TRANSGENIC RODENT MALARIA PARASITES FOR THE EVALUATION OF PROTECTIVE IMMUNITY AGAINST PLASMODIUM FALCIPARUM

by
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ABSTRACT

Malaria is a global health emergency. Current estimates indicate that 3.3 billion people live at risk of infection and that more than half a million lives are lost to the disease annually, most of them from children under the age of 5. Despite extensive research and numerous clinical trials, the development of a fully efficient malaria vaccine remains an elusive goal. The RTS,S vaccine candidate has demonstrated significant potential to prevent clinical disease and severe malaria, but improvements in vaccine efficacy and duration of immunity are still needed. For decades, rodent Plasmodium parasites have been instrumental tools for addressing fundamental questions in malaria biology and immunology. However, their utility for the evaluation of vaccine candidates is limited due to significant differences in antigenic structure between murine and human parasite antigens. In recent years, transgenic rodent parasites expressing human malaria antigens have emerged as practical tools to overcome the limitations of the traditional rodent models. In this dissertation, I present a series of studies assessing protective immune responses against the P. falciparum Circumsporozoite Protein (CSP) using newly generated chimeric rodent malaria parasites. We demonstrate that a monoclonal antibody capable of inhibiting the proteolytic cleavage of CSP strongly inhibits in vivo infection of sporozoites expressing the N-terminus of P. falciparum CSP. In further chapters, we evaluated immune responses induced by full-length recombinant P. falciparum CSP in combination with different adjuvant systems and demonstrate that E. coli-derived recombinant protein is highly immunogenic and can induce considerable antibody and CD4+ T-cell responses that inhibit sporozoite infection. Also, we report the development of several epitope-focused virus-like particles (VLPs) incorporating different domains from the P. falciparum CSP. In this study, we found that a construct incorporating B- and T-cell CSP epitopes conferred sterilizing immunity to mice upon immunization with different adjuvant formulations. In more recent investigations, we assessed antibody and CD8+ T-cell responses, induced by immunizations with a novel cationic liposomal adjuvant, using new parasite chimeras expressing the entire P. falciparum CSP. Importantly, while antibodies against CSP can neutralize sporozoite infection, the induction of CD8+ T-cell responses against the P. falciparum CSP enhances the inhibitory capacity
of humoral responses and induces long-lasting sterilizing immunity. Lastly, the studies here presented provide ample evidence supporting the usefulness of chimeric rodent parasites as platforms for the evaluation of malaria vaccine candidates.

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CHAPTER 1
GENERAL INTRODUCTION
1.1 The burden of malaria

Malaria is a burden to humanity. This disease is one of the leading causes of morbidity and mortality worldwide, imposing major socioeconomic challenges to many developing nations.

Malaria and poverty are inextricably related. The vast majority of cases occur in low-income nations and, within these countries, the communities in which malaria has the highest impact tend to be the poorest and more neglected [1]. The precise estimation of the economic burden that malaria imposes to developing nations is limited by the lack of high-quality data as well as by the need to compound a series of direct and indirect costs [2, 3]. The predicted impact of malaria on economic growth is significant and some of the ways through which this disease thwarts financial development include child and pregnant women mortality; loss of productivity as a result of coping with malaria; inadequate cognitive development and education; and impairment as a consequence of malnutrition [4]. And while impoverished countries face major challenges for preventing, diagnosing and treating the disease, the location and severity of malaria are defined to a significant extent by climate and ecology, not by poverty alone [4]. Nevertheless, it is important to keep in mind that the relationship between malaria and poverty is bidirectional and that the reduction in economic output due to malaria can in turn reduce the ability of populations to deal with the disease [5].

The social costs that malaria imposes are dictated by the changes in human behavior in response to the disease. Indeed, malaria affects numerous aspects of household practices towards fertility, savings and investment rates, crop choices, education and migration decisions [4]. Understanding the social and behavioral components that influence malaria transmission is of utmost importance for the successful implementation of control measures [6].

1.2 The malaria parasite

1.2.1 Life cycle

Malaria is caused by protozoan parasites of the genus *Plasmodium* spp., which are transmitted to humans through the bites of infected mosquitoes. The five *Plasmodium* species that are known to
cause malaria in humans are *Plasmodium falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi*, which is an uncommon zoonosis. *P. falciparum* and *P. vivax* are the most common and widely distributed malaria species, the former being responsible for the large majority of the deaths attributed to the disease.

The *Plasmodium* parasite has a complicated life cycle that spans both humans and mosquitoes (Fig. 1.1). Infection of the human host begins with the inoculation of sporozoites into the skin through the bites of an infected *Anopheles* mosquito. After skin deposition, sporozoites will find and penetrate blood vessels in order to reach the liver. When parasites reach this organ, they proceed to invade hepatocytes and undergo asexual replication before being released into the bloodstream as merozoites. Once in circulation, parasites undergo a series of differentiation and replication steps leading to the production of more infectious merozoites, continuously reentering the blood stage cycle. A fraction of blood stage forms will develop into the sexual stages of the parasite, male and female gametocytes, which are eventually taken up the mosquito during a blood meal. Within the mosquito, gametocytes undergo fertilization and develop into a zygote that matures into an ookinete, a motile form that invades the mosquito midgut. Once ookinetes reach the midgut basal lamina, they transform into oocysts. It is during this stage that parasites divide and generate thousands of sporozoites, which are released into the mosquito circulatory system. Finally, sporozoites invade the salivary glands, where they remain until injected into a new host when the mosquito takes another blood meal.

### 1.2.2 Clinical manifestations

The clinical manifestations of malaria are caused by asexual forms of the parasite as they replicate in the bloodstream. Importantly, the clinical outcomes of the disease depend on a number of different factors. Prior exposure to infection and age are important determinants of disease severity. However, parasite and host genetics, nutritional condition, and geographic and socioeconomic aspects also play a role [7-9]. In individuals with no history of previous exposure, the initial manifestations of malaria—such as fever, headache, myalgia, chills, vomiting and lethargy followed by malarial paroxysms—develop 7 to 15 days after infection and are associated with increased levels of circulating cytokines [10].
If left untreated, otherwise uncomplicated malaria due to *P. falciparum* can result in severe illness and even death. Age is a determining factor of disease severity due to falciparum malaria [11]. In children, severe malaria comprises 3 overlapping conditions: cerebral malaria, metabolic acidosis and severe anemia [7]. Sadly, cerebral malaria and metabolic acidosis have a mortality of 15-20%, with survivors occasionally suffering from neurocognitive sequelae [12]. And while non-immune and semi-immune adults can also develop cerebral malaria and acidosis, other common complications associated with severe disease in adulthood include acute pulmonary edema, renal failure, and jaundice [13]. Further, pregnant women are also at risk of developing placental malaria. This condition is a consequence of parasite sequestration in the placenta and results in the cascade of immunological responses that are deleterious to both the fetus and the pregnant mother [10].

Malaria due to *P. vivax* has been historically regarded as a “benign” and non-life threatening condition. However, this misconception –attributed to erroneous and outdated clinical classifications– has been disproved by recent data demonstrating that *P. vivax* malaria can be associated with morbidity and mortality [14]. Severe manifestations of *P. vivax* malaria include, among others, severe anemia, respiratory distress, renal failure, jaundice, seizures and coma [14, 15]. A distinguishing characteristic of *P. vivax* malaria are the relapses that occur after resolving the primary infection. These recurring episodes have been attributed to the activation of dormant liver stage parasites known as hypnozoites. Different factors have been proposed to trigger the reactivation of hypnozoites, including components of mosquito saliva [16] as well as febrile episodes following *P. falciparum* or other parasitic and bacterial infections [17].

The incidence of clinical cases due to *P. malariae* and *P. ovale* is low despite the fact that these infections can be common and have lower pyrogenic thresholds than *P. falciparum* (reviewed in [18]). In addition, severe manifestations resulting from *P. malariae* or *P. ovale* infections are rare, typically reported as isolated cases of jaundice, splenic rupture, thrombocytopenia, splenomegaly or renal failure [19]. Some of the clinical symptoms due to *P. knowlesi* infection include fevers, chills, headaches,
myalgia, malaise, and poor appetite [20]. Fatal cases of *P. knowlesi* are unusual but have been previously reported [21].

### 1.2.3 Epidemiology and geographical distribution

The 2014 World Malaria Report by the World Health Organization (WHO) indicates that in 2013 there were approximately 198 million cases of malaria (95% uncertainty interval, 124–283 million) and 584 000 malaria deaths globally. The same report points out that about 3.3 billion people in 97 countries are at risk of contracting malaria and that 1.2 billion live at high risk. Noteworthy, 82% of malaria cases and 90% of malaria deaths occurred in the WHO African Region; children under 5 years of age and pregnant women are the most severely affected [22].

The numbers provided above reflect the heavy burden imposed by malaria and the World Malaria Report represents the WHO’s flagship publication for the disease. However, ten years ago, a study by Snow *et al.* indicated that in 2002 the WHO’s assessment of clinical *P. falciparum* malaria likely underestimated a large number of cases that occurred during that year [23]. Using epidemiological, geographical and demographic data, authors of that study calculated that the global number of malaria cases due to *P. falciparum* was probably 50% higher than the one reported by the WHO [23]. Further, a recent opinion by Snow elaborates on the difficulties in measuring the malaria burden in Africa and the need to develop more accurate metrics, which would allow a better assessment of the efficacy of current control efforts [24].

*P. falciparum* is by far the main cause of severe cases and deaths due to malaria. One of the most recent epidemiological studies indicates that in 2010 approximately 2.4 billion people worldwide were living at risk of *P. falciparum* [25]. Using a cartographic approach, another study determined that the global burden during the same year was 451 million cases of clinical *P. falciparum* malaria (as opposed to the 247 million cases estimated by the WHO) [26]. In terms of global geographical distribution, an updated version of the *P. falciparum* spatial limits map indicates that, as of 2010, this species remains endemic to 85 countries.
The recent appreciation of the burden, socioeconomic toll and severity of disease imposed by \textit{P. vivax} has led to an increase in efforts to better understand the global distribution and epidemiology of this long-neglected malaria species. Current estimates indicate that 2.9 billion people live at risk of \textit{P. vivax} malaria \cite{27}, with approximately 80 million to 300 million clinical cases per year \cite{28}. To date, it remains unclear how many deaths can be precisely credited to \textit{P. vivax}. In this regard Baird provides an interesting insight, “Global malaria mortality estimates from any source offer no evidence of species composition—attrition to \textit{P. falciparum} rests solely upon the presumption of death as a very rare outcome of \textit{P. vivax} infection” \cite{14}. In a recent study, 95 countries, through Africa, Asia and the America, were identified as endemic for \textit{P. vivax} \cite{29}. One of the reasons for the wide geographic distribution of \textit{P. vivax} is the species capacity to occur at latitudes that do not allow \textit{P. falciparum} development, such as the Korean Peninsula, China, and southwestern Asia \cite{14}. This is mainly attributed to \textit{P. vivax}'s ability to develop at lower temperatures during sporogony (\textit{P. vivax}'s sporogonic cycle is shorter and its sexual stage is active at lower temperatures than other human malaria species); and ii) it’s the parasite’s capacity to produce hypnozoites in the human host (reviewed in \cite{27}).

Epidemiological data from the other 3 human-infecting malaria species (\textit{P. malariae}, \textit{P. ovale} and \textit{P. knowlesi}) is limited. Little is known about the prevalence and clinical manifestations of \textit{P. malariae} and \textit{P. ovale} infections in populations living in endemic areas. Some of the factors contributing to the lack of knowledge are i) difficulty in differentiating \textit{P. malariae} from \textit{P. falciparum} and \textit{P. ovale} from \textit{P. vivax} by light microscopy; ii) low level blood parasitemias associated with these infections; iii) and the decreased ability of Rapid Diagnostic Tests (RDTs) to detect \textit{P. ovale} compared to other human malaria species \cite{30, 31}. By the same token, no global estimates of prevalence are yet available for \textit{P. knowlesi}. The geographic distribution of these \textit{Plasmodium} species is diverse. \textit{P. malariae} tends to overlap with \textit{P. falciparum} infections and are more common in sub-Saharan Africa and Southeast Asia \cite{32}. However, they have also been described—albeit as infrequent infections— in malaria-endemic regions of Asia, the Middle East, South America and Central America (reviewed in \cite{18}). \textit{P. ovale} has been described in several isolated foci throughout Southeast Asia and Oceania; but evidence suggests that this species is
endemic to sub-Saharan Africa and islands of the western Pacific [19, 33]. Lastly, *P. knowlesi* cases have been described in a number of southeastern Asian countries including Malaysia, Thailand, the Philippines, Myanmar, Singapore, Vietnam, Indonesia, Brunei and Cambodia [20].

1.3 Malaria prevention, diagnosis and treatment

1.3.1 Prevention strategies

1.3.1.1 Insecticide-treated bednets

Insecticide-treated bednets (ITNs) are fundamental tools in the fight against malaria. The 2014 World Malaria Report reveals that, in 2013, 49% of the population at risk of malaria in sub-Saharan Africa had access to ITNs, as opposed to only 3% in 2004 [22]. When used correctly, ITNs represent a practical and cost-effective approach to prevent malaria in highly endemic areas [34].

Due to their low toxicity in mammals and significant insecticidal capacity, pyrethroids are the only substance class recommended by the WHO for treating bednets [35]. The effectiveness of ITNs against the African malaria vectors *Anopheles gambiae* s.s. and *An. funestus* is partly explained by the fact that both species feed indoors and remain inside households after feeding [36]. In addition, the repelling capacity of ITNs also results in less number of mosquitoes entering homes [37].

Over the last 15 years, the effectiveness of ITNs has been threatened by increasing resistance to pyrethroids by malaria vectors. A study in 2011 by Ranson *et al.* indicated that 27 nations in sub-Saharan Africa have reported pyrethroid resistance in *Anopheles* vectors [38]. It is considered that the 2 main mechanisms contributing to resistance are i) changes in the sensitivity of the insecticide’s target (sodium channels of the mosquito’s neuron) and ii) metabolic resistance by detoxification or sequestration of the insecticide before it reaches its target site [39, 40].

Despite the increasing pyrethroid resistance, a recent meta-analysis indicates that ITNs remain a relatively effective tool against malaria vectors in Africa [36]. However, additional research is needed to identify new insecticidal substances and prevent further resistance spread.
1.3.1.2 Indoor residual spraying

Indoor residual spraying (IRS) is an essential approach for malaria control. Historically, the WHO proposed the global eradication of malaria through the use of DDT for household spraying in 1955 [41]. To date, IRS has been adopted in 90 countries worldwide, including 42 out of the 45 malaria-endemic countries in the WHO African region [22]. For some time, the use of IRS was not considered a viable option in areas of stable malaria transmission because of the logistical and economic challenges that such interventions represent [42]. However, the development of new long-lasting formulations would presumably justify such interventions in areas of stable transmission.

The effect of combining the use of ITNs and IRS in order to achieve greater vector control has been the subject of several studies. And while there is conflicting evidence about whether there is an added benefit from combining these 2 approaches, more research is needed to establish the relative contribution of each method under different malaria transmission intensities [42-46].

Similarly to ITNs, resistance mechanisms have been described for the four classes of insecticides currently approved by the WHO for IRS (organophosphates, organochlorines, pyrethroids and carbamates) [41, 47, 48]. However, ongoing efforts are aiming to develop new insecticidal substances and even transgenic mosquitoes that can interrupt malaria transmission [49].

1.3.2 Diagnostic methods

1.3.2.1 The need for accurate diagnostic methods.

The need for accurate diagnostic methods for malaria can be summarized in 4 items [50]:

a) In addition to malaria, several other infections are also accompanied by febrile episodes and related symptoms. Diagnostic methods that can accurately confirm the presence of *Plasmodium* parasites are essential to provide adequate treatment.

b) The detection of subclinical or asymptomatic infections is critical for assessing transmission reduction and reach elimination.

c) Because primaquine -the licensed drug for the radical cure of *P. vivax*- can elicit hemolytic anemia in individuals suffering from Glucose-6-Phosphate dehydrogenase (G6PD) deficiency,
methods that can accurately discriminate between *P. falciparum* and *P. vivax* infections are of the essence.

d) The accurate identification of cases in areas of unstable or low malaria transmission should facilitate the efficient allocation of resources and precisely targeted interventions.

1.3.2.2 *Microscopy.*

Microscopy has been historically regarded as the gold standard for malaria diagnosis. Blood samples can be prepared in two different ways for microscopic examination: thick blood smears and thin blood smears. In thick blood smears, the blood sample is smeared on a glass slide, allowed to dry and then stained using Giemsa stain. For thin blood smears, a smaller blood sample is thinly spread in a glass slide, dried and fixed with methanol prior to Giemsa staining. Treatment with methanol prevents erythrocytes from lysing and parasites can be visualized within red blood cells. This allows the detailed examination of their morphological characteristics and species differentiation.

1.3.2.3 *Rapid Diagnostic Tests*

RDTs consist of immunochromatographic assays for the detection parasite-specific antigens in blood. The tests have been engineered in a user-friendly format that can provide results 5-20 minutes after sampling. Importantly, RDTs can be easily applied in field settings because their implementation requires minimal resources and training. In terms of performance, it is generally accepted that these tests provide reliable results down to approximately 500 *P. falciparum* parasites/µl or 5000 *P. vivax* parasites/µl of blood.

1.3.2.4 *Polymerase Chain Reaction*

Due to its unparalleled sensitivity, Polymerase Chain Reaction (PCR)-based approaches have become a commonly used tool for the diagnosis of different parasitic diseases [51]. PCR assays for detecting malaria infections have been developed more than 20 years ago and have rapidly evolved into more accurate and simple approaches, such as Real-Time PCR and Loop-mediated isothermal amplification (LAMP) [52, 53]. Compared to microscopy and RDTs, PCR-based methods can achieve
significantly greater sensitivity, with detection limits reportedly at <5 parasites/µl and perhaps as low as 0.004 parasites/µl [54].

A summary of the advantages and disadvantages of the above described diagnostic approaches is presented in Table 1.1.

1.3.3 Antimalarial drugs

During the 1880s-1890s, Alphonse Laveran, Ronald Ross and Battista Grassi made the first descriptions about the malaria parasite and its relationship with mosquito vectors. Their seminal observations mark the beginning of our scientific understanding of the biology of this disease and how we treat it. However, it is remarkably interesting that centuries before those early findings by Laveran, Ross and Grassi, two herbal treatments - cinchona bark and qinghao - were used to treat malarial fevers [55]. Time passed and our knowledge significantly evolved. Because malaria meant an obstacle for the growth of developed nations into tropical regions, it became one of the best-characterized pathologies in Western medicine until half of the 20th century [55]. While malaria is no longer a threat to industrialized countries, there are renewed expectations to eradicate this disease from the developing world.

The literature on antimalarial drugs is extensive. The molecular mechanisms by which these compounds exert their anti-parasitic effects, as well as their performance under field conditions and with different populations, have been well characterized. However, the significant majority of recent studies are focused on the emergence and rapid dissemination of drug-resistant parasites. Critically, resistance to every antimalarial drug developed to date has been reported.

Over decades, drugs have been instrumental for reducing the global malaria burden and still remain a critical tool in the fight against this disease. Nevertheless, the worldwide spread of drug-resistant parasites, coupled with the current lack of a fully-efficient malaria vaccine, underscore the need for developing new therapies.
1.4 Malaria vaccine development status

The need for a malaria vaccine is self-evident. The previously discussed matters about prevention, diagnosis and treatment of malaria indicate that, while these approaches are an absolute necessity, they are not sufficient for eradicating this disease. The malaria parasite is a complex organism, capable of evolving and adapting to a continuously changing environment. Thus, it is reasoned that a comprehensive approach, capable to targeting the parasite at multiple developmental stages –both in humans and mosquito vectors- is the key to malaria eradication.

The malaria parasite has a complicated life cycle and this complexity contributes to difficulty in engineering a successful vaccine. Efforts to develop a vaccine have traditionally targeted one of the three major stages of the malaria life cycle: the pre-erythrocytic stage, the blood- (or asexual) stage or the mosquito (or sexual) stage. In the following section I will briefly present the advantages and shortcomings of each vaccine approach, the main challenges that each of them faces and provide an overview of their current stage of development. In addition, I also present a summary of the current status of the WHO global malaria vaccine pipeline (Figure 1.2).

1.4.1 Pre-erythrocytic stage vaccines

Pre-erythrocytic stage vaccines aim to elicit immune responses against the early stages of the parasite in the human host. Immunity induced by these vaccines targets the sporozoites, deposited in the skin by the mosquito vector, and aims to prevent them from establishing a liver infection. These vaccines also target parasites that reach the liver and invade hepatocytes to ultimately prevent the release of merozoites that will infect red blood cells.

There are several advantages of targeting the pre-erythrocytic stages of malaria. From a public health perspective, pre-erythrocytic stage vaccines are appealing because they could not only prevent the onset of disease in the host but also cease the parasite from being further transmitted. In a biological context, pre-erythrocytic vaccines also have significant advantages. The limited amount of sporozoites deposited in the skin by the mosquito vector [56, 57], along with the fact that a small number of those will ultimately infect the liver, validate inducing immune responses that are capable of striking parasites
at this vulnerable stage. Most importantly, the feasibility of conferring sterile immunity to malaria by targeting the pre-erythrocytic stages of the parasite was demonstrated long ago in both rodents and humans immunized with irradiated sporozoites [58, 59].

Conceptually, it is difficult to envision any possible drawbacks or limitations from a pre-erythrocytic vaccine. However, several studies and reviews have addressed the shortcomings of specific vaccine candidates, providing important lessons on the immunological requirements that must be met by a yet-to-be successful formulation. Some of the recurring topics found in literature include -but are not limited to- the inability of vaccine candidates to induce long-lasting protection, the poor representation of the parasite’s genetic diversity and the feasibility of these candidate formulations to induce protective responses in populations living in malaria endemic areas (reviewed in [60]).

To date, RTS,S remains the overall leading malaria vaccine candidate. Originally developed through a joint collaboration between GlaxoSmithKline (GSK) and the Walter Reed Army Institute of Research (WRAIR), this formulation initially consisted of 16 amino acid tetra repeats (Asn–Ala–Asn–Pro, or NANP) from the *P. falciparum* 7G8 Circumsporozoite Protein (CSP) that were fused to the pre-S2 region of the hepatitis B virus surface antigen (reviewed in [61]). The amino acid sequence of the initial construct was later derived from the NF54 *P. falciparum* strain and modified to incorporate T-cell and B-cell epitopes found in the carboxy-terminal (C-terminal) region of CSP (reviewed in [61]). The name RTS,S was coined from the resulting particle: ‘R’ for the CS Repeats, ‘T’ for T-cell epitopes and ‘S’ for HBsAg (the additional ‘S’ is an indication that “the fusion protein is coexpressed in yeast cells with HBsAg, yielding a product that is composed of 25% of the CS-HBsAg fusion protein and 75% of HBsAg” [62]). Initial results of a Phase III clinical trial indicated that RTS,S had a vaccine efficacy of ~55% in children 5-17 months of age and ~31% in infants 6-12 weeks old, when assessed during 12 months after vaccination [63, 64]. However, a follow-up evaluation, at 18 months after immunization, showed that vaccine efficacy decreased to 46% in children and 27% in infants [65]. The latest results of this large-scale trial indicated that RTS,S reduced the number of cases by 36% in children and by 26% in infants, when assessed from month 0 to study end (median follow-up of 48
months for children and 38 months for young infants) [66]. Overall, the results of these clinical trials clearly represent the two main criticisms that RTS,S commonly faces, modest vaccine efficacy and waning immunity.

Two additional pre-erythrocytic vaccine candidates are currently undergoing Phase II clinical trials, i) *P. falciparum* irradiated sporozoites and ii) ChAd63/MVA (CS; ME-TRAP) (simian adenovirus 63 (ChAd63)/modified vaccinia virus Ankara (MVA) expressing the Circumsporozoite (CS) Protein or the Multiple Epitope–Thrombospondin-Related Adhesion Protein (ME-TRAP)) [67]. While immunization with irradiated sporozoites is indeed capable of conferring sterile protection against malaria, important regulatory and logistical barriers need to be overcome before this approach becomes a viable option. Some of the main challenges include the need for large amounts of sporozoites required to confer protection; the difficulty in storing and transporting the parasites to remote areas without compromising their viability; and, perhaps most importantly, the fact that protection is only achievable through repeated intravenous immunizations [68]. In the case of ChAd63/MVA (CS; ME-TRAP), although early studies showed promising immunogenicity, this platform failed to meet expectations and was only modestly efficacious in recent Phase II clinical trials [69, 70].

1.4.2 Blood-stage vaccines

Blood-stage vaccines target the asexual stage phase of malaria, initiated when hepatocytes rupture and release thousands of infectious merozoites into the circulation. Each merozoite infects a red blood cell (RBC) and in the case of *P. falciparum* and *P. vivax* 16 new merozoites are released from a single RBC every 48 hours. Immune responses are not initiated in the draining lymph nodes but the spleen has an essential role (reviewed in [60]).

The induction of immunity against blood stages of *Plasmodium* is justified by the fact that these stages are responsible for death and disease. Thus, the appeal of blood-stage vaccines relies on their potential to prevent fatal cases and severe illness by controlling parasitemia. In addition, it is reasoned that such a vaccine could be administered along with pre-erythrocytic formulations in order to prevent clinical disease caused by parasites that manage to establish blood-stage infection [71].
The development of blood-stage vaccines faces critical immunological challenges. Two of the main difficulties include the parasite’s antigenic diversity - which allows it to effectively evade the immune system - and mechanisms of immune interference evidenced during natural exposure to *Plasmodium* [72, 73].

Currently, the most advanced blood-stage vaccine candidate is the *P. falciparum* apical membrane antigen 1 (AMA1). Despite an early study showed a certain degree of protection in monkeys immunized with AMA1 against challenge with *P. falciparum* [74], the latest clinical trials have reported disappointingly low vaccine efficacy because of the genetic diversity of *P. falciparum* field isolates [75, 76].

