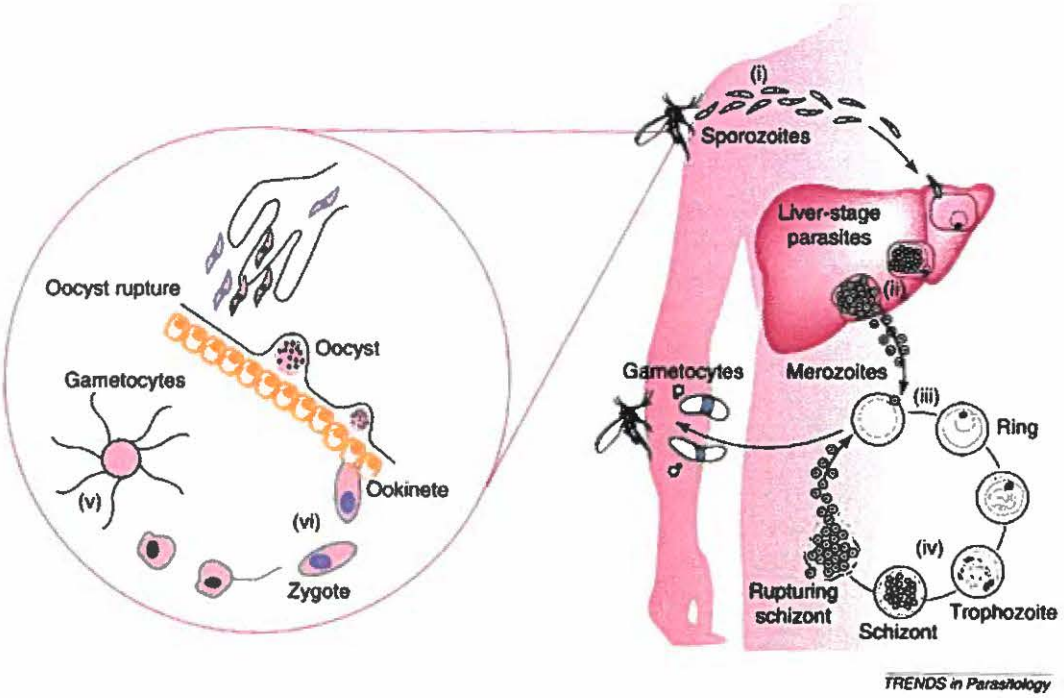
though not sufficient to block further infection entirely. Merozoites that emerge from liver cells are subject to agglutination and opsonization by merozoite-specific antibodies that are able to inhibit invasion of RBCs through receptor blockade. Further, antibodies to variant surface proteins can opsonize and agglutinate infected RBCs (iRBCs) and prevent sequestration (cytoadherence) in small blood vessels. Opsonized merozoites and infected RBCs (iRBCs) can then be phagocytized by macrophages that are activated by IFNγ producing T cells. Complement-fixing antibodies are involved in immune responses to gametocyte and gamete stage antigens, preventing fertilization in the mosquito's midgut after she has taken an infected blood meal. [7,18]

The range of infection with malaria parasites spans from asymptomatic to mild to severe and can even result in death. Disease caused by malaria can be categorized into uncomplicated or complicated (severe). It is generally the asexual erythrocytic stages that are associated with clinical disease. Some aspects of immune-pathogenesis may also be due to the release of toxic waste products such as hemozoin (also known as malaria pigment) accumulated in infected RBCs, released upon RBC lysis and picked up by cells of the immune system. Accumulation of such toxins contributes to the pathophysiology associated with malaria (cdc.gov). Fevers, chills, sweats, headache, nausea and vomiting, bodyaches and general malaise characterize uncomplicated malaria. In the case of *P. falciparum*, this is classically seen every 48 hrs and lasts 6-10 hrs. Severe malaria is characterized by complications arising from organ failure or abnormalities in blood or metabolism. Manifestations include cerebral malaria, severe anemia, low blood pressure, acute

kidney failure, metabolic acidosis, and hypoglycemia, among others. In the case of *P. falciparum* malaria, severe disease during pregnancy as well as recurrent infection in children is of particular concern. (www.cdc.gov).

Fig. 1.1 Lifecycle of *Plasmodium*. Pre-erythrocytic stage life cycle begins with invasion of hepatocytes by sporozoites. (i) and (ii) also show stages of development of liver-stage schizonts targeted by antibodies and cell-mediated immune responses, respectively. Asexual blood stage infection involves invasion of erythrocytes by merozoites and schizogony. (iii) Shows that asexual life cycle can be blocked by immune responses against the merozoite surface and organellar molecules involved in erythrocyte invasion. (iv) Shows antibodies can also target selected VSAs expressed on schizont-infected erythrocytes, effective in preventing (*Plasmodium falciparum*) parasite development, probably by preventing cytoadherence. Sexual life cycle involves differentiation of asexual stages into male and female sexual stages, also known as gametocytes and further sexual development in the mosquito midgut. Gametocytes undergo gametogenesis and produce male and female gametes which fertilize. Zygotes, transform into motile ookinetes which traverse the mosquito midgut wall and develop into oocysts. Sporozoites produced in the oocysts migrate to salivary glands and are injected during next blood feeding cycle. Transmission-blocking: **(v)** antibodies directed against gamete antigens prevent fertilization from occurring. **(vi)** Antibodies against ookinete antigens prevent further development of the sporogonic cycle in the mosquito. (Figure adapted from Targett, 1985).

Fig. 1.1 Lifecycle of *Plasmodium*



1.3 History of malaria:

The rationale for examining the history of malaria is summed up by Randall Packard in the book entitled "*The Making of a Tropical Disease: A Short History of Malaria (2007)*. As stated in the book, "...Malaria policy needs to be informed by history. The history of malaria tells us that malaria cannot be understood or eliminated independently of changes in the societal forces that drive it. This is not to argue that current and past malaria control efforts have had no effect on reducing the burden of malaria. Nor do I believe that malaria cannot be controlled before social and economic impediments to health are completely removed. Rather I argue that the array of biomedical weapons mobilized in the war against malaria needs to be joined with efforts to understand and improve the social and economic conditions that drive the epidemiology of the disease..."

The origins of malaria remain unclear, however, it is highly likely to have emerged among farming populations in the tropical forests of sub-Saharan Africa (The Making of a Tropical Disease, 2007). Malaria is most certainly an ancient disease with first known writings of the symptoms seen in Chinese medical texts from over five thousand years ago. It spread through Greece in 4th century BCE and caused the decline of populations in cities and rural areas and began to be written about extensively. Sanskrit medical texts from India included descriptions of malarial fever and associated it with the bite of insects. Roman writings on the other hand, attributed the disease to swamp lands. In fact, the name malaria comes from Italian for "bad air", a notion that has since been proved false but the name stuck (cdc.gov). The malaria parasite was discovered in 1880 by a French army surgeon

names Alphonso Laveran who noticed parasites in the blood of a patient, a discovery for which he was awarded the Nobel Prize in 1907 (www.cdc.gov). Ronald Ross, a British officer in the Indian Medical Service, was the first to demonstrate that mosquitoes transmitted the parasites and a sporogonic cycle (parasite development in the mosquito) was required for transmission. Ross received the Nobel Prize for his discovery in 1902 (cdc.gov). While Ross's discovery was based on avian parasite studies, it was Giovanni Batista Grassi of Italy, who sketched out the entire transmission cycle of human malaria parasites *P. falciparum*, *P. vivax* and *P. malariae* between 1898 and 99 (www.cdc.gov).

The discoveries of Laveran, Ross and Grassi among others have allowed malaria control efforts to have a biological focus, and will be discussed at length in the following section. Our knowledge of *Plasmodium's* biological systems is still incomplete, which warrants further investigations. However, on the other spectrum of malaria is the ecological systems, influenced greatly by human interventions, such as agricultural expansions of the 19th century that resulted in a resurgence of malaria in many tropical and sub-tropical regions. It will be important to consider the entire picture in future malaria control and elimination policies (The Making of a Tropical Disease, 2007).

1.4 Malaria control and elimination:

In 2008, the roll back malaria partnership adopted strategy that was comprised of three components designed to target disease elimination: (i) aggressive control in high endemic countries, (ii) progressive elimination from low

to mid-endemic countries, and (iii) research into vaccines, next-generation drugs and insecticides among other interventions; with the ultimate goal of eradicating malaria [19,20]. It is important to first examine the efforts and progress that were made with respect to malaria control and elimination in order to understand the rationale behind the 2008 global malaria action plan. The last 100 years have seen remarkable advances in the efforts to reign in malaria, beginning with the discovery of the *Plasmodium* parasite and it's transmission by *Anopheles* mosquitoes. [21,22]. In the first half of the 20th century about 178 countries had endemic malaria with very little control efforts, largely disrupted by the two world wars. Between 1945 and 2010 however, 79 countries were able to eliminate malaria [23,24], [25,26]. Population growth notwithstanding, 50% of the world's population lives in malaria-free areas compared with 30% in 1950 [27,28]. The Global Malaria Eradication Program was introduced in 1955 for vector control (indoor residual spraying), and systematic detection and treatment of cases but was abandoned by 1969 due to financial, administrative and technical issues (WHO, 1969). The 70s and 80s saw a resurgence of malaria due to a collapse of interventions, chloroquine and DDT resistance, as well as political and economic instability [29,30], [31,32].

In spite of the holdups of the 70s and 80s, significant progress towards elimination has been made owing to the widespread use of long-lasting insecticide-treated bednets (LLINs) (up from 3% in 2000 to 53% in 2012) [3,11], artemisinin-based combination therapy (ACTs) and rapid diagnostic tests (RDTs) [11,33]. WHO's World Malaria Report 2013 summarizes the disease burden estimates from 2000-2012. It reports that increased financial investments and political commitment have

resulted in around 3.3 million lives saved during this period. Achievements notwithstanding however, the goal set out by Global Malaria Action Plan (GMAP) of 50% reduction in the number of deaths and cases by 2010, was not met (Roll Back Malaria Partnership, 2008). This led to the creation of an updated set of goals for the end of 2015, which includes a 75% case reduction from levels in 2000 (Roll Back Malaria Partnership, 2011). Currently, significant challenges threaten the ability to meet the goals for 2015, which include a funding plateau, lack of data on increasing incidence rates in some regions, inadequate control tools coupled with the spread of drug and insecticide resistance. An important agenda of GMAP that is a long way from completion is the research and development aspect for development of new control and elimination tools[4,34]. The significance of R&D into new interventions was highlighted in a 2010 model-based evaluation of current intervention strategies that factored in a switch to artimisinin combination therapy (ACT), increased coverage of long-lasting insecticide treated nets (LLINs) as seen since year 2000; along with additional rounds of indoor residual spraying (IRS), mass screening and treatment (MSAT), and also factored in future use of the RTS,S/ASO1 vaccine. Their findings suggest that while it is possible to reduce prevalence to 1% in low and moderate transmission settings using currently available tools; it is necessary to invest in new tools if similar successes are desired in areas of high malaria transmission [1,35].

1.5 Malaria vaccines:

One key R&D agenda outlined by the malaria eradication plan, is vaccines [4,36]. There are no licensed vaccines against *P. falciparum* despite years of research resulting in extensive knowledge gained with respect to development of in vitro parasite culture methods and access to *Plasmodium* whole genome sequence information [6,37,8,11] among other advances. After the collapse of the Global Malaria Eradication Program in 1969, which was followed by almost 25 years of inaction, the idea of a malaria vaccine emerged with initial SPf66 clinical trials showing around 30% efficacy [9,27]. SPf66 was based on small synthetic peptides based on asexual stage antigens chemically linked and formulated with adjuvants. Due to disappointing outcomes in subsequent clinical trials, progress with this particular candidate lost momentum [11,38]. However, since the demonstration of the ability of irradiated sporozoites to confer sterile immunity [13,35], there has been a strong focus on recombinant vaccine development using CSP and other sporozoite surface antigens [14,39]. One particular candidate vaccine based on CSP, named RTS,S combined with a strong adjuvant, has undergone a series of clinical trials and has consistently shown 30-50% efficacy against the disease (reviewed in [16,40,18,41]). RTS,S/ ASO1 entered phase III clinical trials at 11 different sites in the African continent, recruiting 15,000 children, in 2011 [19,42,7,21], and seems poised to become the first licensed malaria vaccine in the market [23,43, 5,25]. Promising as the outlook for the first malaria vaccine appears specifically with regards to reduced mortality rates, it is unlikely to impact malaria transmission or play a significant role in reducing gametocyte prevalence [27,44]. Partial protection, as seen with RTS,S will

require continued support from current control measures such as ITNs and IRS. Furthermore, studies have shown that adults in endemic regions present with pre-existing non-sterile immunity [29,45] as well as older children in endemic regions will serve as asymptomatic reservoir for continued transmission [31,46]. Considering that the target of such RTS,S vaccination protocols in endemic regions would be routine infant immunizations [11,46], the transmission pattern in these regions is likely to be altered. Thus, epidemiological changes that would result from partial elimination within populations due to RTS,S, an anti-infection vaccine in essence, could potentially increase risk of active disease in previously protected adults [11,47].

The complexity of the malaria parasite lifecycle allows for vaccine design that can target multiple different lifecycle stages. The first partially effective vaccine to be developed, dating back to the early 70s, is comprised of pre-erythrocytic vaccines, with RTS,S as the frontrunner. The idea is to prevent disease by blocking further development after sporozoite infection. Parasite density at the sporozoite and liver stage infection is low enough to be considered a bottleneck in the lifecycle and thus, is a viable target for vaccine development [34,48]. Apart from vaccines based on the circumsporozoite protein, other candidate antigens such as TRAP and liver stage antigens LSA1 and LSA3 have also been studied, in particular, as multi-antigen vaccines or in a prime-boost regimen combined with CSP [35,49]. Despite the odds, progress has been made using the irradiated whole sporozoite approach. However, the need for cryopreservation for storage and transport, and intravenous immunization for potent immune responses, suggest that some advances are needed before we have a licensed killed-whole parasite vaccine [36,47]. An alternative

approach under consideration, that has shown promise in humanized mouse studies, is the development of genetically attenuated sporozoite vaccination [37,48]. It is unlikely, that any therapeutic vaccine, with around 30-50% efficacy that persists for 1-2 years will be able to replace current control measures [11,48]. They can however, contribute significantly when used in combination with ITNs and IRS, especially in areas of high transmission, to reduce the burden of disease. Additional measures, specifically, vaccines that can reduce malaria transmission, are key pieces of the puzzle needed to target malaria elimination [27,50].

Another approach of vaccine development that was first theorized in the early 60's, are vaccines that target asexual or blood stage parasites as seen first with immunoglobulins from semi-immune adults, when transferred to infected children, were able to clear parasites [38,51]. Additionally, the rationale for using anti-disease vaccine comes from observations of protection against clinical malaria in young infants as long as maternal antibodies remain available, and also the general ability to control parasitemia after repeat exposures of individuals in endemic areas [35,52,53]. The primary targets of asexual vaccine development, that have undergone extensive preclinical and clinical testing, are antigens on the surface of merozoites such as MSP-1 and AMA-1[54]. There are however, considerable limitations to developing blood-stage vaccines such as the need for diversified immune responses as well as the need for mechanisms to overcome high levels of genetic polymorphisms displayed by the parasites [34]. An important antigen of blood stage infection is PfEMP1 (*P. falciparum* erythrocytic membrane protein 1). While the high degree of antigenic variation makes it a difficult candidate for vaccine development purposes,

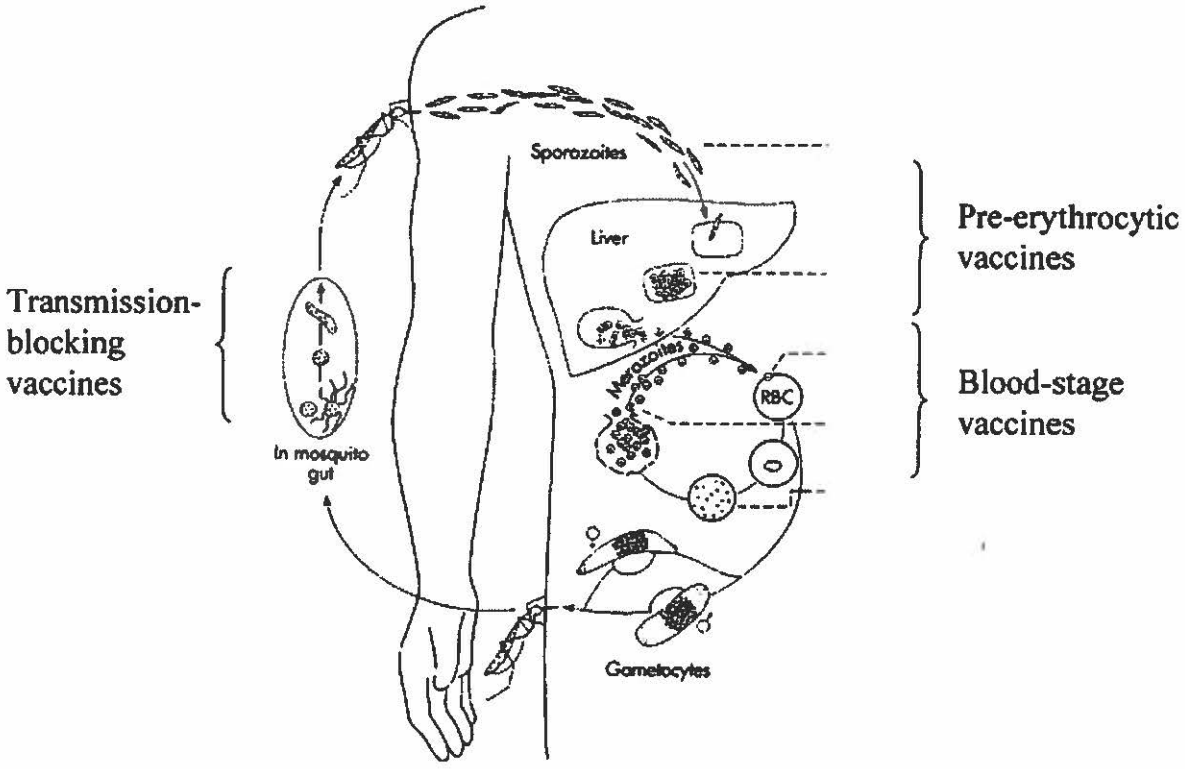
it has been identified to play a key role in severe disease manifestation as well as in malaria-related complications in pregnancy [55], justifying the need for further investigation with this antigen.

Under the broad concept of vaccines that interrupt malaria transmission or VIMT, the malERA Consultative Group on Vaccines suggested that a VIMT could be composed of a few basic components ; (i) a highly effective pre-erythrocytic vaccine that reduces parasite prevalence, (ii) a highly effective erythrocytic vaccine that reduces parasite density and thus, impacts transmission, and finally (iii) transmission-blocking vaccines (TBVs) that target sexual and mosquito stage parasite development in order to prevent vector to host transmission [27]. The first two categories have been covered in the preceding section and with details on progress made in each of the two. It is important to note that both categories are currently only modestly effective and only the first (with RTS,S) is anywhere close to a licensed clinical vaccine. This, among other reasons, highlights the importance of exploring the third option, i.e. TBVs, which will be the focus of the rest of this thesis.

Fig 1.2 Malaria vaccines targeting various lifecycle stages. Lifecycle of a malaria parasite (*Plasmodium falciparum*) showing three phases of the cycle to which vaccines are being developed

(Figure from Carter, 2001)

Fig 1.2 Malaria vaccines targeting various lifecycle stages



Chapter 2: Transmission-blocking vaccines (TBVs)

2.1 Transmission biology

Before we analyze the concept of transmission-blocking (TB) vaccines, it is important to understand the biology of *Plasmodium's* sexual differentiation and development stages in order to make informed decisions on vaccine design. Sexual differentiation into male and female gametocytes begins about 7-15 days after the appearance of parasites in the bloodstream [56]. This process can be further divided into induction, commitment, and stages of progression through maturation, however the molecular mechanisms governing gametocytogenesis are not fully understood [57]. Biological factors such as gene regulation[58] as well as stress factors such as parasite density, anemia, host immune responses, or drug treatment [59], may determine whether parasites develop into gametocytes or remain in the asexual cycle [57]. It is at the schizont stage that parasites become committed to either asexual or sexual development and gender specificity is determined for those undergoing gametocytogenesis [60]. Sex ratio is typically skewed towards a female bias by 5:1 of males, which is postulated to be a balance mechanism as one male gametocyte can form about 8 microgametes during the fertilization step in mosquitoes [61]. Once committed to sexual development, the process of gametocyte formation takes about 10 days in the case of *P. falciparum* parasites (in most other *Plasmodium* species the process lasts about 2 days) [62]. Gametocytes go through 5 stages of maturation in the vertebrate host [63]. The first 4 developmental stages are sequestered in tissues and once terminally differentiated, the stage V gametocytes are released in circulation

where they become infectious to mosquitoes in another 2-3 days [64]. The phase of *Plasmodium's* lifecycle in the mosquito midgut is known as the sporogonic phase and is initiated after a blood meal by the female *Anopheles* mosquito in which both male and female gametocytes are ingested [65]. Once in the mosquitoe's midgut, the process of gametogenesis is initiated, which lasts about 10-20 mins and results in the formation of 1 female gamete from 1 female gametocyte, and as many as 8 male gametes as a result of 3 mitotic divisions of 1 male gametocyte [57]. The signals for exflagellation (the initiation of gamete formation) are largely environmental factors such as a drop in temperature by ~5^0 C [66], alkaline pH in the gut as well as xanthurenic acid, a byproduct of eye pigment synthesis in mosquitoes [67]. A shift in pH was originally thought to be an additional signal but later stated to be an artificial inducer of exflagellation (reviewed in [56]). Post-exflagellation, fertilization between male and female gametes results in zygote formation which is the only diploid stage of the parasite lifecycle [57]. Zygotes then undergo meiotic division resulting in haploid reproduction within oocysts, which leads to the formation of hundreds of sporozoites that migrate to the salivary glands to be transferred to a vertebrate host during a mosquito blood meal, in order to initiate a new infection cycle [65].

The rationale behind designing malaria transmission-blocking vaccines is to target parasite lifecycle stages that develop in the mosquitoes, in order to prevent the transmission to a vertebrate/human host [46]. Target antigens of transmission-blocking immunity belong to the following categories (i) pre-fertilization antigens that are expressed on gametocyte and gamete surfaces and elicit responses that

fully understood, however the involvement of key players of the immune system have been charted, and is key in making informed decisions for the design of TBVs. The important role of antibodies is well established and several studies have demonstrated the ability of sera from individuals in endemic regions to block transmission [40, 73, 74]. Purified IgGs from immune sera of individuals are able to prevent fertilization and further development of zygotes and ookinetes by interfering with specific molecules required for gamete interaction during fertilization [75]. Additionally, TB IgG antibodies can be either neutralizing or complement fixing [42,76] with the latter resulting in the lysis of mature gametes and zygotes. The role of complement was first demonstrated in studies in *P. gallinaceum* wherein the alternative complement pathway caused lysis of newly fertilized zygotes [77]. Studies have further established that immune sera recognizing sexual stage antigens such as Pfs230 and Pfs48/45 do not effectively block parasite development in the absence of complement [78]. In the case of Pfs48/45 however, studies with specific monoclonal antibodies have shown that some are capable of blocking without complement whereas others are complement dependent [73]. Cytokine involvement in transmission-blocking immunity has not yet been fully mapped out, however TNFα and IFNγ that are present in high levels during parasite-induced paroxysm in patients, are also associated with reduced infectivity of gametocyte to mosquitoes [79], [80]. They are also known to contribute to interruptions in gamete fertilization, ookinete motility and parasite penetration of mosquitoes midgut epithelium [81]. Lastly, in addition to the role of antibodies, complement and cytokine, sustenance of antibody levels dependent upon

immunological memory driven by long-lived plasma cells and memory B cells is also an important consideration for effectiveness of TBVs [7,49].

2.3 Target antigens of TBVs:

Sexual stage surface antigens were first identified in 1983 by surface radio-iodination of zygotes and of the four proteins identified at the time. Two of these, Pfs25 and Pfs48/45, will be discussed in further detail. Since the sequencing of the *Plasmodium* genome, many more sexual and mosquito-stage proteins have been identified that are currently being investigated as additional potential vaccine candidates [82]. The first target antigen in question, Pfs25 is a 25 kDa protein expressed post-fertilization on the surface of zygote and ookinete stages of *P. falciparum* and is a cysteine-rich protein made up of four tandem epidermal growth factor (EGF) domains [83]. Pfs25 is believed to function as a group in combination with adjacent surface protein Pfs28, and both are relatively conserved across *Plasmodium* species [45]. Gene knockout studies using a *P. berghei* P25 and P28 resulted in reduced formation of ookinetes confirming their role in mosquito stage parasite development [84]. Pfs25 was first demonstrated as a candidate TBV using monoclonal antibodies and subsequently validated using a vaccinia virus delivery method and also as a recombinant protein expressed in yeast [85, 86]. Various other studies have examined alternative vaccination options with Pfs25 such as dimerized conjugation techniques [87], nasal immunizations with *Pichia pastoris*-produced antigen [88], among others. *Pichia pastoris*-expressed Pfs25 using Montanide ISA 51 adjuvant was also tested in phase 1 human trials and showed moderate blocking of

infection but adjuvant associated reactogenicity resulted in the studies being cut short [89]. More recently, a study with codon harmonized recombinant Pfs25 was expressed in *E. coli* and generated potent transmission-blocking antibodies after appropriate refolding [90] making it a promising platform for further evaluation of the candidate antigen. It is thus apparent that Pfs25 has the potential of being an important TBV candidate provided that the vaccination and delivery platforms are optimized.

A second potential TBV candidate, Pfs48/45, is a cysteine-rich protein belonging to the Pf12 superfamily that also includes Pfs230 and Pfs47, and is highly conserved across *Plasmodium* species [45]. Pfs48/45 is homogenously expressed on the surface of male and female gametocytes and gametes and gene disruption studies have shown that it plays an important role in male gamete fertility and thus, is important for zygote formation and transmission [91]. While the number of pre-fertilization antigens since the discovery of Pfs48/45 and Pfs230 has grown, these two still remain the lead candidates for vaccine development on account of being best characterized and also validated by transmission-blocking assays [86]. An added benefit of pre-fertilization is the potential for natural boosting as these proteins are seen in circulation on gametocyte surfaces in the vertebrate host [92]. There is the likelihood of a detrimental outcome of natural boosting however; i.e. the possibility of immune selective pressure as a result of immune surveillance resulting in reduced immunogenicity and enhanced antigenic diversity [81]. Furthermore, there is the likelihood that natural boosting may not occur for blocking antibodies and might even be detrimental to the binding of transmission-blocking antibodies. Many of the

questions regarding the immune correlates of protection for Pfs48/45 and Pfs230 remain to be answered. As a matter of fact, in comparison to post-fertilization antigen Pfs25, these antigens as vaccine candidates have been explored to a much lesser extent. Studies have attempted to investigate the TBV potential of Pfs48/45, expressed in bacterial [93], vaccinia virus [94] and yeast cells [95] but with limited success attributed, at least in part to loss in conformation due to incorrect folding in a heterologous host. Partial success was obtained with 10-20% correctly-folded protein produced in *Escherichia coli* that had some *P. falciparum* oocyst reduction potential in *A. stephensi* mosquitoes [96]. The blocking efficiency was further enhanced with the co-expression of *E. coli* chaperones resulting in close to 90% of a correctly folded protein [97]. In 2009, using the process of codon harmonization, a properly folded full-length recombinant protein expressed in *E.coli*, demonstrated significant blocking potential in membrane feeding assays [98]. However, clinical-grade vaccines that can be produced and scaled up with ease and can demonstrate potent reduction in transmission are still elusive, which entreats investigation into alternative vaccine development strategies based on current TBV candidates.

Finally, it is important to keep in mind the uniqueness of parasitic infections, when designing vaccines [99]. Antigenic variation, immunodepression and unnatural immune responses demand a multi-pronged approach to targeted vaccine design against diseases such as malaria [100]. These factors necessitate the consideration of transmission-blocking vaccines, the target antigens for which are under relative less immune selection pressure compared with pre-erythrocytic and erythrocytic vaccine candidates, since they are primarily expressed in mosquito stages. That said,

considering the immune exposure of pre-fertilization TBV antigens due to expression on gametocyte surfaces, and the possible down side of natural boosting (discussed earlier in this section), it is also important to design multivalent transmission-blocking vaccines that combine the antigenic properties of various candidates [81]. Factoring in the complications associated with production of individual TBV candidate antigen using the various traditional vaccine platforms (discussed earlier in this section), the need for alternate vaccine development platforms becomes a key requirement. The simplistic design of DNA vaccines might just be the solution to the hurdles faced so far with TBV design.

2.4 DNA vaccines: overview and immune responses:

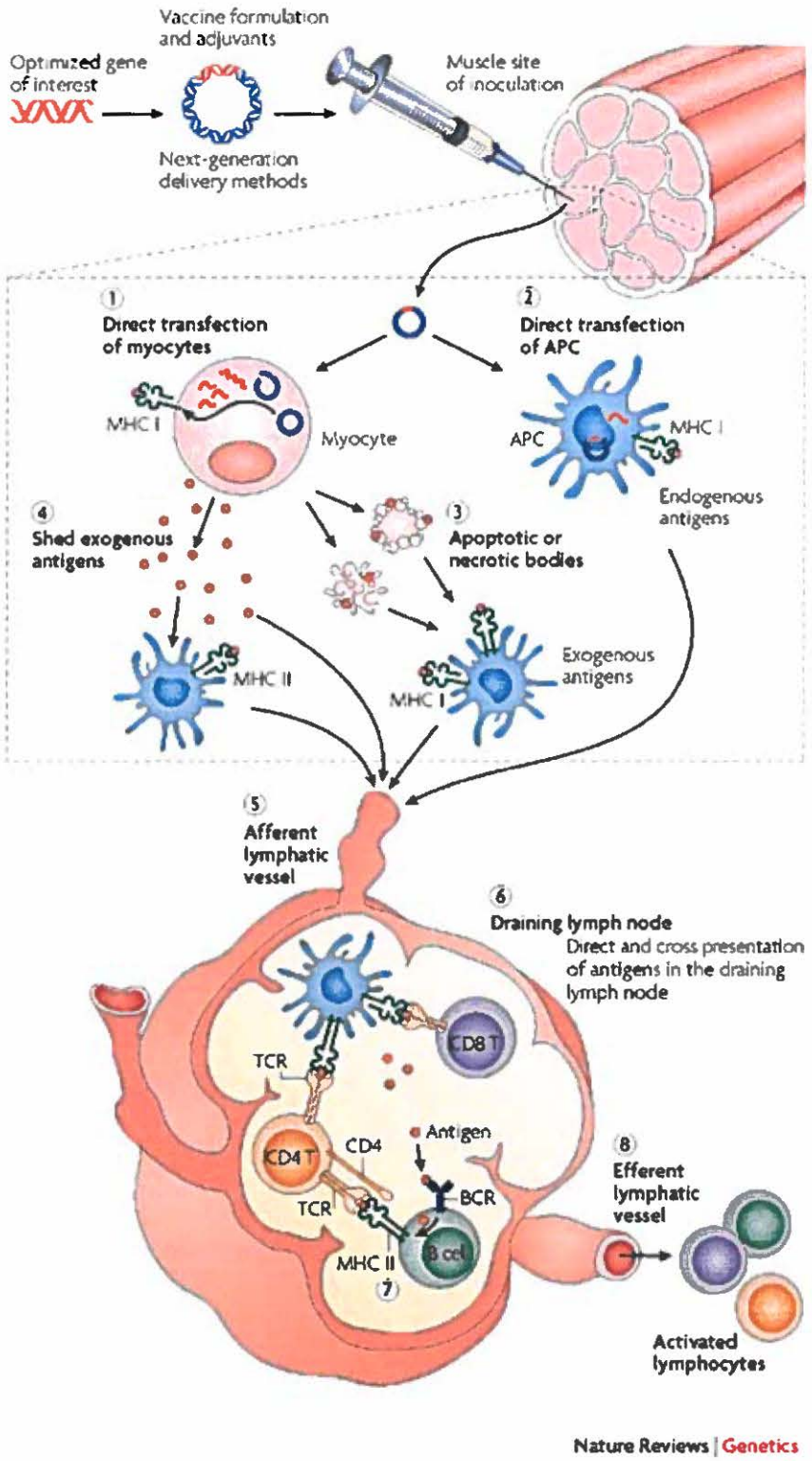
Currently-licensed vaccines for human use are either killed, subunit, or live attenuated pathogens. Limitations seen with such vaccines including skewing of immune responses to either cellular or humoral arm, reduced immunogenicity due to attenuation and safety issues [101]. DNA vaccines provide a novel subunit vaccine platform that allow protein expression in mammalian cells after the introduction of plasmids encoding the antigen of interest [49]. DNA vaccines are able to stimulate all 3 arms of adaptive immunity, namely antibodies, helper T cells and CTLs, while also generating rapid innate responses [102]. In addition, they are easy to design, manipulate and store, which makes them the ideal sentries for complex lifecycles, stage specificity, antigenic variations and immune evasion mechanisms that are the mainstay of parasitic infections [49]. While the immune responses generated differ from one target antigen to another and are host dependent, the general consensus is

that DNA vaccines are quite efficient in generating primary immune responses and also inducing immunological memory [46]. These outcomes are significantly stronger in mice than in nonhuman primates, which warrant further optimization of candidate vaccines [46]. However, recently-licensed veterinary DNA vaccines for pigs and horses suggest that earlier postulations of reduced potency due to size in larger animals and humans may not be entirely accurate and warrants further studies in improving the immunogenicity of DNA vaccines [102].

The simplistic design of a DNA or genetic vaccine involves bacterial vectors encoding a gene of interest from a pathogenic source that is under the control of a strong promoter from cytomegalovirus (CMV) [103]. Injection of DNA vaccines is usually into muscle tissue or skin with a syringe, often with the aid of propulsion devices such as gene guns or electroporators. The outcome is uptake of DNA into cells followed by transcription and translation of the gene of interest, resulting in a diverse array of humoral and cell-mediated immune responses [104]. The mechanism of action of DNA vaccines is believed to involve plasmid DNA entry to the nucleus of transfected cells followed by transcription and translation resulting in protein production, which then undergoes additional post-translational modifications using host cellular machinery [105]. Host-produced antigens are then subjected to antigen presentation in the context of MHC class I and II as detailed in the figure below (Fig 2.1).

Fig. 2.1 The optimized gene sequence of interest (for example, antigenic or immune adjuvant genes) is generated synthetically or by PCR. This sequence is enzymatically inserted into the multiple cloning region of a plasmid backbone, purified, and then delivered to the inoculation site by one of several delivery methods to either the skin, or muscle. Using the host cellular machinery, the plasmid presumably enters the nucleus of transfected keratinocytes (skin delivery) or myocytes (intra-muscular route of delivery) (1) and possibly resident antigen presenting cells (APCs) (2); here, the plasmid components initiate gene transcription, which is followed by protein production in the cytoplasm and the consequent formation of foreign antigens as proteins or as peptide strings. The cell provides endogenous post-translational modifications to antigens that reproduce native protein conformations and the cell customizes the antigens in a similar manner to the pathways induced by live infection with recombinant vectors. (Figure adapted from Kutzler and Weiner, 2008)

Fig. 2.1 Induction of cellular and humoral immunity by DNA vaccines



DNA vaccines encoding *P. falciparum* transmission-blocking antigens, Pfs25 and also Pvs25 (a *P. vivax* homologue of Pfs25), have been studied extensively in mice as well as nonhuman primates, albeit with the notable observation of reduced immunogenicity in higher mammals [47, 48, 106, 107, 51] (described further in Chapter 3). However, these as well as other studies (reviewed in [108]), have emphasized the need for further optimization and enhancement mechanisms. These may include DNA prime-protein boost strategy, sequence optimization for mammalian cell expression, the use of in vivo electroporation, N-linked glycosylation site mutations and effective delivery methods. As far as TBV target antigens are concerned, the pre-fertilization vaccine candidate Pfs48/45 has never been examined on a DNA vaccine platform. The overarching goal of studies reported in this thesis included studies with individual candidate antigens as well as the development of multivalent transmission-blocking DNA vaccines based on Pfs25 and Pfs48/45.

2.5 DNA vaccines: modifications:

The idea of DNA vaccination as an immunization tool was conceived in the early1990s with direct gene transfer studies in mice (reviewed in [100]). Since then, as is the case with any vaccine platform, various modes of optimization have been under review with respect to different target antigens and with the goal of developing a candidate that can be licensed for human use. Codon optimization was conceived as an optimization tool to take into consideration the differences in codon usage in different species. Codon bias in different organisms is based on the most efficient mode of gene expression, with related species using similar codons [49]. Since

DNA vaccines use cells of target organisms for transcription and translation of the antigens, improved gene expression via codon optimization can result in increased protein yield leading to enhanced immune responses [105]. Among the enhancement technologies used to date for DNA vaccines, electroporation (EP)-based DNA delivery has demonstrated up to a 1000-fold increase in DNA delivery potential over traditional mechanisms [109], making it one of the most promising enhancements for DNA vaccines available. EP-mediated DNA delivery relies on an electrical pulse based reversible, short-lived, increase in cell membrane permeability along with an inflammation-based influx of antigen-presenting cells to the site of delivery that result in increased uptake of vaccine as well as processing and presentation of target antigen. [110]. When tested with Pfs25, EP-mediated immunization revealed 100-fold increased immunogenicity in mice and approximately 10-fold enhancement in nonhuman primates [107, 51]. Thus, codon optimized DNA sequences and in vivo EP use in mouse models make a strong case for the future use of these enhancement combinations in clinical studies [107].

Protein glycosylation [which can be asparagine (N)- or serine/threonine (O)-linked] is a form of post-translational modification, which results in the formation and attachment of carbohydrate side chains onto the amino acid backbone of polypeptides. Such modifications seen in all biological systems contribute in a wide range of functions such as protein folding, quality control, surface receptor molecular interactions and epitope recognition [111]. The role of N-linked glycosylation on immunogenicity of DNA vaccines is less obvious in comparison to codon optimization or EP-based delivery, and is highly antigen specific. An influenza

HA antigen based DNA vaccine showed that N-linked glycosylation had little impact on the antibody response against H5 HA antigen [112]. Another study looking at an Ebola virus-based DNA vaccine showed that the outcome varied based on the position of N-glycosylation sites mutated, ranging from deleterious to enhanced immunogenicity [113]. With respect to *Plasmodium* proteins, the presence or absence of N-glycans in itself has been a controversial topic but most current literature suggests that *Plasmodium* forms severely truncated N-glycan precursors at putative glycosylation sites, resulting in carbohydrate sidechains that are significantly different from those seen in mammalian systems [114]. It can be speculated that *P. falciparum* antigens encoded by DNA vaccines when introduced in mammalian cells, are likely to undergo mammalian post-translational modifications. Additionally, such modified malarial antigens with the presence of putative N-glycosylation moieties expressed in mammalian cells are expected to be structurally and functionally different from native *P. falciparum* forms and might even impact ensuing immune responses. Blocking putative N-glycosylation sites might preserve the native structure, conformation and biological function of these antigens, which may influence the immune responses generated against them.

Thus, a combination of enhancement strategies such as codon optimization and in vivo electroporation, while also understanding the role of N-linked glycosylation on immunogenicity, may be key to overcoming the insufficient immune responses seen previously in Pfs25 DNA vaccines studies [47, 48, 107, 51]. In addition, a similar approach is likely to benefit additional targets such as Pfs48/45.

HYPOTHESIS:

We hypothesize that codon optimization for enhanced mammalian expression of encoded genes and mutation of putative N-glycosylation sites on the expressed target antigen, coupled with in vivo electroporation, will markedly influence the immunogenicity of Pfs25- and Pfs48/45- encoded by DNA vaccines.

OBJECTIVES:

The following specific aims were pursued to address the stated hypothesis. Studying the impact of in vivo electroporation, codon optimization, and modifications of N-glycosylation sites in various permutations, of *P. falciparum* TBV candidates, Pfs25 and Pfs48/45, will improve effective immunogenicity with the long-term goal of developing a multivalent transmission-blocking DNA vaccine against human malaria infections.

Specific Aim 1: To evaluate the impact of codon optimization and N-linked glycosylation on functional immunogenicity of Pfs25 DNA vaccines in pre-clinical studies in mice.

Specific Aim 2: To study immunogenicity of Pfs48/45 encoded by DNA vaccines in mice for eliciting potent transmission-blocking antibodies, vaccine optimization approaches including codon optimization, N-linked glycosylation and in vivo electroporation will be evaluated.

Specific Aim 3: To evaluate *P. falciparum* transmission-blocking DNA vaccines encoding Pfs25 and Pfs48/45 by in vivo electroporation in nonhuman primates.

Chapter 3 (Specific Aim 1):

Evaluation of the impact of codon optimization and N-linked glycosylation on functional immunogenicity of Pfs25 DNA vaccines in pre-clinical studies in mice

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Abstract

P. falciparum sexual stage surface antigen Pfs25 is a well-established candidate for malaria transmission-blocking vaccine development. Immunization with DNA vaccines encoding Pfs25 has been shown to elicit potent antibody responses in mice and nonhuman primates. Studies aimed at further optimization have revealed improved immunogenicity by the application of in vivo electroporation and using a heterologous prime-boost approach. The goal of studies reported here was to systematically evaluate impact of codon optimization, in vivo electroporation and N-linked glycosylation, on the immunogenicity of Pfs25 encoding DNA vaccines. The results from this study demonstrated that while both codon optimization and in vivo electroporation greatly improved functional immunogenicity of Pfs25 DNA vaccines, the presence or absence of N-linked glycosylation did not significantly impact vaccine efficacy. These findings suggest that leaving N-glycosylation sites intact on Pfs25 encoded by DNA vaccines is not detrimental to overall transmission-blocking efficacy.

3.1. Introduction

In 2013, there are still 104 countries where malaria is considered endemic and WHO estimated that 3.4 billion people were at risk of disease, with ~207 million cases and ~627,000 deaths reported in 2012, 77% of which were children under the age of 5 [WHO World malaria report 2013]. Of the established *Plasmodium* species that cause human malaria, *P. falciparum* is responsible for the most morbidity and mortality and is thus the major focus of current vaccine development efforts.

However, due to limited understanding of the immune correlates of protection combined with a complex multi-stage parasite lifecycle, progress towards vaccine development has been slow [43]. The malaria eradication research agenda (malERA) initiative of 2011 underscored the need for a multi-pronged approach for malaria control and elimination that includes vaccines targeting infection [44] and transmission along with various other control measures currently in use such as indoor residual spraying and insecticide-treated mosquito nets [5].