### 1.4.3 Mosquito-stage vaccines

Mosquito-stage vaccines (also known as transmission-blocking vaccines) target the sexual stages of the parasites, the male and female gametocytes, as well as the products of fertilization in the mosquito midgut. These vaccines aim to induce immune responses capable of preventing the fusion of gametocytes or the migration of ookinetes out of the midgut, preventing infection in the mosquito. Mosquito-stage vaccines act on a community or altruistic level, as they do not prevent disease in the recipient but in the next susceptible host.

One of the main advantages of transmission-blocking vaccines is that mosquitoes are a critical bottleneck for *Plasmodium*. Compared to the large biomass of asexual forms that can be found in the blood, the relative amount of parasites in the mosquito (especially as midgut-traversing ookinetes) is several logs lower [77]. It is thus reasoned that targeting a limited amount of parasites is more achievable than dealing with exponentially larger numbers. In addition, compared to the asexual stages of the parasites, surface proteins on gametocytes are significantly less polymorphic [78]. Further, the availability of reliable assays for testing transmission-blocking vaccine efficacy are now available greatly facilitates the systematic evaluation of novel vaccine candidates [79].

The main shortcoming of transmission-blocking vaccines is that they do not provide sterilizing immunity to vaccinees. It has been noted that referring to them as altruistic vaccines can “cause a
negative perspective on the utility of these vaccines” [80], complicate regulatory procedures for implementation and even compromise their acceptance by national governments and local communities [78].

To date, the most advanced transmission-blocking vaccine candidates are the *P. falciparum* surface protein 25 (Pfs25) and the *P. vivax* homolog Pvs25. The most recent Phase I clinical trial demonstrated the feasibility of inducing blocking responses using a Pfs25-based vaccine [81]. However, the unexpected reactogenicity to the adjuvant system used and the modest blocking effect achieved indicate that further improvements to this vaccine formulation are needed.

1.5 Malaria vaccine adjuvants

Adjuvants are compounds that boost and modulate the immunogenicity of vaccine antigens. Usually, these substances are added to antigens such as recombinant proteins, subunit vaccines and inactivated organisms, which tend to lack the necessary molecular patterns to trigger a robust immune response [82]. Indeed, while new molecular technologies have enabled the production of highly pure vaccine antigens, safer than previous whole-pathogen approaches, this has resulted in weakened immunogenicity [83].

In the last 20 years, great advances have been made to define and characterize the molecular basis of the adjuvant effect on the immunogenicity and induction of adaptive immune responses. It is now known that this largely depends on the activation of innate immunity receptors and it is recognized that this is one of the important features of most adjuvants (reviewed in [84]). There are several families of innate immune receptors and the best characterized are the so called Toll-Like Receptors (TLRs). TLRs are type I integral transmembrane glycoproteins, defined as a family of pattern recognition receptors (PRRs) expressed on cell surfaces or within endosomes, and they are critically involved in defense against microbial infections. They are expressed in lymphocytes, dendritic cells, and macrophages, and other non-immune cell types like epithelial and endothelial cells (reviewed in [85]). There are 12 different TLRs which respond to distinct pathogen-associated molecular patterns.
(PAMPs) leading to the activation of specific signaling pathways via release of type-I interferon, chemokines, cytokines and co-stimulatory molecules (reviewed in [86, 87]).

In the case of malaria, the development of the RTS,S vaccine candidate has been closely related to the evolution of GSK’s Adjuvant Systems (AS), a technology that has been in progress for about 3 decades. AS, which consist of mixtures of different immunostimulatory substances, appeal to the notion that combination of multiple adjuvants can produce a synergistic effect and therefore enhance the immune response [88]. Therefore, the main challenge for this approach is to identify the best performing combination, both efficient and safe for the targeted populations [89].

Over the years, RTS,S has been tested in combination with different AS in multiple clinical trials. AS04, AS03 and AS02 were among the first formulations to be evaluated, being the latter the one with better protective capacity as well as ability to induce humoral responses and cellular immunity (reviewed in [61]). Still, an even stronger formulation (AS01B) was later introduced, demonstrating up to 50% protection in malaria-naïve individuals and showing significant potential for pediatric use in malaria endemic areas [90]. AS01B is a liposome-based AS that incorporates the immunostimulatory components Monophosphoryl lipid A and a purified fraction of Quil-A called QS21. Importantly, MPL engages the TLR-4 promoting the maturation of antigen presenting cells (APCs), which then prime naïve T cells [88]. The mechanism of action of QS21 is not yet fully understood. However, it is speculated that its lytic nature, perhaps causing the release of intrinsic ‘danger’ signals, has a role [82].

Other adjuvant formulations that have been tested in malaria vaccine clinical trials or undergone pre-clinical evaluation include aluminum salts, water-in-oil emulsions (Montanides: ISA51, ISA720) and oil-in-water emulsions (MF59) [67]. Notably, ISA720 has been tested in several clinical trials, demonstrating remarkable immunogenicity albeit occasional reactogenicity at the site of injection [82]. Clinical studies with MF59 are also extensive but its use has only been approved in one vaccine, Fluad [82]. Further, a number of recent malaria vaccine studies have reported the use of well-defined TLR agonists known to enhance B- and T-cell responses such as Poly I:C, a synthetic double stranded RNA, which is a strong inducer of interleukin (IL)-12 and type I IFNs through activation of innate
immunity via TLR3; the TLR-4 agonist glucopyranosyl lipid adjuvant-stable emulsion (GLA-SE); and CpG-oligodeoxynucleotides, which engage TLR-9.

1.6 Protective immune responses against malaria sporozoites

In this section I will briefly review different components of the immune response that are capable of eliciting and/or mediating protection against sporozoite infection. These responses will be presented in a broad context, however, elaborating upon the underlying molecular mechanisms of immune activation is beyond the scope of this dissertation.

1.6.1 Innate immune responses

Innate immune responses against sporozoites occur in the skin, where the parasites are deposited as the female *Anopheles* mosquito probes for blood. Here, the parasites will likely come into contact with a wide variety of skin-resident cells including mast cells, neutrophils, dendritic cells, macrophages, innate lymphoid cells and T-cells [91]. Mast cells are likely one of the first cell types that sporozoites will find [92]. Characterized by their high content of secretory granules, these cells are filled with a large amount of fast-acting immune modulators capable of causing significant inflammatory reactions [93]. However, the downstream effects of mast cell activation upon sporozoite infection remain unknown [92].

Despite the fact that neutrophils have a critical role in the induction of immune responses to *Leishmania* infection [94], a recent study found that depletion of these cells did not prevent the onset of immune mechanisms against *P. berghei* sporozoite infection [95]. And while it was noticed that neutrophils are rapidly recruited to the site of sporozoite inoculation, their immunological role upon sporozoite infection is yet to be determined [95].

Different studies have demonstrated that the induction of CD8+ T-cell responses against malaria significantly relies on dendritic cells (DCs) [96-101]. However, the specific role of skin-resident DCs has not been yet fully elucidated. In this regard, a recent study showed that live sporozoites directly access the skin-draining lymph node and that CD8+ T-cell priming is carried by DCs that reside in this
compartment [96]. Interestingly, authors did not find evidence supporting a role for skin-derived DCs for CD8+ T-cell priming [96].

There is a paucity of data about the events and immunological consequences that result from the interactions between skin macrophages and sporozoites. Nonetheless, the interplay between liver-resident macrophages (Kupffer cells, KC) and parasites has been better characterized [102-104]. Notably, one of the most recent studies in this area showed that sporozoite cell traversal allows parasites to migrate thorough cellular boundaries on their way to the liver parenchyma and avoid phagocytosis by KC [105].

1.6.2 Adaptive immune responses

1.6.2.1 Humoral responses

Antibodies against malaria sporozoites were first characterized using serum samples from mice vaccinated with irradiated sporozoites [58, 106]. At that time, the mechanisms conferring immune protection were not fully understood but early studies indicated that this was -at least in part- mediated by antibodies [107, 108]. As the field progressed and newer methodologies became available, the Circumsporozoite Protein (CSP) was identified as a major component of the surface membrane of the sporozoite and a potential target of protective antibodies [109-111]. The development of hybridoma technology then allowed the development of anti-CSP monoclonal antibodies, which were shown capable of inhibiting sporozoite infection in vitro and in vivo [112-114].

The basic structure of CSP consists of a central repeat region –specific to each Plasmodium species- flanked by an amino-terminal (N-terminal) portion that contains a conserved proteolytic processing site and a C-terminal flanking region that contains a type I thrombospondin repeat (TSR) motif [115, 116]. Early studies using monoclonal antibodies against CSP demonstrated that the majority were specific for the repeat domain [117]. More importantly, it was also shown that these anti-repeat CSP monoclonal antibodies could protect mice against infection with sporozoites. Further research demonstrated that immunization of mice with synthetic peptides representing the repeat domain of the P. berghei CSP induced antibody responses capable of conferring sterile protection [118, 119]. In
the case of the *P. falciparum* CSP, it was determined that antibodies that inhibited the *in vitro* invasion of sporozoites recognized a linear epitope defined by 3 consecutive repeats (NANP)3 [120]. More recent studies showed that anti-repeat CSP antibodies could provide protection *in vivo* against infection by transgenic *P. berghei* parasites expressing the repeat domain of the *P. falciparum* CSP [121]. A few studies have shown that antibodies targeting the N-terminal region of CSP could inhibit sporozoite infection *in vitro* [122-124]. Recently, our group demonstrated that antibodies against this region of the *P. falciparum* CSP are capable of neutralizing sporozoite infection *in vivo* (Chapter 3). The potentially inhibitory effect of antibodies against CSP’s C-terminus has yet to be demonstrated.

To date, although several different antigens have been identified in the malaria sporozoite, evidence on the protective capacity of antibodies against non-CSP proteins is still limited (reviewed in [125]). However, the expectation is that inducing antibody responses—as well as other arms of immunity—against an array of different antigens could confer stronger protection than responses directed only against a single target.

1.6.2.2 *Cellular responses*

In this sub-section I will summarize what is known about the protective effect of canonical CD4+ and CD8+ T-cells against sporozoite infection. However, it should be noted that other cellular populations and subsets are capable of exerting protective mechanisms against early stages of the malaria parasite. Some of these cells include gamma delta (γδ) T cells [126-128], Natural Killer T (NKT) cells [127, 129, 130] and NK cells [131]. Addressing the mechanisms of protection exerted by these cell types is beyond the scope of this work.

1.6.2.2.1 *CD4+ T-cell responses*

CD4+ T-cells can mediate protection against sporozoite infection through 3 different mechanisms, i) by providing help to B cells for the production of antibodies; ii) by providing help for the development of CD8+ T-cell responses; iii) and by directly exerting anti-parasitic effects. Notwithstanding, because of their tight interplay with other cell types and their requirement for the
activation of competent CD8+ T-cell and B-cell responses, the characterization of CD4+ T-cell effector mechanisms is a difficult task.

Early studies investigating the effector role of CD4+ T-cells demonstrated that the adoptive transfer of a CD4+ T-cell clone capable of recognizing an antigen shared by sporozoites and blood-stages of the P. berghei could confer sterile protection to naïve mice upon sporozoite challenge [132]. Further research showed that CD4+ T-cells play a major protective role in mice immunized with irradiated sporozoites [133]. More recently, it was shown that, in the absence of CD8+ T-cells, CD4+ T-cells can mediate protective immunity against sporozoite infection [134]. However, as authors of that study noted, protection could be attributed to both direct CD4+ T-cell effector mechanisms (directly killing infected cells or producing interferon-gamma (IFN-γ) that could inhibit intrahepatic parasites) and indirect mechanisms (activating macrophage to efficiently clear antibody-neutralized or opsonized parasites).

Studies in malaria-naïve human volunteers have shown that the RTS,S vaccine candidate is capable of eliciting CD4+ T-cell responses that are correlated with protection from sporozoite infection [135, 136]. However, such correlation is weak or non-existent when CD4+ T-cell responses are assessed in malaria endemic areas [137, 138].

1.6.2.2 CD8+ T-cell responses

There is a large body of literature documenting the protective effect of CD8+ T-cell responses against pre-erythrocytic stages of malaria. Certainly, the liver stage is the only opportunity the parasite is subject to recognition and elimination by cytotoxic CD8+ T cells.

Similarly to CD4+ T cells, the protective capacity of CD8+ T cells was first demonstrated in experiments in which the adoptive transfer of antigen-specific CD8+ T-cell clones into naïve mice conferred complete protection against sporozoite-induced infection [139, 140]. Previous studies had shown that mice immunized with irradiated sporozoites were rendered susceptible to infection when CD8+ T cells were depleted [141, 142]. Importantly, subsequent investigations found that, in the presence of CD8+ T cells, the depletion CD4+ T cells did not result in a loss of protective immunity,
indicating that both T-cell mediated mechanisms are likely to be redundant and that the depletion of both T-cell subsets has a much more pronounced effect in abolishing and decreasing protective immunity [133]. Besides irradiated sporozoites, further research showed that protective CD8+ T cells could be also induced by other forms of vaccination such as recombinant bacteria, viral vectors and genetically attenuated P. berghei sporozoites (reviewed in [143]). Notably, CSP was the antigen expressed by both the recombinant bacteria and viruses used in those studies, demonstrating that CD8+ T cells elicited against this protein can confer significant protection against sporozoite infection.

As the field progressed and new technologies became available, important questions about the immunobiology of CD8+ T cells against pre-erythrocytic stages of malaria were answered. A few key findings from recent years include:

- Priming of CD8+ T cells upon irradiated-sporozoite immunization requires IL-4 secreted by CD4+ T cells for the successful induction of effector populations [144].
- While the production of IFN-γ is an important mechanism by which CD8+ T cells exert their anti-parasite effects, IFN-γ-independent protective immunity has been described (reviewed in [143]).
- CD4+ T cells are critical for the expansion and survival of CD8+ T cells induced by irradiated-sporozoite immunization [145].
- CD8+ T-cell priming occurs in the skin draining lymph node, where sporozoites must migrate in order to induce robust responses [96, 98].

As opposed to the extensive amount of data supporting the protective role of CD8+ T-cells in murine models, limited evidence exists about their role in human malaria due to methodological and ethical constrains. A number of studies have reported an absence of significant CSP-specific CD8+ T-cell responses in individuals immunized with the RTS,S vaccine candidate (reviewed in [146]). Heterologous prime-boost immunization regimens using viral vectors have showed some potential for inducing CD8+ T-cells, but there is limited evidence about the protective efficacy of these responses (reviewed in [147]). Lastly, the most recent trial with the irradiated sporozoite vaccine approach
detected CD8+ T-cell responses in a 7 out 12 protected subjects, with the remaining 5 having low or non-detectable responses [68]. Importantly, these responses were only achievable through 4 or 5 doses of intravenous sporozoite injections but not when the parasites were delivered subcutaneously or intradermally [68, 148]. The suggestion has been made that the poor immunogenicity and infectivity of these parasites is due to their decreased viability.

1.7 Transgenic rodent parasites to evaluate malaria vaccine candidates

Rodent and non-human primate malaria parasites have been critical for answering fundamental questions in malaria biology and are instrumental models for the pre-clinical evaluation of vaccine candidates. Nevertheless, a basic limitation of murine malaria species is that rodent orthologues are antigenically distinct from those in Plasmodium species that cause disease in humans. This is a crucial weakness of rodent models as conclusions drawn from rodent malaria antigens, while conceptually important, are limited as they cannot account for significant differences in the antigenic structure of murine and human parasite antigens.

The generation of transgenic rodent parasites expressing human malaria antigens has recently emerged as a practical way to overcome the limitations of the traditional murine models (reviewed in [149]). During the last fifteen years, newly developed transgenic parasite strains have been used for the pre-clinical evaluation of vaccine candidates against P. falciparum and P. vivax malaria [121, 150-156]. Notably, these parasite strains have facilitated the systematic evaluation of different immunizing routes, antigen constructs, adjuvant formulations and immunization regimens.

Studies of the last several decades have demonstrated that antibodies and CD8+ T cells, and probably also CD4+ T cells, are important effectors of immune responses against pre-erythrocytic stages. While it is possible to generate protective immunity entirely based on antibodies alone or CD8+ T cells exclusively, the conditions to achieve sterile immunity in these conditions is extremely rigorous, as very large responses are required prior to infection by the parasite. It appears that a more realistic expectation is for vaccination to induce antibodies as well as T-cell mediated immunity, so that their
individual contributions can have an additive effect. This is reasonable to expect as antibodies and T cells act at different time points of the malaria pre-erythrocytic stage, antibodies neutralize sporozoite infection and T cells inhibit the development of intra-hepatocytic parasites. As none of these mechanisms is *per se* entirely sufficient, it is not possible to assign them an established protective value or preference. Instead, the logical approach is to consider the induction of both protective mechanisms as critical to achieve an efficient malaria vaccine.

1.8 Thesis objectives

While significant progress has been made towards eradicating malaria, current tools and treatments are not yet able to prevent the loss of thousands of young lives every year. The complexity and adaptability of the malaria parasite requires a combination of multiple approaches to defeat this affliction. Prevention methods, along with antimalarial drugs, have been undeniably useful in the reduction of malaria incidence and mortality rates over the past decade. Further, the significant increase in the proportion of suspected malaria cases receiving a diagnostic test has also contributed to the recent decline in malaria deaths [23]. Still, an efficient malaria vaccine is needed to overcome the shortcomings of the previous control efforts and finally eliminate the disease.

The main focus of this dissertation is to characterize protective immune responses against the CSP of human malaria parasites through the use of newly developed chimeric rodent parasites. The specific aims of this work and rationale are the following:

1. **To develop and characterize chimeric *P. berghei* strains expressing the N and C-terminal regions of the *P. falciparum* CSP as well as a chimeric line expressing the full-length *P. falciparum* CSP.**

   *Rationale:* Chimeric sporozoites expressing specific regions or the full-length *P. falciparum* CSP can be useful tools for assessing protective immune responses against this protein *in vivo.*
II. **To assess the protective effect of antibodies against the N-terminal region of the *P. falciparum* CSP.**

**Rationale:** A number of studies have reported that antibodies against the N-terminal region of the *P. falciparum* CSP can inhibit sporozoite invasion of *in vitro* cultured hepatocytes. Using a newly developed chimeric rodent parasite, I demonstrate that antibodies targeting this region can also inhibit sporozoite infection *in vivo.*

III. **To evaluate the protective efficacy and immune responses elicited by CSP-based formulations in combination with different adjuvant systems.**

**Rationale:** The newly generated chimeric rodent parasites can be used as platforms for the pre-clinical evaluation of newly developed CSP-based antigenic constructs as well to characterize novel vaccine adjuvants. In 3 independent studies, I will show the usefulness of chimeric rodent parasites to assess:

i. The immunogenicity and protective effect of recombinant *P. falciparum* CSP expressed in *E. coli* or in the yeast *P. pastoris* administered with poly(I:C)LC or GLA-SE as adjuvants.

ii. The immunogenicity and protective effect of virus-like particles (VLPs) carrying selected B- and T-cell epitopes from the *P. falciparum* and *P. vivax* CSPs administered in combination with a number of different adjuvant systems.

iii. The immunogenicity and protective effect of vaccinations with recombinant *P. falciparum* CSP in combination with a novel cationic liposomal adjuvant, which has been shown to induce robust CD8⁺ T-cell responses when administered with soluble antigens.
Figure 1.1. The *Plasmodium* life cycle. (A) Anopheline mosquito injects *Plasmodium* sporozoites into the host dermis during a blood meal. (B) Upon reaching a blood vessel, sporozoites travel to the liver where they invade hepatocytes. (C) After asexual replication and development inside the hepatocyte, thousands of merozoites are released into the bloodstream. (D) Merozoites infect red blood cells during cycles of asexual replication. (E) A proportion of asexual forms develop into female and male gametocytes. (F) Through another blood meal, a mosquito ingests gametocytes into its midgut. (G) Fertilization of gametes occurs in the mosquito midgut with the formation of ookinetes and later the oocysts. (H) Sporozoites released from the oocyst migrate to the salivary gland of the mosquito and are released during the next blood meal.

Figure adapted from Portugal S, et al. Superinfection in malaria: *Plasmodium* shows its iron will. EMBO Rep. 2011 Dec 1;12(12):1233-42. doi: 10.1038/embor.2011.213. Reproduced with permission from the publisher.
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<th>Diagnostic Approach</th>
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<td><strong>Microscopy</strong></td>
<td>- inexpensive to perform</td>
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<td>- allows quantifying parasitemia</td>
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<td>- can detect other blood-borne infections</td>
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<td><strong>Rapid Diagnostic Tests</strong> (RDTs)</td>
<td>- simple to perform and interpret</td>
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<td>- highly sensitive for <em>P. falciparum</em> detection</td>
<td>- variable performance depending on manufacturing conditions</td>
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<td>- no need for electrical power</td>
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<td><strong>Polymerase Chain Reaction (PCR)</strong></td>
<td>- extremely high sensitivity</td>
<td>- requires specialized equipment, reagents and personnel</td>
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<td>- very accurate speciation</td>
<td>- high cost</td>
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Table 1.1. Advantages and disadvantages of malaria diagnostic methods.
Figure 1.2. Global malaria vaccine pipeline.

1.9 References


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CHAPTER 2
DEVELOPMENT AND CHARACTERIZATION OF TRANSENIC RODENT MALARIA PARASITES EXPRESSING ANTIGENS OF THE PLASMODIUM FALCIPARUM CIRCUMSPOROZOITE PROTEIN
2.1 Abstract

Transgenic rodent parasites are useful tools for the pre-clinical evaluation of malaria vaccines. Over the last decade, several studies have reported the development and use of transgenic rodent parasites expressing *P. falciparum* antigens for the assessment of vaccine-induced immune responses, which traditionally have been limited to *in vitro* assays. However, it has been observed that the genetic manipulation of rodent *Plasmodium* species can have detrimental effects on the parasite’s infectivity and development. In this chapter, we describe the generation and characterization of new, fully-infective *P. berghei* chimeric strains expressing selected regions of the *P. falciparum* Circumsporozoite Protein (CSP) as well as the full-length CSP protein. These transgenic lines were developed using standardized transfection protocols and evaluated for their viability and infectivity throughout different stages of their life cycle. Importantly, all of the chimeric parasite strains infect and develop in mosquitoes at comparable efficiency to wild-type *P. berghei* ANKA parasites. In fact, exposing mice to the bites of 3 infected mosquitoes results in the development of patent blood-stage parasitemia, indicating that the transgenic lines produce highly infectious sporozoites and efficiently express *P. falciparum* antigens, as shown by reactivity of monoclonal antibodies against the *P. falciparum* CSP. Overall, the transgenic parasites we developed represent a robust model for evaluating protective immune responses against *P. falciparum* vaccines based on CSP.
2.2 Introduction

Despite significant advances in our understanding of the biology and immunogenicity of *Plasmodium* parasites, a safe and fully effective vaccine against malaria has not been developed.

Malaria vaccine development has traditionally targeted one of three stages of the *Plasmodium* parasite life cycle: i) pre-erythrocytic stages, from sporozoite inoculation through mosquito bites to hepatocyte infection; ii) asexual blood stages, from the release of merozoites within an infected hepatocyte to their continuous infection of red blood cells; iii) sexual stages, from the development of blood-stage gametocytes in the mammalian host to the development of sporozoites in the mosquito. Among these strategies, pre-erythrocytic vaccines are appealing as they have the potential to prevent infection and clinical disease, as well as block transmission.

The Circumsporozoite Protein (CSP), the most abundant protein on the surface of *Plasmodium* sporozoites, is one of the best-studied malaria antigens and a leading vaccine candidate. Studies in animal models and human volunteers have shown that antibodies and T-cell responses against CSP can inhibit sporozoite infection in mammalian hosts and provide protective immunity [1, 2]. Human vaccine trials using the CSP-based malaria vaccine candidate RTS,S—currently the most successful subunit malaria vaccine formulation—have shown that this formulation is capable of preventing the development of clinical malaria in 30-50% of vaccinees and to reduce the incidence of severe disease in children living in malaria endemic areas [3, 4]. Notwithstanding, it is apparent that RTS,S does not confer protection to a large proportion of vaccinees and its protective efficacy wanes significantly after 1-2 years [3-5]. These results may, to some extent, be explained by the over-reliance of RTS,S on a single antigen, CSP. And while CSP remains the most studied target of pre-erythrocytic malaria vaccines, additional antigens capable of eliciting a protective immune response have been identified [6]. It is expected that more complex subunit vaccines incorporating multiple antigens, including CSP, may greatly improve protective efficacy [7].

Rodent and non-human primate malaria parasites have been valuable for addressing basic questions in malaria biology and are useful models for the pre-clinical evaluation of vaccine candidates.
Nevertheless, a fundamental limitation of murine malaria species is that rodent orthologues (i.e. functionally similar genes) are antigenically distinct from human-infecting *Plasmodium* species. This is a critical drawback of rodent models as conclusions based on rodent malaria antigens, while valuable from a conceptual perspective are limited, as they cannot account for significant differences in the antigenic structure of murine and human parasite antigens. The development of transgenic rodent parasites expressing human malaria antigens has emerged as a practical solution to this problem (reviewed in [8]). Over the last few years newly developed transgenic parasite strains have been used for the pre-clinical evaluation of vaccine candidates against malaria pre-erythrocytic stages [9-12]. Importantly, these parasite strains have facilitated the systematic evaluation of different immunizing routes, antigen constructs, adjuvant formulations and immunization regimens. And while the optimization of transfection protocols for rodent *Plasmodium* species has significantly simplified genetic manipulation [13, 14], the substitution and/or deletion of essential genes or regulatory sequences can negatively impact the parasite’s infectivity and life cycle [10, 15, 16]. This is an important obstacle because transgenic parasite strains with decreased viability and virulence are not adequate tools to evaluate vaccine candidates.

In this chapter, I describe the development and characterization of novel rodent malaria parasites expressing different antigens of the *P. falciparum* CSP. Importantly, the methods here described have been applied by our group and others to assess the viability and infectivity of transgenic *P. berghei* parasites throughout their life cycle [9, 11, 12, 17]. Lastly, the careful selection of highly infectious strains is essential for the stringent evaluation of malaria vaccine candidates *in vivo*.

### 2.3 Materials and methods

#### 2.3.1 Development of transgenic *P. berghei* parasites

##### 2.3.1.1 *P. berghei* expressing the N-terminal region of the *P. falciparum* CSP

The transgenic *P. berghei* strain expressing the N-terminal region of the *P. falciparum* CSP was generated using the plasmid pR-CSPfNT, which carries the N-terminal region of the *P. falciparum* CSP.
This plasmid was derived from plasmid pIC-CSPfNT, which resulted from the replacement of the *P. berghei* CSP N-terminus with the N-terminal region of the 3D7 strain of *P. falciparum* CSP. Briefly, a 570-bp restriction fragment encompassing base pairs 65 to 634 of the *P. berghei* csp gene was excised from pIC-CSwt [18] using the restriction enzymes PflMI and EagI and then replaced with a fragment comprising the *P. falciparum* CSP N-terminal region. The *P. falciparum* CSP N-terminus was excised within a 579-bp PflMI-EagI restriction fragment from plasmid pPfNT (Genscript, Piscataway Township, NJ), synthesized to comprise the *P. falciparum* CSP N-terminal region flanked by a PflMI restriction site at its 5' end and 372 bp from the *P. berghei* CSP (base pairs 267 to 639) at its 3' end. Thus, the csp gene in the resulting plasmid, pIC-CSPPfNT, consists of the *P. berghei* CSP signal sequence (base pairs 1 to 69) followed by the N-terminal region of *P. falciparum* CSP (base pairs 70 to 285) and the remainder of the *P. berghei* CSP (base pairs 285 to 1032). We then excised the hybrid csp gene from pIC-CSPfNT as a KpnI-XhoI fragment and inserted it into the transfection plasmid, pR-CSPfNT. KpnI and SacI were used to release the inserted fragment from pR-CSPfNT prior to transfection of WT *P. berghei* (ANKA strain) parasites, as previously described [19].

Transgenic parasites were selected in Swiss Webster mice by treatment with pyrimethamine (MP Biomedicals, Solon, OH) in drinking water (0.07 mg/ml). Pyrimethamine-resistant parasites were then cloned by limiting-dilution. Successful recombination at the 5' and 3' ends of the locus was verified by PCR. The primers used to confirm 5' integration were 5'-F (TCACCCCTCAAGTTGGGTAAAA) and PbPfNT-R (TTATATAAATTAGTGCTGCAATTATCA); the primers to verify integration at the 3' end were 3'-F (TGTAATAATGTTATGGTTGTGC) and 3'-R (GTGCCATTACGACTTTGCT). To verify that the cloned parasite population did not have contaminating WT *P. berghei* parasites, we developed a PCR assay using primers which flank the replaced N-terminal CSP sequence and then digested the resulting product using AflIII. This restriction site is not present in the WT *P. berghei* CSP sequence but was inserted by replacement with our synthetic construct. The primers used for this PCR analysis were 5'-F and the reverse primer PbWT NT (ACAATCCACAACCACAGC). Lastly, DNA isolated from the
cloned chimeric parasites was sequenced to confirm the replacement of the \textit{P. berghei} N-terminal region with the \textit{P. falciparum} CSP N-terminus sequence.

2.3.1.2 \textit{P. berghei} expressing the C-terminal region of the \textit{P. falciparum} CSP

The transgenic \textit{P. berghei} strain expressing the C-terminal region of the \textit{P. falciparum} CSP was generated using the plasmid pR-CSPfCT, which carries the C-terminal region of the \textit{P. falciparum} CSP. This plasmid was derived from plasmid pIC-CSPfCT, which resulted from the replacement of the \textit{P. berghei} CSP C-terminus with the C-terminal region of the 3D7 strain of \textit{P. falciparum} CSP. Briefly, a 306-bp restriction fragment encompassing base pairs 715 to 1020 of the \textit{P. berghei} CSP gene was excised from a modified version of pIC-CSwt [18] using the restriction enzymes SexAI and PacI and then replaced with a fragment comprising the \textit{P. falciparum} CSP C-terminal region. The \textit{P. falciparum} CSP C-terminus was excised as a 312-bp SexAI-PacI restriction fragment from plasmid p PfCT (GenScript, Piscataway Township, NJ), synthesized to comprise the \textit{P. falciparum} CSP C-terminal region. Thus, the CSP gene in the resulting plasmid, pIC-CSPPfCT, consists of the \textit{P. berghei} N-terminal and repeat regions (base pairs 1 to 786) and the remainder of the \textit{P. falciparum} CSP (base pairs 787 to 1026). We then excised the hybrid CSP gene from pIC-CSPPfCT as a KpnI-XhoI fragment and inserted it into the transfection plasmid, pR-CSPfCT. KpnI and SacI were used to release the inserted fragment from pR-CSPfCT prior to transfection of WT \textit{P. berghei} (ANKA strain) parasites, as previously described [19]. Transgenic parasites were selected in Swiss Webster mice (NCI, Frederick, MD) by treatment with pyrimethamine (MP Biomedicals, Solon, OH) in drinking water (0.07 mg/ml). Pyrimethamine-resistant parasites were then cloned by limiting-dilution. Successful recombination at the 5' and 3' ends of the locus was verified by PCR. The primers used to confirm 5' integration were 5'-F (TCACCCTCAAGTTGGGTAAAA) and PbPfCT-R (GCAGAGCCAGGCTTTATTCT); the primers to verify integration at the 3' end were 3'-F (TGTAACAAAATGTGTATGTGTGTGTGC) and 3'-R (GTGCCCATATTACGACTTTTGC). To verify that the cloned parasite population did not have contaminating WT \textit{P. berghei} parasites, we developed a PCR assay using primers that flank the SexAI restriction site and then digested the resulting product with this enzyme. This restriction site is not present in the WT \textit{P. berghei} CSP sequence but was inserted by replacement
with our synthetic construct. The primers used for this PCR analysis were PbWT NT-F (TGTTACAATGAAGAATGATAATAATTGTATTGA) and Pb 3’UTR-R (TTTGGACATATTCATTTTAGA). Lastly, DNA isolated from the cloned chimeric parasites was sequenced to confirm the replacement of the *P. berghei* C-terminal region with the *P. falciparum* CSP C-terminus sequence.

2.3.1.3 *P. berghei* expressing the full-length *P. falciparum* CSP

We developed 2 transgenic lines expressing the full-length *P. falciparum* CSP. The first of these transgenic strains, termed P.b.-P.f. CSP-FL, was generated by excising a 784-bp restriction fragment from the *csp* gene of the previously described plasmid pIC-CSPPfNT [20]. This segment was then replaced with a 943-bp fragment obtained from plasmid pHZ-PfCSP upon digestion with BbsI and PacI. Therefore, the *csp* gene (1188 bp) in the resulting plasmid, pIC-CSPFULL, incorporates a full-length *P. falciparum* 3D7 CSP in which the signal sequence has been replaced with the one of the *P. berghei* CSP (base pairs 1 to 69). Then, we excised the *csp* gene from pIC-CSPFULL as a KpnI-XhoI fragment and inserted it into the transfection plasmid, pR-CSPFULL. Lastly, KpnI and SacI were used to linearize the recombinant fragment from pR-CSPFULL prior to transfection of *P. berghei* ANKA parasites as previously described [21].

P.b.-P.f. CSP-FL chimeric parasites were selected in Swiss Webster mice by treatment with pyrimethamine (MP Biomedicals, Solon, OH) in drinking water (0.07 mg/ml). Drug-resistant parasites were cloned by limiting-dilution. The successful recombination at the 5’ and 3’ ends of the modified locus was verified by PCR. Recombination of the transfected DNA fragment at the 5’ and 3’ ends from the modified locus was verified by PCR. The primers used to confirm 5’ integration were 5’-F (TCACCCTCAAGTTGGGTAAAA) and PbPfNT-R (TTATATAATAGTGCCTGCATTATCA); the primers to verify integration at the 3’ end were 3’-F (TGTTAAAATGTGTGTGTGTGC) and 3’-R (GTGCCATTACGACTTTTGCT). DNA isolated from chimeric parasites was sequenced to verify the sequence of the replaced CSP.

Similarly to the P.b.-P.f. CSP-FL transgenic line, an additional chimera, P.b.-P.f. CSP-FL CD8CT, was also developed by excising a 784-bp restriction fragment, encompassing base pairs 246
to 1029, from the plasmid pIC-CSPPfNT [20] using restriction enzymes BbsI and PacI (New England Biolabs, Ipswich, MA). This portion was then replaced with a 943-bp fragment, which was released using the same restriction enzymes from the plasmid pHZ-PfCSP. Thus, the csp gene (1188 bp) in the resulting plasmid, pIC-CSPlFL-CD8CT, consists of a full-length *P. falciparum* 3D7 CSP in which the signal sequence has been replaced with the one of the *P. berghei* CSP (base pairs 1 to 69). In addition, a single base-pair in the csp gene of plasmid pIC-CSPlFULL was replaced to incorporate a cytotoxic epitope that is not present in the *P. falciparum* 3D7 strain. This change was introduced in base pair 1079 by site-directed mutagenesis using a QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, La Jolla, CA). We then excised the csp gene from the resulting plasmid, termed pIC-CSPlFL-CD8CT, as a KpnI-PacI fragment and inserted it into the transfection plasmid, pR-CSPlFL-CD8CT. Lastly, XhoI and KasI were used to linearize pR-CSPlFL-CD8CT prior to transfection of GFP-Luciferase *P. berghei* ANKA parasites [22], as previously described.

P.b.-P.f. CSP-FL CD8CT transgenic parasites were selected as described above. The primers used to confirm 5' integration were PbCS5'-F (TGCCCTATTCTCATATTTACCAC) and hDHFR5'UTR-R (CACCATTTTTGAAAGATTAATTTGA); the primers to verify integration at the 3' end were PfCSP-F (TGAATGGTCCCCATGTAGTG) and PbCS3'UTR-R (CGCCTTATGTGCAATTGCT). Finally, DNA isolated from chimeric parasites was sequenced to verify the correctness of the replaced gene.

### 2.3.2 Biological characterization of transgenic *P. berghei* parasites

#### 2.3.2.1 Exflagellation of male gametocytes

Development of viable male gametocytes in blood was qualitatively assessed by means of an exflagellation assay. Briefly, 1.5 µl of blood from a mouse with patent blood parasitemia was incubated at room temperature for 20 min with 1.5 µl of heparin (40 U/ml in 1X phosphate-buffered saline [PBS]) and 7 µl of complete ookinete medium (RPMI 1640 medium with 10% fetal calf serum, 25 mM HEPES, 2 mM glutamine, 0.2 mM hypoxanthine, 12 mM sodium bicarbonate, 1 µM xanthurenic acid). After incubation, the mixture was placed on a slide and observed under a light microscope (X 100 magnification).
2.3.2.2 Assessment of midgut oocyst development

Development of oocysts in *Anopheles stephensi* female mosquito midguts was assessed 14 days post-blood meal. Midguts were dissected in sterile PBS and stained for 15 minutes in a 0.1% mercury chromate solution (Sigma, St. Louis, MO). The number of oocysts per midgut was then determined by counting under a light microscope (X 20 magnification).

2.3.2.3 Salivary gland infection rate and mosquito sporozoite yield

To assess the mosquito infection rate of the developed transgenic parasites, salivary glands of *An. stephensi* female mosquitoes were isolated 21 days after feeding them on infected mice. Salivary glands were carefully dissected, placed on a microscope slide and observed under a light microscope (X 40) for the presence of sporozoites.

To determine the average number of salivary gland sporozoites per mosquito, salivary glands were isolated and disrupted using a plastic pestle to release the parasites. The amount of sporozoites in solution was then determined by counting under a light microscope (X 40 magnification) and this value was then adjusted to the number of mosquitoes dissected.

2.3.2.4 Development of blood-stage forms upon infectious mosquito bites

The overall infective capacity of the newly generated transgenic lines was assessed by feeding infected *An. stephensi* mosquitoes on naïve mice to then determine if this results in blood-stage parasitemia. Briefly, 3 infected mosquitoes were allowed to feed on 5 to 8 weeks-old C57BL/6 female mice (Charles River, Frederick, MD) for 5 minutes. Four days later, daily blood smears were taken and observed under a microscope. Smears were fixed with methanol and then stained with a 10% Giemsa stain solution (Sigma, St. Louis, MO).

2.3.3 Immunofluorescence assay

An indirect immunofluorescence assay (IFA) was used to assess the reactivity of the newly developed transgenic rodent malaria parasites against monoclonal antibodies targeting specific regions of the *P. falciparum* CSP. In brief, 10 µl of a sporozoite suspension (4 x 10^5 to 6 x 10^5 sporozoites/ml) was air dried on poly-L-lysine-covered slides (Tekdon Inc., Myakka City, FL) and incubated for 30 min
at room temperature with 1 µg/ml of monoclonal antibody. Slides were then washed with PBS-1% bovine serum albumin (BSA) and incubated for 30 min at room temperature with a secondary-antibody solution [AlexaFluor 488 F(ab’)2 fragment of goat anti-mouse IgG(H+L); 2 mg/ml; Invitrogen]. Green-fluorescent sporozoites were visualized under an upright fluorescence microscope (Nikon Eclipse 90i).

2.4 Results

2.4.1 The *P. berghei*-*P. falciparum* CSP transgenic parasites develop normally throughout their entire life-cycle

We generated transgenic rodent malaria parasite by genetic transformation of *P. berghei* ANKA parasites using the Nucleofector technology for transfection [22]. In these transgenic strains, the wild-type (WT) *P. berghei* N-terminal region, the C-terminal region or the full-length CSP has been replaced with its *P. falciparum* counterpart. We did not modify the WT *P. berghei* CSP signal sequence (amino acids 1 to 23) in any of the newly developed chimeric lines. In the *P. berghei* transgenic strain expressing the N-terminal region of the *P. falciparum* CSP (P.b.-P.f. CSP NT), amino acids 24 to 92 of the *P. berghei* CSP have been replaced with residues 25 to 97 from the *P. falciparum* 3D7 CSP (Fig. 2.1). In the *P. berghei* chimera expressing the C-terminal region of the *P. falciparum* CSP (P.b.-P.f. CSP CT), amino acids 263 to 340 of the *P. berghei* CSP have been replaced with residues 318 to 397 from the *P. falciparum* 3D7 CSP (Fig. 2.2). Lastly, we generated 2 nearly-identical chimeras expressing the full-length *P. falciparum* CSP. In the first of these full-length CSP chimeras (P.b.-P.f. CSP-FL), amino acids 24 to 340 of the *P. berghei* CSP have been replaced with residues 25 to 397 from the *P. falciparum* 3D7 CSP (Fig. 2.3). In the second full-length CSP chimera (P.b.-P.f. CSP-FL CD8CT), the replaced fragment spans the same amino acids as in P.b.-P.f. CSP-FL. However, in P.b.-P.f. CSP-FL CD8CT, the alanine residue (A) at position 361 of the *P. falciparum* 3D7 CSP has been replaced by a glutamic acid residue (E) to create a cytotoxic epitope in the CSP’s C-terminal region (Fig. 2.4). The correctness of all the replaced DNA sequences was verified by genomic sequencing of the transgenic lines.
We characterized all of the developed transgenic strains for their ability to produce male gametocytes that exflagellate normally, develop in *An. stephensi* mosquitoes and result in blood-stage parasitemia upon sporozoite infection delivered by mosquito bites. Successful exflagellation of male gametocytes was verified in all of the generated chimeric lines. Further, all of the chimeric parasite strains infect and develop in mosquitoes at comparable efficiency to WT *P. berghei* ANKA parasites. In addition, all mice developed blood stage parasites by 5 days after feeding 3 infected mosquitoes on them, as observed in with mice infected with WT *P. berghei* (Table 2.1).

2.4.2 Monoclonal antibodies against the *P. falciparum* CSP recognize the transgenic *P. berghei*-*P. falciparum* CSP parasites

To verify that the sporozoites of the developed transgenic strains display the replaced CSP sequences, we performed a series of IFAs using monoclonal antibodies specific against different regions of the *P. falciparum* CSP. For these assays, we used the anti-*P. falciparum* CSP N-terminus antibody mAb5D5 [20], the anti-*P. falciparum* CSP repeat region antibody mAb2A10 [23] and the anti- *P. falciparum* CSP C-terminus antibody mAb1E9 (kindly provided by Dr. Carole Long). As expected, mAb5D5 specifically recognized the P.b.-P.f. CSP NT chimera, mAb1E9 bound the P.b.-P.f. CSP CT chimera, and all monoclonal antibodies recognized the 2 full-length *P. falciparum* CSP replacement transgenic lines (Fig. 2.5).

2.5 Discussion

For many years, rodent malaria parasites have been useful tools to evaluate and characterize immune responses against *Plasmodium*. Indeed, rodent malaria models allow the assessment of *in vivo* immune responses, facilitate the evaluation of cellular immunity in the liver and secondary lymphoid organs, enable the systematic study of multiple immunological parameters and, since they cannot infect humans, do not represent a biohazard. Nevertheless, while the conclusions reached with these platforms are conceptually valuable, rodent malaria parasites are different from those that infect humans and cannot account for differences in the antigenic structure between murine and human
Plasmodium species. Over the last decade, transgenic rodent malaria parasites have emerged as a practical alternative to overcome the limitations of traditional rodent malaria models. Importantly, these transgenic parasites incorporate the advantages of the rodent platform but also open the possibility of evaluating in mice malaria vaccine constructs which can be then tested directly in humans. Thus, these chimeric lines enable the pre-clinical evaluation of several antigens, adjuvants, immunization schedules and regimens, which means that multiple trials can be done in a relatively short period of time to down-select promising vaccine candidates for future studies.

We generated 4 transgenic P. berghei strains expressing select regions or the entire P. falciparum CSP, which remains to date the main focus of several pre-erythrocytic malaria vaccine trials. All of the developed chimeras produce viable gametocytes and successfully infect An. stephensi mosquitoes, in which their development is comparable to that of WT P. berghei. Further, feeding infected mosquitoes on naïve C57BL/6 mice results in the development of patent blood-stage parasitemia. This is an important feature of the chimeric parasites we have developed, as vaccine efficacy should be evaluated using a natural route of sporozoite inoculation, which allows the participation of all components of the immune response in protection. Importantly, a previously developed chimeric line, similar to our full-length P. falciparum CSP strains, was impaired in its capacity to develop in mosquitoes and is defective at infecting mice [10]. And while further studies aimed at improving the infectivity of these parasites, they failed to achieve consistent sporozoite infections when mice were challenged by mosquito bites [24].

The fact that transgenic parasites are recognized by monoclonal antibodies according to their CSP epitope specificity demonstrates that these chimeric sporozoites adequately express the introduced P. falciparum CSP sequences. We reason that the availability of chimeric sporozoites expressing different CSP regions should facilitate the evaluation of immune responses targeting specific domains of this protein. Certainly, these transgenic parasites can be used to identify regions of CSP that are preferentially targeted by vaccine-induced T cells and/or antibodies and to define new antigenic determinants recognized by protective responses specific for the N- and C-terminal domains.
The following chapters of this dissertation elaborate on the applicability of the developed transgenic parasites for the identification of new targets of protective responses and how they can be used for the pre-clinical evaluation of novel malaria vaccine candidates. However, in a number of experiments we take advantage of a previously developed transgenic *P. berghei* strain expressing the repeat domain of the *P. falciparum* CSP [25]. And while this chimera remains a useful tool for assessing humoral responses against CSP’s repeat region, full-length *P. falciparum* CSP transgenic strains might represent a better alternative for evaluating broader vaccine-induced protective mechanisms targeting multiple CSP domains.
Figure 2.1 Development of chimeric P.b.-P.f. CSP-NT parasites. (A) Scheme representing the strategy used for replacing the CSP gene of *P. berghei* (ANKA) with a hybrid CSP containing the *P. falciparum* (3D7) N-terminal region. The annealing sites of the primers used to verify recombination by PCR are indicated below. Restriction sites shown are K – KpnI; P-PfMI; A-AfIII; E-EagI; Xh – XhoI; S – SacI. (B) To verify the successful cloning of the chimeric strain, a 1400 bp PCR fragment was amplified from the CSP gene using primers 5’F and PbWT NT and then digested with AfIII, which does not digest the WT CSP gene. Genomic DNA from *P. berghei* (ANKA) was used as control. (C) Using genomic DNA from P.b.-P.f. CSP-NT parasites, 5’ and 3’ integration into the CSP genomic locus was verified by PCR. Primers 5’F and PbPf NT-R yield an 850 bp product; primers 3’F and 3’R yield a 1000 bp product. (D) Amino acid sequence of CSP in the P.b.-P.f. CSP-NT parasite. *P. berghei* amino acids are depicted in gray text while the *P. falciparum* residues are represented in black text.
Figure 2.2 Development of chimeric P.b.-P.f. CSP-CT parasites. (A) Scheme representing the strategy used for replacing the CSP gene of *P. berghei* (ANKA) with a hybrid CSP containing the *P. falciparum* (3D7) C-terminal region. The annealing sites of the primers used to verify recombination by PCR are indicated below. Restriction sites shown are K – KpnI; P – PacI; Se – SexAI; Xh – XhoI; S – SacI. (B) To verify the successful cloning of the chimeric strain, a 1400 bp PCR fragment was amplified from the CSP gene using primers PbWT NT-F and Pb 3’UTR-R and then digested with SexAI, which does not digest the WT CSP gene. Genomic DNA from *P. berghei* (ANKA) was used as control. (C) Using genomic DNA from P.b.-P.f. CSP-CT parasites, 5’ and 3’ integration into the CSP genomic locus was verified by PCR. Primers 5’F and PbPf CT-R yield an 1625 bp product; primers 3’F and 3’R yield a 1000 bp product. (D) Amino acid sequence of CSP in the P.b.-P.f. CSP-CT parasite. *P. berghei* amino acids are depicted in gray text while the *P. falciparum* residues are represented in black text.
Figure 2.3 Development of chimeric P.b.-P.f. CSP-FL parasites. (A) Scheme representing the strategy for replacing the CSP gene of P. berghei (ANKA) with a hybrid P. falciparum (3D7) CSP incorporating the P. berghei signal sequence. The annealing sites of the PCR primers used to verify recombination at the CSP locus are indicated below. Restriction sites shown are B – BbsI; K – KpnI; P – PciI; S – SacI; X – XhoI. (B) Using genomic DNA form P.b.-P.f. CSP-FL parasites, 5’ and 3’ integration at the CSP locus was verified by PCR. Primers 5’-F and PbPf NT-R yield an 850 bp product; primers 3’-F and 3’-R yield a 1000 bp product. (C) Amino acid sequence of CSP in the P.b.-P.f. CSP-FL parasite. The P. berghei signal sequence is depicted in green text while the P. falciparum residues are represented in red text. For reference, the amino acid that was replaced in the additionally developed chimera (P.b.-P.f. CSP-FL CD8CT) is underlined.
Figure 2.4. Development of chimeric P.b.-P.f. CSP-FL CD8CT parasites. (A) Scheme representing the strategy for replacing the CSP gene of GFP-Luciferase *P. berghei* (ANKA) with the *P. falciparum* (3D7) CSP incorporating the *P. berghei* signal sequence. The star symbol (★) represents the location of the introduced nucleotide change to generate the CD8+ T-cell epitope DYENDIEKKI. The annealing sites of the PCR primers used to verify recombination at the CSP locus are indicated below. Restriction sites shown are B – BbsI; K – KpnI; Ka – KasI; P – PacI; X – XhoI. (B) Using genomic DNA form P.b.-P.f. CSP-FL CD8CT parasites, 5’ and 3’ integration at the CSP locus was verified by PCR. Primers PbCS5’-F and hDHFR5’UTR-R yield a 1000 bp product; primers PfCSP-F and PbCS3’UTR-R yield a 2100 bp product. (C) Amino acid sequence of CSP in the P.b.-P.f. CSP-FL CD8CT parasite. The *P. berghei* signal sequence is depicted in green text while the *P. falciparum* residues are represented in blue text. The amino acid replacement (Ala (A) to Glu (E)) introduced to generate the cytotoxic epitope is underlined.
Table 2.1 Developmental characteristics and infectivity of *P. berghei*-P. *falciparum* CSP transgenic parasites in *A. stephensi* mosquitoes

<table>
<thead>
<tr>
<th>Parasite</th>
<th>% Midgut infected</th>
<th>Oocysts/midgut</th>
<th>% Salivary gland infected</th>
<th>Sporozoites/Salivary gland (x 10³)</th>
<th>% of infected mice after mosquito bites(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. b.-P. f. CSP NT</td>
<td>78.64</td>
<td>67.11</td>
<td>80</td>
<td>10</td>
<td>100%</td>
</tr>
<tr>
<td>P. b.-P. f. CSP CT</td>
<td>80.72</td>
<td>70.76</td>
<td>85</td>
<td>11</td>
<td>100%</td>
</tr>
<tr>
<td>P. b.-P. f. CSP-FL</td>
<td>70.58</td>
<td>83.25</td>
<td>85</td>
<td>10</td>
<td>100%</td>
</tr>
<tr>
<td>P. b.-P. f. CSP-FL CD8CT</td>
<td>87.50</td>
<td>60.33</td>
<td>70</td>
<td>9</td>
<td>100%</td>
</tr>
<tr>
<td><em>P. berghei</em> ANKA</td>
<td>75.14</td>
<td>62.25</td>
<td>66</td>
<td>10</td>
<td>100%</td>
</tr>
</tbody>
</table>

\(^a\) Mean of 3 experiments, with at least 20 mosquitoes examined in each experiment.