Malaria transmission-blocking vaccines (TBVs) target the parasites lifecycle stages that develop in the mosquito vector with the goal of reducing or interrupting transmission and further spread of the disease [45]. The primary mode of action of TBVs is via generation of antibodies that target parasite sexual stage antigens expressed in the mosquito midgut. In the case of *P. falciparum*, vaccine candidates include pre-fertilization antigens, Pfs230 and Pfs48/45, as well as post-fertilization antigens such as Pfs25 and Pfs28 [46]. So far, vaccine approaches based on recombinant protein-adjuvant formulations have met with limited success due to the complex conformational nature of these *P. falciparum* TBV antigens, often resulting in improperly folded, unstable proteins using recombinant expression systems [46]. In this respect, DNA vaccines, encoding specific TBV target antigens have offered a useful alternative to traditional platforms as seen in murine [47] and nonhuman primate models [48] using *P. falciparum* TBV antigen-based DNA vaccines. Additional benefits that strengthen the case for use of DNA vaccines include ease of design and sequence modification, stability and transportability [49]. Studies in mice with DNA plasmids encoding Pfs25 showed high TBV activity with >95% oocyst

reduction [47]. Similar studies in rhesus macaques on the other hand, after four immunizations doses revealed only modest immunogenicity. However, heterologous boosting with recombinant protein improved immunogenicity significantly [48]. DNA vaccine delivery using in vivo electroporation (EP) further improved outcomes and studies in mice revealed that a 2-log lower dose of plasmid was capable of generating anti-Pfs25 antibodies comparable to immunization without EP in mice [50]. Further investigation using a nonhuman primate model (olive baboons, *Papio anubis*) also suggested a dose-dependent enhancement in antibody titers and improved functional blocking using EP combined with a heterologous prime-boost regimen [51].

Pfs25 and Pfs28 are highly conserved surface proteins found on the surface of zygotes and were present during transformation into ookinetes [52,53,69]. Pfs25 contains four epidermal growth factor (EGF) domains and is GPI-anchored on the surface of ookinetes and contains a secretory N-terminal signal sequence [83]. In addition, the amino acid sequence of Pfs25 contains three putative N-linked glycosylation sites. Post-translational modifications such as N-glycosylation have been known to play a crucial role in the folding, stability and functional integrity of proteins and are especially relevant when using mammalian host for antigen expression [76,115].

The impact of N-linked glycosylation has been extensively studied with respect to viral virulence and immune evasion (reviewed in [116]). In particular, the impact of mutations in putative glycosylation sites in DNA vaccines encoding viral antigens has been examined with respect to cellular and humoral immune

responses [117, 118, 119]. In the case of *Plasmodium* however, understanding the impact of N-glycosylation on immunogenicity has not been explored extensively. Glycosylation of proteins in *Plasmodium* has remained highly controversial [120, 121] and a recent study has suggested formation of severely truncated N-glycan side chains in *Plasmodium* due to the absence of glycosyltransferases required for precursor side chain generation [114]. A few studies have examined the role of N-glycosylation for immunogenicity of *Plasmodium* antigens. An unglycosylated MSP-1 vaccine proved more efficient in monkey experiments when challenged with a lethal dose of *P. falciparum* [122]. On the other hand, a study with *E. coli* and *P. pastoris* derived AMA-1 showed that both glycosylated and unglycosylated antigens were immunologically and functionally equivalent and unaffected by posttranslational modifications [123]. A more recent study on Pfs48/45 expressed in plant systems suggested that aberrant glycosylation at putative N-glycosylation sites was functionally detrimental and recommended producing non-glycosylated antigens to preserve the biological activity and native conformation of target proteins [124]. In contrast, comparing glycosylated and non-glycosylated variants of PfAMA1 produced in *Nicotiana benthamiana* in rabbits suggests that the presence of N-linked glycosylation might increase vaccine efficacy [125]. The varied immunogenicity outcomes with different viral and *Plasmodium* antigens emphasize the importance of determining the impact of N-linked glycosylation on an individual case basis.

The aim of this study was to investigate whether the antigenic product of a Pfs25 DNA vaccine is N-glycosylated in mammalian cells and if so, does such unnatural glycosylation of Pfs25 has any impact on the stability and functional

immunogenicity parameters. Combined with codon optimization and in vivo EP, the N-glycosylation status of Pfs25 may suggest ways to further improve the effectiveness of DNA vaccines for further development.

3.2. Materials and Methods

3.2.1 DNA plasmids

DNA vaccine vector VR1020 (Vical Inc. San Diego, CA) was used to prepare three different plasmid constructs, each encoding Pfs25 lacking N-terminal signal and C-terminal anchor sequences [47]. The first contained a wild-type coding sequence (Pfs25WT), the second contained Pfs25 codon optimized for optimum expression in mammalian cells (Pfs25SYN), and the third contained codon optimized Pfs25 wherein all the 3 putative N-linked glycosylation sites were mutated from N to Q (Pfs25MUT). Plasmid DNA (<30 EU/mg), purified by Aldevron (Fargo, ND) and supplied at 2.5 mg/ml concentrations was used for all immunizations (Fig. 3.2.1).

3.2.2. Immunization dose and scheme

Five-seven week old female BALB/c mice (NCI, Bethesda, MD) divided into 4 groups per vaccine construct, received 3 intramuscular doses of DNA at four-week intervals. Mice were bled prior to each dose and one month after each immunization. Group 1 received 25 ug DNA/mouse without electroporation (no EP). Groups 2, 3, and 4 were immunized with EP at 25 ug, 2.5 ug and 0.25 ug dose/mouse, respectively. Electroporation was administered using ICHOR pulse generator and TriGrid Electrode Array (Ichor Medical Systems Inc. San Diego, CA) as

described in [126]. DNA was injected using 0.3cc U-100 Insulin syringes (BD Biosciences, NJ) and followed approximately 10 seconds later by electrical pulse at an amplitude of 250 V/cm of electrode spacing (2.5 mm spacing was used). Mice were anaesthetized for immunization using Isoflurane solution and DNA doses were administered in 20ul PBS in the anterior tibialis muscle. Immunization studies were conducted in three independent experimental replicates (Fig. 3.2.1).

3.2.3 Mammalian cell transfections studies

DNA plasmids (Pfs25 WT, SYN and MUT) were transfected into mammalian HEK293T cells using Megatran 1.0 transfection reagent (OriGene Technologies, Rockville, MD) as per product protocol at 2 different concentrations (1 ug/ml and 0.5 ug/ml). Cell medium was changed 4-5 hours post transfection and subsequently maintained with or without tunicamycin (5 ug/ml) (Sigma-Aldrich, St. Louis, MO), an N-glycosylation blocking inhibitor, for 48 hours. Supernatants (culture media) and cells were tested for protein expression by western blotting.

3.2.4 Recognition of recombinant Pfs25 (rPfs25) protein by Western blot analysis

Western blot analysis was performed using ECL method according to manufacturer's protocol (Amersham Biosciences, Piscataway, NJ). rPfs25 [90] was electrophoresed on a 12.5% polyacrylamide gel under non-reduced and reduced conditions and transferred to nitrocellulose membrane. Membranes were blocked with 5% milk-PBST (PBS + 0.1% Tween 20) and cut into strips. Individual strips were incubated for 1 hour with pooled immune sera collected after 3 DNA

immunizations at 1:4000 dilutions (groups immunized w/ EP) and processed as described in [106].

3.2.5 Antibody analysis

Immune sera were analyzed for end-point titers, antibody isotypes and avidity using ELISA. 96 well Immulon-2 plates coated with 100ul/well of 1.5 ug/ml rPfs25 in carbonate buffer (pH 9.6) were used and the assays were performed as previously described [48]. To determine avidity of antibodies, plates were incubated for 15 mins with NaSCN (0, 1, 2, 4, 8 M) after primary antibody incubation and washed prior to incubation with secondary antibody and complement of the remaining ELISA steps. Binding of antibody to antigen after NaSCN treatment was expressed as a percent of total binding in the absence of NaSCN. For antibody isotype analysis, mouse sera were tested at 1:250 (no EP group) and 1:4000 (EP groups) dilutions. The various secondary antibodies used were peroxidase-conjugated goat anti-mouse IgM, IgG_1, IgG_{2a}, IgG_{2b} and IgG_3 (Invitrogen, Carlsbad, CA). Immunofluorescence assays (IFA) were conducted using *P. falciparum* zygote/ookinete-rich parasite preparations that were spotted onto multi-well slides, and fixed with methanol, and used per protocol instructions[106].

3.2.6 In vivo transmission-blocking analysis

Transgenic *P. berghei* parasites expressing Pfs25 (TrPfs25Pb) [127] were used to detect the blocking potential of Pfs25-specific immune sera in vivo. Groups of BALB/c mice immunized with 25 ug DNA (SYN and MUT groups, EP and no EP) after

three DNA doses were divided into test and control groups. Test mice were infected with 10^6/transgenic *P. berghei* (TrPfs25Pb) and control mice received wild type *P. berghei* (Pb WT) parasites. 5 days after infection, mice were used to infect starved *An. stephensi* mosquitoes (25-30 per mouse). Fed mosquitoes were maintained at 19^0 C and 80-90% relative humidity, and dissected 9-10 days post blood feeding to enumerate midgut oocysts [50].

3.2.7 Total IgG purification, parasite culture and standard membrane feeding assays (SMFA)

Total IgG was purified from pooled mouse sera collected after 3 DNA immunizations (25ug DNA; WT, SYN and MUT DNA groups; no EP and EP) using protein A-Sepharose beads as described [90]. Mature, stage V gametocytes of *P. falciparum* (NF54) produced in vitro [128] were used in SMFAs. Gametocytes were mixed with varying concentrations of purified IgG (50-1000 ug/ml) and human RBCs to a final 50% hematocrit and 0.3% gametocytemia. This mixture was fed over 5-6 hrs to 25-30 starving female *An. gambiae* (*Keele* strain) mosquitoes (4-5 days old) as described [51]. All SMFAs included negative controls of IgG from non-Pfs25-immunized mice sera pools and normal human sera. Transmission-blocking activity was determined by calculating reduction in the percent of infected mosquitoes and also reduction in the number of oocysts per midgut as described [50].

3.2.8 Statistical analysis

Antibody end-point titers were defined as serum dilution with absorbance higher than the average absorbance of pre-immune sera+3 standard deviations (SD). Percent inhibition of oocyst development and mosquito infectivity differences were analyzed as described [50]. Statistical analysis was performed using the GraphPad Prism software package.

3.3. Results

3.3.1 Evidence for N-linked glycosylation of Pfs25 in mammalian cells

Mammalian cells (HEK293T) transfected with Pfs25 WT, SYN and MUT DNA were analyzed by western blotting (using pooled anti-Pfs25 antibodies obtained from mice immunized with Pfs25 for primary incubation) to determine protein expression and N-linked glycosylation (Fig. 3.3.1). Expressed Pfs25 protein was detected in both supernatants4 and cell lysates indicating partial secretion of protein out of the cytoplasm. On a per cell basis, WT and SYN DNA revealed higher levels of protein expression compared with MUT DNA. Size of Pfs25 expressed using MUT plasmid (19 kDa) was a lower molecular weight as compared to 25 kDa of protein from WT= and 23 kDa from SYN-transfected cell. Tunicamycin (TN) treatment affected the size of expressed protein from WT and SYN plasmids comparable to that from MUT plasmid. As expected, tunicamycin treatment had no effect on protein expressed from MUT plasmid (Fig. 3.3.2)

3.3.2 Antibody analysis

Immune sera obtained from three independent immunization replicates were analyzed for antibody end-point titers and the averages after 3 DNA doses are shown (Fig. 3.3.2). EP groups showed significantly higher titers than no EP groups. Even at a 10-fold lower immunization dose, WT DNA and SYN DNA (2.5 ug DNA dose with EP) outperformed no EP groups. Additionally, we were interested in investigating the immunomodulatory effect of N-linked glycosylation in inducing antigen-specific antibody responses. Our findings show that antibody titers with SYN DNA were consistently higher than with MUT DNA. SYN DNA titers were also higher than WT DNA titers, suggesting the benefits of codon optimization. In one experimental replicate, at a dose of 25ug/mouse by EP, the average end point titers with SYN DNA were ~300,000 whereas with both WT DNA and MUT DNA, the average titers were ~150,000.

Furthermore, antibody responses after the first DNA immunization from all three experimental replicates were examined to assess benefits of EP over no EP after initial priming DNA dose. As seen in Fig. 3.3.3, the percent of mice responding after a single dose immunization with 25 ug DNA EP (WT, SYN and MUT) ranged between 90-100%. Even at a 10-fold lower 2.5 ug DNA dose, EP groups had 60-80% mice responding. In contrast, no EP groups immunized with 25 ug DNA had only 20-50% of the mice responding after priming dose.

We also compared antibody avidity in sera from mice immunized with the 25ug DNA dose, without or with EP (WT, SYN and MUT DNA groups). NaSCN concentrations resulting in 50% loss of bound antibodies were not significantly

different among WT, SYN and MUT DNA groups and when comparing EP to no EP groups. Concentrations of NaSCN required for 50% dissociation of bound antibodies ranged from 1-1.5 M for no EP groups and from 2.6-3.0 M for EP groups; and were not statistically different between any of the groups (Fig. 3.3.4).

In addition, we wished to explore whether mutating N-glycosylation sites would skew isotypes of elicited antibody responses. Sera from mice immunized with different vaccine plasmids without or with EP were analyzed. A balance of IgG1 and IgG2a isotypes was seen across all groups (Fig. 3.3.5). To further confirm antigen specificity of induced antibodies, polyclonal sera from all three groups with EP were evaluated by western blotting using rPfs25 and by IFA using *P. falciparum* zygote/ookinate stages. All the sera from mice immunized with WT, SYN and MUT plasmids recognized the non-reduced and reduced forms of Pfs25 expressed in *E.coli* (non-glycosylated) and the recognition was not impaired for recognition by antibodies generated against the antigen modified by N-linked glycosylation(Fig. 3.3.6a). Furthermore, sera from all EP immunization groups were also specific for Pfs25 protein present on the surface of post-fertilization parasite stages (zygotes and ookinete) as revealed by IFA (Fig. 3.3.6b).

3.3.3 In vivo transmission-blocking activity of immune sera after 3 DNA immunizations

Mice immunized with 3 DNA doses of SYN and MUT DNA (25ug, no EP and EP) were challenged using transgenic *P. berghei* parasites expressing Pfs25 (TrPfs25Pb) followed by in vivo transmission to *An. stephensi* mosquitoes (Fig. 3.3.7). Both SYN and MUT test group mosquitoes showed drastically reduced

numbers of oocysts and percent of infected mosquitoes with transgenic parasites in the test groups (0-8% infectivity) compared with the control groups infected with wild type *P. berghei* parasites. Mice immunized with SYN and MUT DNA with EP or no EP revealed potent transmission-blocking activity suggesting that a three-dose immunization is sufficient to elicit potent transmission-blocking antibody responses. Non-vaccinated mice likewise challenged with transgenic and wild type *P. berghei* were included to establish that those parasites were comparable otherwise, in their ability to infect mosquitoes.

3.3.4 Standard membrane feeding assays (SMFA) with purified IgGs from immune sera

In order to evaluate transmission-blocking differences between WT, SYN and MUT DNA groups (no EP and EP), SMFAs were conducted with varying concentrations of purified IgG from immune sera pooled from mice of each group in two of the three experimental replicates. In both experimental replicates, purified IgG from EP groups tested at 1 mg/ml and 500 ug/ml (WT, SYN and MUT DNA) showed potent transmission-blocking (ranging from 96-100%). Transmission-blocking with antibodies from WT, SYN and MUT DNA EP groups at 250 ug/ml also showed significant blocking (80-99%). In contrast to EP groups, no EP groups in one experimental replicate showed significant blocking only at 1 mg/ml concentration of IgG for SYN DNA group (88%), whereas the blocking was slightly improved in another replicate, with 94-98% blocking at 1 mg/ml and 500 ug/ml IgG concentrations. This is possibly an outcome of the overall higher end point titers for all groups seen in this experimental replicate. Taken together, the results showed

significantly improved blocking for EP groups. Further evidence for antibody dose-dependent transmission-blocking activity is evident from reduced blocking seen at lower (100 ug/ml and 50 ug/ml) IgG used in SMFA (Table 3.3.1)

3.4. Discussion

DNA vaccines are still in their nascent stages with respect to research and development, and we still do not entirely understand the mechanisms with which they induce immune responses. However, considering the challenges faced by various other subunit vaccine development strategies requiring adjuvant formulations and complex production stages; and the potential benefits of using DNA vaccines with its simplistic design and approach [49, 102], especially when considering resource limited settings [129], further investigation are warranted to understand the mechanisms involved and also to improve the immunogenicity of candidate DNA vaccines. Previous studies have revealed potent immunogenicity of Pfs25 DNA vaccines in mice, especially after electroporation [47,50]. However, similar studies in rhesus macaques without EP [48] and in baboons [51] revealed a need for heterologous protein boost for optimum transmission-blocking effectiveness of induced antibodies. In the present study, we undertook a systematic evaluation of the comparative immunogenicity of DNA vaccines based on native Pfs25 sequence (WT), codon optimized for optimal expression in mammalian cells (SYN) and codon optimized Pfs25 sequence wherein all three putative N-glycosylation sites were mutated (MUT). We wished to determine a) whether codon optimization for mammalian expression improves immunogenicity of Pfs25 DNA vaccines, b)

whether Pfs25 undergoes N-linked glycosylation when expressed in mammalian cells, and c) if the presence of N-glycosylation impacts the functional immunogenicity outcome.

In vitro transfection studies demonstrated that Pfs25 encoded by DNA vaccines is post-translationally modified in mammalian cells resulting in the addition of N-glycan side chains at putative N-glycosylation sites, which is unlike the native state of the protein in the parasite. Mutating putative N-glycosylation sites blocked the addition of N-glycan side chains to the polypeptide backbone. Results from cells transfected with tunicamycin-treated WT and SYN plasmids also confirmed that Pfs25 expressed in mammalian cells undergoes N-linked glycosylation. Evaluation in mice revealed that codon optimized Pfs25 plasmid with N-glycosylation sites intact (SYN) was most potent in eliciting highest antibody titers when compared with native Pfs25 sequence or with a codon optimized N-glycosylation site mutant sequence (MUT), suggesting that the mutations did not confer benefits for Pfs25 specific antibody enhancement. We do not know if these in vivo differences are due to enhanced expression of Pfs25 from SYN DNA or due to reduced expression and / or reduced stability of Pfs25 encoded by MUT DNA. Despite differences in antibody titers between SYN and MUT immunized mice, in vivo transmission-blocking studies using transgenic *P. berghei* parasites in a mouse model showed no difference in blocking by antibodies induced by SYN and MUT DNA plasmids encoded antigens, with both groups capable of ~95-99% blocking. Because of the nature of this in vivo assay that does not allow testing of varying

antibody concentrations, we further evaluated functional activity using an ex vivo SMFA.

Findings from SMFAs testing different concentrations of purified IgGs showed no significant differences in the transmission-blocking among the various groups, with blocking potential plateauing at approximately 250ug/ml total serum IgG. A significant difference between groups was seen with enhanced immunogenicity when comparing EP with no EP procedures, reasserting the benefits of electroporation in improving the immunogenicity of DNA vaccines. Comparing ELISA end point titers and percent responder mice, our results suggest a 10- fold vaccine dose sparing effect by EP compared with no EP approach. Furthermore, the quality of antigen-antibody binding as determined by avidity assays and western blotting did not reveal significant differences among immunization groups as expected. Antibody isotype analysis indicated a consistent pattern across the groups with a more or less balanced IgG1/IgG2 response, which was not altered by codon optimization, EP or N-glycosylation site mutations.

All of these finding when put together indicate that Pfs25 produced in mammalian cells is N-glycosylated, however the modifications are not detrimental to the immunogenicity of the antigen and Pfs25-specific immune sera are able to recognize glycosylated and unglycosylated forms of the antigen. Furthermore, the functional transmission-blocking potential of Pfs25-specific immune sera induced by the N-glycosylated form of protein was not compromised. While our N-glycosylation mutational studies were not extended to determine which of the three putative sites are glycosylated and whether introducing different mutation

combinations would result in different outcomes. Our studies however do suggest that leaving glycosylation sites intact does not compromise immunogenicity, and additional mutational studies are unlikely to inform strategies for improving the immunogenicity of Pfs25 DNA vaccines. These findings are in contrast to the Pfs48/45 studies in *N. benthamiana* that suggest that aberrant N-linked glycosylation of *Plasmodium* proteins by mammalian post-translational modification machinery is likely to be detrimental to immunological outcomes of the antigen [124]. On the other hand, our finding align well with post-translational modification studies with PfAMA-1 produced in *P. pastoris* that showed that immunogenicity and functional responses to PfAMA-1 were not altered by post-translational modifications [123]. However, unlike the results of another PfAMA-1 study in *N. benthamiana* [125], we did not see any significant enhancement in immunogenicity resulting from N-glycosylation.

Our findings, reviewed in light of previous studies with varied outcomes, highlight the importance of examining the role of N-glycosylation on the immunogenicity of DNA vaccine candidates on a case-by-case basis. Our studies also highlight the importance of extending these immunogenicity evaluation studies to understand the specific immune correlates of protection underlying individual candidate vaccines so that novel enhancement methods can be utilized for immunogenicity studies. While the role of N-glycosylation needs to be analyzed on an individual antigen basis, the EP-based delivery method has now been established as an invaluable tool for enhanced immunogenicity. EP allows the possibility of accommodating combinations of multiple plasmid molecules without affecting total

DNA dose; a feature that can be exploited for developing a multi-lifecycle stage, multi-antigen DNA vaccine [109]. The safety of EP technology has also been reproducibly demonstrated in various pre-clinical studies [50, 51], suggesting that further functional immunogenicity enhancement studies for DNA vaccines will need to be built upon the advantages presented by EP delivery.