\(^b\) Three infected mosquitoes were allowed to feed on C57BL/6 mice (n=5) for 3 minutes. Blood stage parasitemia was assessed 5 days later.
Figure 2.5 Recognition of air-dried transgenic sporozoites by monoclonal antibodies against the *P. falciparum* CSP. Chimeric sporozoites were stained with 1 µg/mL of monoclonal antibodies against the N-terminus (mAb5D5), repeat region (mAb2A10) or C-terminus (mAb1E9) of the *P. falciparum* CSP. No cross-reactivity was observed against wild-type *P. berghei* sporozoites. Abbreviations: CSP, circumsporozoite protein; mAb5D5, monoclonal antibody 5D5; mAb2A10, monoclonal antibody 2A10; mAb1E9, monoclonal antibody 1E9; P.b.-P.f. CSP-FL, *P. berghei*–*P. falciparum* CSP full-length; P.b.-P.f. CSP-NT, *P. berghei*–*P. falciparum* CSP N-terminus; P.b.-P.f. CSP-CT, *P. berghei*–*P. falciparum* CSP C-terminus.
2.6 References


CHAPTER 3
PROTEOLYTIC CLEAVAGE OF THE P. FALCIPARUM CIRCUMSPOROZOITE PROTEIN IS A TARGET OF PROTECTIVE ANTIBODIES

The following Chapter has been previously published in its entirety in
The Journal of Infectious Diseases:
3.1 Abstract

Studies in animals and human volunteers demonstrate that antibodies against the repeat-region of the *Plasmodium* Circumsporozoite Protein (CSP) abrogate sporozoite infection. However, the realization that the N- and C- terminal regions flanking the repeats play essential roles in parasite infectivity raised the possibility that they could be targeted by protective antibodies. We characterized a monoclonal antibody (mAb5D5) specific for the N-terminus of the *P. falciparum* CSP which inhibits the proteolytic cleavage of the CSP, a key requirement for parasite infection of hepatocytes. Adoptive transfer of mAb5D5 strongly inhibits the *in vivo* infection of sporozoites expressing the N-terminus of *P. falciparum* CSP, and this protection is greatly enhanced when combined with anti-repeat antibodies. Our results show that antibodies interfering with molecular processes required for parasite infectivity can exert a strong *in vivo* protective activity and indicate that pre-erythrocytic vaccines against *Plasmodium* should include the CSP N-terminal region.
3.2 Introduction

The Circumsporozoite Protein (CSP) is the major surface protein of Plasmodium sporozoites, forming a dense coat on the parasite’s surface. This protein plays a critical role in the journey of Plasmodium parasites from the mosquito to the mammalian host and it is expressed in sporozoites of rodent, primate and human Plasmodium species. The basic structure of the CSP consists of a central repeat domain flanked by an N-terminal portion containing a conserved proteolytic cleavage site and a C-terminal flanking region containing a type I thrombospondin repeat (TSR) motif. As the parasites migrate from the mosquito salivary glands to the liver of the mammalian host, the CSP undergoes major conformational changes \[1, 2\]. Sporozoites injected into the skin display a folded CSP, such that the N-terminal region masks the C-terminal TSR. Upon reaching the liver, this protein undergoes proteolytic cleavage of the N-terminal domain, exposing the C-terminus region, in what appears to be a critical requirement for hepatocyte invasion by sporozoites \[1, 2\].

CSP has been intensively studied as a vaccine candidate, as it is well established that antibodies and T-cell responses against this antigen inhibit infection in mammalian hosts and provide protective immunity in experimental animal models and humans \[3, 4\]. The malaria vaccine candidate RTS,S is a virus-like particle (VLP) consisting of the central repeat and C-terminal regions of the \(P. falciparum\) CSP fused to a Hepatitis B Virus Surface Antigen (HBVsAg). A number of phase 2 and 3 vaccine trials showed that 30-50% of children were protected after vaccination \[5-9\]. These studies also indicated that anti-CSP antibody titers specific for the repeat domain strongly correlate with the protection observed among vaccinees. The protective efficacy of the RTS,S vaccine candidate is encouraging and suggests that further improvements of this subunit vaccine may provide increased protection. Importantly, the RTS,S vaccine does not include the CSP N-terminal region, which contains important functional domains such as a ligand-binding domain \[10\] and a proteolytic processing site \[2\], and may be an important target for protective antibodies.

Here we describe the characterization of a monoclonal antibody, mAb5D5, which binds to a linear epitope located in the N-terminal region the \(P. falciparum\) CSP and prevents its enzymatic
processing. Using *P. berghei* transgenic parasite strains expressing a chimeric CSP which contains the N-terminal region of *P. falciparum* [11], we demonstrate that mAb5D5 strongly inhibits sporozoite infection *in vivo*. Moreover, we show that combining mAb5D5 with antibodies against the repeat domain of the *P. falciparum* CSP greatly enhances the *in vivo* neutralization of sporozoites.

Taken together, these data demonstrate that the CSP N-terminus represents a promising target for antibody-based protection against parasite infection of hepatocytes, and indicates that this CSP region should be included as part of a CSP-based, pre-erythrocytic vaccine construct.

3.3 Materials and methods

3.3.1 Antibody development

Antibodies were prepared by Precision Antibody, a division of A&G Pharmaceutical Inc. (Columbia, MD) using proprietary technology and mouse immunization protocols. Mice were immunized with full-length recombinant CSP (rCSP) [12] and serum titers determined by ELISA. Splenocytes were harvested and fused once titers exceeded 1:50,000. Hybridoma supernatants were screened for reactivity to rCSP by ELISA. A total of 14 clones were selected for mAb production and IgG was purified using a Protein G column followed by a buffer change to PBS.

3.3.2 Epitope specificity analysis

The fine epitope specificity of mAb5D5 was determined using two types of ELISAs.

i) Direct binding to short *P. falciparum* CSP peptides. An ELISA with CSP 15-mer peptides overlapping by 11 amino acids (Genscript, Piscataway Township, NJ), spanning the entire length of the protein (*P. falciparum*, 3D7 strain), was used to determine the epitope specificity. Briefly, MaxiSorp® ELISA plates (Thermo Scientific Nunc, Rochester, NY) were coated with 100 microliters of a synthetic CSP peptides (1 µg/ml) and incubated overnight at room temperature. The wells were then washed and incubated with phosphate buffered saline (PBS) and 1% bovine serum albumin (BSA) (Sigma-Aldrich, Saint Louis, MO) (PBS-1% BSA) for one hour at room temperature. Then, the wells were washed and incubated for one hour at room temperature with 250 ng/ml of mAb5D5 in PBS-1% BSA.
After another washing step, the plate was incubated for one hour at room temperature with a peroxidase-labeled goat anti-mouse (IgG H+L) secondary antibody (KPL, Gaithersburg, MD) at 0.5 µg/ml in PBS-1% BSA. The assay was developed using a horseradish peroxidase substrate kit (KPL, Gaithersburg, MD), according to the manufacturer’s specifications.

ii) Competitive ELISA by short synthetic CSP peptides against *P. falciparum* recombinant CSP.

To further corroborate the mAb5D5 epitope specificity, we sought to inhibit antibody binding to rCSP [12] using short synthetic peptides. In brief, ELISA plates coated with 100 microliters of rCSP (200 ng/ml) were incubated overnight at room temperature. Then, the plates were incubated with PBS-1% BSA for an hour at room temperature. mAb5D5 (15 ng/ml) was incubated for 2 hours at 37°C with different concentrations of selected CSP15-mer peptides in a total volume of 100 microliters of PBS-1% BSA. Then, the antibody-peptide mixtures were transferred to the CSP-coated ELISA plate and incubated for one hour at room temperature. The plated was then washed and incubated with the peroxidase-labeled secondary antibody and developed as described above.

3.3.3 Transgenic parasites

*P. berghei* NK65 strain expressing the repeat domain and a portion of the N-terminal region of *P. falciparum* CSP [11], P.b.-P.f. CSP–R, was kindly provided by Dr. Elizabeth Nardin (New York University). A new transgenic strain, derived from *P. berghei* ANKA strain expressing the N-terminus of *P. falciparum*, was generated using the plasmid pR-CSPfNT, which carries the N-terminal region of the *P. falciparum* CSP. This plasmid was derived from plasmid pIC-CSPfNT, which resulted from the replacement of the *P. berghei* CSP N-terminus with the N-terminal region of the 3D7 strain of *P. falciparum* CSP. Briefly, a 570-bp restriction fragment encompassing base pairs 65 to 634 of the *P. berghei* csp gene was excised from pIC-CSwt [13] using the restriction enzymes PflMI and EagI and then replaced with a fragment comprising the *P. falciparum* CSP N-terminal region. The *P. falciparum* CSP N-terminus was excised within a 579-bp PflMI-EagI restriction fragment from plasmid pPfNT (Genscript, Piscataway Township, NJ), synthesized to comprise the *P. falciparum* CSP N-terminal region flanked by a PflMI restriction site at its 5’ end and 372 bp from the *P. berghei* CSP (base pairs 267 to
639) at its 3' end. Thus, the csp gene in the resulting plasmid, pIC-CSPPfNT, consists of the *P. berghei* CSP signal sequence (base pairs 1 to 69) followed by the N-terminal region of *P. falciparum* CSP (base pairs 70 to 285) and the remainder of the *P. berghei* CSP (base pairs 285 to 1032). We then excised the hybrid csp gene from pIC-CSPPfNT as a KpnI-XhoI fragment and inserted it into the transfection plasmid, pR-CSPPfNT. KpnI and SacI were used to release the inserted fragment from pR-CSPPfNT prior to transfection of WT *P. berghei* (ANKA strain) parasites, as previously described [14].

Transgenic parasites were selected in Swiss Webster mice by treatment with pyrimethamine (MP Biomedicals, Solon, OH) in drinking water (0.07 mg/ml). Pyrimethamine-resistant parasites were then cloned by limiting-dilution. Successful recombination at the 5' and 3' ends of the locus was verified by PCR. The primers used to confirm 5' integration were 5'-F (TCACCTCTAACTGTTGGGTAAAA) and PbPfNT-R (TTATATAATTAGTCCTGCATTATCA); the primers to verify integration at the 3' end were 3'-F (TGTAATAATGTGTGTTGTGTGTGC) and 3'-R (GTCGCCATTACGACTTTGCT). To verify that the cloned parasite population did not have contaminating WT *P. berghei* parasites, we developed a PCR assay using primers which flank the replaced N-terminal CSP sequence and then digested the resulting product using AflIII. This restriction site is not present in the WT *P. berghei* CSP sequence but was inserted by replacement with our synthetic construct. The primers used for this PCR analysis were 5'-F and the reverse primer PbWT NT (ACAATCCACACACCACACGC). Lastly, DNA isolated from the cloned chimeric parasites was sequenced to confirm the replacement of the *P. berghei* N-terminal region with the *P. falciparum* CSP N-terminus sequence.

**3.3.4 Mosquito infection, parasite development and sporozoite infectivity of the *P. berghei*-*P. falciparum* CSP N-terminus**

*Anopheles stephensi* female mosquitoes were fed on Swiss Webster mice infected with blood stages of cloned *P. berghei*-*P. falciparum* CSP N-terminus (P.b.-P.f. CSP-NT) parasites. The development of viable male gametocytes in blood was qualitatively determined by means of an exflagellation assay as described previously [15] and performed prior to mosquito feeding. The development of oocysts in mosquito
midguts was assessed 14 days post-blood meal. The midguts were dissected in sterile PBS and stained in a 0.1% mercury chromate solution.

The in vivo infectivity of chimeric P.b.-P.f. CSP-NT sporozoites was assessed in C57BL/6 mice. The liver parasite load was measured by reverse transcription followed by quantitative real-time PCR (RT-qPCR) upon intravenous challenge of mice with $10^4$ chimeric sporozoites [16]. To evaluate the infectivity of P.b.-P.f. CSP-NT sporozoites by mosquito bite, 3 infected *A. stephensi* mosquitoes were allowed to feed on mice for 3 minutes. The development of blood-stage parasites was then determined by performing daily blood smears.

### 3.3.5 Immunofluorescence assay

An indirect immunofluorescence assay (IFA) was used to characterize the reactivity of mAb5D5 against both live and air-dried P.b.-P.f. CSP-NT sporozoites. In brief, for live-sporozoite IFAs, $4 \times 10^4$ parasites were incubated on ice with different concentrations of mAb5D5. Sporozoites were then washed 3 times with cold PBS-1% BSA, resuspended in 0.2 ml and placed into the well of the Lab-Tek chamber (Thermo Scientific Nunc, Rochester, NY). The chamber was then spun at 300 g for 2 min and, after discarding the supernatant, 0.2 ml of PBS-4% paraformaldehyde (Sigma, Saint Louis, MO) were added. Samples were incubated for 1 hour at room temperature, washed 3 times with PBS and incubated with secondary antibody for 30 min. Samples were then washed and green-fluorescent sporozoites were visualized using a Nikon Eclipse 90i fluorescent microscope. IFAs using air-dried sporozoites were performed as previously described [15]. *P. falciparum* (3D7) sporozoites were obtained from *A. stephensi* -mosquitoes infected with in vitro culture gametocytes of *P. falciparum*. IFA was performed as described in the preceding items.

### 3.3.6 Pulse-Chase Metabolic Labeling Experiments

Pulse-Chase metabolic labeling experiments were performed as previously described [2]. Briefly, freshly dissected *P. falciparum* sporozoites were metabolically labeled in DMEM without Cys/Met, 1% BSA and 400 µCi/ml L-[35S]-Cys/Met for 1 hour at 28°C and then kept on ice or chased at 28°C for 2 hours in DMEM with Cys/Met and 1% BSA in the presence of the indicated
concentrations of mAb5D5, in the presence of the isotype control mAb3D11 (specific against the *P. berghei* CSP repeat region [17]) or in the absence of antisera. Chased sporozoites were lysed and labeled CSP was immunoprecipitated with mAb2A10 (specific against the *P. falciparum* CSP repeat region [18]) conjugated to sepharose, eluted from the beads and analyzed by SDS-PAGE followed by autoradiography.

### 3.3.7 Inhibition of sporozoite infectivity by passive transfer of monoclonal antibodies

Monoclonal antibodies were passively transferred into C57BL/6 mice by i.v. injection. Immediately after antibody transfer, mice were challenged with $10^4$ sporozoites, delivered by tail vein injection into each mouse. Forty-two hours after challenge, livers were harvested to assess the parasite burden by RT-qPCR. In addition to the newly developed chimeric P.b.-P.f. CSP-NT strain, these experiments were performed using another *P. berghei*-P. *falciparum* chimera which also contains the amino acid sequence recognized by mAb5D5. This chimera, “P.b.-P.f. CSP–R”, expresses the repeat region and 23 residues upstream of the repeats of the *P. falciparum* 7G8 strain [11].

### 3.3.8 Data analysis

Data were plotted using Graph Pad Prism 4 software. Unless otherwise stated, data were compared for significance using a Mann-Whitney test.

### 3.3.9 Study approval

Animal studies were conducted at The Johns Hopkins University. Five- to 8-week old female C57BL/6 or Swiss Webster mice were purchased from NCI (Frederick, Maryland). All experimental procedures involving mice were approved by the Institutional Animal Care and Use Committee of The Johns Hopkins University.

### 3.4 Results

#### 3.4.1 mAb5D5 targets an epitope in the N-terminal region of the *P. falciparum* CSP

A series of monoclonal antibodies were isolated after immunization of mice with a full-length *P. falciparum* recombinant CPS (rCSP) [12]. Early experiments indicated that these antibodies bind to rCSP, as
assessed by ELISA and western blot analysis (data not shown). In initial studies, long synthetic peptides representing the N-terminal, repeat or C-terminal regions of the *P. falciparum* CSP were used to determine the epitope specificity of selected anti-CSP monoclonal antibodies (Supplementary Fig. 3.1). One of these antibodies, mAb5D5, recognized the peptide representing the N-terminal region of *P. falciparum* 3D7 CSP, residues 26 to 99. The fine epitope specificity of mAb5D5 was then defined by ELISA using immobilized 15-mer overlapping synthetic peptides representing residues 25 to 97 of the *P. falciparum* 3D7 CSP. Using this assay, we found that mAb5D5 bound to peptides $^{77}$DGNNEDNEKLRPKH$^{90}$ and $^{81}$EDNEKLRPKHKKSLK$^{95}$, which represent a region spanning amino acids 81 to 90 in the *P. falciparum* 3D7 CSP (Fig. 3.1A). Further confirmation of this epitope specificity, was obtained from assays in which these synthetic peptides were used in soluble form, to inhibit the binding of mAb5D5 to immobilized rCSP. Consistent with the ELISA results using immobilized peptides, we found that peptides $^{77}$DGNNEDNEKLRPKH$^{90}$ and $^{81}$EDNEKLRPKHKKSLK$^{95}$ inhibited the binding of mAb5D5 to rCSP. No such effect was observed with the peptides flanking these peptides, namely $^{77}$GENDDGNNEDNEKLR$^{87}$ and $^{85}$KLRPKHKKSLKQPAD$^{99}$ (Fig. 3.1B). Taken together, these results indicate that the core sequence recognized by mAb5D5 in CSP is an epitope defined by the 11 amino acid peptide $^{81}$EDNEKLRPKH$^{91}$ of the *P. falciparum* 3D7 CSP.

### 3.4.2 mAb5D5 recognizes the *P. falciparum* CSP expressed in live sporozoites and inhibits CSP processing

To determine whether this antibody recognized native CSP as expressed in sporozoites, we performed indirect immunofluorescence assays (IFAs) using *P. falciparum* sporozoites. The results of these assays using air-dried fixed and live sporozoites clearly indicated that mAb5D5 binds to the native *P. falciparum* CSP and, most important, that this epitope is exposed in live parasites (Fig. 3.2).

Given that the binding of mAb5D5 site lies in close proximity to Region I, which contains the CSP proteolytic processing site, we performed pulse-chase metabolic labeling experiments to determine if this antibody could inhibit processing of CSP by *P. falciparum* sporozoites. These sporozoites were metabolically labeled using 35S-Cysteine/Methionine and then chased in the presence
of mAb5D5 or an isotype control antibody. We found that 100 µg/ml and 50 µg/ml of mAb5D5 had a strong inhibitory effect on CSP processing (Fig. 3.3). These results further highlight mAb5D5's fine epitope-specificity and indicate that antibody binding to, or close to, the proteolytic cleavage site sterically inhibit CSP processing.

3.4.3 The 5D5 antibody strongly inhibits infection of hepatocytes

Since the regulated cleavage of CSP is an important step for efficient hepatocyte invasion by sporozoites, we next investigated whether mAb5D5 had an inhibitory effect on sporozoite infectivity in vivo. For this purpose, we performed infectivity assays using *P. berghei* parasites expressing a chimeric CSP in which the N-terminal region of the *P. falciparum* replaced the native *P. berghei* N-terminal sequence, P.b.-P.f. CSP-NT (Supplementary Fig. 3.2A-3.2C). We did not modify the *P. berghei* CSP's signal sequence (amino acids 1 to 23), thus in the chimeric CSP amino acids 24 to 92 of the *P. berghei* CSP have been replaced with residues 25 to 97 from the *P. falciparum* 3D7 CSP (Fig. 3.1C). The transgenic parasites develop normally in mosquitoes and efficiently infect naïve C57BL/6 mice after intravenous injection of sporozoites (Supplementary Fig. 3.2D). In addition, all mice developed blood-stage parasites by 5 days after feeding 3 infected mosquitoes on them, as also observed with mice infected with WT *P. berghei* (Supplementary Table 3.1). Furthermore, mAb5D5 (1 µg/ml) recognized live chimeric P.b.-P.f. CSP-NT sporozoites by IFA, thus confirming its epitope specificity. A weak cross-reactivity, possibly due to low sequence homology, was only observed with WT *P. berghei* parasites when using high concentrations of mAb5D5 (100 µg/ml) (data not shown). These results further corroborate the specificity of mAb5D5 and the recognition of an antigenic determinant which is displayed on the surface of live parasites.

To evaluate the in vivo protective effect of mAb5D5, we passively transferred 300 µg of this antibody into naïve C57BL/6 mice which were then challenged with the transgenic *P. berghei* strain, P.b.-P.f. CSP-NT. We found that mice receiving mAb5D5 and challenged with the P.b.-P.f. CSP-NT sporozoites had nearly 1 log reduction in parasite burden in the liver, compared to naïve controls (Fig. 3.4A). Control mice receiving an irrelevant antibody, mAb2F6, had parasite loads comparable to those
of naïve controls. We also evaluated the protective effect of this antibody using a different strain of transgenic *P. berghei* sporozoites that express part of the N-terminal region (residues 97-111) and the repeat domain of *P. falciparum* 7G8 CSP, (P.b-P.f. CSP-R) [11] (Fig. 3.1C). As determined by IFA, the sporozoites of this transgenic strain are also recognized by mAb5D5 (data not shown). As observed with P.b.-P.f. CSP-NT sporozoites, when this antibody was passively transferred into mice subsequently challenged with P.b.-P.f. CSP-R sporozoites, it reduces by approximately 2 logs the parasite burden in the liver (Fig. 3.4B). The fact that mAb5D5 can inhibit sporozoite invasion in two different chimeric strains, rules out the possibility that the observed inhibitory effect could be due to spurious reactivity of mAb5D5 with the repeat domain of *P. falciparum* or *P. berghei* CSP. All together, these results confirm the epitope-specificity and remarkable neutralizing capacity of mAb5D5 against sporozoites bearing the N-terminal region of the *P. falciparum* CSP.

### 3.4.4 Enhanced *in vivo* sporozoite-neutralization by combining mAb5D5 and mAb2A10

Next, we investigated whether the antibody-mediated sporozoite neutralization could be enhanced by combining antibodies that recognize different epitopes of the *P. falciparum* CSP. First, we performed passive transfer experiments to compare the neutralizing capacities of mAb5D5 and mAb2A10, specific for the repeat domain [18]. Recipient mice and control mice were challenged with the P.b.-P.f. CSP-R parasite strain, expressing both the N-terminal and repeat domain epitopes. As expected, both antibodies inhibited parasite infection although in repeated experiments we consistently observed that mAb5D5 displayed a higher inhibitory activity than mAb2A10 (Fig. 3.5). To determine whether the neutralizing effect of these antibodies was additive, we compared liver parasite burden of mice that received 25 µg of mAb5D5 or 100 µg of mAb2A10 to that of mice receiving both antibodies i.e., mAb5D5 and mAb2A10. When compared to mice that received a single antibody, passive transfer of mAb5D5 together with mAb2A10 resulted in significantly enhanced inhibition of parasite infection (Fig. 3.6).
3.5 Discussion

In this study we report the characterization of a mAb specific for the *P. falciparum* CSP, which recognizes an epitope located in the N-terminal region of *P. falciparum*. This antibody binds to a linear epitope defined by the sequence \(^8\)EDNEKLRKPKH\(^9\), which is exposed on the surface of live sporozoites. Using transgenic *P. berghei* parasites, expressing the N-terminal region of the *P. falciparum* CSP we demonstrate, for the first time, that a single monoclonal antibody against the N-terminal region of the CSP region can neutralize sporozoite infectivity *in vivo*. Importantly, we show that mAb5D5 blocks the enzymatic processing of CSP as it recognizes an epitope adjacent to the proteolytic cleavage site of the CSP N-terminal region. These findings are in full agreement with previous studies demonstrating that proteolytic processing of the CSP is a required step for an efficient sporozoite invasion of hepatocytes [1, 2]. Finally, our results are also in agreement with *in vitro* observations reporting that polyclonal antibodies raised against the N-terminal region of *P. falciparum* CSP can inhibit sporozoite invasion of *in vitro* cultured hepatocytes [10, 19-21].

The protective effect of RTS,S in humans appears to be largely mediated by antibody responses elicited against CSP's repeat region [5-9, 22]. Our data indicate that the neutralizing capacity of anti-CSP antibodies is enhanced when combining antibodies of different epitope specificity, suggesting that vaccines inducing anti-CSP antibodies responses of broad epitope specificities may result in increased protective efficacy. Our results strongly suggest that the N-terminal region of the *P. falciparum* CSP should be included in RTS,S or similar CSP-based vaccine candidates as it is apparent that antibodies against this important region can have a major inhibitory effect on sporozoite infectivity. For years it has been suggested that the dominant antibody response to the CSP repeat region could represent a parasite strategy to divert the host immune response by preventing the recognition of regions critical importance for parasite invasion. The availability of rodent parasites expressing distinct domains of human parasite orthologues should help answer some of these questions and advance research to study the immunogenic properties of the different CSP regions. Further studies should seek to determine whether induction of multi-domain protective antibody responses
can be better induced by immunizing with full-length recombinant CSP or with independent constructs representing individual CSP domains. In fact, while mAb5D5 recognizes a linear epitope and live sporozoites, it remains to be determined whether a similar antibody could be induced by native parasite protein.

Finally, our study shows that research aimed at defining the functional roles that molecules or specific domains may have on the parasite’s biology and infection can provide critical information for identifying functionally important molecular domains that could be targeted by vaccine-induced protective antibody responses.

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3.7 Acknowledgements

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Figure 3.1 Epitope specificity of the mAb5D5 determined by ELISA. (A) Direct binding of mAb5D5 to overlapping synthetic peptides representing the entire N-terminus of P. falciparum CSP. mAb5D5 specifically binds to the peptides 77DGNNEDNEKLR90 and 81EDNEKLRPKHKKLK95. (B) Competition ELISA between synthetic peptides and rCSP for mAb5D5 binding. Preincubation with different concentrations of synthetic peptides 77DGNNEDNEKLR90 and 81EDNEKLRPKHKKLK95 was able to inhibit binding of mAb5D5 to P. falciparum CSP; no effect was observed with the flanking peptides 73GENDDGNNEDNEKLR87 and 85KLRKPKHKKLKQPAD99. Results are expressed as optical density at 405 nm. (C) Comparison of CSP N-terminal region amino acid sequences beginning after the signal sequence of P. berghei (ANKA), P. falciparum (3D7), P.b.-P.f. CSP-NT, and P.b.-P.f. CSP-R. The P.b.-P.f. CSP-NT amino acid sequence was derived from DNA sequencing of clonal transgenic parasites. Underlining denotes the core sequence recognized by mAb5D5. Abbreviations: CSP, circumsporozoite protein; ELISA, enzyme-linked immunosorbent assay; mAb5D5, monoclonal antibody 5D5; OD, optical density; P.b.-P.f. CSP-NT, P. berghei–P. falciparum CSP N-terminus; P.b.-P.f. CSP-R, P. berghei–P. falciparum CSP repeat domain; rCSP, recombinant CSP.
Figure 3.2 Recognition of air-dried and live sporozoites by mAb5D5. *P. falciparum* (3D7), chimeric *P.berghei-P. falciparum* CSP-NT and
*P. berghei* (ANKA) sporozoites were stained with mAb5D5 (1 µg/mL) live or after air-drying. Abbreviations: CSP, circumsporozoite protein; mAb5D5, monoclonal antibody 5D5; *P.berghei-P. falciparum* CSP N-terminus.
Figure 3.3 CSP processing is inhibited by mAb5D5. (A) P. falciparum sporozoites were metabolically labeled with 35S-Cys/Met and then kept on ice (pulse, P) or chased for 2 hours in the absence (C) or presence of the indicated concentrations (µg/mL) of mAb5D5 or an isotype control (mAb3D11). Sporozoites were then lysed, and CSP was immunoprecipitated and analyzed by SDS-PAGE and autoradiography. Molecular mass is indicated in kilodaltons on the left-hand side of the autoradiograph. (B) Densitometry analysis of scanned film using ImageJ software. Shown for each chased sample is the ratio of the density of the high MW CSP band to the low MW CSP band. A ratio of 1 indicates that the density of the top and bottom bands is the same. These results are representative of 2 independent experiments. Abbreviations: CSP, circumsporozoite protein; mAb5D5, monoclonal antibody 5D5; MW, molecular weight; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.
Figure 3.4 Protection against challenge with chimeric sporozoites by passive transfer of mAb5D5. 300 µg of mAb5D5 were inoculated intravenously into C57BL/6 mice immediately prior to injection of $10^4$ sporozoites. Forty hours later, livers were harvested, RNA extracted, and liver parasite burden was determined by RT-qPCR. Passive transfer of mAb5D5 had a significant protective effect against *P.berghei*-*P.falciparum* CSP-NT chimeric sporozoites (A), as well as *P.berghei*-*P.falciparum* CSP-R (B), compared to mice that received irrelevant antibody (mAb2F6) or to naïve controls. Mean ± SEM; n = 5, results are representative of 2 independent experiments. *P < .05; **P < .01. Abbreviations: CSP, circumsporozoite protein; mAb5D5, monoclonal antibody 5D5; ns, not significant; *P.berghei*-*P.falciparum* CSP-NT, *P.berghei*-*P.falciparum* CSP N-terminus; *P.berghei*-*P.falciparum* CSP-R, *P.berghei*-*P.falciparum* CSP repeat domain; rRNA, ribosomal RNA; RT-qPCR, quantitative real-time polymerase chain reaction; SEM, standard error of the mean.
Figure 3.5 Comparison of the sporozoite-neutralizing effect of mAb5D5 and mAb2A10. A total of 300 µg of mAb5D5 or mAb2A10 were inoculated intravenously into C57BL/6 mice immediately prior to injection of $10^4$ P. berghei–P. falciparum CSP-R sporozoites. Passive transfer of both monoclonal antibodies had a significant protective effect compared to mice-naive controls. Mean ± SEM; n = 5, results are representative of 2 independent experiments. **P < .01. Abbreviations: CSP, circumsporozoite protein; mAb, monoclonal antibody; ns, not significant; P. berghei–P. falciparum CSP repeat domain; rRNA, ribosomal RNA; SEM, standard error of the mean.

Figure 3.6 Enhanced inhibitory effect upon combination of antibodies of different epitope specificity. The antibodies mAb5D5 (25 µg) and mAb2A10 (100 µg) were administered combined or independently to assess their additive effect against $10^4$ P. berghei–P. falciparum CSP-R sporozoites. Mean ± SEM; n = 5, results are representative of 2 independent experiments. *P < .05. Abbreviations: CSP, circumsporozoite protein; mAb, monoclonal antibody; P. berghei–P. falciparum CSP repeat domain; rRNA, ribosomal RNA; SEM, standard error of the mean.
3.8 References


CHAPTER 4
FULL-LENGTH PLASMODIUM FALCIPARUM CIRCUMSPOROZOITE PROTEIN ADMINISTERED WITH LONG-CHAIN POLY(I·C) OR THE TOLL-LIKE RECEPTOR 4 AGONIST GLUCOPYRANOSYL LIPID ADJUVANT-STABLE EMULSION ELICITS POTENT ANTIBODY AND CD4+ T CELL IMMUNITY AND PROTECTION IN MICE

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4.1 Abstract

The Plasmodium falciparum circumsporozoite (CS) protein (CSP) is a major vaccine target for preventing malaria infection. Thus, developing strong and durable antibody and T cell responses against CSP with novel immunogens and potent adjuvants may improve upon the success of current approaches. Here, we compare four distinct full-length P. falciparum CS proteins expressed in Escherichia coli or Pichia pastoris for their ability to induce immunity and protection in mice when administered with long-chain poly(I·C) [poly(I·C)LC] as an adjuvant. CS proteins expressed in E. coli induced high-titer antibody responses against the NANP repeat region and potent CSP-specific CD4+ T cell responses. Moreover, E. coli-derived CS proteins in combination with poly(I·C)LC induced potent multifunctional (interleukin 2-positive [IL-2+], tumor necrosis factor alpha-positive [TNF-α+], gamma interferon-positive [IFN-γ+]) CD4+ effector T cell responses in blood, in spleen, and particularly in liver. Using transgenic Plasmodium berghei expressing the repeat region of P. falciparum CSP [Pb-CS(Pf)], we showed that there was a 1- to 4-log decrease in malaria rRNA in the liver following a high-dose challenge and ~50% sterilizing protection with a low-dose challenge compared to control levels. Protection was directly correlated with high-level antibody titers but not CD4+ T cell responses. Finally, protective immunity was also induced using the Toll-like receptor 4 agonist glucopyranosyl lipid adjuvant-stable emulsion (GLA-SE) as the adjuvant, which also correlated with high antibody titers yet CD4+ T cell immunity that was significantly less potent than that with poly(I·C)LC. Overall, these data suggest that full-length CS proteins and poly(I·C)LC or GLA-SE offer a simple vaccine formulation to be used alone or in combination with other vaccines for preventing malaria infection.
4.2 Introduction

Malaria infection with *Plasmodium falciparum* causes more than 600,000 deaths annually as well as significant morbidity worldwide [1]. A range of efforts to control and treat malaria include public health measures such as insecticide-treated bed nets, indoor residual spraying, and widespread usage of antimalarial drugs. Despite the impact of these approaches, the most cost-effective solution to prevent infection and to ultimately control the malaria endemic is to develop a vaccine.

Currently, the most advanced vaccine tested in humans against *P. falciparum* infection is RTS,S, which targets the circumsporozoite (CS) protein (CSP), the major and most abundant antigen expressed on the surfaces of infectious sporozoites. RTS,S given with the AS01 adjuvant (RTS,S/AS01) shows ~30% protection against clinical disease and severe malaria [2, 3]. Thus, while these first results in phase III trials with RTS,S/AS01 are encouraging, there may be additional approaches for further optimizing the breadth, potency, and duration of immunity against the CSP using different immunogens or more-potent adjuvants. In terms of antigen design, RTS,S is comprised of a truncated form of CSP containing the central repeat region, NANP, which is a target for antibody-mediated neutralization, as well as CD8+ and CD4+ T cell epitopes at the C-terminal end. This truncated CS protein is then fused to the hepatitis B virus surface antigen, creating an immunogenic particle. Therefore, using a more-full-length CSP, including the N-terminal end and the R1 region of CSP as well as the minor repeat region (NVDP), might favor broader antibody responses than against the NANP repeat region alone [4–8]. Moreover, a full-length CSP may provide additional T cell epitopes, leading to increased breadth of cellular immunity, which could also enhance protection. Another approach is to enhance the humoral and cellular immune responses by altering the type of adjuvant given with the full-length-CSP-based protein vaccine.

Early studies in mice showed that protection was associated with high antibody titers [9–11]. The next generation of malaria vaccines combined CSP with more-potent adjuvants, like *Pseudomonas aeruginosa* exotoxin A, monophospholipid A (MPL), mycobacterial cell wall skeleton, or squalene (Detox; Ribi Immunochem) [12–14], which resulted in high antibody titers; however, they failed to
confer sufficient protective efficacy [15–17]. In contrast, studies using irradiated sporozoites for vaccination have shown a critical requirement for gamma interferon (IFN-γ) and cellular immunity in mediating protection against malaria [18, 19]. Accordingly, optimizing CD4+ T cell-derived IFN-γ production after RTS,S vaccination by altering the adjuvant formulation enhanced protection [20–22]. Using the Toll-like receptor 4 (TLR4) ligand MPL and saponin (QS-21) in an oil-water emulsion (AS02) or liposome (AS01B or -E) formulation led to a strong antibody response and increased CD4+ T cell immunity compared to levels induced with older formulations with alum and MPL [21, 23, 24]. Collectively, these data highlight the importance of adjuvant formulations in optimizing immunity and protection.

In this study, long-chain poly(I·C) [poly(I·C)LC] and the TLR4 agonist glucopyranosyl lipid adjuvant-stable emulsion (GLA-SE) were compared as adjuvants when they were administered with a number of CS proteins expressed in the yeast Pichia pastoris or in Escherichia coli, to which we refer as full length but which lack the glycosylphosphatidylinositol (GPI) signal region and contain various numbers of the NANP repeat region. Poly(I·C)LC is a unique formulation of long-chain double-stranded RNA, polyinosinic-poly(C), and carboxy methylcellulose that has extended in vivo activity compared to that of poly(I·C). Poly(I·C)LC mediates innate signaling through TLR3 and melanoma differentiation-associated protein 5 (MDA-5), leading to activation of dendritic cells and induction of interleukin 12 (IL-12) and type I interferons (IFNs) [25–27]. In addition, poly(I·C) has been shown to promote T cell survival and enhance germinal-center formation through the generation of CD4+ T follicular helper (Tfh) cells [28]. As an immune adjuvant, poly(I·C)LC has been shown to elicit strong humoral and cellular immunity when administered with a variety of protein- or dendritic cell-targeting vaccines in a number of mouse and nonhuman primate (NHP) studies [29–35].

GLA is a synthetic and therefore homogeneous variant of the TLR4 agonist lipid A, formulated in a stable oil-in-water emulsion (SE) [36]. Strong Th1 immune responses induced by protein antigens in combination with GLA-SE have been observed in mouse models of tuberculosis [37], leishmaniasis [38], and influenza [39]. Additionally, GLA-SE showed an adjuvant activity similar
to or enhanced relative to that of MPL-SE (one of the adjuvant components in AS01) in various animal models, such as mice, guinea pigs, and nonhuman primates [40, 41].

Here, we compare four full-length P. falciparum CS proteins expressed in E. coli or in the yeast P. pastoris given with poly(I-C)LC or GLA-SE as an adjuvant and determine their relative immunogenicities and levels of protection using an in vivo mouse challenge model. Furthermore, one of the CS proteins and poly(I-C)LC was tested in NHPs. Together, these data provide a potential simple vaccine formulation for inducing potent and protective P. falciparum CSP responses.

4.3 Materials and Methods

4.3.1 Animals

Six- to 8-week-old C57BL/6 mice were obtained from The Jackson Laboratories (Bar Harbor, ME) and maintained in the Vaccine Research Center Animal Care Facility (Bethesda, MD) under pathogen-free conditions. Male, Indian rhesus macaques were stratified into comparable groups on the basis of age and weight. The animals were housed at the School of Medicine, Comparative Medicine and Veterinary Resources, University of Maryland. All experiments were conducted according to the guidelines of the National Research Council under protocols approved by the Institutional Animal Care and Use Committee at the National Institutes of Health.

4.3.2 Reagents

Four P. falciparum CS proteins were provided by the PATH Malaria Vaccine Initiative (MVI; Washington, DC) and provided to us in a blind manner (they were designated CSP1, CSP2, CSP3, and CSP4). These four proteins are called full length in the text, but they lack the GPI signal region and contain various numbers of the repeat region. After the immune studies were completed, it was revealed to us that two proteins were expressed in E. coli (CSP1 and CSP2) and two were expressed in P. pastoris (CSP3 and CSP4). CSP1 and CSP2 were provided to the MVI from Gennova (Pune, India) and WRAIR (Silver Spring, MD), respectively. Protein concentration was measured using a bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL). Endotoxin contaminants were
determined using the Limulus amebocyte lysate (LAL) assay (Lonza, Walkersville, MD). If endotoxin levels exceeded 2 endotoxin units (EU)/ml, endotoxin was removed by two-phase extraction with Triton X-114. Small amounts of endotoxin were detected in the E. coli-derived proteins. Thus, all four proteins, including the two expressed in yeast, which had no detectable endotoxin, were treated with Triton X-114. All proteins used for immunization had final endotoxin levels below 1 EU/ml. After removal of endotoxin, protein concentration was determined to confirm that all groups of mice received the same amount of protein. *P. falciparum* CSP15-mer peptides overlapping by 11 amino acids (aa) and spanning the entire length of the protein (*P. falciparum* 3D7 strain) were synthesized by GenScript. Each batch of CSP was tested for accurate size using SDS-PAGE.

### 4.3.3 Antibodies

The following anti-mouse antibodies for flow cytometry were purchased from BD Pharmingen: purified anti-CD28 (37.51), PerCP–Cy55–anti-CD3 (145-2C11), and Alexa 700–anti-CD4 (RM4-5). The following antibodies were purchased from BioLegend: allophycocyanin (APC)–Cy7–anti-CD8 (53-6.7). LIVE/DEAD fixable violet dead cell stain (ViViD) was purchased from Molecular Probes, and staining was performed as described by Perfetto et al. [42]. Intracellular staining was performed according to the BD Cytofix/Cytoperm kit instructions using APC–anti-IFN-γ (XMG1.2), phycoerythrin (PE)–Cy7–anti-tumor necrosis factor alpha (anti-TNF-α) (MP6-XT22), and PE–anti-IL-2 (MQ1-17H12), which were purchased from BD Biosciences, and Alexa Fluor 488–anti-IL-10 (JES5-16E3), which was purchased from eBioscience.

For staining of nonhuman primate (NHP) cells, the following anti-human antibodies were purchased from BD Pharmingen: purified anti-CD49d (9F10), APC–Cy7–anti-CD3 (SP34-2), PE–Cy7–anti-TNF-α (MAb11), PE–anti-IL-2 (MQ1-17H12), PE–Cy5–anti-CD95 (DX2), and fluorescein isothiocyanate (FITC)–anti-IFN-γ (B27). PacificBlue–anti-CCR7 (TG8/CCR7) was purchased from BioLegend. Anti-CD45RA–R phycoerythrin-Texas Red (ECD) (clone 2H4LDH11LDB9) was purchased from Beckman Coulter, and Qdot605–anti-CD4 (S3.5) was purchased from Invitrogen. The
conjugates Alexa 680–anti-CD28 (CD28.2) and Qdot655–anti-CD8 (RPA-T8) were produced in-house by the laboratory of Mario Roederer (NIAID, NIH, Bethesda, MD).

4.3.4 Immunizations

C57BL/6 mice were immunized with 2 μg or 20 μg of CSP, with or without 50 μg poly(I-C)LC (Oncovir, Inc., Washington, DC) or 5 μg GLA-SE (Infectious Disease Research Institute, Seattle, WA) which are the optimal doses for these adjuvants. Naïve mice or mice immunized with the adjuvant alone served as controls. The vaccines were administered subcutaneously (s.c.) in both hind footpads in a total volume of 50 μl per foot. Animals were immunized at weeks 0 and 3 or 0, 3, and 6.

Rhesus macaques were immunized with 100 μg of *P. falciparum* CSP mixed with 1 mg of poly(I-C)LC. A total volume of 1 ml was injected s.c. into the deltoid area of the upper arm. For boosting, the opposite arm was used. Animals were immunized at weeks 0, 5, and 16.

4.3.5 Analysis of CD4+ T cell responses

Cells from mice were harvested from spleens at various times postvaccination, and single-cell suspensions (2 × 10^6 cells/well) from individual mice were incubated for 5 h with anti-CD28, 10 μg/ml Brefeldin A, and 2 μg/ml CSP peptide pool. Cells were stained with the viability dye LIVE/DEAD fixable violet dead cell stain (ViViD), CD4, and CD8, followed by intracellular staining for CD3, IFN-γ, IL-2, IL-10, and TNF-α using the BD Cytofix/Cytoperm kit according to the manufacturer's instructions. For intrahepatic-lymphocyte isolation, the liver was perfused with phosphate-buffered saline (PBS) before being processed, and lymphocytes were isolated by Percoll density centrifugation. Stimulation and staining were performed as described for splenocytes in this section.

For analysis of NHP peripheral blood mononuclear cells (PBMCs), cryopreserved cells were thawed and rested overnight at 37°C. The next day, cells (2 × 10^6 cells/well) were stimulated for 5 h with anti-CD49d and anti-CD28, 10 μg/ml Brefeldin A, and 2 μg/ml CSP peptide pool. Cells were stained in warm medium with CCR7, followed by surface staining with a LIVE/DEAD fixable aqua dead cell stain kit (Invitrogen), CD45RA, CD95, CD4, and CD8 in PBS. Intracellular cytokine staining was performed using IFN-γ, IL-2, CD3, and TNF-α with the BD Cytofix/Cytoperm kit according to
the manufacturer’s instructions. Cells were resuspended in 1% paraformaldehyde, acquired on a modified BD LSR II flow cytometer, and analyzed using FlowJo software (Tree Star), Pestle, and SPICE (Mario Roederer, NIAID, NIH).

4.3.6 Detection of CSP-specific antibodies

Serum samples from immunized mice and NHPs were analyzed for IgG antibodies specific for the repeat region ([NANP]₆ peptide) of *P. falciparum* CSP by the Malaria Serology Laboratory at WRAIR. Plates were read at 414 nm, and endpoint titers were calculated at an optical density (OD) of 1.0. For analysis of total CSP-specific IgG1 and IgG2a titers, enzyme-linked immunosorbent assay (ELISA) plates were coated with CSP1 protein at a concentration of 1 μg/ml and washed. Serially diluted serum samples from immunized or control mice were added in duplicate for 2 h. After being washed, samples were incubated with either anti-mouse IgG1-horseradish peroxidase (HRP) or anti-mouse IgG2a-HRP. Plates were then developed with 3,3′,5,5′-tetramethylbenzidine substrate-chromogen (Dako) and read at 450 nm. Midpoint 50% effective concentration (EC50) titers were calculated using 4PL curve fitting.

4.3.7 Immunofluorescent antibody (IFA) assay

To determine whether serum bound to sporozoites, slides (Tekdon Inc.; poly-L-lysine coated) were coated with a 10-μl suspension of sporozoites of *P. berghei* expressing the repeat region of CSP from *P. falciparum* [Pb-CS(Pf)] at a concentration of 4 × 10⁵ to 6 × 10⁵ sporozoites/ml and air dried. Serum samples from immunized mice or NHPs were diluted in PBS-1% bovine serum albumin (BSA) prior to their addition to the slides (10 μl of sample) and then incubated for 30 min at room temperature. Slides were then washed with PBS-1% BSA, and 10 μl of a secondary-antibody solution [Alexa Fluor 488 F(ab’)2 fragment of goat anti-mouse IgG(H+L) (2 μg/ml; Invitrogen) and FITC-labeled goat anti-monkey IgG(H+L) (KPL) for mice and NHPs, respectively] was added for 30 min at room temperature; then slides were washed with PBS-1% BSA. Fluorescent sporozoites were visualized using an upright fluorescence microscope (Nikon Eclipse 90i). The lowest titer at which
sporozoites were visualized was scored as positive. Binding of serum antibodies to sporozoites was scored as +++ (very good), ++ (good), + (weak), or − (no) binding.

4.3.8 Sporozoite challenge

Mice were challenged intravenously (i.v.) with $1.5 \times 10^4$ transgenic Pb-CS(Pf) sporozoites, kindly provided by E. Nardin [10]. Approximately 40 h later, mice were euthanized to assess parasite burden in livers. Parasite loads were determined by quantitative PCR (qPCR) for *P. berghei* 18S rRNA [43]. For the assessment of blood-stage parasitemia, mice were challenged i.v. with $1 \times 10^3$ of the transgenic Pb-CS(Pf) sporozoites. Starting on day 4, blood smears were taken and observed under a microscope. Smears were fixed with methanol (for 30 s) before being stained with a 10% Giemsa stain solution (Sigma-Aldrich) for 15 min.

4.3.9 Serum transfer

Serum was collected from individual mice 2 weeks after three immunizations with 20 μg CSP1 and poly(I·C)L C, and 0.5 ml was transferred i.v. immediately prior to challenge.

4.3.10 Isolation of PBMCs from NHPs

PBMCs were isolated from fresh blood by Ficoll density centrifugation using LeucoSep tubes (Greiner Bio One) according to the manufacturer's instructions. After several washes, cells were cryopreserved.

4.3.11 Statistics

The majority of the data and statistical analysis were created using Prism software (GraphPad) and a Mann-Whitney test. Differences were found to be significant when *P* was less than 0.05 or 0.01. Data are represented as means + standard deviations (SD) or as geometrical means, as indicated in the figure legends. Bar and pie charts of cytokine production were created using FlowJo software (Tree Star), Pestle, and SPICE (Mario Roederer, NIAID, NIH).
4.4 Results.

4.4.1 *E. coli*-derived CSP induces robust CD4+ T cell cytokine responses

The goal of this blinded study was to compare the immunogenicities of four full-length CSPs (CSP1 to -4) in combination with different adjuvants. CSP1 and CSP2 were produced in *E. coli*, while CSP3 and CSP4 were expressed in the yeast *P. pastoris*. The amino acid sequences of CSP2 and CSP3, derived from the 3D7 strain of the malaria parasite, share strong homology at the amino- and carboxy-terminal regions but differ in their numbers of NANP repeats (Fig. 4.1). CSP1 and CSP4 are derived from the India strain IND637HDD1, contain all 37 NANP repeats, and share strong amino acid sequence homology. The 3D7 strain has a deletion in the N-terminal region (aa 91 to 97), whereas this region is intact in the India strain. We first selected poly(I·C)LC as an adjuvant based on its potency for generating T cell and antibody responses shown in prior studies by our lab and others [34, 35].

C57BL/6 mice were immunized twice, 3 weeks apart, with 2 μg or 20 μg of CSP with or without 50 μg poly(I·C)LC. Four weeks after the second immunization, CD4+ T cell responses in the spleens were assessed by multiparameter flow cytometry. Mice immunized with poly(I·C)LC or any of the CSPs without adjuvant had no measurable CSP-specific CD4+ T cell cytokine responses (Fig. 4.2A). In contrast, 2 μg of CSP1 and CSP2 administered with poly(I·C)LC induced robust CD4+ T cell cytokine responses, producing IFN-γ, IL-2, and TNF-α (Fig. 4.2B). Such responses were increased ∼2- to 3-fold by using 20 μg of CSP with poly(I·C)LC (Fig. 4.2C). Remarkably, both CSPs produced in yeast (CSP3 and CSP4) failed to induce significant antigen-specific CD4+ T cell responses with poly(I·C)LC, even at the higher dose (20 μg).

4.4.2 *E. coli*-derived CSP induces antibodies against the NANP repeat region

Since antibodies against CSP are critical for protection, we assessed responses against the NANP repeat region following immunization of all vaccine groups. Consistent with the data in Fig. 4.2, CSP1 and CSP2 vaccines produced in *E. coli* elicited significantly more potent antibody responses than did CSP3 and CSP4 expressed in yeast (Fig. 4.3A to C). Higher antibody titers were observed in mice that received the higher dose (20 μg) of CSP and poly(I·C)LC. Taken together, the *E. coli*-derived
proteins were more potent for CSP-specific antibody and CD4+ T cell immunity in this study with poly(I·C)Lc or with CpG as an adjuvant (data not shown). Therefore, CSP1 was selected for further testing.

4.4.3 Protection requires three immunizations with CSP and poly(I·C)Lc

We next sought to establish the optimal antigen dose and number of immunizations required to induce protection against a challenge. Because mice are not susceptible to *P. falciparum*, we used a recombinant *P. berghei* parasite expressing the *P. falciparum* CSP repeat region [Pb-CS(Pf)] [10] as a challenge for our *P. falciparum* CSP-vaccinated mice. Mice were immunized two or three times at 3-week intervals with 20 μg of CSP1 and poly(I·C)Lc. Four weeks after the last immunization, mice were challenged i.v. with 1.5 × 104 Pb-CS(Pf) parasites. Forty hours later, livers of infected mice were harvested and parasite-derived 18S rRNA was measured by real-time qPCR (RT-qPCR). Mice that received two immunizations with 20 μg of CSP1 and poly(I·C)Lc showed a modest but significant (∼0.5 log) reduction (P < 0.01) of parasite-derived 18S rRNA levels compared to naive mice or mice that received poly(I·C)Lc or CSP1 alone (Fig. 4.4A). Moreover, three immunizations with 20 μg CSP1 and poly(I·C)Lc resulted in an ∼2- to 4-log reduction in 18S rRNA (Fig. 4.4A) compared to levels in mice immunized with protein alone, poly(I·C)Lc alone, or CSP1 protein and poly(I·C)Lc (two immunizations). A separate series of experiments sought to determine whether the dose of CSP1 influenced protection. We compared three immunizations with 2 or 20 μg of CSP1 and poly(I·C)Lc. As shown in Fig. 4.4B, the higher dose of CSP1 provided a significant (P = 0.005) (∼2-log) reduction in parasite load in the livers of mice compared to those with the lower dose of CSP1. Overall, these data show that three immunizations with the higher dose (20 μg) of CSP1 and poly(I·C)Lc provided significant reduction of parasite load against a high-dose i.v. challenge with sporozoites.

4.4.4 Antibody against CSP correlates with protection

In the next series of experiments, we sought to determine the immunological correlates of protection following immunization with CSP1 and poly(I·C)Lc. Prior studies in mice and humans showed that antibodies and CD4+ T cells play a role in protection [20, 22, 44–48]. In Fig. 4.3, we show
that two immunizations with 2 or 20 μg of CSP1 induced potent antibody responses against the NANP repeat region. To extend this analysis, we assessed CSP-specific antibody titers at both doses of CSP after two or three immunizations. As shown in Fig. 4.5A, the greatest antibody response was detected with the higher dose of CSP1 given three times, consistent with improved protection in this group (Fig. 4.4). To substantiate a direct role of antibodies in mediating protection, pooled serum from mice immunized three times with 20 μg CSP1 and poly(I·C)LC was transferred into naïve recipients, and then the mice were challenged. As a negative control, serum from naïve mice was transferred to naïve mice. As a positive control, mice immunized three times with 20 μg of CSP1 and poly(I·C)LC were challenged at the same time. Naïve mice that received serum from CSP1- and poly(I·C)LC-vaccinated animals showed an ~1-log reduction in parasite burden compared to mice that received control serum or naïve mice (Fig. 4.5B). However, this protection was not as robust as that seen in animals immunized with CSP1 and poly(I·C)LC. This could be due to dilution of transferred antibodies in the blood of recipient animals or to a contribution of vaccine-elicited CD4+ T cells absent in passively transferred animals. Nevertheless, these data show that humoral immunity is sufficient to confer some protection against this high-dose challenge.