Fig. 3.2.1. Immunization dose and schedule

N = 5 F balb/c mice per group Total = 12 grps	WT		SYN		MUT	
DNA Plasmid	Pfs25WT		Pfs25SYN		Pfs25MUT	
Electroporation (EP)	-	+	-	+	-	+
DNA Dose (ug)	25	25	25	25	25	25
		2.5		2.5		2.5
		0.25		0.25		0.25

Immunization experiments conducted as 3 independent replicates

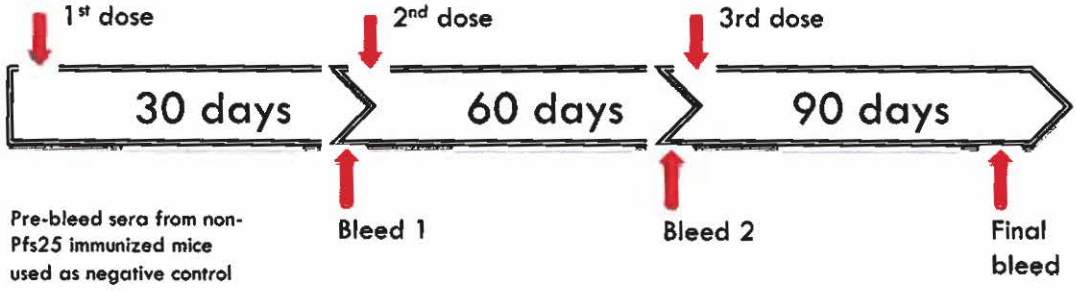

Fig 3.3.1. Evidence for Pfs25 N-linked glycosylation by in vitro mammalian cell transfections and Western blotting. Expression of Pfs25 was investigated after in vitro transfection of mammalian HEK293T cells with DNA plasmids and cultured in the presence or absence of tunicamycin (TN). Cell supernatant (a) and lysate (b) were analyzed by SDS-PAGE under non-reducing conditions. Lane 1 – standard protein markers. Lane 2 – rPfs25 protein control. Lane 3 and 4 – No DNA negative control. Lane 5 through 10 – WT, SYN or MUT DNA transfected samples, treated in the presence (+) or absence (-) of TN.

Fig. 3.3.1. Evidence for Pfs25 N-linked glycosylation by in vitro mammalian cell transfection

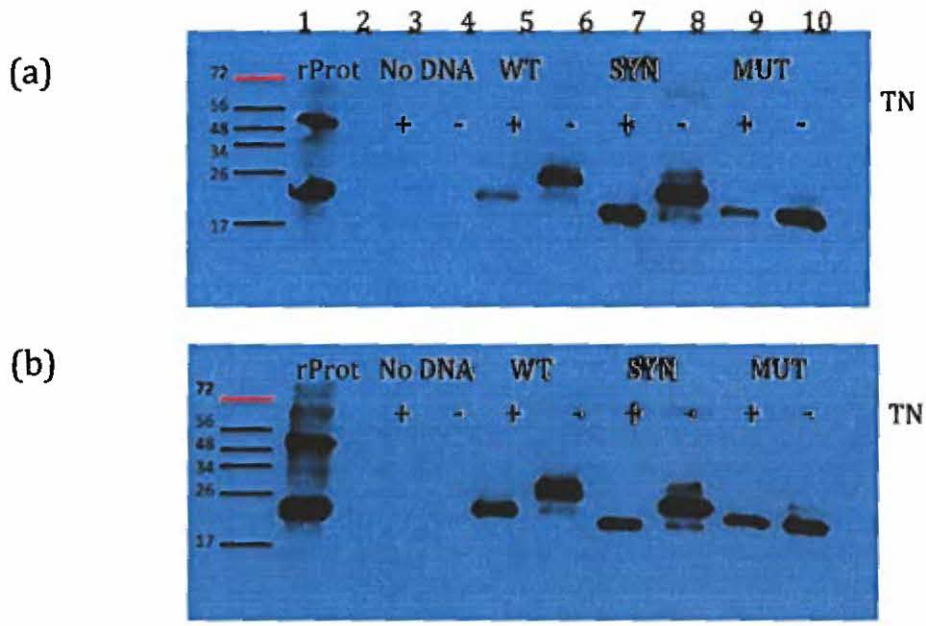

Fig. 3.3.2 Analysis of antibody titers by ELISA from mice (n=5) immunized with different DNA vaccine constructs. Data shown are for three independent experimental replicates. Sera collected after three DNA immunizations were evaluated: (a), (b) and (c) show average end-point from experimental replicate 1, 2 and 3, respectively. End-point titers were defined as serum dilutions giving an absorbance (405nm) higher than that with pre-immune sera + 3SD. Statistically significant differences in immune responses between groups were determined by student t tests at $p < 0.003 - 0.05$, indicated by (*). The error bars indicate SD.

Fig. 3.3.2 Antibody endpoint titers by ELISA

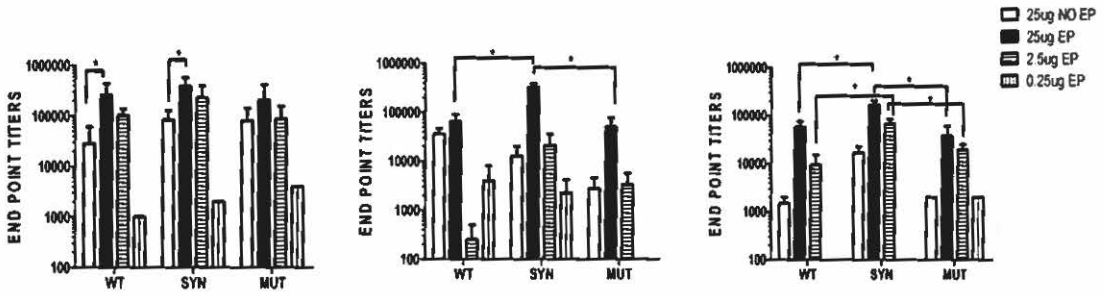

Fig. 3.3.3 Percent responder mice after primary immunization. Immune sera obtained from all groups after primary immunization were analyzed at 1:100 dilution. The percentage of responding mice was determined based on positive ELISA absorbance reading over pre-immune negative control + 3SD. Data shown are averages of three experimental replicates (n=15 mice per vaccine dose group). The error bars indicate SD.

Fig. 3.3.3 Antibody responses after primary immunization

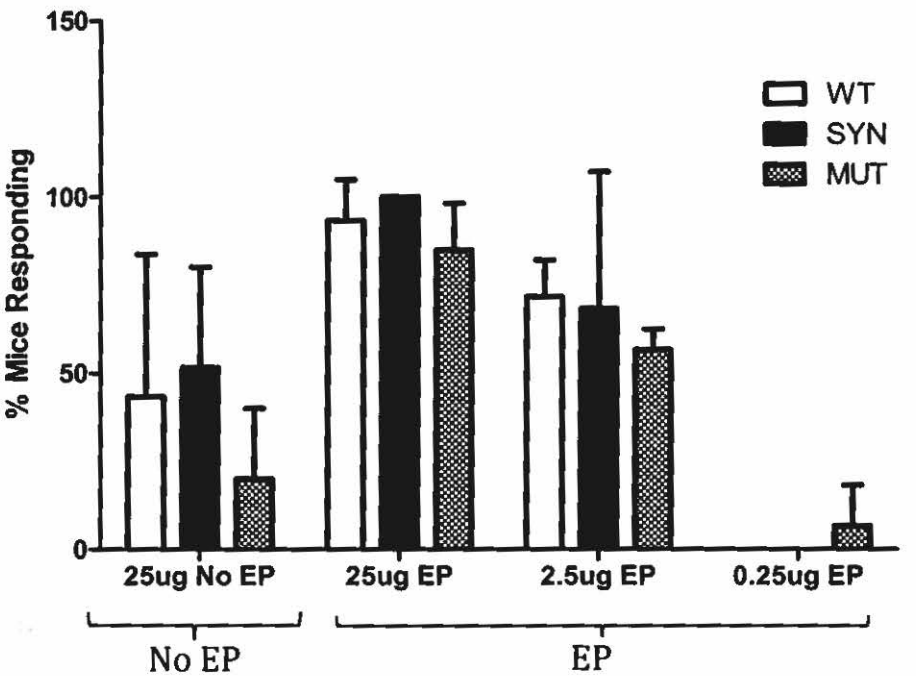

Fig. 3.3.4 Antibody avidity determination. The plots show molar concentrations of NaSCN required to reduce antigen-antibody binding of individual mice sera from WT, SYN and MUT DNA immunized groups (No EP and EP). Zero molar represents the baseline with 100% total binding. Molar concentration in parenthesis represents the average NaSCN concentration for 50% binding dissociation for each group.

Fig. 3.3.4 Antibody avidity determination by NaSCN dissociation

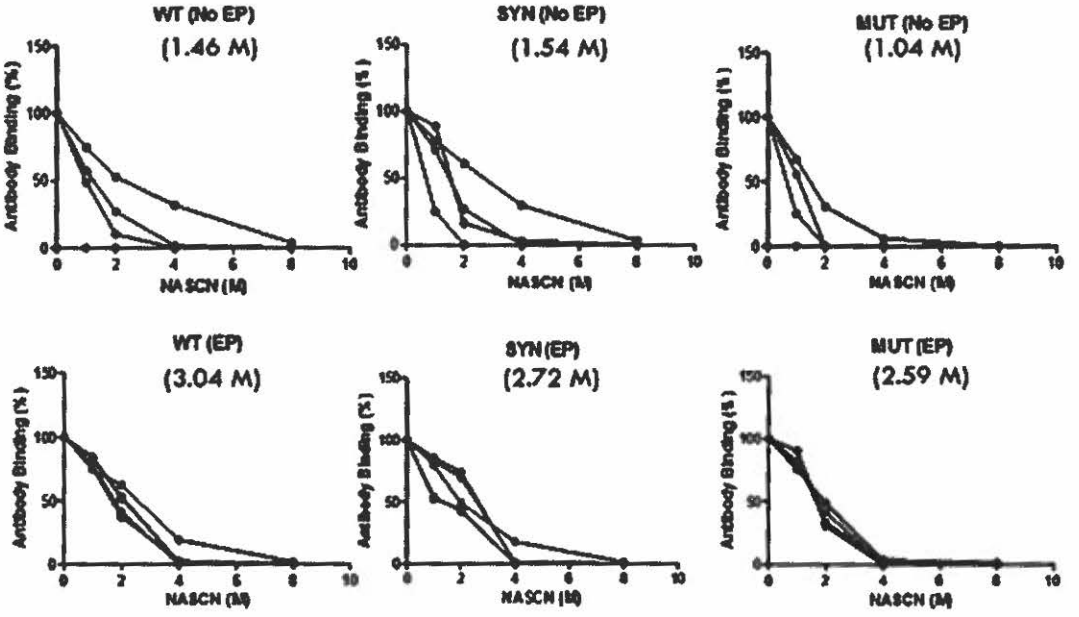

Fig. 3.3.5 Comparison of IgG isotypes. Sera obtained from mice immunized with WT, SYN and MUT DNA, (a) 25ug No EP at 1:500 dilution or (b) 25ug EP at 1:4000 dilution were analyzed for IgM, IgG_1, IgG_{2a}, IgG_{2b} and IgG_3 isotypes. The error bars indicate SD.

Fig. 3.3.4 Comparison of IgG isotypes

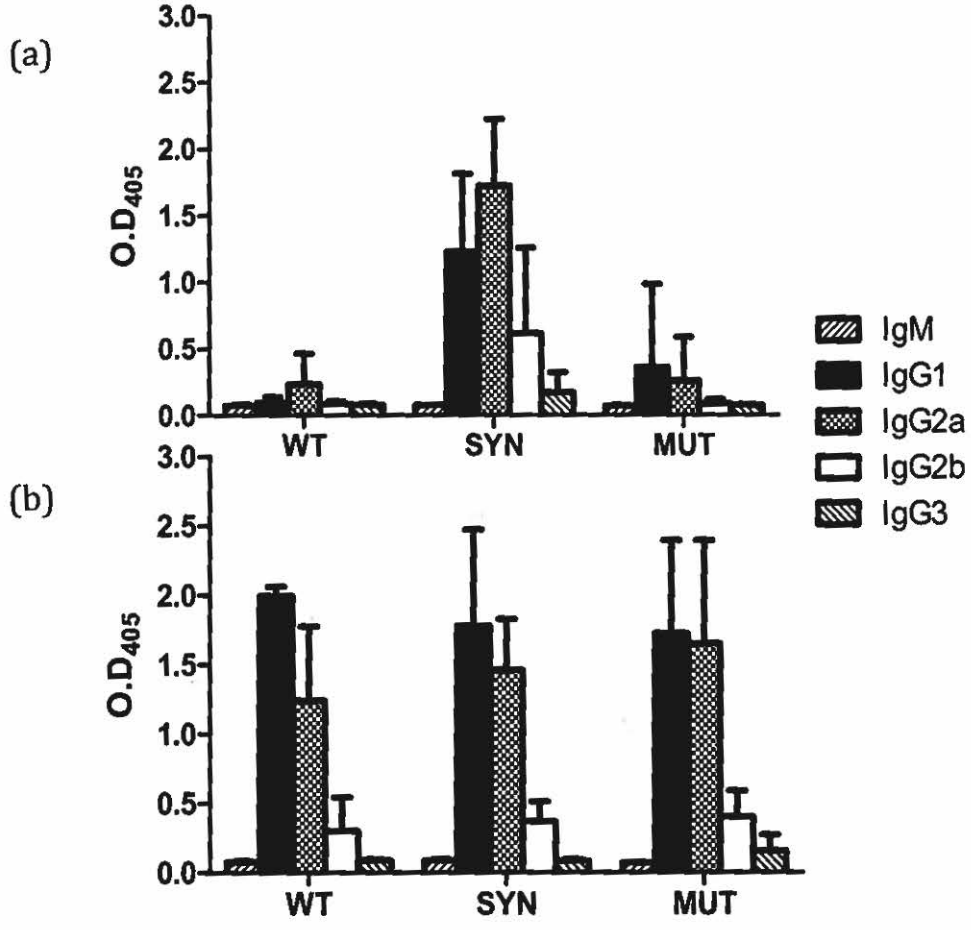

Fig 3.3.6 Recognition of Pfs25 by western blotting and immunofluorescence assays (IFA). (a) Recognition of rPfs25 by DNA vaccine-immunized sera. Pooled immune sera from groups of mice (n=5) immunized with WT, SYN and MUT DNA (25 ug EP) were tested for recognition of non-reduced and reduced forms of rPfs25 protein by western blotting. (b) Parasite surface recognition by IFA using sera from WT, SYN and MUT DNA (25 ug EP) immunized mice. Panel on the right shows pre-immune sera.

Fig. 3.3.6 Recognition of Pfs25 by western blotting (a) and immunofluorescence assays using purified gametes (b)

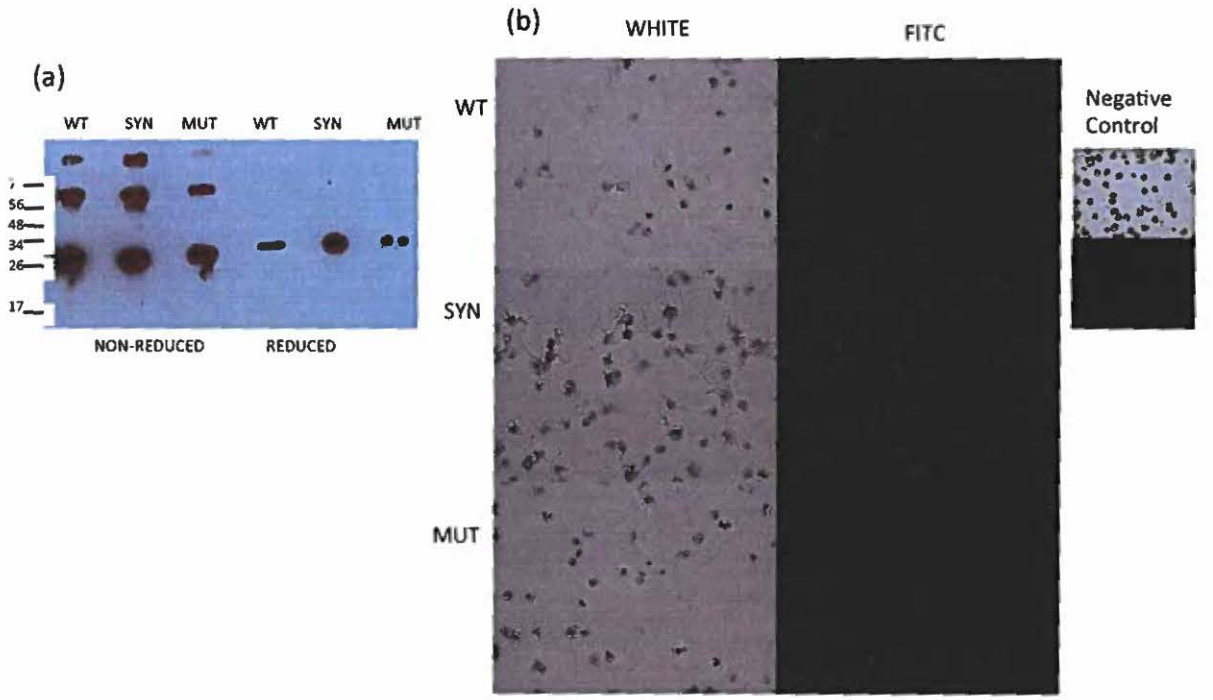

Fig. 3.3.7 Evaluation of in vivo transmission-blocking activity using TrPfs25Pb-murine model. SYN and MUT DNA immunized mice (three DNA doses of 25ug each, No EP and EP) were divided into control and test animals (n=2 per group). Control mice were infected with PbWT parasites and test mice were infected with TrPfs25Pb parasites (10^6 IP). Day 5 after infection, starved *An. stephensi* mosquitoes (n=25-30) were allowed to feed on control and test mice. Eight to ten days later, mosquitoes were dissected to assess infectivity and transmission-blocking activity. Non-immunized mice served as additional experimental controls for infection with PbWT and TrPfs25Pb parasites. The percent of mosquitoes infected in each group is indicated above each data set. The horizontal bars indicate median oocyst numbers.

Fig. 3.3.7 Evaluation of in vivo transmission-blocking activity using TrPfs25Pb murine model

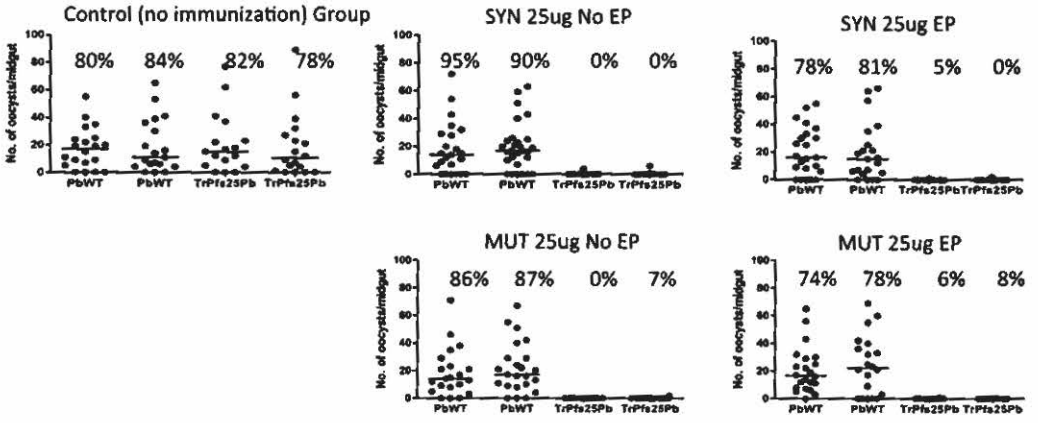

Table 3.3.1 Membrane Feeding Assays. Transmission-blocking activity of purified IgG from pooled immune sera of WT, SYN and MUT DNA

Table 3.3.1

Membrane Feeding Assays. Transmission blocking activity of purified IgG from pooled immune sera of WT, SYN and MUT DNA

EXP. No.	IgG (ug/ml)	NMS (control)		WT	SYN	MUT
1	500	(18/21) 6.5 [4.9](0-25)	No EP	^97.2 (2/21) 0 [0.14](0-9) *	97.6 (2/20) 0 [0.12](0-3) *	99.43 (1/19) 0 [0.04](0-1) *
			EP	98.6 (2/19) 0 [0.07](0-1) *	99 (1/21) 0 [0.05](0-2) *	99.4 (1/22) 0 [0.03](0-1) *
	250	(14/17) 9 [5.8](0-21)	No EP	99.15 (1/18) 0 [0.05](0-2) *	98.75 (3/25) 0 [0.08](0-1) *	99.57 (1/23) 0 [0.05](0-1) *
			EP	100 (0/22) 0 [0.01](0-0) *	100 (0/20) 0 [0.01](0-0) *	99.15 (1/20) 0 [0.03](0-2) *
2	1000	(16/19) 16 [11.4](0-120)	No EP	59.7 (11/19) 5.5 [4.6](0-79)	89.1 (12/24) 0 [1.25](0-12) *	36.9 (18/21) 7 [7.2](0-61)
			EP	96.7 (5/21) 0 [0.3](0-4) *	99.5 (2/22) 0 [0.06](0-1) *	96.9 (9/24) 0 [0.3](0-2) *
	500	(18/20 111 (70.8](0-235)	No EP	NB (20/20) 113 [114.7](61-251)	NB (17/18) 96 [89.5](0-220)	NB (19/20) 105 (80.02](0-243)
			EP	97.6 (10/19) 2 [1.76](0-19) *	98.2 (11/21) 2 [1.2](0-7) *	99.5 (7/20) 0 [0.4](0-3) *
	250	(19/21) 82.5 [60.8](0-234)	No EP	NB (19/19) 85 [82.8](29-192)	NB (18/18) 89 [88.1](31-208)	NB (19/20) 88 (68.5](0-171)
			EP	93 (14/21) 8.5 [4.2](0-31) *	96.3 (10/23) 8 [2.28](0-25) *	97.7 (10/21) 2 [1.35](0-11) *
	100	(17/20) 28 [18.5](0-69)	EP	NB (17/19) 24 [20.1](0-80)	33 (16/20) 22.5 [12.4](0-35)	54.1 (16/22) 18 [8.5](0-36) *
	50	(21/24 24.84 [12](0-103)	EP	NB (14/18) 28 [14.4](0-63)	NB (16/20) 24.5 [15](0-69)	NB (16/19) 23 [15.6](0-65)

^ %TBA (no. infected/ total) Median (Geometric Mean](Range of oocysts)
%TBA = 100 – [Geometric Mean of oocysts in test groups/ Geometric Mean of oocysts in normal mouse IgG) x 100]
* = Significant blocking against NMS control (Mann Whitney Test , p value <0.0001-0.015].
NB (no blocking) = infectivity higher than NMS control

Chapter 4 (Specific Aim 2):

Studying the immunogenicity of Pfs48/45 encoded by DNA vaccines in mice for eliciting potent transmission-blocking antibodies. Vaccine optimization approaches including codon optimization, N-linked glycosylation and in vivo electroporation will be evaluated.

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Abstract:

Pfs48/45, a pre-fertilization gametocyte surface antigen of *P. falciparum* is an important candidate for the development of a malaria transmission-blocking vaccines. Expression of Pfs48/45 on the surface of gametocytes and presentation to the immune system during natural infection in the human host also makes it ideal for natural boosting further validating its role as a transmission-blocking vaccine (TBV) candidate. Due to the conformational nature of this protein however, it has been difficult to produce a fully folded and functionally active protein using various protein expression platforms. In this study, we demonstrate for the first time that full-length Pfs48/45 expressed using DNA vaccines are able to induce potent transmission reducing responses using SMFAs. We have successfully generated DNA vaccines for comparison of immunogenicity responses between a natural Pfs48/45 sequence (WT) with one that is codon optimized (SYN) and two N-glycosylation mutant forms (MUT1 and MUT2). Our findings suggest that Pfs48/45 as a DNA vaccine is a viable TBV candidate whose functional immune responses are significantly enhanced by the use of codon optimization and in vivo electroporation. Furthermore, we demonstrate that leaving N-glycosylation sites intact on a Pfs48/45 sequence encoded by DNA vaccine is beneficial in comparison to N-glycosylation lacking mutant forms with respect to transmission-blocking responses.

4.1. Introduction

The death toll with malaria was estimated at 1300 children under 5 yrs per day for 2012 (WHO World Malaria Report 2013). While global mortality rates are on a steady decline, it is imperative that additional intervention tools and techniques be developed and deployed to maintain the current progress before funding for such measures begins to dwindle. An important intervention that is likely to play a critical role in reducing transmission i.e. malaria TBVs, have yet to become available for human use [27]. RTS,S, an anti-sporozoite vaccine, is currently the only candidate to have advanced to clinical phase 3 trials [23], whose efficacy is presently estimated at around 50% [44]. While it would add an important tool to existing control measures; RTS,S in its present form is unlikely to interrupt malaria transmission in mid- to high-endemic areas [1]. An alternative vaccine development strategy that has recently been termed as vaccines that interrupt malaria transmission (VIMT) [4] is likely to be a key player in the goal of achieving global malaria elimination [34].

A key component of vaccines to reduce transmission is to target parasite sexual stages in the human host as well as developmental stages in the mosquito vector [34]. Key TBV candidates for the human malaria parasite *Plasmodium falciparum* include gametocyte and gamete surface antigens such as Pfs48/45 and Pfs230 as well as zygote/ookinete surface antigens Pfs25 and Pfs28 [82]. First described in the early 1980s [53, 130], Pfs48/45 belongs to a family of proteins characterized by six conserved cysteines (6-Cys proteins) [131] and plays an essential role in parasite male gamete fertility [91]. Pfs48/45 is postulated to work alongside Pfs47 (a female-specific paralog of Pfs48/45) and Pfs230 in contributing to

recognition and fertilization involving male and female gametes [132, 133]. Pfs48/45 specific antibodies have been shown to reduce transmission by inhibiting zygote formation [73, 134, 135]. Because of their biosynthetic origin in the circulating intra-erythrocytic gametocytes antigens such as Pfs48/45 and Pfs230 are able to induce antibody responses in naturally exposed individuals [136, 135, 137] allowing for the possibility of natural boosting of immunity.

However, unlike extensively evaluated Pfs25 [89], Pfs48/45 has met with limited success due to its comparatively larger size and difficulties in expressing properly folded protein that presumably requires correct pairing of 16 cysteine residues. Early attempts at protein expression in bacteria, vaccinia virus and yeast [93, 94, 95] were unsuccessful due to improper protein folding. Since then however, some progress has been made which includes *E. coli* expression of partial protein (containing 10 of the 16-cysteine C-terminal residues) that is able to induce blocking antibodies [97]. Further progress was reported using codon harmonization for heterologous expression of protein in *E. coli* which resulted in a full length, conformational protein capable of inducing transmission-blocking antibodies in mice and olive baboons [98]. Despite these initial successes, reproducibility, stability and conformational integrity continue to thwart further vaccine development based on recombinant protein – adjuvant formulations.

An alternative vaccine development platform that has not yet been explored with Pfs48/45 antigen is DNA vaccine. The ability of DNA vaccines to naturally induce a broad range of immune responses along with ease of manufacturing, stability, storage and transport make them an ideal platform for diseases such as

malaria [138], [49]. With respect to malaria transmission-blocking, considerable success has been made in developing Pfs25 based DNA vaccines in rodent and nonhuman primate studies [47,48,50,51,106]. While the initial studies struggled with the issues of low immunogenicity [57], [48], [106], significant advances were made with the use of in vivo electroporation delivery and heterologous prime-boost strategy in mice and nonhuman primates [50], [51]. Additional optimization of immunogenicity has addressed combining codon optimization, in vivo electroporation and examining the impact of N-linked glycosylation on the outcome of Pfs25 DNA vaccines (Datta et al., manuscript in preparation, Chapter 3). *Plasmodium* proteins do not contain N-glycans due to lack of key enzymes needed for N-glycosylation [114], [121]. Studies on asexual stage vaccine development using non-native expression system, have examined the impact of N-glycosylation on the immunogenicity of MSP-1 and AMA-1 [122], [123], [125]. A recent study has also looked at the role of N-glycosylation in plant produced Pfs48/45 [124], suggesting a preference for the absence of N-glycosylation for vaccine development using *Plasmodium* antigens.

The goal of the present study was to systematically examine Pfs48/45 using a DNA vaccine platform for the first time as well as to test immunogenicity optimization approaches including codon optimization, in vivo electroporation, mutations affecting N-linked glycosylation, and a heterologous prime-boost delivery, in order to develop a viable vaccine candidate for future pre-clinical and clinical studies. In addition to a native Pfs48/45 gene sequence (Pfs48/45WT), we evaluated a codon-optimized sequence (Pfs48/45SYN) and sequence mutations that block any N-linked glycosylation. For the latter, two different mutational strategies

were designed, the first (Pfs48/45MUT1) changed all putative N's to either D, K or T and the second (Pfs48/45MUT2) uniformly changed the S/T of the NXS/T glycosylation site to A, with the intended outcome of blocking N-linked glycosylation in both cases.

4.2. Material and Methods

4.2.1 DNA plasmids

DNA vector VR1020 (Vical Inc. San Diego, CA) was used to prepare four different plasmid constructs, each encoding Pfs48/45 (minus N-terminal signal and C-terminal anchor sequences). Additionally, VR1020 encoding Pfs25SYN (minus N-terminal signal and C-terminal anchor sequences and codon optimized for optimal expression in mammalian cells) was also included in these studies. Construct one contained the native coding sequence of (Pfs48/45WT), the second was a codon optimized version for enhanced expression in mammalian cells (Pfs48/45SYN), and the third and fourth constructs were N-glycosylation mutant versions of the SYN plasmid with all 7 putative glycosylation sites altered to prevent N-glycosylation. In the case of construct three (Pfs48/45MUT1), sequence modifications to block N-linked glycosylation included $N_{50}\rightarrow D$, $N_{131}\rightarrow D$, $T_{192}\rightarrow A$, $N_{204}\rightarrow T$, $N_{254}\rightarrow K$, $S_{301}\rightarrow A$, $N_{303}\rightarrow D$ in all NXS/T sites. The fourth construct was generated by changing all S/T of the NXS/T site to A (Pfs48/45MUT2) (Fig. 4.2.1). Plasmid DNA (<30 EU/mg) was purified and supplied at 2.5 mg/ml (Aldevron, Fargo, ND).

4.2.2 Mammalian cell transfection studies

DNA plasmids (Pfs48/45 WT, SYN, MUT1 and MUT2) were used to transfect mammalian HEK293T cells using Megatran 1.0 (OriGene Technologies, Rockville MD) transfection reagent as per product protocol. DNA was transfected at 2.5 ug/ml and cell medium was changed at 3 hours post transfection, subsequently maintained without or with tunicamycin (2.5 ug/ml) (Sigma-Aldrich, St. Louis, MO), an N-glycosylation blocker. After 48 hours of treatment, supernatant (culture media) and cell lysate were tested for protein expression by western blotting under non-reducing and reducing conditions.

4.2.3 Immunization dose and schedule

5-7 week old female Balb/c mice (NCI, Bethesda) were used for immunization studies. Each plasmid DNA was tested in 4 groups of mice (n=5 per group). Group 1 received 50 ug DNA/mouse without electroporation (no EP), group 2 received 50 ug/mouse with EP, group 3 received 25 ug/mouse with EP and group 4 received 5 ug/mouse with EP. Two additional groups were included in the studies, one received Pfs25 SYN DNA at 50ug/mouse with EP and the other one received a combined dose of Pfs48/45 SYN and Pfs25 SYN at 50 ug each/mouse. All groups received 3 intramuscular DNA doses, whereas the 25 ug and 5 ug immunization group for each of the 4 DNA constructs also received an intraperitoneal protein boost using 10 ug *E. coli* produced recombinant protein adsorbed with alum, all given at 4 week intervals with bleeds collected prior to each dose and one month after final boost. EP was administered using ICHOR pulse generator and TriGrid

Electrode Array (Ichor Medical Systems Inc. San Diego, CA) as described in [126]. DNA was injected using 0.3cc U-100 Insulin syringes (BD Biosciences, NJ) and followed approximately 10 seconds later by electrical pulse at an amplitude of 250 V/cm of electrode spacing (2.5 mm spacing used). Mice were anaesthetized for immunization using Isoflurane solution and DNA doses were administered in 20ul PBS in the anterior-tibialis muscle.

4.2.4 Antibody analysis

Antibody analysis was conducted to determine primary responses after single DNA immunization; end point titers after 3 DNA immunizations as well as after protein boost; avidity of antibody binding as well as antibody isotypes after DNA immunization and after protein boost. ELISAs were conducted using 96-well Immulon-2 plates coated with 100 ul/well of 1.5 ug/ml rPfs25 in carbonate buffer, (pH 9.6) were used and the assays were performed as previously described [48]. To determine avidity of antibodies, plates were incubated for 15mins with NaSCN (0, 1, 2, 4, 8 M) after primary antibody incubation and washed prior to incubation with secondary antibody and remaining ELISA steps. Binding of antibody to antigen after NaSCN treatment was expressed as a percent of total binding in the absence of NaSCN. For antibody isotype analysis, mouse sera were tested at 1:250 (no EP group) and 1:4000 (EP groups) dilutions. The various secondary antibodies used were peroxidase-conjugated goat anti-mouse IgM, IgG_1, IgG_{2a}, IgG_{2b} and IgG_3 (Invitrogen, Carlsbad, CA).

4.2.5 Standard membrane feeding assays (SMFAs)

Total IgG was purified using protein A-Sepharose beads, from pooled mouse sera collected after 3 DNA immunization (50 ug DNA; WT, SYN and MUT DNA groups; no EP and EP) as described [90] to test in SMFAs. Three individual SMFAs were conducted to test the following groups- (i) no EP immunized groups (WT, SYN, MUT1 and MUT2) at 1 mg/ml and 0.5 mg/ml purified IgG after 3 DNA immunizations. (ii) EP immunized groups (Pfs48/45 WT, SYN, MUT1 and MUT2 as well as Pfs25 SYN and Pfs25+Pfs48/45 combined) at 1 mg/ml, 0.5 mg/ml and 0.25 mg/ml, after 3 DNA immunizations. (iii) EP immunized groups (WT, SYN, MUT1 and MUT2) at 1 mg/ml, 0.5 mg/ml and 0.25 mg/ml after protein boost.

4.2.6 Statistical analysis

Antibody end point titers were defined as serum dilution with absorbance higher than the average absorbance of pre-immune sera+3 standard deviations (SD). The percent inhibitions of oocyst development and mosquito infectivity differences were analyzed as described [50]. Statistical analysis was performed using the GraphPad Prism software package.

4.3. Results

4.3.1 Evidence for N-linked glycosylation of Pfs48/45 in mammalian cells

To determine protein expression and N-linked glycosylation, mammalian (HEK293T) cells were transfected with Pfs48/45 WT, SYN, MUT1 and MUT2 plasmids and analyzed 48 hrs later by western blotting (using pooled anti-Pfs48/45

antibodies obtained from mice immunized with Pfs48/45 for primary incubation). In all cases, Pfs48/45 was detected in the cell lysate alone, suggesting that the expressed protein was not secreted out of the cytoplasm. In addition, a clear protein band could only be detected under reducing conditions, suggesting the protein produced in mammalian cells was highly aggregated. WT and SYN DNA showed higher protein expression on a per cell basis in comparison to MUT1 and MUT2, and the size of Pfs48/45 expressed using WT and SYN DNA was close to 72 kDa. In comparison the size of protein expressed by Pfs48/45 MUT1 and MUT2 DNA was around 56kDa, suggesting a difference in size that can be attributed to the lack of N-linked glycosylation in the latter two groups. Tunicamycin (TN) treatment resulted in a shift in size of expressed protein from WT and SYN plasmids comparable to that from MUT1 and MUT2 plasmids. However, TN treatment had no effect on the expressed protein from MUT1 and MUT2 plasmids, as expected (Fig. 4.3.1).

4.3.2 Antibody responses in Pfs48/45 WT-, SYN-, MUT1- and MUT2 DNA-immunized groups

Immune sera from WT, SYN, MUT1 and MUT2 (50ug no EP and EP, 25 ug and 5ug) groups were examined after the first DNA immunization to determine primary antibody responses as well as to assess any difference in sero conversion between EP and no EP modes of immunization. When tested at 50 ug and 25 ug dose by EP all four vaccine constructs revealed 100% of the mice responding by sero-conversion after a single vaccine dose. Even at 5ug with EP 60-100% mice were responsive, whereas immunization with 50 ug without EP only demonstrated about 20-60%

response after a primary injection. Additionally, immune reactivity (as determined by OD reading over negative control + 3SD) was very low for no EP groups and showed a dose-dependent trend for EP groups (Fig. 4.3.2).

End point titers after 2nd and after 3rd DNA immunizations are shown in Fig. 4.3.3. For each of the 4 immunization groups (WT, SYN, MUT1 and MUT2) titers for EP groups at 50 ug and 25 ug were higher than no EP at 50 ug. Data analysis also revealed that the titers in the 5 ug EP groups, a 10-fold lower immunization group were either comparable or higher than 50 ug no EP for all DNA immunization groups. Comparing titers between different constructs revealed that SYN DNA at 50 ug, without and with EP, had significantly higher titers than WT DNA. Titers for both MUT1 and MUT2 were lower but not significantly different compared to SYN DNA and matched the levels of WT group. End point titers for groups that received protein boosts (25 ug and 5 ug EP) displayed a 10-fold increase in titers, however no differences were observed in the titers among the four DNA constructs (Fig. 4.3.3).

4.3.3 Antibody responses for co-immunization groups

End point titers were also examined for Pfs25SYN immunized group as well as the cocktail immunization group with the goal of determining the outcome of co-immunizations. Comparing groups immunized with 50 ug EP of Pfs25SYN alone or Pfs48/45SYN alone, along with combined Pfs25 and Pfs48/45 SYN immunizations revealed no significant differences in specific antibody titers resulting from co-immunizations. End point titers for Pfs25 SYN DNA immunized group were significantly higher than of the Pfs48/45 SYN immunized group (Fig. 4.3.4).

4.3.4 Antibody avidity

Antibody avidity was determined for 50 ug EP groups of all four constructs after 3 DNA immunizations and for 5 ug EP groups after protein boost. For the DNA immunized groups, avidity for SYN DNA (1.87 M) was slightly higher than of antibodies from mice administered with WT, MUT1 and MUT2 (0.87, 1.1, 1.59 M respectively). This difference persisted after a protein boost with SYN DNA (2.1 M) showing higher avidity compared with the rest (1.37 M, 1.9 M and 1.33 M respectively). However the differences in avidity between immunization groups, as well as before and after protein boost, were not statistically significant (Fig. 4.3.5).

4.3.5 Antibody isotypes

Antibody isotypes were also examined to determine whether mutating N-glycosylation sites would result in skewing isotypes of elicited antibodies and if so, would the skewing differ for the two different forms of N-glycosylation mutants, likely due to unknown conformation-specific epitope processing and presentation. Our results indicate a balanced IgG2a/IgG1 response for WT and SYN DNA groups but a skewing towards IgG2a for both mutant groups. This isotype distribution is consistent for no EP and EP groups and persisted even after a protein boost (Fig. 4.3.6).

3.6 Standard Membrane Feeding Assays (SMFAs)

SMFAs were conducted to determine the functional activity of immune sera from all four groups (WT, SYN, MUT1 and MUT2) immunized with 3 doses of 50 ug

DNA given with EP or no EP and the group immunized with 5 ug DNA by EP after the protein boost. In these studies we also compared transmission-blocking antibody induced by Pfs25SYN alone or in combination with Pfs48/45. IgG was purified from pooled immune sera from mice collected from all groups of mice and tested at varying concentrations. Mice immunized with SYN plasmid DNA vaccine (3 doses) by EP exhibited higher blocking activity (88%, 75% and 40% at 1.0, 0.5 and 0.25 mg/ml, respectively) than mice immunized with WT, MUT1 and MUT2 plasmids. Even in the no EP group, IgG from SYN group revealed 60% blocking activity at 1 mg/ml as compared to 50%, 50% and 35% with MUT1, MUT2 and WT plasmids, respectively. Interestingly, mice previously immunized with 5 ug doses by EP with all four vaccine constructs exhibited >90% blocking after a single protein boost suggesting a role for heterologous prime-boost in effective immunogenicity of Pfs48/45 DNA vaccine (Fig. 4.3.7).

Further, Pfs25 SYN and the co-immunization group Pfs25+Pfs48/45 were tested after 3 DNA immunizations, at 1 mg/ml, 0.5 mg/ml and 0.25 mg/ml IgG concentration. SMFA data from 50 ug EP Pfs25SYN immunized group showed 96-99% blocking for 1 mg/ml, 0.5 mg/ml and 0.25 mg/ml DNA groups, similar to that seen in other studies with Pfs25 DNA vaccines (Datta et al., manuscript in preparation). IgGs from group with combined immunization of Pfs25 and Pfs48/45 also showed 95-99% blocking, which suggests that a multivalent transmission-blocking DNA vaccine has potential that warrants further investigation (Fig. 4.3.8)

4.4 Discussion

Pfs48/45 is an important target of transmission-blocking immunity to *Plasmodium* parasites and has shown potential as a viable target for blocking parasite infectivity to mosquitoes in studies using specific monoclonal antibodies [73, 52, 130]. Having been established as a viable candidate for TBV development, various technical limitations have restricted progress towards a clinical grade Pfs48/45 vaccine. Limitations include the lack of evidence on conformational structure, such that target epitopes involved in transmission-blocking immunity remain unknown, restricting the ability of using recombinant vaccine design technology to induce targeted immune responses [45]. DNA vaccines provide a platform that allows in vivo expression of encoded antigen employing host's transcription and translation machinery after introduction of plasmid DNA [105]. While this platform has been extensively explored for Pfs25, another potential TBV candidate [57, 48, 106, 50, 51]; this is the first time Pfs48/45 DNA vaccines have been examined in pre-clinical studies. The goal of studies presented here was to investigate the contributions of (i) codon optimization [108], (ii) in vivo electroporation [110], (iii) heterologous prime-boost regimen [139], (iv) the role of N-linked glycosylation [121] on the immunogenicity of Pfs48/45 DNA vaccines in pre-clinical studies in mice. Considering the need for developing multivalent TBVs targeting different lifecycle stages, we also endeavored to determine the outcome of co-immunization with Pfs25 and Pfs48/45 DNA vaccines.