4.4.5 Poly(I·C)LC induces higher CD4+ Th1 cell immunity than GLA-SE

The RTS,S vaccine has been formulated with the TLR4 ligand MPL and QS-21 in either a liposomal solution (AS01) or oil-in-water emulsion (AS02). Thus, to compare poly(I·C)LC (which signals through TLR3 and MDA-5) to an adjuvant with some commonality to AS02, we choose GLA-SE. This formulation consists of a synthetic form of the TLR4 agonist lipid A, which has been shown to provide adjuvant activity that is similar to, if not better than, that of MPL-SE [49].

Mice were immunized three times with 2 μg or 20 μg of CSP1 formulated with either poly(I·C)LC or GLA-SE, and the CD4+ T cell responses were analyzed in the blood, spleen, and liver 7 to 14 days after the third vaccination. At this peak time point, the frequencies of IFN-γ-producing CD4+ T cells in the blood (Fig. 4.6A) were similar when we compared the 2-μg and 20-μg doses of CSP1 protein using either poly(I·C)LC or GLA-SE as an adjuvant. However, CD4+ IFN-γ responses
were significantly higher when poly(I·C)Lc was used than when GLA-SE was used for both doses of CSP1 (Fig. 4.6A). Moreover, in spleen (Fig. 4.6B) and liver (Fig. 4.6C), CD4+ T cell responses were also significantly higher using poly(I·C)Lc than using GLA-SE. Mice immunized with either adjuvant alone or CSP1 alone showed no detectable CD4+ T cell responses (data not shown). Lastly, we did not detect any CD8+ T cell responses (data not shown), consistent with the absence of a P. falciparum CSP-specific CD8+ T cell epitope in C57BL/6 mice.

To extend this analysis, we assessed the quality of the CD4+ T cell cytokine response based on the relative proportions of cells producing IL-2, IFN-γ, and TNF-α, either alone or simultaneously. The quality of the CD4+ T cell response may be an important metric because of evidence that multifunctional CD4+ T cells correlate with protection [20, 23, 50]. As shown in Fig. 4.6D, ~50% of the total cytokine-producing responses were multifunctional, producing IL-2, TNF-α, and IFN-γ, with another ~25% making IFN-γ and TNF-α without IL-2. Thus, both adjuvants elicit the induction of CD4+ T cells that secrete two critical effector cytokines that may mediate parasite killing [51–53].

### 4.4.6 Poly(I·C)Lc and GLA-SE confer comparable levels of protection correlating with antibody titers

We next compared antibody responses induced with 2 or 20 μg of CSP1 and GLA-SE to poly(I·C)Lc following three immunizations. In contrast to the magnitudes of CD4+ T cell cytokine responses, which were significantly different, the total titers of IgG against the NANP repeats measured with the two adjuvants were not significantly different (Fig. 4.7A, upper left). Of note, the lower dose (2 μg) of CSP1 consistently induced ~2- to 5-fold-lower total antibody titers than the higher dose (20 μg) for both adjuvants. Similar results were seen after analyzing IgG1 and IgG2a antibody titers (Fig. 4.7A). In terms of protection, there was a 2- to 3-log reduction of 18S rRNA after challenge with Pb-CS(Pf) in mice immunized with 20 μg of CSP1 with poly(I·C)Lc compared to that in naïve mice, consistent with the results of previous experiments (Fig. 4.4). Immunization with 20 μg of CSP1 and GLA-SE resulted in similar levels of protection (Fig. 4.7B). Thus, in a setting of equivalent
antibody titers, there was no significant difference in protection using poly(I·C)LC or GLA-SE as an adjuvant.

To determine whether humoral or cellular immune responses correlated with protection, prechallenge antibody titers (Fig. 4.7C) and CD4+ T cell cytokine responses (Fig. 4.7D) were plotted against the level of protection achieved upon consecutive challenge. There was a highly significant correlation between CSP-specific antibody titers and the level of 18S rRNA measured in livers after parasite challenge (Pearson $r = -0.7$) (Fig. 4.7C). In contrast, there was no correlation between the frequency of IFN-γ-producing CD4+ T cells in the blood and the level protection after parasite challenge (Pearson $r = -0.02$) (Fig. 4.7D). Finally, to confirm that our vaccine-elicited CSP-specific antibodies had binding activity against the intact parasite, we used an IFA assay to visualize direct antibody binding to sporozoites (Table 4.1). Consistent with the CSP-specific antibody titers, poly(I·C)LC and GLA-SE induced strong and comparable IFA titers when they were administered with 20 μg of CSP1 protein. Taken together, these data show that poly(I·C)LC and GLA-SE differ in their capacities to induce CD4+ /Th1 immune responses after immunization with CSP; however, they induce comparable antibody responses that correlate best with protection.

While reduction in parasite load in the liver following a high-dose i.v. challenge may have important clinical consequences by reducing the prepatent period and time to infection [20, 54, 55], the most important and clearest clinical outcome is sterilizing immunity. Thus, mice vaccinated with CSP1 and poly(I·C)LC were i.v. challenged with a low dose of $1 \times 10^3$ Pb-CS(Pf) sporozoites and monitored daily by blood smear to establish the onset of infection. As shown in Fig. 4.8, all naive mice and mice immunized with poly(I·C)LC alone developed parasitemia within 5 days. In contrast, 5 out of 10 mice immunized with CSP1 and poly(I·C)LC did not develop parasitemia during the 15 days of follow-up. Also, the prepatent period was significantly delayed ($P < 0.05$) in mice that received CSP1 and poly(I·C)LC (5.8 days) compared to mice that received poly(I·C)LC alone or naive animals (4.6 days and 4.8 days, respectively) (Fig. 4.8A; Table 4.2). Analysis of prechallenge sera from mice vaccinated with CSP1 and poly(I·C)LC revealed that protected mice from this group displayed
significantly higher antibody titers than the nonprotected mice from this same vaccine group (Fig. 4.8B). These data are consistent with human trials showing that a very high level of antibody is required for sterilizing immunity [20, 56].

4.4.7 CSP and poly(I·C)LC induce humoral and cellular responses in NHPs

Based on the results from the mouse studies, we determined whether CSP1 and poly(I·C)LC could induce potent antibody and CD4+ T cell responses in NHPs. NHPs are a more useful animal model than mice for testing vaccine adjuvants because of greater similarities to humans, with respect to innate immune pathways and TLR expression. Rhesus macaques received either two or three immunizations of 100 μg CSP1 and poly(I·C)LC, and CD4+ T cell and antibody responses were assessed at different time points after immunization. For CD4+ T cell cytokine responses, a batch analysis was done on frozen PBMCs from various time points. As shown in Fig. S4.1 in the supplemental material, as with the mouse data in Fig. 4.2, the frequency of CSP-specific cytokine-producing CD4+ T cells is increased in NHPs that received three immunizations rather than only two immunizations (see Fig. S4.1A and B in the supplemental material), with a substantial portion of multifunctional cells secreting IFN-γ, IL-2, and TNF-α or IFN-γ and TNF-α (Fig. S4.1C and D). There were also robust CSP antibody titers that were maximal after two immunizations with CSP1 and poly(I·C)LC. Waning antibody titers could be boosted with a third immunization but did not exceed the levels that were reached after two immunizations (Fig. S4.1E and F). Moreover, in the IFA assay, sera from animals that received three immunizations contained antibodies with a stronger binding capacity than those from animals that received two immunizations (see Table S4.1 in the supplemental material). Therefore, we conclude that the CSP1 protein and poly(I·C)LC are strongly immunogenic in NHPs.

4.5 Discussion

Currently, the RTS,S vaccine administered with AS01 is the most advanced malaria vaccine, providing ~30 to 50% protection in humans [2, 3]. The recent results of the pivotal phase III clinical
efficacy trial were a major breakthrough in establishing that a protein/particle-based vaccine with a well-formulated adjuvant system could achieve some protective efficacy against a parasite infection and confirms this approach as a first step toward developing a successful malaria vaccine. In the study presented here, we compared various full-length CS proteins for their ability to induce strong immunity and protection. Such proteins were provided by four partners, in partnership with MVI, to enable direct comparison and down-selection of one or more proteins for further development, thereby providing a potentially simpler formulation than RTS,S/AS01 with comparable or improved efficacy.

The data presented here using poly(I·C)LC as an adjuvant show a striking difference between the immunogenicities elicited by E. coli-derived CSP and yeast-derived CSP. The relatively low responses to the yeast-derived protein were surprising, since RTS,S (as well as other successful protein-based vaccines) is produced in yeast [57] and one of the yeast-derived proteins used in this study when given with Montanide did elicit potent antibody titers in mice (data not shown), showing that at least for antibody responses, the yeast-derived protein was immunogenic. We speculate that differences in the amino acid sequences or mannosylation sites of CSPs expressed in yeast and E. coli may have accounted for the limited immunogenicity. Indeed, one of the yeast-derived proteins induced higher antibody and CD4+ T cell responses when the same amino acid sequence was expressed in E. coli (data not shown). However, modifications in the mannosylation sites did not significantly alter the immunogenicity of yeast-expressed CSP (data not shown). Therefore, the mechanistic basis for the differences in immunogenicity of yeast- and E. coli-expressed CSPs used in this experiment is not entirely clear. In view of these results, CSP1 was used with the two adjuvants, poly(I·C)LC and GLA-SE, for the remainder of the studies.

Both poly(I·C)LC and GLA-SE elicited strong and comparable levels of CSP-specific IgG antibody titers, which conferred similar degrees of protection against high-dose sporozoite challenge. However, poly(I·C)LC induced a substantially higher frequency of CSP-specific CD4+ T cell cytokine responses than GLA-SE. Moreover, such responses were comprised of a large percentage of multifunctional cells secreting IFN-γ, IL-2, and TNF-α, which have been proposed to contribute to
antimalarial protection after RTS,S/AS01 vaccination [20, 23]. Of note, while vaccination with CSP (P. vivax) and GLA-SE in mice has been shown to induce a robust CD4+ T cell effector response [58], the same vaccine induced monofunctional (IL-2 only) CD4+ T cell responses in NHPs [49]. In contrast, we show that CSP and poly(I·C)LC induced robust multifunctional Th1 CD4+ T cell responses in both mice and NHPs. These data highlight potential differences in the magnitudes and quality of CD4+ T cell responses induced in mice and NHPs and are consistent with the results of other studies showing poly(I·C) or poly(I·C)LC to be an especially potent adjuvant in NHPs for such responses [34, 59]. Finally, it is notable that despite the dramatic differences in the CD4+/Th1 responses between poly(I·C)LC and GLA-SE in mice, the antibody titers were similar. These data highlight the possibility that adjuvants can differentially mediate effects on antibody and Th1 immunity, which is currently being investigated.

In terms of correlates of protection, both antibody and CD4+ T cells have been described to occur in mice or humans with CSP vaccines. Here, we show that the antibody titers using poly(I·C)LC or GLA-SE strongly correlate with protection (Fig. 4.7C). Moreover, serum transfer from CSP-immunized animals reduced the parasite burden after high-dose i.v. challenge. Thus, antibodies are necessary and sufficient in this model. Of note, protection was best with the higher dose of CSP (20 μg), which induced an ∼1-log increase in antibody titer compared to that induced by the lower dose of protein (2 μg). It is notable that, in humans, 25 μg or 50 μg of RTS,S induced antibody titers better than and comparable to those induced by 10 μg, suggesting some dose effect similar to what we observed in this study [60]. This suggests that there may be a threshold for the amount of CSP-specific antibody required to provide or increase protection. Indeed, when we analyzed antibody titers from protected and nonprotected animals (Fig. 4.7C and 4.8B), there was a threshold for the amount of antibody required to mediate protection after low- or high-dose challenge. This is consistent with data from humans in which anti-CSP antibody titers needed to be above 40 EU/ml [56] to induce protection. In addition, we show that nonprotected mice had a significantly delayed prepatent period compared to the control animals (Table 4.2). Accordingly, studies using RTS,S and different adjuvants
in humans also show that the prepatent period can be delayed. At present, it still is not clear whether this delay might play a role in improving the clinical outcome of a malaria infection [20, 54, 55].

Regarding the role of cellular immunity in mediating protection, previous studies have shown that CD4+ T cells specific for *P. yoelii* CSP eliminate infected hepatocytes in vitro and, when adoptively transferred, mediate protection *in vivo* [61, 62]. The protective role of CD4+ effector cells against malaria was further substantiated in other murine and human malaria models [22, 63, 64]. In data not shown, antibody depletion of CD4+ T cells in mice immunized with CSP1 and poly(I·C)LC at the time of sporozoite challenge resulted in only modestly reduced protection. Hence, in our model, the data support a far more critical role for CSP antibodies in mediating protection than for CD4+ effector T cells. Nevertheless, human studies using the AS02 or AS01 adjuvant showing that improved CD4+ T cell responses were associated with improved protection after challenge with *P. falciparum* [20, 22] provide evidence for the importance of these T cells. Therefore, the ability of poly(I·C)LC to elicit such potent CD4+ T cells in blood, liver, and spleen may be of importance in humans through a variety of mechanisms. Indeed, IFN-γ has been shown to inhibit the development of liver-stage malaria in various animal models [52, 65]. That would provide a potential advantageous role for poly(I·C)LC, based on the high frequency of those cells detected in the livers of immunized mice.

There is also strong evidence for cytotoxic CD8+ T cells in mediating protective immunity against liver-stage malaria [18, 46, 66, 67]. As our study used a strain of mice in which there was no major histocompatibility complex (MHC) class I recognition of *P. falciparum* CSP peptides, we did not detect any CD8+ T cell responses. However, we and others have previously shown that type I IFN is essential for mediating cross-priming of protein in mice [68, 69], highlighting the potential advantage of poly(I·C)LC over other adjuvants [25]. Nevertheless, while adjuvants that induce type I IFN should be preferred to induce CD8+ T cell responses upon protein vaccination, virus-based vaccines seem preferable if robust CD8+ T cell immunity is required. Improving how the protein is formulated and targeting it to specific dendritic cell subsets may ultimately improve the capacity of such vaccines to consistently and efficiently induce CD8+ T cells.
In conclusion, we show that a full-length *P. falciparum* CS protein when combined with poly(I-C)LC or GLA-SE induces potent immunity and protection in mice. Moreover, poly(I-C)LC is an effective adjuvant for eliciting such responses in NHPs, providing a predictive model for what might occur in humans [20, 21, 70, 71]. It is possible that the increased breadth of CSP responses with a full-length CSP (compared to the CSP responses with truncated RTS,S) and/or the enhanced CD4+ Th1 immunity that would potentially be elicited by poly(I-C)LC would enhance protection and/or durability. Whether a full-length protein vaccine with an adjuvant would substantially improve the outcome over the current platforms of RTS,S/AS01 and RTS,S/AS02, which have the advantages of being particles with well-formulated adjuvants and of being safe and scalable, remains a question for further development. Our study shows that a protein platform can provide effective protection against malaria. The key question for future studies is whether this protection will also provide a prolonged durability compared to that of the RTS,S vaccine.

### 4.6 Acknowledgements

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We thank MVI for funding and facilitating this comparative study by identifying and accessing the proteins and ensuring agreement of all partners on the final study design. We thank Gennova Biopharmaceuticals and WRAIR for providing the proteins CSP1 and CSP2, respectively.
Figure 4.1 Graphic diagrams of the characteristics of four different CSPs. Full-length CSP consists of the signal peptide (SP), two highly conserved motifs at the N- and C-terminal ends of the protein, region 1 and region 2 plus (R1 and R2), tandem repeats (37 NANP repeats), and the GPI anchor signal (GPI). CSP1 and CSP2 were produced by expression in *Escherichia coli*, CSP3 and CSP4 by expression in *P. pastoris*.
Figure 4.2 CD4+ T-cell responses in mice immunized with full-length CSP and poly-ICLC. C57BL/6 mice (n = 4) were immunized twice, 3 weeks apart, with either 2 μg or 20 μg of various CSPs and 50 μg poly(I·C)LC. Naive mice and mice that received poly(I·C)LC alone or 20 μg of each protein alone served as controls. Four weeks later, T cells in spleens were analyzed for cytokine production by flow cytometry. Frequencies of CSP-specific IFN-γ, IL-2, TNF-α, or IL-10-producing CD4+ T cells are shown for protein-only and control mice (A) and mice that received either 2 μg (B) or 20 μg (C) of CSP with poly(I·C)LC. Data are expressed as means ± SD and are representative of two independent experiments. *, P < 0.05. The color of the asterisk indicates the group to which the value is being compared.
Figure 4.3 Antibody responses in mice immunized with full-length CSP and poly-ICLC. C57BL/6 mice (n = 4) were immunized as described in the legend of Fig. 4.2. At the time of tissue harvest, sera were collected and analyzed for CSP-specific IgG antibodies in protein-only and control mice (A) and mice that received either 2 µg (B) or 20 µg (C) of CSP with poly(I:C)LC. Antibodies were measured against part of the repeat region ([NANP]₆). Data points are graphed as geometrical means of endpoint titers and are representative of two independent experiments. *, P < 0.05. The color of the asterisk indicates the group to which the value is being compared.
Figure 4.4 Mice immunized with 3 times with 20 μg CSP1 and poly-ICLC significantly inhibit sporozoite infection. C57BL/6 mice (n = 6) were immunized two or three times, 3 weeks apart, with 2 or 20 μg of CSP1 and 50 μg poly(I·C)LC. Naive mice and mice that received poly(I·C)LC alone or 20 μg of CSP1 alone served as controls. Four weeks later, mice were challenged with 1.5 × 10⁴ Pb-CS(Pf) sporozoites. Parasite burden was determined 40 h postchallenge by counting the copies of 18S rRNA in the livers by RT-PCR. Shown are comparisons of levels of protective immunity in mice that received either two or three immunizations of 20 μg CSP1 and poly(I·C)LC (A) or three immunizations with 2 μg or 20 μg CSP1 and poly(I·C)LC (B). Data points are graphed as geometrical means and are representative of two independent experiments *, P < 0.05; **, P < 0.01. The color of the asterisk (A) or the length of the bar (B) indicates the group to which the value is being compared.
Figure 4.5 Anti-CSP antibodies mediate protection in mice immunized with CSP1 and poly-ICLC. (A) C57BL/6 mice (n = 4 to 6) were immunized two or three times, 3 weeks apart, with 2 or 20 μg of CSP1 and 50 μg poly(I·C)LC. Naive mice and mice that received poly(I·C)LC alone or 20 μg of CSP1 alone served as controls. Two weeks later, sera were collected and CSP-specific IgG antibodies were determined. Data points are graphed as means ± SD. *, P < 0.05. The values for all control groups are significantly lower than those for vaccine groups. (B) Serum from mice immunized three times with CSP1 and poly(I·C)LC or serum from naive mice was transferred into naive mice at the time of challenge with 1.5 × 10^4 P. berghei CS(Pf) sporozoites. Vaccinated mice (CSP1 and poly(I·C)LC) served as a positive control. Forty hours postchallenge, parasite burden was determined by counting the copies of 18S rRNA in the livers by RT-PCR. Black bars show the geometrical means. **, P < 0.01. Results are representative of at least two independent experiments.
Figure 4.6 Polyfunctional analysis of CD4+ T cells in mice immunized with CSP and poly(I·C)LC or GLA-SE. C57BL/6 mice (n = 6 to 18) were immunized three times, 3 weeks apart, with the indicated doses of CSP1 with either 50 μg poly(I·C)LC or 5 μg GLA-SE as the adjuvant. Mice that received poly(I·C)LC, GLA-SE, or CSP1 alone, mice that received CSP1 and SE, or naive mice served as controls (not shown). CD4+ T cells in blood, spleen, and liver were analyzed for cytokine production by flow cytometry. (A) Frequencies of CSP-specific IFN-γ-producing CD4+ T cells in the blood 7 days after the third immunization; (B, C) frequencies of CSP-specific IFN-γ-, IL-2-, TNF-α-, or IL-10-producing CD4+ T cells in the spleen (B) and liver (C) 2 weeks after three immunizations; (D) relative proportions of each individual combination of IFN-γ-, IL-2-, or TNF-α-producing cells in the spleen and liver 2 weeks after three immunizations. ND, not determined (because the frequency was too low). The black circle represents the percentage of CD4+ T cells that make IFN-γ. Bars show the means ± SD. *, P < 0.05; **, P < 0.01. All control groups had values below the limit of detection. The color of the asterisk indicates the group to which the value is being compared. Results are representative of two independent experiments.
**Figure 4.7** Antibody titers, sporozoite challenge and correlation of humoral and cellular responses with parasite liver burden in mice immunized with poly-I:ClLC or GLA-SE. C57BL/6 mice (n = 6) were immunized three times, 3 weeks apart, with the indicated doses of CSP1 together with either 50 μg poly(I·C)LC or 5 μg GLA-SE. Mice that received poly(I·C)LC, GLA-SE, or CSP1 (20 μg) alone or naive mice served as controls. (A) Two weeks after the third immunization, sera were collected and CSP-specific IgG, IgG1, and IgG2a antibodies were measured. Bars are graphed as means ± SD. All control groups showed significantly lower antibody titers than the vaccine groups. (B) Four weeks after the third immunization, mice were challenged with 1.5 × 10^4 Pb-CS(Pf) sporozoites. Forty hours postchallenge, parasite burden was determined by counting 18S rRNA copies in the livers by RT-PCR. Black bars show the geometrical means. The matrix (B) represents significances among various immunization groups. *, P < 0.05; **, P < 0.01; ***, P < 0.001. (C) Correlation between antibody titers and parasite burden in the liver. (D) Correlation between frequency of IFN-γ-producing CD4+ T cells in the blood (day 7) and parasite burden in the liver. (C and D) As indicated, Pearson’s r was calculated. Results are representative of at least two independent experiments.
Vaccine Binding of serum antibodies to sporozoites as determined by fluorescence intensity at an antibody dilution of:

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Table 4.1 Antibodies in sera from mice that were immunized with a high dose of CSP1 and adjuvant showed a strong binding capacity to sporozoites in vitro.

a Serum samples that were collected 2 weeks after the third immunization (as described in the legend of Fig. 4.6) were pooled by group. The serum pools at different dilutions were incubated on slides coated with fixed sporozoites. The slides were washed and incubated with a secondary fluorescently labeled antibody. Finally, slides were analyzed under a microscope. +++ very good binding; ++, good binding; +, weak binding; –, no binding. Results are representative of two independent experiments.
Figure 4.8 High anti-CSP antibody titers and sterile protection are significantly associated. C57BL/6 mice (n = 10 per group) were immunized three times, 3 weeks apart, with 20 μg of CSP1 together with 50 μg poly(I·C)LC as the adjuvant. Mice that received poly(I·C)LC or naive mice served as controls. (A) Five weeks later, mice were challenged i.v. with $1 \times 10^3$ Pb-CS(Pf) sporozoites. Starting on day 4, daily blood smears were taken and analyzed under a microscope. Kaplan-Meier plots show the time to detection of parasites in the blood for each vaccine group after the challenge. (B) Sera collected 3 weeks before challenge were analyzed for CSP-specific IgG antibodies. Endpoint titers are grouped by whether mice went on to be protected from challenge. *, P < 0.05. Results are representative of two independent experiments.
Table 4.2 Vaccination with CSP1 and poly(I·C)LC prevents 50% of mice from developing parasitemia after a low-dose sporozoite challenge.

C57BL/6 mice (n = 10/group) were immunized three times, 3 weeks apart, with 20 μg CSP1 together with 50 μg poly(I·C)LC as the adjuvant. Mice that received poly(I·C)LC only or naive mice served as controls. Five weeks after the last immunization, mice were challenged i.v. with $1 \times 10^3$ Pb-CS(Pf) sporozoites. Starting at day 4, daily blood smears were taken and observed under a microscope to look for the onset of parasitemia. Results are representative of two independent experiments.