Findings from in vitro transfection studies demonstrated that Pfs48/45 encoded by DNA vaccine does undergo N-glycosylation in mammalian cells using

host translational machinery. The presence of this form of post-translational modification on the native Pfs48/45 backbone (Pfs48/45 WT and SYN DNA) was confirmed by mutating putative N-glycosylation sites in Pfs48/45 (Pfs48/45 MUT1 and MUT2), which prevented the additional of N-glycan side chains as demonstrated by a smaller protein band by western blotting. Blocking the formation of N-glycan side chains using tunicamycin further confirmed that the size difference between glycosylated and unglycosylated mutants was indeed due to modification of polypeptide backbone by the addition of N-glycan side chains at putative glycosylation sites. Further, we demonstrate here the ability to utilize two different techniques to generate N-glycosylation mutants. Gene sequence of Pfs48/45 has 7 putative N-glycosylation sites. In one mutant form (Pfs48/45MUT1), 3 N's were altered to D's, one to K and one to T. These particular substitutions were chosen based on the natural polymorphism at these sites as revealed by comparison of sequences in other *Plasmodium* species. In two instances putative N-glycosylation sites were conserved across species thus prompting us to leave N residues intact and instead mutating S/T residues at the third position by A. In the second mutant form (PFs48/45MUT2), the S/T at the third position was altered to A in all 7 putative N-glycosylation sites. The rationale for testing two different N-glycosylation mutant constructs was to determine which N-glycosylation mutation altered functional blocking responses to Pfs48/45, and whether the differences can be attributed to N-glycosylation changes or to alterations in structural integrity of the protein due to sequence mutations.

Results of our antibody end point titer analysis revealed no significant differences in the induction of antibodies induced by glycosylated and unglycosylated forms of antigens encoded by DNA vaccines. The quality of binding was also not affected as seen by antibody avidity analysis. An unexpected and interesting observation however, was the difference in the isotype presentation between N-glycosylation and N-glycosylation mutant groups. Both WT and SYN DNA had a balanced IgG1/IgG2a response whereas both MUT1 and MUT2 DNA presented a skewing towards IgG2a isotypes. Furthermore, SMFA data revealed significantly higher blocking responses by SYN DNA compared with MUT1 or MUT2 DNA. There is a possibility that this difference in functional responses may be attributed to the difference in isotype presentation. Similar studies examining the role of N-linked glycosylation in Pfs25 DNA vaccines did not note a difference in functional responses between SYN and MUT DNA. However, in those studies, in addition to end point titers and antibody avidity, the isotype presentation for both immunization groups was a balanced IgG1/IgG2a (*Datta et al. manuscript in preparation*). Of note, between N-glycosylated groups (WT and SYN), use of codon optimization proved advantageous as the titers for SYN DNA were significantly higher and the differences were seen in functional blocking responses as well. In all cases, in vivo EP was able to significantly enhance antibody end point titers as well as functional blocking responses. A protein boost using *E. coli* produced recombinant Pfs48/45, was also able to boost antibody titers as well as functional responses for all immunization groups, making a case for incorporating a heterologous prime-boost regimen for DNA vaccine delivery. And finally, end point titers in Pfs48/45SYN DNA-immunized

groups were consistently lower than Pfs25SYN DNA-immunized groups. However, co-immunization with Pfs25SYN and Pfs48/45SYN revealed no detrimental effect of one immunization group on the outcome of the other. In fact, functional blocking responses of co-immunized group were comparable to blocking by Pfs25 SYN alone. These findings are important for considering multivalent transmission-blocking DNA vaccines, as it is important to determine whether one antigen would interfere with the functional outcome of another when co-immunized.

The studies reported here demonstrated for the first time, that codon optimized Pfs48/45 is a viable vaccine candidate whose immunogenicity is enhanced by in vivo EP and heterologous prime boost with recombinant protein. Our findings also revealed an advantage to leaving N-linked glycosylation sites intact in the case of Pfs48/45 DNA vaccines, an outcome that might be attributed to the balanced IgG1/IgG2a isotype presentation compared to an IgG2a skewing for N-glycosylation mutant. And finally, we demonstrate here the feasibility of using multivalent transmission-blocking DNA vaccines, to be explored further with the goal of developing a clinical-grade vaccine for human trials.

Fig 4.2.1 Immunization dose and schedule

N = 5 F balb/c mice per group Total = 18 grps	WT		SYN		MUT1		MUT2		Pfs25	COMBINED
DNA Plasmid	Pfs48/45WT		Pfs48/45SYN		Pfs48/45MUT1		Pfs48/45MUT2		Pfs25SYN	Pfs48/45SYN+ Pfs25SYN
Electroporation (EP)	-	+	-	+	-	+	-	+	+	+
DNA Dose (ug)	50	50	50	50	50	50	50	50	50	50 + 50
		25		25		25		25		
		5		5		5		5		
rProtein boost (10ug)	rPfs48/45		rPfs48/45		rPfs48/45		rPfs48/45			

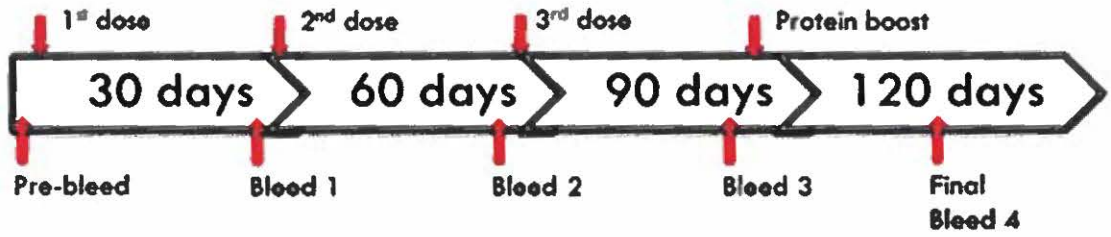


Fig 4.3.1 Evidence for Pfs48/45 N-linked glycosylation by in vitro mammalian cell transfections and Western blot analysis. Recognition of Pfs48/45 by in vitro transfection of mammalian HEK293T cells with DNA plasmids and cultured in the presence or absence of tunicamycin (TN). Cell lysates were analyzed by SDS-PAGE under reducing conditions. Lane 1 – no DNA control. Lane 2 through 10 – WT, SYN or MUT1 or MUT2 DNA transfected samples, treated in the absence (-) or presence (+) of TN.

Fig. 4.3.1 Western blot evidence for Pfs48/45 N-linked glycosylation by in vitro mammalian cell transfections

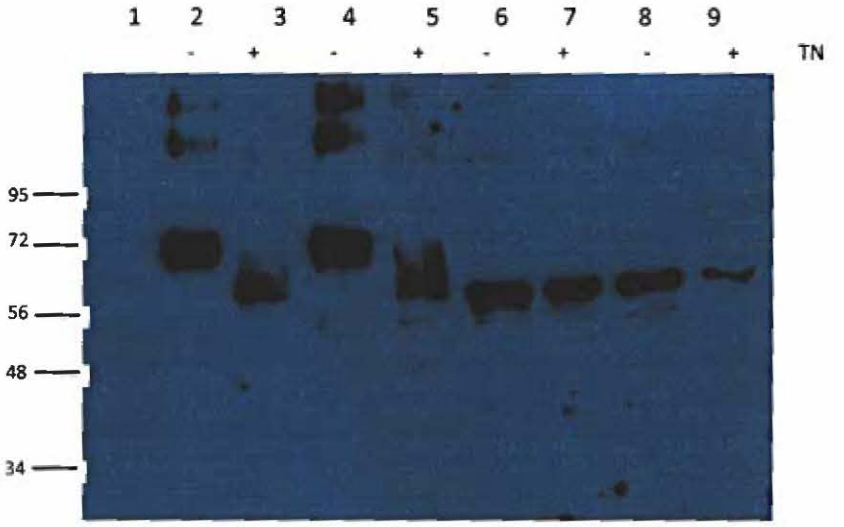


Fig. 4.3.2. Antibody responses after primary immunization. Immune sera obtained from all immunization groups after primary immunization were analyzed at 1:100 dilution. The percent of responding mice (indicated over bars) was determined based on positive ELISA absorbance reading (indicated by bars on the X axis) over preimmune negative control + 3SD

Fig. 4.3.2 Immune responses after primary immunizations

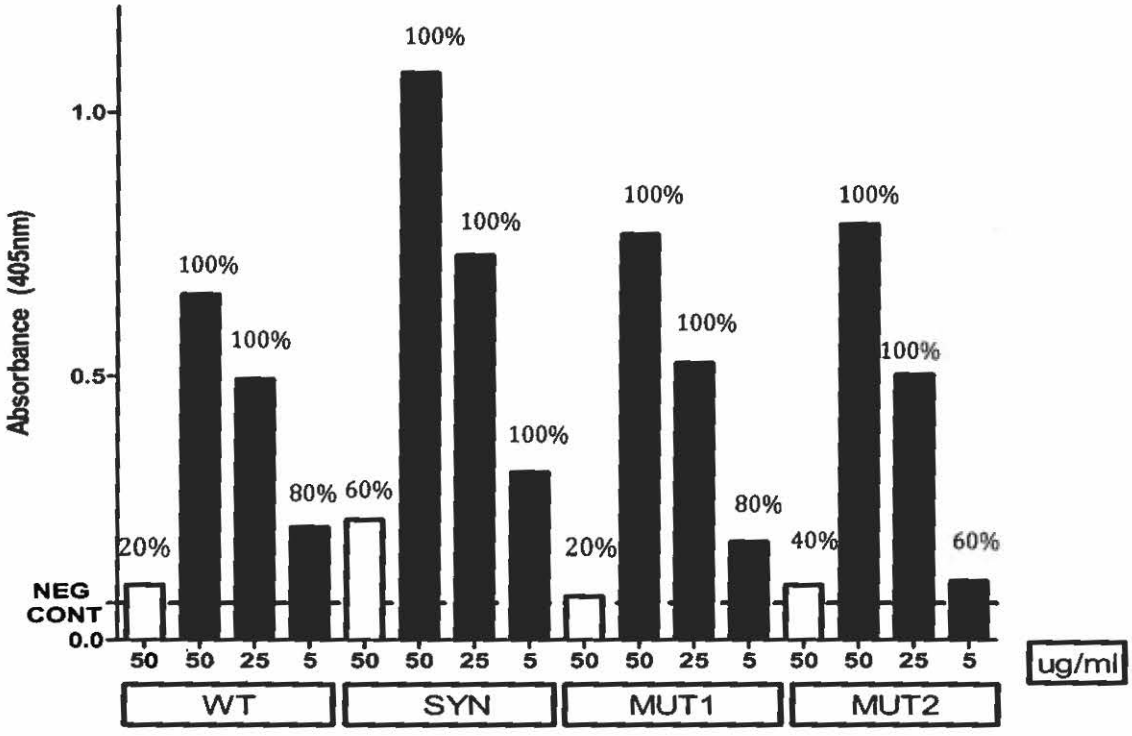


Fig. 4.3.3. Analysis of ELISA antibody titers from mice (n=5) immunized with different DNA vaccine constructs. (a) Average end point titers after 2 DNA immunizations (b) average end point titers after 3 DNA immunizations (c) average end-point titers after a protein boost. End point titers were defined as serum dilutions giving an absorbance (405nm) higher than that with pre-immune sera + 3SD. Statistically significant differences in immune responses between groups were determined by student *t* tests at $p < 0.001 - 0.05$, indicated by (*). The error bars indicate SD.

Fig. 4.3.3. Analysis of ELISA antibody titers of immunized with different DNA vaccine constructs

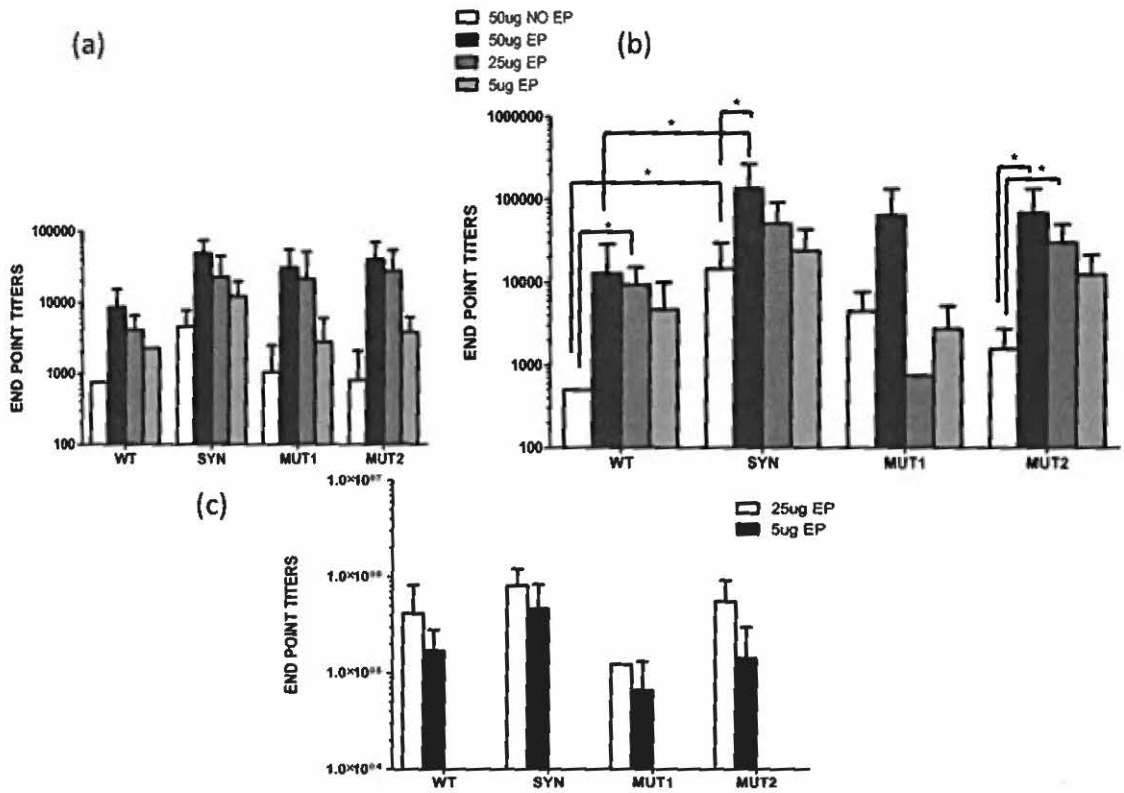


Fig. 4.3.4. Analysis of antibody titers by ELISA from mice (n=5) in co-immunization groups. Bar 1: Pfs25 immunization group, bar 2: antibody titers against Pfs25 in co-immunization group, bar 3: Pfs48/45 immunization group, bar 4: antibody titers against Pfs48/45 in co-immunization group. End point titers were defined as serum dilutions giving an absorbance (405nm) higher than that with pre-immune sera + 3SD). The error bars indicate SD.

Fig. 4.3.4 Analysis of antibody titers in co-immunization groups

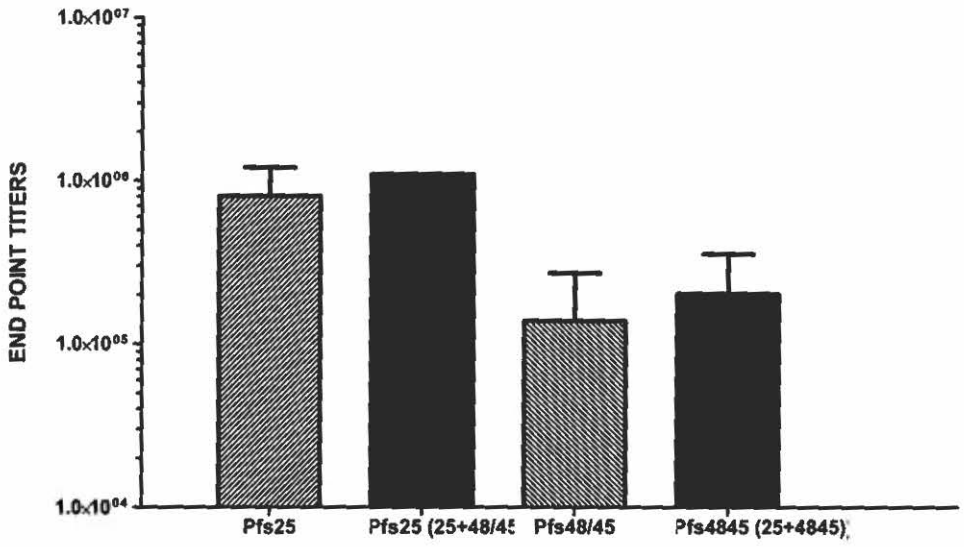


Fig. 4.3.5. Determination of antibody avidity. The plots show molar concentrations of NaSCN required to reduce antigen-antibody binding of individual mice sera from WT, SYN and MUT1 and MUT2 DNA immunized groups (with EP). (a) After 3 DNA immunizations, and (b) after protein boost. Zero molar represents the baseline with 100% total binding. Molar concentrations in parentheses represent the average NaSCN concentration for 50% binding dissociation for each group.

Fig. 4.3.5 Antibody avidity

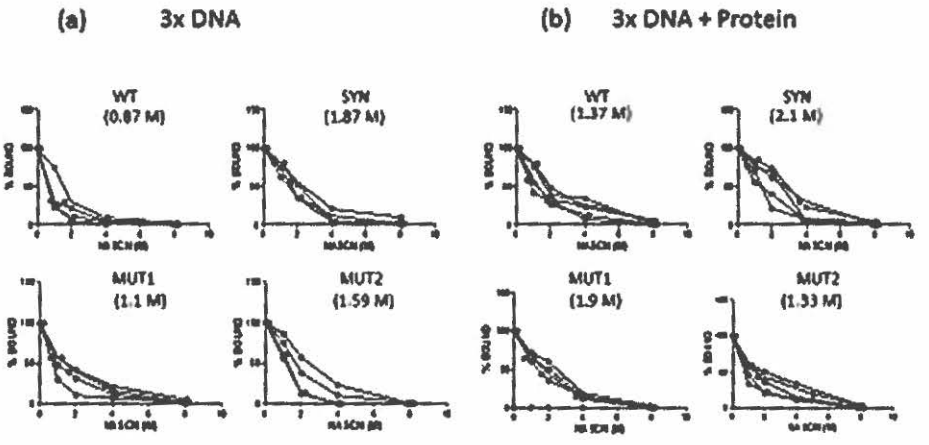


Fig. 4.3.6. Comparison of IgG isotypes. Sera obtained from mice immunized with WT, SYN and MUT DNA via (a) no EP 50 ug DNA groups after 3 DNA immunizations, (b) EP 50 ug DNA groups after 3 DNA immunizations, and (c) EP 5 ug DNA groups after a protein boost were analyzed for IgM, IgG_1, IgG_{2a}, IgG_{2b} and IgG_3 isotypes. The isotypes are represented as a proportion of total IgG1. The error bars indicate SD.

Fig. 4.3.6 Comparison of IgG isotypes

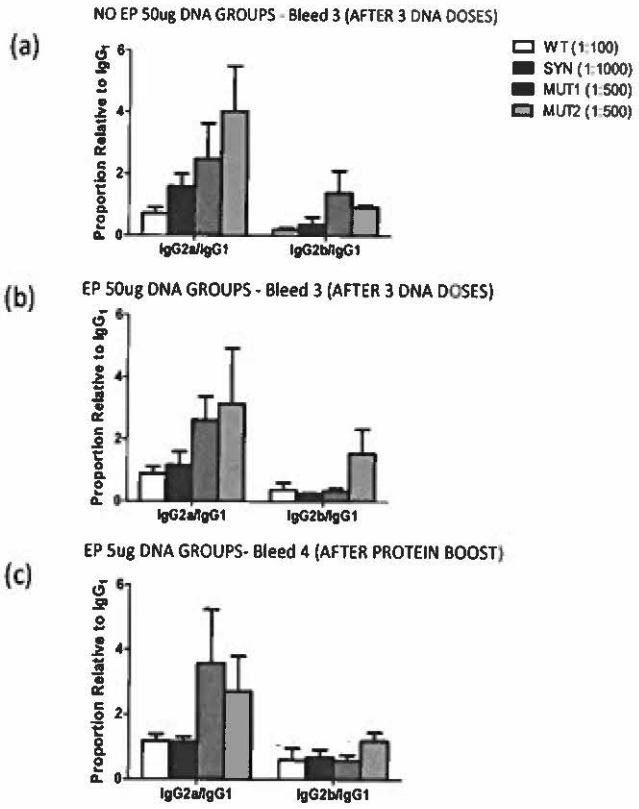


Fig. 4.3.7. Standard membrane feeding assays. Transmission-blocking activity of purified IgG from pooled immune sera of mice immunized with WT, SYN and MUT1 and MUT2 DNA via (a) no EP tested at 1.0 and 0.5 mg/ml, (b) EP tested at 1.0, 0.5 and 0.25 mg/ml, (c) After protein boost immunization groups tested at 1.0, 0.5 and 0.25 mg/ml. Bars represent median oocyst counts.

Fig. 4.3.7. Standard Membrane Feeding Assays

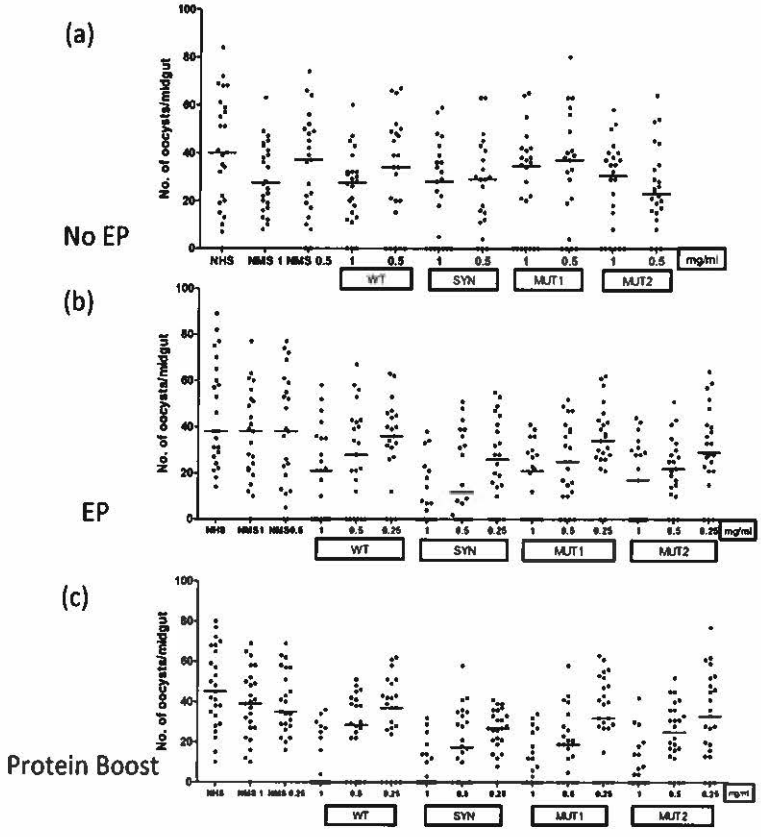
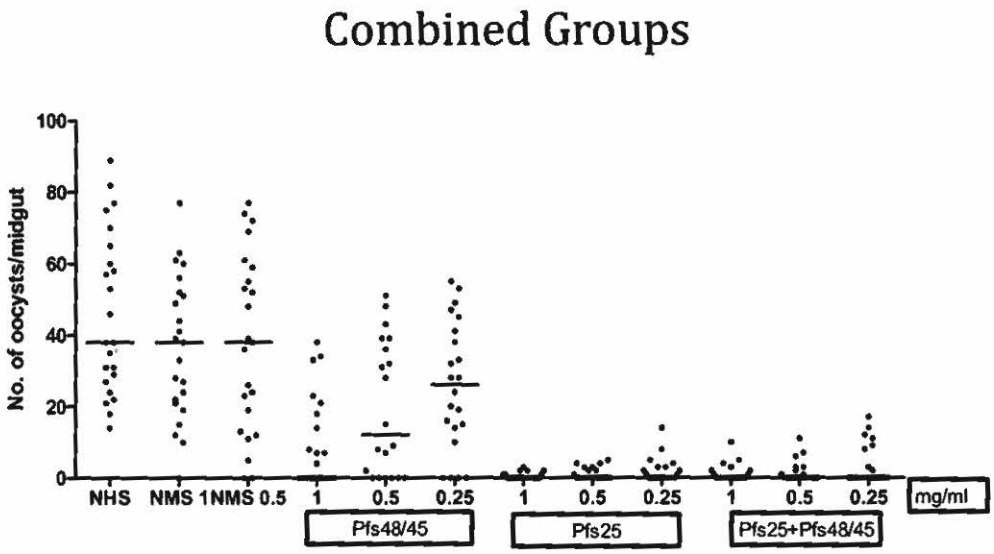


Fig. 4.3.8. Standard Membrane Feeding Assays



Chapter 5 (Specific Aim 3):

Evaluation of *P. falciparum* transmission-blocking DNA vaccines encoding Pfs25 and Pfs48/45 by in vivo electroporation in nonhuman primates

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Abstract:

Pfs25 and Pfs48/45 are two most important transmission-blocking vaccine candidates of the human malaria *P. falciparum*. While Pfs25 has been examined extensively in the past using various expression platforms including DNA vaccines in rodents and nonhuman primates; Pfs48/45 has been less well studied, with no rodent or nonhuman primate studies using a DNA vaccine platforms conducted to date. Here we report comparative immunogenicity studies of Pfs25 and Pfs48/45 DNA vaccine candidates in rhesus macaques with the eventual goal of developing multivalent transmission-blocking DNA vaccines for future clinical trials. Our studies demonstrate that both Pfs25 and Pfs48/45 are capable of inducing potent transmission-blocking responses individually as well as in combination and that neither antigen interferes with the immune responses generated by the other. We were also able to confirm the benefits of codon optimization and in vivo electroporation as well as leaving N-linked glycosylation sites intact, as seen in our rodent studies with Pfs25 and Pfs48/45 DNA vaccines. These findings indicate for the first time in nonhuman primate studies that combining multiple TBV candidates can be used as a viable vaccine development tool against *P. falciparum*.

5.1. Introduction

DNA vaccines were first described in the early 1990's and generated much interest due to their simplistic design and ability to induce cellular and humoral immune responses [103,140-144]. DNA vaccines also offer a promising platform wherein a single plasmid or multiple plasmids in a cocktail encoding multiple antigens of

interest, can potentially be used as a multistage, multi-immune response vaccination approach against malaria [145,49]. Of particular interest to our studies are vaccines targeting sexual stages to prevent malaria transmission, known as transmission-blocking vaccines (TBVs) (reviewed in [46]). Pfs25, a zygote-ookinete surface antigen and Pfs48/45, a gametocyte and gamete surface protein, are two well-established P. *falciparum* TBV target antigens [69]. Of these, Pfs25 has been tested in previous studies using a DNA vaccine platform with varying degrees of success in rodent and nonhuman primates [47,48,50,51]. Administration of Pfs25 DNA plasmid using in vivo electroporation was further found to enhance functional immunogenicity [50]. Pfs48/45 on the other hand, has never been evaluated using DNA immunizations except for studies presented in Chapter 4 of this thesis.

Since the promising early beginnings of DNA vaccine studies further vaccine development has been hampered, owing largely to the low potency seen in primate studies and a few phase I clinical trials [146]. Exact mechanisms of poor immunogenicity of DNA vaccines in *Homo sapiens* are not known and further studies aimed at understanding immune correlates will guide further DNA vaccine optimizations [147]. In particular, more nonhuman primate studies are warranted owing to their phylogenetic closeness to humans [148] and their presumed ability to mimic the outcomes expected in humans [146].

The objective of the study reported here was to conduct comparative immunogenicity studies with *P. falciparum* TBV antigens (Pfs25 and Pfs48/45) in nonhuman primates. In particular we investigated the contributions of (i) codon optimization [108], (ii) in vivo electroporation [110], (iii) heterologous prime-boost

regimen [139], (iv) the role of N-linked glycosylation [121] and (v) the impact of co-immunization with Pfs25 and Pfs48/45 in *Macaca mulatta* (rhesus macaques). The underlying goal was to determine factors that may contribute and accelerate DNA vaccine development for malaria transmission reduction.

5.2. Materials and Methods

5.2.1 DNA plasmids

DNA vector VR1020 (Vical Inc. San Diego, CA) described in [47] encoding Pfs25 or Pfs48/45 optimized for mammalian cell expression, lacking signal and anchor sequences were constructed (Pfs25SYN or Pfs48/45SYN). Additionally, an N-glycosylation mutant form of the optimized Pfs48/45 sequence was developed where all 7 putative N-glycosylation sites were mutated (Pfs48/45MUT). Sequence modifications to block N-linked glycosylation include N_{50}→D, N_{131}→D, T_{192}→A, N_{204}→T, N_{254}→K, S_{301}→A, N_{303}→D in all NxS/T sites. Plasmid DNA (<30 EU/mg) was purified by Aldevron (Fargo, ND) and supplied at a 2.5 mg/ml concentration (Fig 5.2.1).

5.2.2 Animals

Twenty rhesus macaques (*Macaca mulatta*) of Chinese origin housed at the Tulane National Primate Research Center (TNPRC) were used in this study. TNPRC animal care facilities are accredited by the American Association for Accreditation of Laboratory Animal Care and licensed by the U.S. Department of Agriculture. All

tested at 5 mg/ml and 1 mg/ml and from groups 3, 4, and 5 were tested at 10, 2, 1 and 0.5 mg/ml. SMFAs were conducted by mixing different concentrations of purified IgG with mature *P. falciparum* parasites (at 0.3% gametocytemia) and human erythrocytes (at 50% hematocrit), and feeding the final mix to 4-5 day old *Anopheles gambiae* (Keele strain) mosquitoes that were starved for 4-6 hours. Blood fed mosquitoes were then maintained for 8-10 days at 26° C and 80% relative humidity. Mosquitoes were dissected and midguts were extracted and stained with 0.1% mercurochrome for oocyst enumeration by microscopy.

5.2.9 Statistical analysis

All statistical tests were conducted using Graphpad Prism software. Antibody end point titers were defined as serum dilutions giving an absorbance higher than the average OD value at 405nm of pre-immune sera plus 3SD. statistically significant differences in antibody responses between groups were determined using ANOVA and Mann-Whitney U test. P values of < 0.05 were considered significant. Statistical significance of MFA data was calculated using Kruskal-Wallis test (for oocyst variation between pre-immune and immunized groups).

5.3. Results

5.3.1 Effect of DNA immunization without and with EP and protein boost, on Pfs25 antibody responses

Immunogenicity of Pfs25 SYN DNA vaccine was evaluated in two groups of rhesus macaques (Group 1 and 2) to compare antibody titers without and with EP.

After 3 DNA immunizations, average end point titers for the EP group was a log higher than for the no EP group. After a final boost with rPfs25, end point titers for the no EP group increased to levels comparable to those in the EP group (Fig. 5.3.1).

5.3.2 Pfs48/45 antibody response after immunization with SYN and MUT Pfs48/45 DNA vaccines without and with EP and protein boost

Two groups (Group 3 and 4) of rhesus macaques were immunized with Pfs48/45SYN and Pfs48/45MUT respectively to compare the antibody titers generated by glycosylated and unglycosylated antigens encoded by DNA vaccines. After 3 DNA immunizations the average end point titers were not significantly different between the two immunization groups. There was a slight boosting effect seen after recombinant protein immunization in both groups however the differences between the two groups or within the groups after DNA immunization compared to after protein boost were not significant (Fig 5.3.1).

5.3.3 Assessment of antibody responses after combined immunization with Pfs25 and Pfs48/45

Group 5 received a cocktail of Pfs25SYN and Pfs48/45SYN DNA to determine feasibility of co-immunization with two separate sexual stage antigens. This group was developed with the eventual goal of evaluating a multi-antigen vaccine targeting various transmission stages. Antibodies generated after 3 DNA immunizations and after a protein boost were tested in ELISAs against each individual antigen (Pfs25 and Pfs48/45). The end point titer analysis revealed no

5.3.7 Transmission-blocking efficacy of anti-Pfs48/45 antibodies

To determine functional blocking responses elicited by Pfs48/45SYN (group 3) and Pfs48/45MUT (group 4) immunizations, purified IgGs from each group were tested at 10, 2, 1 and 0.5 mg/ml. Initially IgG tested at 10 mg/ml showed nearly 100% blocking in both groups before and after protein boost (data not shown). At 2 mg/ml IgG blocking remained highly potent for both groups (95-97% after 3 DNA doses, 97-99% after protein boost. Even at 1 mg/ml IgG blocking for Group 3 and 4 remained high (82-84% after immunization with DNA and 84-86% after protein boost). Both groups displayed reduced blocking potential at 0.5 mg/ml IgG. (Fig. 5.3.3 c and d). These studies, conducted for the first time using Pfs48/45 DNA vaccines in nonhuman primates, demonstrate potent blocking with codon optimized Pfs48/45 (SYN DNA) suggesting that Pfs48/45 DNA vaccines provide a safe and effective platform for transmission-blocking vaccine development. No differences in functional responses were seen between DNA vaccines encoding glycosylated (SYN DNA) and unglycosylated (MUT DNA) antigens, making a case for leaving putative glycosylation sites intact on Pfs48/45 encoded DNA vaccines.

5.3.8 SMFA for combined groups

IgG from Pfs25+Pfs48/45 cocktail immunization group (group 5) were also tested in SMFAs to determine the impact of co-immunization on the functional immunogenicity of each individual antigen. IgG tested at 10 mg/ml showed nearly 100% blocking before and after protein boost (data not shown). At 2 mg/ml blocking remained highly (~96% after immunization with DNA and ~98% after

protein boost (Fig. 5.3.3 e). At 1 mg/ml IgG showed slightly reduced blocking (71.7% after DNA immunizations and 80% after protein boost) and blocking potential was significantly reduced at 0.5 mg/ml IgG. The findings of Pfs25 and Pfs48/45 co-immunization reported here are the first time TBV candidates have been tested together, with the goal of developing a multivalent transmission-blocking vaccine in the future. The results indicate that co-immunizations are not particularly detrimental to the functional immunogenicity outcome of either antigen. Even though a small reduction in blocking efficacy was observed at 1 mg/ml when compared to individual immunization groups, the reduction was not statistically significant. However, further studies are warranted to evaluate the outcome of co-immunization of various vaccine candidates before considering human trials (Fig 5.3.3 e).

5.4. Discussion

Malaria vaccines targeting development of parasite stages in the female *Anopheles* mosquitoes represent an effective approach for interrupting malaria transmission [45]. In general, it has been rather difficult to produce various TBV target antigens in correctly folded conformations, a key requirement for functional immunogenicity responses. Only Pfs25 has been studied extensively in rodent, nonhuman primate, and limited phase I human trials using various vaccine platforms such as vaccinia virus, *P. pastoris* and *E. coli* [86, 89, 90], with varying degree of success. Pfs48/45 has been less well explored owing to its larger size and the inability to generate a full-length correctly folded antigen in the monomeric form [97,

[98]. These difficulties continue to underscore the need to explore alternative development paths that are technically less challenging (i.e. vaccines that are stable, safe, easy to store and transport), can induce effective immune responses without extensive vaccine manipulations and can facilitate development of a multivalent vaccine targeting different lifecycle stages of the parasite in vertebrate hosts as well as mosquito vectors. DNA vaccines satisfy many of these attributes, however remain in need of clear mechanistic approaches to optimize their potency in larger vertebrates [102]. One mechanism that has been suggested as a contributing factor is reduced transfection efficiency and low levels of antigen expression in larger animals [149]. It has also been noted that size alone does not determine reduced efficacy in larger animals, based on various animal studies such as protective efficacy of WNV DNA vaccine seen in horses [150] and therapeutic melanoma vaccine in dogs [151]. It seems reasonable to speculate that DNA vaccines can be expected to meet their initial proposed potential with improved understanding of immune correlates of protection and mechanisms needed to enhance immunogenicity outcomes [102], [108]. It is crucial however that more experimental undertakings to improve the DNA vaccine platform be designed in larger animal models with more immune-physiological relatedness to humans in comparison to the commonly used laboratory rodent strains [152].

To this end, we have previously reported Pfs25 DNA vaccine studies in rhesus macaques to examine the outcome of a DNA prime-recombinant protein boost regimen [48] and in olive baboons to study the outcome of in vivo EP, putative N-glycosylation site mutations and recombinant protein boost [51]. On the other hand,

Pfs48/45, an important TBV target antigen has not been evaluated as a DNA vaccine in any animal model. The studies presented here were aimed to elucidate comparative functional immunogenicity analysis of the following vaccines in rhesus macaques: (i) Pfs25 SYN DNA no EP or with EP (groups 1 and 2) (ii) Pfs48/45 SYN and Pfs48/45MUT by EP (groups 3 and 4); and (iii) immunogenicity outcomes of combines Pfs25SYN and Pfs48/45SYN immunizations (group 5).

Our findings revealed that Pfs25 delivered with EP (group 2) exhibited 10-fold higher ELISA antibody titers in comparison to no-EP DNA delivery (group 1) before a protein boost. Superiority of EP was further stressed by the fact that a protein boost was needed with the no-EP group to achieve comparable antibody titers. A similar boosting effect of protein was seen with previous rhesus trial using Pfs25 administered without EP and in baboons employing EP delivery of unglycosylated form of Pfs25 [51]. The quality of antibody binding, as determined by avidity data also presented an advantage of EP delivery over no-EP delivery for Pfs25 immunization groups. This advantage persisted after a protein boost, however both no EP and EP groups also showed an improvement in antibody avidity resulting from a protein boost suggesting a clear advantage for EP DNA delivery, aided by heterologous prime boost. When comparing functional responses between plasmid constructs EP DNA delivery showed potent blocking after DNA immunization, which remained high after the protein boost. In contrast, transmission blocking with no-EP group was significantly lower than in the EP group after DNA immunization but was significantly boosted after the protein boost. Overall, our studies confirmed earlier finding in rodent [50], (Datta et al., manuscript

in preparation) and nonhuman primate studies [51] that suggest a comparative advantage for EP delivery of Pfs25 DNA vaccine using a codon optimized Pfs25 sequence. Our studies also call for further evaluation of recombinant protein used for heterologous boosting of Pfs25 and currently, our lab is exploring this avenue in further rhesus studies using a highly immunogenic codon-harmonized *E. coli* produced rPfs25 protein [90].

The studies conducted here, with Pfs48/45 DNA vaccines, report for the first time immunogenicity outcomes in nonhuman primates. We examined a codon optimized, N-glycosylation site intact Pfs48/45 sequence (SYN) against a codon optimized but N-glycosylation mutant sequence (MUT). The gene sequence of Pfs48/45 has 7 putative N-glycosylation sites. Of these 3 N's were altered to D's, one to K and one to T. These mutations were determined based on sequence comparison with other *Plasmodium* species and selecting for alterations at those N sites seen across species. In two instances, putative N-glycosylation site were conserved across species. This suggested a crucial role in protein structural integrity. In these instances, we altered the S/T at the third position to A based on the premise that recognition of the entire NxS/T sequon is required for N-glycosylation to take place. However, results from rhesus immunizations with Pfs48/45 SYN and MUT DNA revealed no significant differences in antibody end point titers, quality of antibody-antigen binding or functional blocking responses in SMFA. There was a dose dependent blocking response for both group, shown by high blocking activity at 1 to 10 mg/ml IgG concentrations tested in SMFA. As with Pfs25 EP immunizations, recombinant protein boosting did not significantly alter the antibody or blocking

responses of either Pfs48/45 SYN or MUT DNA immunized groups. These findings demonstrated for the first time, the transmission reducing potential of Pfs48/45 DNA vaccines and warrant further exploration of Pfs48/45 as a TBV candidate using the DNA vaccine platform. Furthermore, antibody avidity data for Pfs48/45SYN and Pfs48/45MUT (group 3 and 4) revealed no significant differences, suggested no benefit of N-glycosylation mutant group. The 50% dissociation rate of antibody binding (avidity) for both group 3 and 4 was also not significantly enhanced after a protein boost, which may be attributed to low efficacy of the recombinant Pfs48/45 protein used.

Our final experimental group was designed to explore the possibility of a multi-antigen transmission-blocking DNA vaccine combining both Pfs25 and Pfs48/45 antigens. Antibodies obtained after co-immunization with both DNA plasmids, when tested against each individual antigen showed no significant difference as compared to antibodies from groups immunized with individual antigens. Additionally, all the other immunogenicity parameters in the combination groups were not compromised and, support the feasibility of combining multiple antigens for effective immunizations. Further studies will be required to determine whether combined immunization with Pfs25 and Pfs48/45 leads to any additional and/or synergistic functional potency of immune sera.

Based on the all the results from these experiments, we conclude that Pfs25 DNA vaccines continue to be a viable TBV candidate that warrants further investigation for immunogenicity enhancement. Potential ways ahead could include next-generation DNA plasmid design, adjuvant use or nanoparticle-based delivery

[146], [147]. Pfs48/45 has also, for the first time using a DNA vaccine platform, shown promise as a TBV approach. Further understanding of the immune correlates of protection of these transmission-blocking antigens is warranted. In the case of Pfs48/45, levels of antibodies generated in the immunized animal as well as transmission-blocking activity appeared to be lower than that with Pfs25. We do not know whether this is due to insufficient amount of antibodies required to target parasite development in a relatively short duration in the mosquito midgut after a blood meal [57], [75]. Considering the outcome of these studies, the potential benefits of designing TBVs targeting different parasite lifecycle stages in the mosquitoes, and the relative ease of designing cocktail DNA vaccines and combined immunization studies using Pfs25 and Pfs48/45 need to be explored further with the ultimate goal of developing highly potent transmission-blocking DNA vaccines that can target malaria elimination in endemic regions, in combination with current control measures [27].

Fig. 5.2.1 Immunization dose and schedule for SYN and MUT DNA constructs. Shown are also Pfs48/45 sequences showing mutations affecting N-glycosylation.

N = 4 per group	Group 1	Group 2	Group 3	Group 4	Group 5
DNA Plasmid	Pfs25SYN	Pfs25SYN	Pfs48/45SYN	Pfs48/45MUT	Pfs25SYN +Pfs48/45SYN
Electroporation	-	+	+	+	+
DNA Dose	2.5mg	2.5mg	2.5mg	2.5mg	2.5mg each
rProtein boost	rPfs25	rPfs25	rPfs48/45	rPfs48/45	rPfs25+rPfs4845

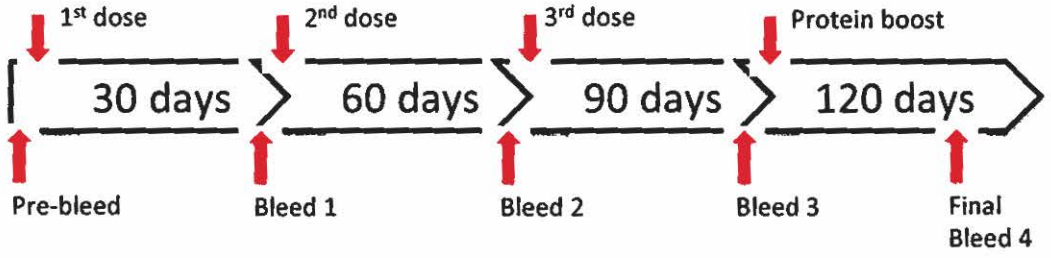


Fig. 5.3.1. Analysis of antibody titers by ELISA in non-human primates (n=4) immunized with different DNA vaccine constructs. Sera collected after three DNA immunizations were evaluated. End point titers were defined as serum dilutions giving an absorbance (405 nm) higher than that with pre-immune sera + 3SD. Statistically significant differences in immune responses between groups were determined by student t tests at $p < 0.05$, indicated by (*). The error bars indicate SD.