<table>
<thead>
<tr>
<th>Immunization</th>
<th>No. of infected/no. of challenged mice</th>
<th>Prepatent period (days)</th>
<th>Protection (%)</th>
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<tr>
<td>20 μg CSP + poly(I·C)LC</td>
<td>5/10</td>
<td>5.8</td>
<td>50</td>
</tr>
<tr>
<td>Poly(I·C)LC</td>
<td>10/10</td>
<td>4.6</td>
<td>0</td>
</tr>
<tr>
<td>None (naive mice)</td>
<td>10/10</td>
<td>4.8</td>
<td>0</td>
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</tbody>
</table>
4.7 References


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CHAPTER 5
P. FALCIPARUM AND P. VIVAX EPITOPE-FOCUSED VLPS ELICIT STERILE IMMUNITY TO BLOOD STAGE INFECTIONS

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*contributed equally to this work
5.1 Abstract

In order to design *P. falciparum* preerythrocytic vaccine candidates, a library of circumsporozoite (CS) T and B cell epitopes displayed on the woodchuck hepatitis virus core antigen (WHcAg) VLP platform was produced. To test the protective efficacy of the WHcAg-CS VLPs, hybrid CS *P. berghei*/*P. falciparum* (Pb/Pf) sporozoites were used to challenge immunized mice. VLPs carrying 1 or 2 different CS repeat B cell epitopes and 3 VLPs carrying different CS non-repeat B cell epitopes elicited high levels of anti-insert antibodies (Abs). Whereas, VLPs carrying CS repeat B cell epitopes conferred 98% protection of the liver against a 10,000 Pb/Pf sporozoite challenge, VLPs carrying the CS non-repeat B cell epitopes were minimally-to-non-protective. One-to-three CS-specific CD4/CD8 T cell sites were also fused to VLPs, which primed CS-specific as well as WHcAg-specific T cells. However, a VLP carrying only the 3 T cell domains failed to protect against a sporozoite challenge, indicating a requirement for anti-CS repeat Abs. A VLP carrying 2 CS repeat B cell epitopes and 3 CS T cell sites in alum adjuvant elicited high titer anti-CS Abs (endpoint dilution titer >1x10^6) and provided 80–100% protection against blood stage malaria. Using a similar strategy, VLPs were constructed carrying *P. vivax* CS repeat B cell epitopes (WHc-Pv-78), which elicited high levels of anti-CS Abs and conferred 99% protection of the liver against a 10,000 Pb/Pv sporozoite challenge and elicited sterile immunity to blood stage infection. These results indicate that immunization with epitope-focused VLPs carrying selected B and T cell epitopes from the *P. falciparum* and *P. vivax* CS proteins can elicit sterile immunity against blood stage malaria. Hybrid WHcAg-CS VLPs could provide the basis for a bivalent *P. falciparum*/*P. vivax* malaria vaccine.
5.2 Introduction

Malaria is an important tropical parasitic disease that kills more people than any other communicable disease with the exception of tuberculosis. The causative agents in humans are four species of *Plasmodium* protozoa: *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*. Of these, *P. falciparum* (*Pf*) is the most lethal. The vast majority of deaths occur among young children in Africa. *P. vivax* is the most prevalent species outside of sub-Saharan Africa and responsible for approximately 50% of all malaria cases worldwide [1]. Malaria is a public health problem today in more than 106 countries, inhabited by a total of 3.4 billion people- 50% of the world's population. Worldwide prevalence of the disease is estimated to be on the order of 135–287 million clinical cases each year. Mortality due to malaria is estimated to be in the range of 473,000–789,000 each year [2]. The *P. falciparum* malaria parasite has 14 chromosomes, an estimated 5,300 genes (many of which vary extensively between strains) and a complex four-stage life cycle as it passes from a mosquito vector to humans and back again. Furthermore, the natural *P. falciparum* infection does not result in immunity, and partial immunity occurs only after years of recurring infections and illnesses. Therefore, a vaccine must outperform the immune response to the natural infection. This complexity and the lack of suitable animal models have impeded vaccine development against both *P. falciparum* and *P. vivax*.

All stages of the *P. falciparum* malaria life cycle have been targeted for vaccine development, however, only preerythrocytic stage (i.e., the circumsporozoite (CS) protein [3] and the multiepitope (ME)-thrombospondin-related adhesion protein (TRAP) [4]) immunogens have been shown to elicit significant clinical efficacy. Only one CS vaccine candidate has reached phase III clinical trials, known as RTS,S, which targets the CS protein’s NANP repeat B cell epitopes and C-terminal T cell domains by fusing them to the hepatitis B surface antigen (HBsAg) [3]. RTS,S has been in development for over two decades and tested in multiple experimental and field trials [3,5–10]. In brief, protective efficacy for 3 doses of RTS,S formulated in a combination of three relatively potent adjuvants is reported as between 30 and 50% as judged by preventing clinical and severe malaria and the level of protection is dependent on malaria transmission intensity, age and time since vaccination [11]. Development of
RTS,S is a significant achievement and demonstrates that a recombinant subunit vaccine containing only isolated B and T cell epitopes from a single CS protein delivered on a heterologous carrier can elicit protection in humans. However, it is generally acknowledged that “second generation” vaccines will be necessary for full implementation of a malaria vaccine intended for all at-risk populations (i.e., endemic populations, travelers to endemic regions and the military) [3]. The search for second generation preerythrocytic vaccine candidates has included use of the entire CS protein, addition of other preerythrocytic antigens, new adjuvants, DNA delivery, viral vectors, prime-boost strategies, etc., with little success to date [12]. The development of the RTS,S vaccine has concentrated on formulation optimization using the same antigen construct for over two decades.

Our CS epitope-focused approach has been to test multiple constructs on alternative carrier platforms, chosen because they are more immunogenic than the HBsAg, namely the hepadnavirus nucleocapsid proteins (i.e., HBcAg and WHcAg), to carry CS-derived B and T cell epitopes [13–15]. In the current study 2 CS repeat B cell epitopes (NANP-based and NVDP-based), singly or combined, were genetically inserted onto the WHcAg carrier as were 3 CS-derived non-repeat B cell epitopes and 1 to 3 well-defined human T cell domains. Hybrid VLPs were screened based on VLP self-assembly, expression level, antigenicity, immunogenicity and most importantly protective efficacy in an infectious \textit{in vivo} model. Rodent malaria (\textit{P. berghei}) parasites bearing the extended repeat region of the \textit{P. falciparum} CS protein have been developed as an important preclinical tool for evaluating the efficacy of human CS protein-based vaccine candidates \textit{in vivo} [16]. Chimeric Pb/Pf sporozoites are fully infectious in mice and can be neutralized by Abs and/or T cells specific for the portions of the \textit{P. falciparum} CS protein replacing the \textit{P. berghei} CS protein. Employing the WHcAg combinatorial VLP technology combined with the Pb/Pf hybrid sporozoite challenge system has allowed us to develop and test a variety of WHcAg-CS hybrid VLPs. Selected VLPs carrying Pf-specific CS repeat, but not non-repeat B cell epitopes were capable of eliciting sterile immunity against blood stage infection. Using a similar strategy we also developed a hybrid VLP carrying \textit{P. vivax} CS repeat B cell epitopes and immunized
mice challenged with Pb/Pv hybrid sporozoites [17] demonstrated full protection from blood stage malaria.

5.3 Materials and methods

5.3.1 Animals

The (B10xB10.s) F1 mice used in VLP screening and immunogenicity evaluation were obtained from the breeding colony of the Vaccine Research Institute of San Diego (VRISD). The B6 mice used for protection studies were obtained from NCI (Fredrick, MD). The rabbits used for immunogenicity testing were New Zealand White rabbits obtained from ProSci Inc. (Poway, California). All animal care was performed according to National Institutes of Health standards as set forth in the Guide for the Care and Use of Laboratory Animals (2011). Animals at all facilities were monitored at least weekly.

5.3.2 Ethics Statement

Experimental procedures involving F1 mice were carried out at Explora BioLabs (San Diego, CA), where they were housed, and were conducted by VRISD and VLP Biotech researchers under approval of the Explora BioLabs Institutional Use Committee (Protocol Number EB13-028, approved for the currently described studies). Experimental procedures involving B6 mice were carried out at Johns Hopkins University and were approved by the Institutional Animal Care and Use Committee of the Johns Hopkins University (Protocol Number MO13H123, approved for the currently described studies). The experiment involving rabbits was approved by ProSci Institutional Animal Care and Use Committee (paper protocol on file approved 11/5/2009). Humane endpoints were used: in the blood-stage challenge studies, mice were monitored daily and euthanized when they were infected by malaria parasites. Animals were euthanized by CO₂ inhalation in accordance with the AMVA Guidelines on Euthanasia.
5.3.3 Recombinant WHcAg hybrid VLP Construction

The WHcAg and hybrid WHcAg VLPs were expressed from the pUC-WHcAg vector expressing the full-length WHcAg protein codon optimized for expression in *E. coli* [18]. The sequence for WHcAg (accession NC_004177) was cloned into the pUC19 vector. For inserting heterologous B cell epitopes, EcoRI-XhoI restriction sites were engineered into the open reading frame between amino acids 78 and 79 of the core protein gene. The engineered restriction sites add a Gly-Ile-Leu linker on the N-terminal side and a Leu linker on the C-terminal side of the inserted epitopes. For fusing heterologous T cell epitopes, an EcoRV restriction site was engineered at the 3' end of the WHcAg gene, which adds an Asp-Ile linker between the WHcAg gene and the fused epitopes. Epitopes were cloned into the VLP gene using synthetic oligonucleotides comprising the desired epitope coding sequence and the appropriate engineered restriction sites. The WHc(C61S) point mutation to reduce carrier antigenicity was constructed by PCR using primer mismatches to create point mutations. All WHcAg constructs were transformed into Alpha-Select competent *E. coli* (Bioline USA, Inc.) and confirmed by DNA sequencing. Inserted B cell epitope sequences were exactly matched to the CSP sequence from clone 7G8 of *P. falciparum* used in the construction of the CS(Pf) or the VK210 repeat from Salvador I strain of *P. vivax* used in the construction of the Pb/Pv hybrid sporozoites, respectively. The inserted T cell epitopes had only one conservative mismatch, i.e., Cys 283 of the CS protein in the new Pb-Pf-CSP-CT hybrid sporozoites (see below and S5.1B Fig) is a Ser in the VLPs with the TH and 3T T cell epitope sequences (see S5.2A Fig).

5.3.4 Purified Proteins and Synthetic Peptides

The VLP particles were expressed in Alpha-Select *E. coli* cells grown in Terrific Broth (Teknova, Hollister, CA). Cells were lysed by passage through an EmulsiFlex-C3 (Avestin, Ottawa, ON, Canada) and the lysate heated to 65°C for approximately 10 min, then clarified by centrifugation. The WHcAg particles were selectively precipitated by the addition of solid ammonium sulfate to approximately 45% saturation (277 g/L) and the precipitates were collected by centrifugation. Precipitated VLPs were redissolved in minimum buffer (10mM Tris, pH 8), dialized against the same
buffer and applied to a Sepharose CL4B column (5x100cm). Finally, VLPs were formulated in 20mM Tris, pH8, 100mM NaCl. Endotoxin was removed from the core preparations by phase separation with Triton X-114 [19,20]. Briefly, the VLP solution was made 1% Triton X-114 and incubated at 4°C for 30 min with mixing, incubated at 37°C for 10 min, centrifuged at 20KxG for 10 min and the protein recovered in the upper phase. This was repeated for 4 extractions. The purified VLPs were 0.2um sterile-filtered, characterized and aliquoted. Characterization typically includes custom ELISA, native agarose gel electrophoresis, PAGE, heat stability, circular dichroism and dynamic light scattering as previously described [18,21].

Recombinant CS protein was produced from the CS27 IV C clone (MRA-272, MR4, ATCC Manassas, VA) obtained through the Malaria Research and Reference Reagent Resource Center (www.mr4.org) and deposited by Photini Sinnis [22]. The open reading frame was moved to the pQE-60 vector (Qiagen) and transformed into M15 E. coli cells (Qiagen). Integrity of the gene was confirmed by DNA sequencing before purification by standard methods. Briefly, LB medium, supplemented with 2 g/L glucose, 25 μg/ml Kanamycin and 50 μg/ml Ampicilin, was inoculated with a 1:40 dilution of overnight culture. Bacteria were grown at 37°C to an A600 of 0.8–1.0, isopropyl β-D-1-thiogalactopyranoside added to a concentration of 100 mg/L, grown 3 hours longer and harvested by centrifugation. Cells were suspended in lysis buffer (25 mM Tris, pH 8, 0.3 M NaCl, 10 mM imidazole) and lysed by a single passage through an Avestin EmulsiFlex-C3 (Ottawa,ON, Canada). The lysate was clarified by centrifugation at 48,000 X G for 30 min, and applied to a nickel column (BioRad Profinity IMAC). Unbound proteins were removed by elution with the lysis buffer, then bound proteins were eluted in the same buffer containing 100 mM imidazole. This procedure yielded approximately 10mg of pure protein per liter of cultured bacteria.

Synthetic peptides derived from the WHcAg or CS sequences were synthesized by Eton Biosciences (San Diego, CA).
5.3.5 Immunizations and serology

Groups of mice were immunized intraperitoneally (i.p.) with the WHcAg hybrid VLPs (usually 10–20 μg) emulsified in incomplete Freund’s adjuvant (IFAd) for both antibody production and T cell experiments. The dose was varied when other adjuvants were used, i.e., saline (200 μg) and alum (100 μg). For antibody experiments, mice were bled retro-orbitally and sera pooled from each group. Periodically individual mouse sera were tested to confirm the fidelity of the pooled sera results. Anti-WHc and anti-insert immunoglobulin G (IgG) antibodies were measured in murine sera by an indirect solid-phase ELISA by using the homologous WHcAg (50 ng/well) or synthetic peptides (0.5 μg/well), representing the inserted sequence, as solid-phase ligands as described previously [15]. Serial dilutions of both test sera and preimmunization sera were made and the data are expressed as antibody titers representing the reciprocals of the highest dilutions of sera required to yield an optical density at 492 nm (OD 492) three times an equal dilution of preimmunization sera. IgG isotype-specific ELISAs were performed by using IgG1-, IgG2a-, IgG2b- and IgG3-specific peroxidase-labeled secondary antibodies (Southern Biotechnology, Birmingham, AL). Rabbits were immunized with WHcAg hybrid VLPs (200 μg in IFAd) and boosted either with 200 μg in saline or 100 μg in IFAd.

5.3.6 IFA

Indirect immunofluorescence assays (IFA) using both live and air-dried sporozoites were used to characterize and titrate antibody responses. Briefly, for live-sporozoite IFAs, 40,000 parasites were incubated on ice with different sera dilutions. Sporozoites were then washed 3 times with cold PBS with 1% BSA, suspended in 0.2 ml and placed into the well of a Lab-Tek chambered coverglass (Thermo Scientific Nunc, Rochester, NY). The chamber was then spun at 300 x G for 2 min and, after discarding the supernatant, 0.2 ml of PBS with 4% Paraformaldehyde (Sigma, Saint Louis, MO) were added. Samples were incubated for 1 h at room temperature, washed 3 times with PBS and incubated with secondary antibody [AlexaFluor 488 F(ab’)_2 fragment of goat anti-mouse IgG(H+L); 2 mg/ml; Invitrogen] for 30 min. Samples were then washed and green-fluorescent sporozoites were visualized.
using a Nikon Eclipse 90i fluorescent microscope. IFAs using air-dried sporozoites were performed as previously described [17].

5.3.7 **In vitro** T cell cytokine assays

Spleen cells from groups of 3 each of (B10xB10.s) F1 mice were harvested and pooled 4–6 weeks after immunization with the various WHcAg hybrid VLPs. Spleen cells (5×10⁵) were cultured with varying concentrations of WHcAg, CS or synthetic peptides derived from WHcAg or CS protein. For cytokine assays, culture supernatants were harvested at 48 h for IL-2 determination and at 96 h for interferon-gamma (IFNγ) determination by ELISA. IFNγ production was measured by a two-site ELISA using mAb 170 and a polyclonal goat anti-mouse IFNγ (Genzyme Corp., Boston, MA).

5.3.8 Development of *P. berghei* chimeric parasites expressing the C-terminal region of the *P. falciparum* CS protein (*Pb/Pf-CSP-CT*)

The new transgenic strain derived from *P. berghei* ANKA strain expressing the C-terminus of *P. falciparum* was generated using the plasmid pR-CSPfCT, which carries the C-terminal region of the *P. falciparum* CSP. This plasmid was derived from plasmid pIC-CSPfCT, which resulted from the replacement of the *P. berghei* CSP C-terminus with the C-terminal region of the 3D7 strain of *P. falciparum* CSP. Briefly, a 306-bp restriction fragment encompassing base pairs 715 to 1020 of the *P. berghei* CSP gene was excised from a modified version of pIC-CSwt [23] using the restriction enzymes SexAI and PacI and then replaced with a fragment comprising the *P. falciparum* CSP C-terminal region (S5.1A Fig). The *P. falciparum* CSP C-terminus was excised as a 312-bp SexAI-Paci restriction fragment from plasmid pPfCT (Genscript, Piscataway Township, NJ), synthesized to comprise the *P. falciparum* CSP C-terminal region. Thus, the CSP gene in the resulting plasmid, pIC-CSPPfCT, consists of the *P. berghei* N-terminal and repeat regions (base pairs 1 to 786) and the remainder of the *P. falciparum* CSP (base pairs 787 to 1026). We then excised the hybrid CSP gene from pIC-CSPPfCT as a KpnI-XhoI fragment and inserted it into the transfection plasmid, pR-CSPfCT. KpnI and SacI were used to release the inserted fragment from pR-CSPfCT prior to transfection of WT *P. berghei* (ANKA strain) parasites, as previously described [24]. Transgenic parasites were selected in Swiss Webster mice (NCI, Frederick, MD) by treatment with pyrimethamine (MP
Biomedicals, Solon, OH) in drinking water (0.07 mg/ml). Pyrimethamine-resistant parasites were then cloned by limiting-dilution. Successful recombination at the 5' and 3' ends of the locus was verified by PCR. The primers used to confirm 5' integration were 5'-F (TCACCCTCAAGTTGGGTAAAA) and PbPfCT-R (GCAGAGCCAGGCTTTATTCT); the primers to verify integration at the 3' end were 3'-F (TGTAATAATGTGTATGTTGTCG) and 3'-R (GTGCCCATACGACTTTTGCT). To verify that the cloned parasite population did not have contaminating WT P. berghei parasites, we developed a PCR assay using primers that flank the SexAI restriction site and then digested the resulting product with this enzyme. This restriction site is not present in the WT P. berghei CSP sequence but was inserted by replacement with our synthetic construct.

The primers used for this PCR analysis were PbWT NT-F (TGTTACAATGAAGGAAATGATAATAATTGTAT) and Pb 3'UTR-R (TCTTTTGGACATATATTCATTTTAGCA). Lastly, DNA isolated from the cloned chimeric parasites was sequenced to confirm the replacement of the P. berghei C-terminal region with the P. falciparum CSP C-terminus sequence. The sequence of the hybrid CS protein is provided in S5.1B Fig.

5.3.9 In vivo protection assays

To measure liver parasite load, C57Bl/6 mice were challenged i.v. with 10,000 Pb/Pf or Pb/Pv hybrid sporozoites. Forty-eight hours later livers were harvested to assess the parasite load by RT-PCR as previously described [25]. We assessed sterile protection by monitoring the mice for development of blood-stage parasites after feeding by infected Anopheles stephensi mosquitoes. Briefly, prior to challenging mice, the percentage of infected mosquitoes was determined by choosing at least 10 mosquitoes from the pool and examining each salivary gland for the presence of sporozoites. Based on this information, the number of sporozoites used for the challenge was determined. The mice were anesthetized by i.p. injection of 250 μl of 2% avertin prior to feeding Pb/Pf- or Pb/Pv-infected A. stephensi mosquitoes for five minutes. After feeding, all mosquitoes were examined for the presence of blood in their gut to determine the number that took a blood meal. Daily blood smears were performed starting at 4 days after challenge. For measuring protection mediated by antibodies, Pb/Pf (described as CS(Pf) in [16]) or Pb/Pv [17] hybrid sporozoites were used for the challenge when VLPs targeting P. falciparum and P. vivax epitopes, respectively, were used as immunogens. For assessing protective
efficacy of T cell epitopes, the new Pb-Pf-CSP-CT hybrid sporozoites described above were used for the challenge.

5.4 Results

5.4.1 Immunogenicity of VLPs carrying repeat versus non-repeat CS B cell epitopes

A number of interesting candidate epitopes outside the CS repeat domain have been described. For example, non-repeat CS B cell epitopes which have been shown to elicit in vitro neutralizing antibodies include: aa93–113 (lysine (K)-rich region), aa112-123 (conserved N1), and aa298-315 [26–28]. Similarly, a high percentage of adults and lesser numbers of children living in malaria endemic areas possess antibodies specific for CS C-terminal sequences that represent CD4⁺ and CD8⁺ recognition sites for human and murine T cells (i.e., UTC, TH3.R and CS.T3 regions) [29]. For several reasons the consideration of these non-repeat, CS B cell epitopes for vaccine design has been marginalized. Firstly, the immunodominance of the NANP and NVDP repeats and the established neutralizing efficacy of anti-CS repeat antibodies has reduced interest in non-repeat B cell epitopes somewhat [30–32]. Secondly, the induction of high titer CS-specific antibodies to non-repeat epitopes has been difficult with most immunogens. Our WHcAg platform technology allows insertion of virtually any CS sequence onto WHcAg. The resulting immunogens elicit high titer antibody even if the CS sequence is cryptic on the native CS protein. The Pb/Pf sporozoite technology allows evaluation of efficacy of these candidate vaccines by inserting the Pf B and T cell candidate epitopes in the CS protein of Pb sporozoites. The combination of these technologies permitted us to overcome the problems that have prevented analysis of the protective efficacy of CS non-repeat B and T cell sites in the past.

We produced, characterized and examined the immunogenicity of hybrid-WHcAg VLPs carrying the NANP/NVDP repeat epitopes and three selected non-repeat CS-specific B cell epitopes: the N1 region (aa 112–123); the K-rich region (aa 93–113); and the aa 298–315 region (see Fig 5.1) in comparison to full length rCS protein. Although the T cell response to CS protein is highly genetically
restricted in mice and humans [33,34], we avoided this problem by immunizing a high responder strain (H-2b). Immunization with the full length rCS protein elicited very high antibody production to the 2 repeat epitopes, NANP and NVDP. However, consistent with a cryptic nature of the non-repeat CS B cell epitopes, immunization with rCS protein elicited no antibody to the CS298-315 region, extremely low antibody production to the N1 region (i.e., 1:1000 titer) and relatively low antibody production to the K-rich region (i.e., 1:125,000 titer) after primary and secondary immunization (Table 5.1). In contrast, both repeat and non-repeat B cell regions “excised” from the CS protein and inserted onto hybrid WHcAg VLPs elicited high levels of anti-insert antibodies (i.e., at least 1:3x106 titers) (Table 5.1). Furthermore, the repeat and non-repeat anti-insert antibodies bound rCS protein in ELISAs. The repeat and non-repeat anti-insert antibodies also bound dry, hybrid Pb/Pf sporozoites to varying degrees as demonstrated by immunofluorescence assays (IFA) (Table 5.1). Interestingly, only the repeat-specific anti-insert antibodies (i.e., NANP/NVDP-specific) bound live sporozoites. These observations suggest that the three non-repeat B cell epitopes on the CS protein may be cryptic on intact, viable sporozoites.

5.4.2 Protective efficacy of VLPs carrying repeat versus non-repeat CS B cell epitopes

We also performed immunization/challenge experiments to determine the protective efficacy of hybrid WHcAg VLPs carrying the 2 repeat B cell epitopes (NANP/NVDP) and the three non-repeat B cell epitopes described above. As shown in Fig 5.2, immunization (2 doses of 20 and 10 μg) with VLPs carrying the repeat B cell epitopes protected mice challenged with 10,000 Pb/Pf sporozoites at a level of 98% in terms of parasite 18S rRNA copies detected in liver compared to mice immunized with a control hybrid WHcAg VLP carrying an irrelevant insert from the hepatitis B virus (HBV). In contrast, immunization (3 doses of 20, 10, and 10 μg) with the hybrid WHcAg VLPs carrying each of the three non-repeat B cell epitopes provided little to no protection (0–44%) against Pb/Pf sporozoite challenge despite the fact that high levels of anti-insert antibodies were present in the immunized mice (Fig 5.2 and Table 5.1). These results suggest that the non-repeat B cell epitopes may be cryptic on viable sporozoites in vivo. The results also suggest that it may not be productive to include these three
non-repeat B cell epitopes in a CS-VLP vaccine candidate. A caveat to this interpretation is that the non-repeat B cell epitopes in the context of the VLPs may not represent the epitope structures present within the native CS protein, although anti-non-repeat Abs do bind rCS and dry sporozoites.

5.4.3 Confirmation that CS repeat antibodies are predominant in providing protection

As an alternate approach to addressing the question of the importance of repeat vs. non-repeat CS-specific antibodies, we performed an experiment using rCS as the immunogen rather than hybrid VLPs. Mice were immunized with 2 doses of rCS (20 μg/10 μg) and the resulting antisera were pooled and pre-incubated with the 10,000 Pb/Pf sporozoites used for the challenge. This antiserum provided significant protection compared to sporozoites pre-incubated in normal mouse sera (NMS). However, if the anti-rCS antiserum was pre-adsorbed with repeat-containing VLPs (ΔNANP, NVDP) prior to being added to the 10,000 sporozoites, the protective efficacy was largely lost (S5.3 Fig).

5.4.4 WHc-Mal-78-UTC elicits protective Abs in rabbits

To examine protective efficacy of antisera from a second species, two rabbits were immunized with a VLP carrying the 2 CS repeat B cell epitopes and a malaria-specific human T cell epitope (UTC), designated WHc-Mal-78-UTC (Fig 5.3A). The antisera were passively transferred into naïve murine recipients. The recipients of anti-VLP rabbit sera were either challenged intravenously (i.v.) with 10,000 Pb/Pf sporozoites and parasite burden in the liver determined (Fig 5.3B) or challenged by the bites of infected mosquitoes and blood-stage parasitemia monitored over a 10–14 day period (Fig 5.3C). As shown in Fig 5.3, both rabbits (#73 and #74) produced high titer anti-NANP, anti-NVDP and anti-rCS Abs detected by ELISA and by IFA on hybrid sporozoites (Fig 5.3A). Antisera (0.5 ml) from both rabbits were passively transferred (i.v.) to naïve mice and the mice were immediately challenged with 10,000 Pb/Pf sporozoites (i.v.). Forty hours later the parasite liver burdens were determined. Passively transferred anti-VLP sera from both rabbits significantly reduced the parasite liver burden as compared to control rabbit sera, although rabbit #74 sera was most effective (Fig 5.3B). It is notable that rabbit #74 was primed with WHc-Mal-78-UTC emulsified in IFAd but boosted with the VLP in saline, whereas, rabbit #73 was primed and boosted in IFAd. This suggests that there may be no advantage
to the use of potent adjuvants after the primary injection of the VLPs. Rabbit #74 serum was chosen to passively transfer (0.2 ml) to murine recipients, which were challenged with the bites of from 3 to 12 Pb/Pf-infected mosquitoes over a five minute time frame. Blood stage parasitemia was monitored for the next 10–14 days. All 21 mice receiving the anti-WHe-Mal-78-UTC rabbit sera were totally protected from blood stage parasitemia regardless of exposure to 3, 6 or 12 infected mosquitoes. The 8 control mice exposed to 3 or 6 infected mosquitoes demonstrated infection by day 4 or 5 (Fig 5.3C). However, it is interesting to note that the adoptive transfer of 0.2 ml of a 1:3 dilution of rabbit #74 sera failed to protect mice against blood stage infection. These studies demonstrate that the protective efficacy elicited by WHe-Mal-78-UTC VLPs can be mediated solely by anti-CS repeat Abs, but a threshold level of protective Abs is required.