Fig. 5.3.1 Analysis of antibody titers by ELISA from immunization with different DNA vaccine constructs

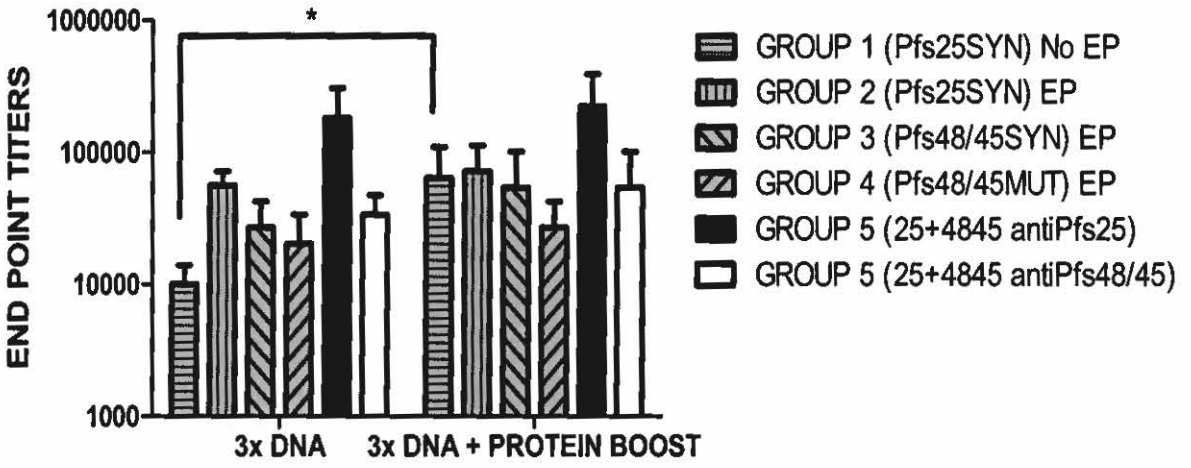


Fig. 5.3.2. Determination of antibody avidity. The plots show molar concentrations of NaSCN required to reduce antigen-antibody binding of individual rhesus sera. Zero molar represents the baseline with 100% total binding. Molar concentrations in parentheses represent the average NaSCN concentrations for 50% binding dissociation for each group.

Fig. 5.3.2 Antibody avidity

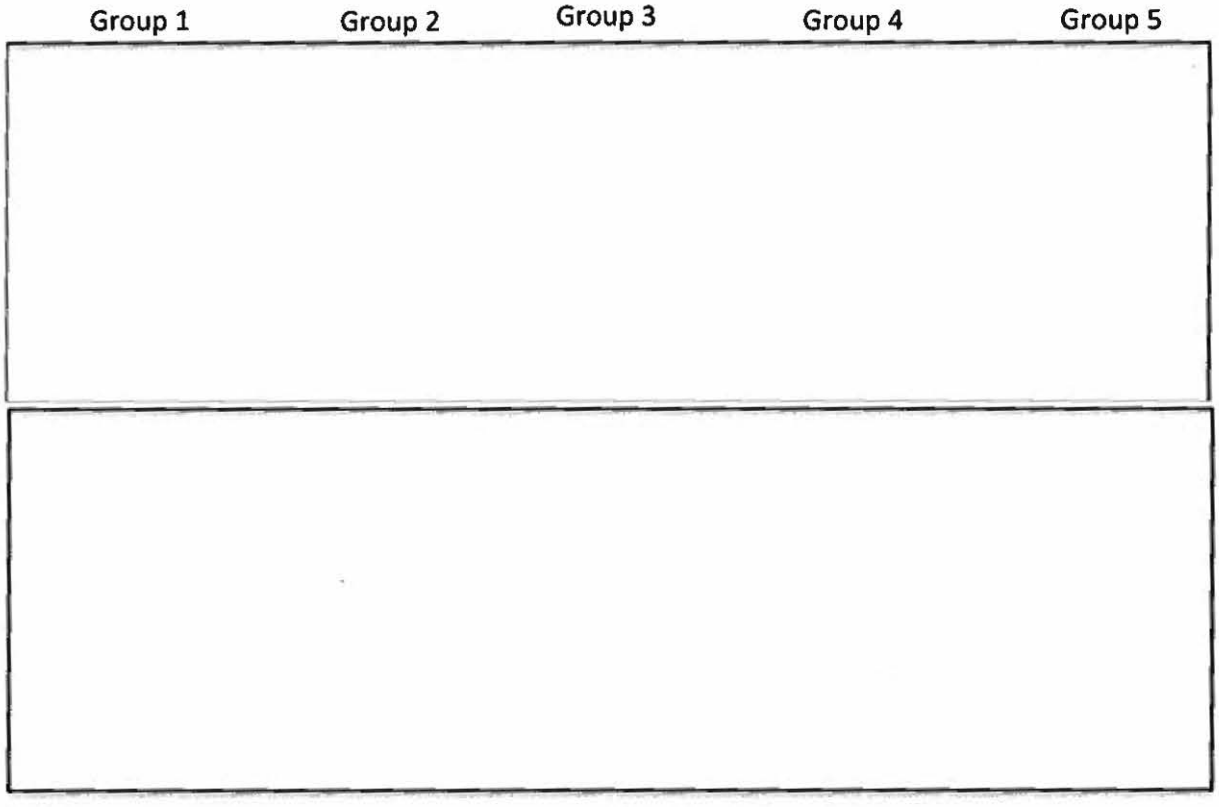
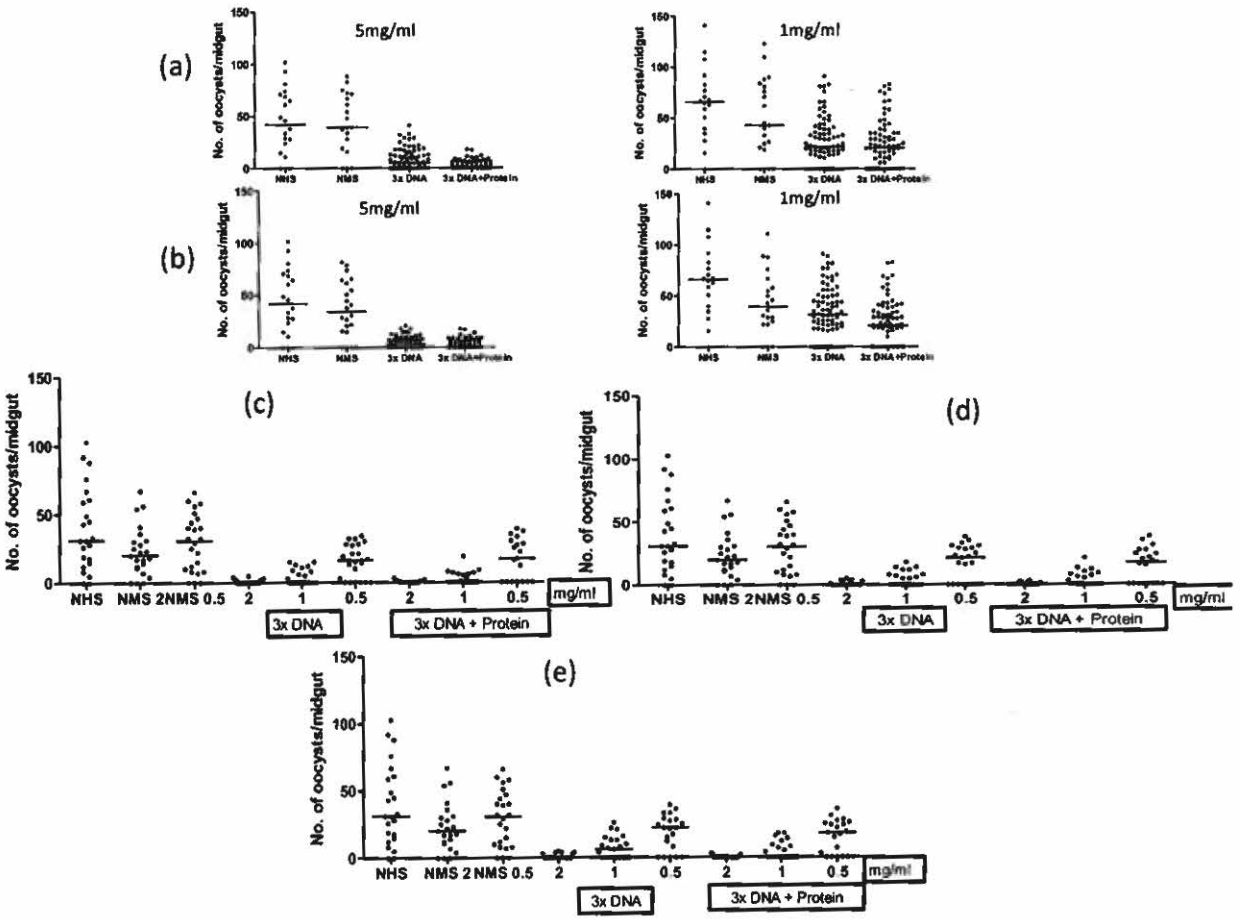


Fig. 5.3.3. Standard membrane feeding assays. Transmission-blocking activity of purified IgG from immune sera of rhesus groups (a) Pfs25 no EP, (b) Pfs25 EP, (c) Pfs48/45SYN EP (d) Pfs48/45MUT EP, and (e) Pfs25SYN+Pfs48/45SYN EP. Bars represent median oocyst counts.

Fig. 5.3.3. Standard Membrane Feeding Assays



Chapter 6: Conclusions and Future Directions

The studies described in this thesis were proposed with the intention of evaluating parameters that might likely influence functional immunogenicity of malaria transmission-blocking DNA vaccines encoding two most potent target antigens, Pfs25 and Pfs48/45. The parameters investigated included: a) the role of N-linked glycosylation on the immunogenicity of *P. falciparum* TB DNA vaccine candidates, b) the outcome of DNA vaccine enhancement mechanisms such as codon optimization and in vivo EP on the immunogenicity of target antigens Pfs25 and Pfs48/45 and, c) evaluation of a multi-antigen TBV combining Pfs25 and Pfs48/45 antigens in nonhuman primates to guide future clinical trials and expected outcomes.

While, native proteins expressed in the parasites do not undergo N- linked glycosylation, when DNA sequences encoding the same proteins are expressed in mammalian cells as would be the case for DNA vaccines, these proteins do undergo extensive N-linked glycosylation. These conclusions were suggested by larger protein size when expressed in transfected mammalian cells and smaller size when expressed using DNA with N-glycosyloation site mutations or in the cells treated with tunicamycin. Whether such protein modifications will have an impact on their immunogenicity is not known and needs to be evaluated empirically. An important conclusion that can be drawn from the findings reported here is that N-linked glycosylation at putative N-glycosylation sites on Pfs25 and Pfs48/45 gene sequence does not negatively impact the immunogenicity outcome from DNA vaccines encoding these antigens. While the Pfs25 studies reported no significant differences

in the functional responses to glycosylated and unglycosylated antigens in mice and rhesus monkeys; Pfs48/45 in fact showed a slight improvement in functional responses to glycosylated antigens, at least in mice. Primarily, this suggests that mutating putative glycosylation sites on *Plasmodium* proteins is not always advantageous for functional immune responses; in spite of the lack of such glycan side chains on proteins produced by the native *Plasmodium* host. Our findings also stress that such effect of N-glycosylation must be examined independently for each individual antigen of interest for vaccine development studies.

Furthermore, our studies provide clear evidence towards the advantages of using optimized sequences that is specific to the target host and also confirm the benefits of using in vivo electroporation for DNA vaccine immunizations. While this had been suggested for Pfs25 DNA vaccines, the outcomes for Pfs48/45 DNA vaccines are reported here for the first time. In addition, our studies provide a comparative analysis of the various enhancements and alterations (codon optimization, in vivo electroporation and N-glycosylation mutations) for both TB vaccine candidates (Pfs25 and Pfs48/45) individually and in combination. As a result, our studies suggest further enhancements approaches to improve the immunogenicity outcomes based on the use of codon-optimized sequences with glycosylation sites intact and utilizing in vivo electroporation.

Another novel finding from these studies is the outcome of co-immunization with Pfs25 and Pfs48/45 DNA vaccines. We were able to demonstrate that neither antigen interferes with the functional outcomes of the other in rodent and human primate studies. These studies provide a scientific basis for designing multivalent

TBVs targeting different lifecycle stages of the parasite (i.e. both pre- and post-fertilization stages), which would increase the likelihood of preventing the development of parasites in the mosquito vector, further preventing the chances of human transmission. These finding also support the development of TB DNA vaccines based on *P. vivax* antigens as well with the purpose of creating multi-species, multivalent malaria transmission-blocking DNA vaccines.

Finally, from the findings reported in this thesis we can conclude that various enhancement tools evaluated for TB DNA vaccines are able to generate potent transmission-blocking responses in mice and nonhuman primates. However, in many cases, they required a heterologous protein boost thus highlighting their limitation and suggest further approaches to optimize their immunogenicity for inducing strong functional responses prior to contemplating human trials. For this, we consider that it is important to design and develop second-generation DNA vaccine backbones that include stronger promoters, protein secretion signals and other in-built enhancers including genetic adjuvants. It is also crucial to consider targeting gene delivery for increased plasmid uptake by antigen presenting cells. Needless to say, further research into understanding the mechanism of action and immune correlates of DNA vaccines is warranted if we are to consider informed decisions in vaccine design and delivery.

The outcomes from these studies do however, make a strong case for continued research on the DNA vaccine platform, particularly for malaria vaccines as it provides a more cost-effective, stable, development and user-friendly platform for the fight against an infectious agent that specifically targets populations in

resource- poor settings for effective and sustainable program for preventing and eliminating malaria transmission.

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CAREER PROFILE:

A PhD candidate in parasitology and tropical infectious diseases seeking opportunities in applicable field research as well as project management experience in international infectious disease initiatives.

EDUCATION:

2010 – 2015	**Doctor of Philosophy,** Parasitology and Tropical Medicine	Tulane School of Public Health and Tropical Medicine, New Orleans, LA
2008 - 2009	**Master of Science,** Biotechnology	Georgetown University, Washington, DC
2004 - 2008	**Bachelor of Technology,** Biotechnology	Dr. D.Y. Patil University, Navi Mumbai, Maharashtra, India

REVELANT EXPERIENCE:

8/2010-1/2015 **Tulane School of Public Health and Tropical Medicine**, New Orleans, LA

Doctoral Candidate, Parasitology and Tropical Medicine, Dept. of Tropical Medicine.

- Developed a mock NIH-R21 grant on *Safety and Immunogenicity trials of Ag85B-ESAT-6 and mucosal adjuvant R192G as a booster vaccine. Studies including latent TB infection and HIV co-infection* for a Vaccine Biology course.
- Developed a mock NIH-R21 grant on the *Involvement of CD4+CD25+ regulatory T cells in Tuberculosis reactivation triggered by Helminth co-infection* for the departmental Preliminary Exam Proposal that was unanimously approved by the exam committee.
- Developed a policy memo on *Corruption in India's public sector healthcare system* for a Health Economics in Developing Countries course.
- Designed a monitoring and evaluation plan for a malaria prevention program based in Uganda for a Malaria M&E course.
- Designed a policy memo addressed to India's revised National Tuberculosis Control Programs DOTS strategy for increasing access and enrollment as well as to reduce non-compliance for an International Health Policy course.

RESEARCH EXPERIENCE:

8/2010-1/2015 **Tulane School of Public Health and Tropical Medicine**, New Orleans, LA

Doctoral Candidate, Parasitology and Tropical Medicine, Dept. of Tropical Medicine. Advisor: Nirbhay Kumar

- Development of *Plasmodium falciparum* transmission-blocking DNA vaccines encoding Pfs25 and Pfs48/45 candidate antigens. Investigating the role of N-linked glycosylation on the immunogenicity of Pfs25 and Pfs48/45
- Testing potential enhancement mechanisms such as electroporation, novel adjuvant formulations and co-immunization in rodent and rhesus models.

- Examining immune correlates involved in transmission-blocking DNA vaccines as well as the mechanisms of innate immunity against *Plasmodium* sexual stage antigens.
- Development of new techniques for better assessment of transmission reduction activity such as transgenic parasite models that use luciferase assays for mosquito midgut infectivity measurement.
- Investigating nanoparticle based DNA delivery methods as alternatives to electroporation based DNA vaccine delivery.

7/2009-7/2010 **Johns Hopkins Bloomberg School of Public Health,** Baltimore, MD
(Continued at **Tulane School of Public Health,** New Orleans, LA following PIs transition)
Laboratory Manager, Malaria Research Institute, Dept. of Molecular Microbiology and Immunology. Principal Investigator: Nirbhay Kumar

- Cultured *Plasmodium falciparum* parasites in vitro.
- Maintained *Anopheles gambiae* and *A. stephensi* mosquito breeding colonies.
- Maintained standard operating procedures and protocols and managed general technical requirements of the lab, including equipment records and upkeep.
- Managing lab reagents and supplies, placing lab orders and maintaining financial records.
- Provided training on general malaria lab techniques spanning in vitro blood, cell and tissue work, insectary tasks and surgical procedures on rodents.
- Conducted immunological and molecular experimental procedures with rodent, non-human primate and human samples pertaining to transmission-blocking vaccine studies.

1/2009-4/2009 **Johns Hopkins University,** Baltimore, MD
Georgetown Graduate Intern, Office of Technology Transfer

- Reviewed the office's infectious disease portfolio for unlicensed, patented (or patent pending) inventions for potential commercializing opportunities.
- Responsible for technology and patent reviews and analysis followed by market surveys.
- Created market summaries for valuable cases and identified potential licensing partners for promising cases.

3/2008- 7/2008 **D.Y. Patil University,** Navi Mumbai, India
Student Research Scholar, Department of Microbiology

- Project: *"Isolation of Actinomycetes from Marine Ecosystems and Screening for Metabolites"*

6/2007-8/2007 **Centre for Research in Mental Retardation (CREMERE),** Mumbai, India
Summer Research Intern, Genetics Lab

- Karyotype and chromosomal analysis of patient samples to identify understand the genetic manifestation of different forms of mental illness.

12/2006-1/2007 **GeneOmbio Technologies**, Pune, India
Summer Research Intern, Molecular Biology Laboratory

6/2005-8/2005 **Dey's Medical Pharmaceuticals Pvt Ltd.,** Calcutta, India
Laboratory Assistant, Quality Control Department

TEACHING EXPERIENCE:

Tulane School of Public Health and Tropical Medicine, New Orleans, LA
Teaching Assistant, Dept. of Tropical Medicine

8/2014-12/2014 Immunology, Lecturer: Geetha Bansal, PhD
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ABSTRACTS AND SEMINARS:

11/2012 **Datta, Dibyadyuti**, Coban, Cevayir, Hannaman, Drew, Kumar, Nirbhay. "*Protein Glycosylation and Immunogenicity of DNA vaccines.*" Oral and Poster Presentation at **American Society of Tropical Medicine and Hygiene (ASTMH)** 61st Annual Meeting in Atlanta, GA

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2/2012 Presented "*Protein glycosylation and immunogenicity – studies in progress*" at the **New Orleans Protein Folding Intergroup (NOProFIG)**, a consortium of various New Orleans area research laboratories.

AWARDS and LEADERSHIP:

Tulane School of Public Health and Tropical Medicine, New Orleans, LA
11/2012 Travel Award recipient for the **61st Annual ASTMH** conference in Atlanta, GA. Presented research data on "*Protein Glycosylation and Immunogenicity of DNA vaccines* " in an oral and poster format
7/2012- Current Founding member and Book Club coordinator, **SPHTM Interdisciplinary Book Club**

Dr. D.Y. Patil University, Navi Mumbai, India
8/2004-3/2008 Elected Students Representative to the Students Council for all 4 years of college
2/2007 Chair, Administrative and Public Relations committee of the Annual Cultural Festival
8/2006 Founding and organizing committee member, Annual inter-collegiate Science Festival Awarded certificate for *Outstanding Organization and Leadership Skills* by the Dean for contributions at the Science Festival

SOCIETY MEMBERSHIPS:

The American Society of Tropical Medicine and Hygiene (ASTMH)
The American Society for Advancement of Sciences (AAAS/Science)

TECHNICAL and LANGUAGE SKILLS:

Statistical analysis of scientific research data using GraphPad Prism and SAS software's
Office tools: Microsoft Word, Excel and PowerPoint
English (fluent), Hindi (fluent). Bengali (fluent), Spanish (basic)

VOLUNTEER and EXTRACURRICULAR ACTIVITIES:

Assisted with math, English, arts and crafts sessions at Centre for Research in Mental Retardation (CREMERE), Mumbai, India

Participant in fund raisers and 5Ks for Imagine No Malaria and Relay for Life

Member of Salsa India Dance Company performance group and Lindy Hop performance group at Dance Quarter, NOLA.