5.4.5 Addition of CS-specific T cell domains

An important goal is to add CS-specific T cell sites to vaccine candidates in order to prime CS-specific CD4+/CD8+ T cells as well as elicit CS-specific neutralizing antibodies. For this purpose, we added 1, 2 or all 3 (i.e., UTC, TH.3R, and CS.T3) well characterized human T cell domains to a standard hybrid WHeAg VLP carrying the 2 CS specific repeats (i.e. WHe-Mal-78). The T cell domains were added to the C-terminus of the hybrid WHeAg VLPs and all 3 hybrid VLPs were successfully produced and were shown to be approximately equally immunogenic in terms of anti-NANP and anti-NVDP antibody production (S5.4 Fig). In order to determine the contribution of CS-specific T cells to the protective efficacy of candidate VLP vaccines, the established protective efficacy of anti-NANP/NVDP antibodies had to be excluded. For that purpose, we constructed a hybrid WHeAg VLP carrying only the 3 T cell regions and devoid of the neutralizing CS repeat B cell epitopes designated WHe-Ct-3T. As shown in Fig 5.4, immunization with WHe-Ct-3T primed both WHeAg-specific and CS protein-specific CD4+ T cells as determined by cytokine production elicited by splenic T cells cultured with a panel of WHeAg and CS protein-specific proteins and peptides. Also note that WHe-Ct-3T immunization elicited low level Ab production to rCS and the TH.3R site, which is also a B cell epitope in addition to a CD4+ T cell epitope. Because the Pb/Pf hybrid sporozoites used in the
previous studies do not contain the *P. falciparum* T cell domains, a new transgenic Pb sporozoite (Pb/Pf-CSP-CT) containing the complete C-terminus (i.e., aa318-397) from the Pf CS protein was produced (Fig 5.1; S5.1 Fig). Therefore, we were able to perform an immunization/challenge experiment with WHc-Ct-3T VLPs. Although WHc-Ct-3T was immunogenic for both CS-specific B and CD4+ T cell epitopes (Fig 5.4), no protection against a 10,000 Pb/Pf-CSP-CT sporozoite challenge was elicited.

### 5.4.6 Effect of the 3 CS-specific T cell domains on protective efficacy

Because anti-NANP-specific Abs play a dominant role in protection, we compared the protective efficacy of a standard VLP (WHc-Mal5-78) containing only the 4 NANP repeats, which was previously shown to elicit significant protection against a Pb/Pf sporozoite challenge, with a VLP containing the NANP(NVDP(NANP)3 B cell insert in the loop of WHcAg plus all 3 T cell domains (WHc-Mal-78-3T) inserted at the C-terminus of WHcAg. Groups of 6 mice were primed and boosted with WHc-Mal5-78 or WHc-Mal-78-3T formulated either in saline only (200 μg VLPs), alum (100 μg VLPs) or Montanide ISA720 (50 μg VLPs) (Fig 5.5). Both VLPs elicited significant reduction in parasite liver burden (at least 90% reduction in parasite 18S rRNA copies in liver) in all three formulations compared to naïve challenged control mice (Fig 5.5A). However, the VLP carrying the three T cell domains (WHc-Mal-78-3T) elicited statistically superior protection in saline (99.1% vs 95% protection) and in alum (99.2% vs 91.7% protection) compared to the (NANP)4 B cell only-containing VLP (WHc-Mal5-78). Both VLPs were equally protective when formulated in Montanide ISA720 (Fig 4.5A). Anti-CS, anti-NANP, and anti-NVDP antibodies were measured by ELISA and IFAs were performed to determine if differential antibody levels would explain the superior protective efficacy of the WHc-Mal-78-3T VLP formulated in saline and alum (Fig 5.5B). No significant serological differences were noted between the two VLPs. However, there was a trend towards higher titer anti-NVDP repeat antibodies in the WHc-Mal-78-3T-immunized groups, especially when formulated in alum. This was expected because the WHc-Mal5 VLP does not contain the NVDP repeat. However, the polyclonal anti-NANP antibodies elicited by the WHc-Mal5 VLP demonstrated cross-reactivity for the (NVDP)2 peptide. IgG isotype testing also revealed no significant differences between anti-CS
antibodies elicited by WHc-Mal5 and WHc-Mal-78-3T VLPs. This suggests that malaria-specific CD4+ T cells primed by immunization with the WHc-Mal-78-3T VLP may have contributed to the greater efficacy either indirectly by providing an additional source of T helper cell function or, more likely, by directly exerting a negative effect on liver stage development via cytokine production. Although the hybrid sporozoites used for challenge did not contain the Pf T cell domains engineered into the WHc-Mal-78-3T VLPs, the 3 T cell domains of *P. falciparum* and *P. berghei* share a significant degree of homology as shown in S5.5 Fig. In any event, the superior performance of the WHc-Mal-78-3T VLP elevated this VLP to a primary vaccine candidate.

### 5.4.7 A WHcAg-CS VLP in alum elicits sterile immunity to blood stage malaria

WHc-Mal-78-3T performed well in terms of reducing parasite load in the liver after a 10,000 Pb/Pf sporozoite challenge (up to 99.98% reduction, Fig 5.5), however, to determine if this level of reduction in liver burden is sufficient to yield full protection from blood stage parasitemia an immunization/challenge experiment monitoring blood stage parasitemia as the final endpoint is required because a single surviving sporozoite infecting the liver can result in a blood stage infection [35]. For this experiment we modified WHc-Mal-78-3T by a point mutation (C61S) in the WHcAg, which eliminated the intermolecular disulfide bond at residue 61. The C61S mutation in WHcAg-hybrid VLPs was chosen because it can reduce anti-WHc (carrier-specific) antibody production and/or increase anti-insert antibody production.

Groups of 10 mice each were immunized and boosted with 100 μg of the WHc(C61S)-Mal-78-3T VLP either formulated in alum, alum+QS-21, or primed with an emulsion of Montanide ISA 720 (50%) and boosted in alum. The control group was primed with 100 μg of WHcAg (no insert) emulsified in Montanide ISA 720 and boosted in alum (Fig 5.6). Six weeks after the boost mice were challenged by exposure to the bites of 12 Pb/Pf-infected mosquitoes for 5 minutes. This method of challenge was chosen because it represents a more physiologically relevant route of infection as compared to i.v. injection of sporozoites. Blood was sampled over the next 14 days and examined for parasitemia. As shown in Fig 5.6, 10 of 10 WHcAg-immunized control mice became positive for blood
stage malaria within a mean of 4.4 days. In contrast, 0 of 9 mice immunized with WHc(C61S)-Mal-78-3T formulated in alum+QS-21 became infected; 1 of 10 mice immunized in Montanide/alum became infected; and 2 of 10 mice immunized in alum became infected. The 3 of 29 mice in the experimental groups that did become infected demonstrated delayed parasitemia (mean of 6.0 days), suggesting a possible elimination of 99% of sporozoites given that 90% elimination is required to obtain a one day delay in developing a patent blood stage infection. The serology of each group pre-challenge and of the survivors three months post-challenge is shown in S4.1 Table. Although anti-CS Ab titers decreased over time, anti-CS Abs were still in excess of 1x10^6 endpoint titers three months post-challenge in all adjuvant groups. The apparent lack of a boost to the anti-CS Ab titers may reflect the low immunogenicity of the protein in the context of the parasite infection.

**5.4.8 Preliminary evaluation of a VLP carrying *P. vivax* CS epitopes**

A hybrid WHcAg VLP carrying 2 copies each of both variants of type 1 (VK210) CS repeat epitopes from *P. vivax* parasites was constructed (WHc-Pv-78). In *vivo* protective efficacy was evaluated using hybrid *P. berghei/P. vivax* (Pb/Pv) sporozoites expressing the repeat region of the *P. vivax* CS protein (both VK210 variants) [17]. Immunization with 2 doses of varying amounts of WHc-Pv-78 VLPs in either incomplete Freund's adjuvant (IFAd) or alum elicited high titer anti-CS PV repeat antibodies as detected on solid phase peptide and verified by IFA assay on Pb/Pv hybrid sporozoites (S4.2 Table). Immunization with 100 μg of WHc-Pv-78 in IFAd elicited extremely high titer anti-CS Pv repeat antibodies (1.5x10^8). This immunization schedule was chosen to examine the protective efficacy of WHc-Pv-78 VLPs against experimental liver infection as well as blood stage infection with hybrid Pb/Pv sporozoites in mice. As shown in Fig 5.7A, immunization with WHc-Pv-78 VLPs provided 99% protection in terms of parasite 18S rRNA copies detected in the liver compared to mice immunized with the WHcAg carrier after challenge with 10,000 Pb/Pv sporozoites. To determine if this level of reduction in liver parasite burden was sufficient to provide sterile immunity to blood stage infection, WHc-Pv-78 VLP-immunized and WHcAg-immunized mice were challenged by exposure to the bites of 10 Pb/Pv-infected mosquitoes for 5 minutes. Whereas 4 of 5 control mice became infected
in a pre-patent period of 4.5 days, 0 of 4 WHc-Pv-78-immunized mice were infected over an observation period of 14 days (Fig 5.7B).

5.5 Discussion

An epitope-focused approach was utilized to present selected B and T cell epitopes from the CS protein of the *P. falciparum* malaria parasite on the heterologous WHcAg carrier platform. Two repeat and three non-repeat B cell epitopes from the CS protein were inserted into the WHcAg carrier. Although all hybrid VLPs elicited high levels of anti-insert Abs, only hybrid VLPs carrying the CS repeat B cell epitopes (NANP and NVDP) provided significant protection of the liver (98%) against an experimental challenge with hybrid Pb/Pf sporozoites in mice. Whereas, anti-CS repeat and anti-CS non-repeat Abs bound dry Pb/Pf sporozoites, only anti-CS repeat Abs bound viable sporozoites. This data suggests that the 3 non-repeat B cell epitopes are poorly expressed or cryptic on viable sporozoites. Addition of 3 well-defined malaria-specific human T cell domains to the hybrid VLPs enhanced protective efficacy in the liver as well as primed malaria-specific CD4+ T cell cytokine production. However, immunization with hybrid VLPs carrying only the 3 malaria-specific T cell domains was unable to provide protection, indicating that anti-CS repeat Abs are necessary for protection. In fact, anti-CS repeat Abs are sufficient for protection against liver stage as well as blood stage infection as demonstrated by sterile immunity to blood stage infection following adoptive transfer of rabbit anti-VLP antiserum. Furthermore, active immunization with a hybrid VLP, designated WHc(C61S)-Mal-78-3T, elicited sterile immunity to blood stage infection in 26 of 29 mice and delayed parasitemia in the remaining 3 mice, depending on adjuvant formulation. The alum + QS-21 formulation was the most efficient adjuvant and yielded 100% protection from blood stage infection. The results indicate that immunization with an epitope-focused VLP containing selected B and T cell epitopes from the *P. falciparum* CS protein formulated in adjuvants acceptable for human use can elicit sterile immunity against blood stage malaria if sufficient anti-CS protective Abs are produced. The
appropriate adjuvant formulation to achieve protective Ab levels in humans as well as VLP dose will need to be determined in clinical trial.

In previous studies we demonstrated that CS repeat B cell epitopes from both *P. berghei* and *P. yoelii* murine parasites inserted onto the HBcAg conferred 80–100% protection against blood stage infection in immunized mice [13,14]. We extrapolated that strategy to *P. falciparum* CS-derived B and T cell epitopes and produced HBcAg-CS hybrid VLPs [designated V12.PF3.1[15]/ICC1132[36]] that were highly immunogenic in rodents and non-human primates, respectively. Unfortunately, a flawed Phase IIa clinical trial, in which a suboptimal dose of ICC1132 (5 μg equivalent of CS repeat B cell epitope) given in a single injection without a boost, did not permit the realistic efficacy of this hybrid VLP to be determined in humans [37]. In this current study we developed a species variant of the HBcAg, the WHcAg, as a platform for *P. falciparum*/*P. vivax* CS epitopes in order to avoid the disadvantages of using a carrier derived from a human pathogen [38]. This is especially important for a malaria vaccine because HBV and malaria are co-endemic in many regions of the world and chronic HBV carriers are often immune tolerant to both HBcAg and HBsAg (note that the HBsAg is used as a carrier in the RTS,S vaccine). Additional modifications to the WHc(C61S)-Mal-78.3T vaccine candidate compared to ICC1132 are: the use of the full length WHcAg to accommodate the encapsidation of ssRNA as a TLR7 ligand, which enhances immunogenicity [39]; incorporation of additional malaria-specific T cell domains; and mutation of the WHcAg cysteine 61, which eliminates intermolecular disulfide bonds common to both WHcAg and HBcAg. The C61S mutation in hybrid VLPs can reduce anti-WHc (carrier-specific) Ab production and/or increase anti-insert Ab production. For these reasons the WHcAg is a superior choice to the HBcAg as a VLP platform for malaria CS epitopes. It would be useful to directly compare CS-based vaccine candidates, including the industry-standard RTS,S, in a standardized hybrid Pb/Pf challenge model as a preclinical selection tool. A number of CS-based vaccines have been developed recently [40–43]. Typically, protective efficacy has been determined using different challenge methods and different chimeric rodent parasites, making comparisons difficult. For example, hybrid Pb/Pf parasites used herein express an extended CS repeat
region from the Pf CS protein [16], whereas, other hybrid Pb/Pf parasites used for challenge experiments [40,42] contain the full-length Pf CS protein [44].

In the absence of head-to-head comparative studies to date, the WHc(C61S)-Mal-78-3T candidate embodies a number of unique characteristics that may be advantageous in comparison to other CS-vaccine candidates. The enhanced immunogenicity and protective efficacy of WHc(C61S)-Mal-78-3T suggests that the suboptimal performance of a preerythrocytic vaccine candidate is not likely due to the selection of the CS repeat region as a target or to a paucity of B cell epitopes, but rather to insufficient production of protective Abs. For example, the RTS,S vaccine shares similar CS-specific B and T cell epitopes with WHc(C61S)-Mal-78-3T but the carrier moieties are markedly different. WHc(C61S)-Mal-78-3T efficiently self-assembles into hybrid VLPs, which are stable even at 65°C, whereas the HBsAg-based RTS,S requires the addition of excess native HBsAg particles. Compared to the HBsAg, hepadnavirus nucleocapsids are inherently more immunogenic in mice and humans during natural infection or after immunization [45–48], are less susceptible to MHC restricted non-responsiveness and can function as T cell-independent immunogens [45,48]. Finally, use of the WHcAg would circumvent HBV-specific immune tolerance present in populations endemic for HBV that are often endemic for malaria as well [38]. As a practical matter, WHcAg-CS VLPs are produced in high yields in bacteria and are extremely heat stable, therefore, production costs are relatively low and no cold-chain is required.

The immunogenicity and protective efficacy of WHcAg hybrid VLPs carrying P. vivax CS repeat B cell epitopes demonstrates the power and flexibility of the WHcAg VLP combinatorial technology, especially when combined with the hybrid Pb/Pv sporozoite technology [17] used for challenge experiments. In fact, WHc-Pv-78 VLPs represent the first example of a P. vivax immunogen capable of eliciting sterile immunity to blood stage infection in this hybrid Pb/Pv challenge model after active immunization. It is notable that passive transfer of 400 μg of the P. vivax CS-specific Mab 2F2 was not able to confer sterile immunity against a 5 min exposure to the bites of 4 Pb/Pv-infected mosquitoes [17]. The failure of Mab 2F2 to transfer sterile immunity demonstrates that Pb/Pv
sporozoites are highly infectious and represent a stringent model to evaluate the protective efficacy of
*P. vivax* CS-targeted immunogens such as WHc-Pv-78. The CS repeat epitopes and variants from other
*P. vivax* strains can also be inserted onto WHcAg-CS hybrid VLPs. Further development of *P. falciparum*
and *P. vivax*-specific WHcAg-CS hybrid VLPs would allow their use either separately or combined in
a bivalent malaria vaccine, depending on the regional malaria threat.
**Figure 5.1** Targeted T and B cell epitopes on CS protein. (A) Schematic representation of the existing (upper panel) and novel (lower panel) hybrid Pb/Pf sporozoites. Native *P. berghei* sequence is indicated by light shading and transgenic *P. falciparum* sequence by dark shading. Neutralizing or presumptive neutralizing B-cell epitopes are denoted by black bars and human and murine CD4+ T cell epitopes by white bars. The “repeats” epitope (140–159) is part of a much larger motif, delineated by the dotted line. Numbering is based on the amino acid sequence of CSP from the 7G8 clone. (B) VLPs described in the manuscript. S5.2 Fig presents the amino acid sequences of the epitopes. In summary, Mal: NANP and NVDP repeats; Mal5: NANP repeat only; Ct: carboxy-terminal; TH: TH.3R and UTC epitopes; 3T: insertion of all 3 T cell epitopes; C61S: Cys-to-Ser mutation at position 61 of WHcAg; Pv: *P. vivax* B cell repeats. Each of the epitopes is genetically inserted into the sequence of the VLP gene and a fully-assembled VLP consists of 120 homodimers, meaning each VLP presents 240 copies of the inserted epitope(s).
Table 5.1 Characterization of WHc-CS VLPs carrying repeat and non-repeat B cell epitopes. The listed WHcAg hybrid VLPs and full length rCS protein were used to immunize mice (2 doses; 20 μg and 10 μg in IFAd). Secondary antisera were pooled, serially diluted and analyzed by ELISA for binding to: solid phase WHcAg; repeat peptides (NANP) and (DPNANPNV); non-repeat peptides N1, Krich, CSP298-315; and rCS. Endpoint dilution titers are shown. Antisera were also evaluated by IFA on dry or viable sporozoites. The protective efficiency after in vivo challenge with 10,000 Pb/Pf sporozoites of mice immunized with the listed WHc-CS VLPs is also shown.
Figure 5.2 Comparison of protective efficacy of WHc-CS VLPs. Groups of mice were immunized with WHc-HBV negative control and CS-repeat (WHc-Mal-78) VLPs (2 doses of 20 μg and 10 μg in IFAd): CS-non-repeat VLPs (3 doses of 20 μg, 10 μg, 10 μg in IFAd). From 2 to 3.5 months after the last immunization dose all mice were challenged with 10,000 Pb/Pf sporozoites. Parasite 18S rRNA copy number in the liver was determined by qPCR 40 hours after infection. Circles represent individual mice and the bars represent mean values. % protection is based on mean values in comparison to the WHc-HBV negative control.
Figure 5.3 Immunogenicity of WHc-Mal-78-UTC in rabbits and protective efficacy of anti-VLP sera. (A) Animals were primed with 200 μg of WHc-Mal-78-UTC emulsified in IFAd and boosted at week 6 with 100 μg emulsified in IFAd (rabbit 73) or 200 μg in saline (rabbit 74). Serum was collected at the indicated time points and endpoint titers against NANP, NVDP and rCSP determined by ELISA. The sporozoite-specific IFA assay was performed on 18 week antisera and is represented by a star-shaped point. (B) Protection against liver stage Pb/Pf infection. Mice were injected with 500 μl of indicated rabbit antisera and challenged with 10,000 sporozoites i.v. shortly after receiving the antisera. Liver burden was determined by qPCR 40 hours after challenge. Control, normal mouse sera. P values are for Mann-Whitney U-test comparing in each case the 5 mice per group, ns = not significant at the 0.05 significance level. (C) Protection against blood stage Pb/Pf infection. 200 μl of sera from rabbit #74 or from a control naive rabbit were passively transferred to groups of 7 or 4 mice, respectively, by i.v. injection. Mice were then challenged by allowing 3, 5, 6 or 12 mosquitoes infected with Pb/Pf sporozoites to feed on the mice for 5 min. Mice were bled daily starting on day 4 post-challenge and blood-stage infection assessed by microscopy on stained blood smears.
Figure 5.4 WHc-Ct-3T primes malaria-specific as well as WHcAg-specific CD4+ T cells. To assess T cell priming, three mice were immunized with WHc-Ct-3T (a single 20 μg dose in IFAd) and 10 days later spleen cells were harvested and cultured as pools with varying concentrations of the indicated recall antigens. Culture supernatants were collected at day 2 for determination of IL-2 and day 4 for determination of IFNγ. The minimum concentration of each antigen necessary to yield detectable cytokine is shown. For antibody production, mice were immunized (20 μg/IFAd) and boosted (10 μg/IFAd).
Figure 5.5 Comparison of protective efficacy and serology for WHc-Mal5-78 (Mal5) vs WHc-Mal-78-3T (Mal-3T). Groups of 6 mice each were immunized with WHc-Mal5-78 or WHc-Mal-78-3T formulated in either saline, alum or Montanide ISA-720 and given a single booster injection. After the boost mice were challenged with 10,000 hybrid sporozoites. (A) Liver burden was assessed by determining parasite 18S rRNA copies in the liver. Circles represent individual mice, boxes represent mean values. P values are for Mann-Whitney U-test between groups for each formulation, with each group having six mice, except the Mal-3T in alum group, which had five mice. (B) Post-boost antibody levels in pooled sera were determined by ELISA using rCSP, NANP or NVDP peptides as solid phase ligands. IFA titers were performed on dry Pb/Pf sporozoites.
Figure 5.6 Protective efficacy of WHc(C61S)-Mal-78-3T immunization against P. berghei/P. falciparum blood stage malaria infection. Groups of ten mice were primed and boosted with 100 μg of WHc(C61S)-Mal-78-3T formulated in alum (250 μg/dose), alum + QS21 (20 μg/dose) or emulsified in Montanide ISA720 (50% vol/vol) and boosted in alum as indicated. (A) Time line showing schedule of prime, boost and challenge with 12 mosquitoes infected with Pb/Pf hybrid sporozoites allowed to feed on the mice for 5 min. After feeding, mosquitoes were examined for blood in the gut, confirming that multiple mosquitoes had fed on each animal. (B) Graphic representation of the percentage of mice remaining protected (i.e., free of blood stage parasites) during the 14 day monitoring period. (C) Tabular summary of results. One mouse from the Alum+QS21 group died before challenge.
Figure 5.7 Protective efficacy of WHc-Pv-78 immunization against P. berghei/P. vivax malaria infection. Mice were primed and boosted with 100 μg of WHc-Pv-78 or WHcAg emulsified in IFA (50% vol/vol). (A) Mice were challenged with 10,000 Pb/Pv sporozoites injected in the tail vein and liver infection determined by qPCR. P value is for the Mann-Whitney U-test with four mice in the WHc-Pv-78 group and five mice in the control WHc group. (B & C) Mice were exposed to the bites of 10 Pb/Pv-infected mosquitoes for 5 min, after which, mosquitoes were examined for blood in the gut to confirm that multiple mosquitoes had fed on each animal. Malarial infection was determined by blood smear during the 14 day monitoring period. Results are depicted in tabular (B) and graphic (C) forms.
5.6 References


CHAPTER 6
PROTECTIVE EFFICACY OF IMMUNE RESPONSES ELICITED BY FULL-LENGTH CIRCUMSPOROZOITE PROTEIN IN COMBINATION WITH A NOVEL CATIONIC LIPOSOMAL ADJUVANT FORMULATION
6.1 Abstract

Despite several years of extensive research, the development of a fully effective malaria vaccine has yet to be accomplished. RTS,S, currently the most advanced vaccine candidate, has shown potential to prevent a large number of malaria cases and deaths but significant improvements in protective efficacy are still needed. Importantly, several studies have shown that RTS,S induces protective antibody and CD4⁺ T-cell responses but its capacity to elicit CD8⁺ T cells is limited. In this study, we evaluated the protective effect of immune responses elicited by a novel cationic liposomal adjuvant system in combination with recombinant *P. falciparum* Circumsporozoite Protein (CSP). Using newly developed chimeric rodent malaria parasites expressing the full-length *P. falciparum* CSP, we demonstrate that this liposome-based protein-in-adjuvant formulation is capable of inducing robust antibody and CD8⁺ T-cell responses that strongly inhibit sporozoite infection and confer long-lasting sterilizing immunity. Importantly, the chimeric sporozoites we developed allowed us to generate evidence indicating that parasite-specific CD8⁺ T cells significantly enhance the protective effect of anti-CSP antibodies. Although CD8⁺ T cells have been long proven critical mediators of protective immunity against pre-erythrocytic malaria, the induction of cytotoxic responses by soluble proteins has been challenging. Our findings indicate that formulations capable of inducing strong antibody as well as CD8⁺ T-cell responses have significant potential for malaria vaccine development.
6.2 Introduction

Despite significant increased research efforts over the last 25 years, the fight against malaria still faces numerous challenges. Vector control methods such as insecticide-treated bednets and indoor residual spraying are presently threatened by mosquito resistance. In addition, the rapid spread of artemisinin resistance by *Plasmodium falciparum* in the Greater Mekong region represents a serious risk to malaria eradication and has urged the WHO to launch emergency response programs [1]. Thus, there is a critical need for new tools that can overcome the risks posed to the current malaria control efforts. It is acknowledged that the development of a fully-efficient malaria vaccine would be the most cost-effective solution to prevent infection and ultimately eradicate this disease. However, despite many years of extensive research and several clinical trials, the goal of a fully protective vaccine has remained elusive.

To date, RTS,S is the most advanced malaria vaccine candidate. This subunit vaccine is based on a portion of the *P. falciparum* Circumsporozoite Protein (CSP), incorporating the repeat and C-terminal regions of this protein, which are then fused to the hepatitis B virus surface antigen. Initial results of a Phase III clinical trial indicated that RTS,S had a vaccine efficacy of ~55% in children 5-17 months of age and ~31% in infants 6-12 weeks old, when assessed during 12 months after vaccination [2, 3]. However, the latest results of this large-scale trial showed that RTS,S-induced immunity wanes over time, reducing the number of cases by 36% in children and by 26% in infants, when assessed from month 0 to study end (median follow-up of 48 months for children and 38 months for infants) [4]. Nevertheless, RTS,S was capable of preventing an important amount of malaria-related hospital admissions and severe anemia cases [4]. And while this findings indicate that RTS,S can significantly contribute to malaria control when used with other prevention measures, improvements in vaccine efficacy are still needed.

Several studies have demonstrated that RTS,S is capable of inducing robust anti-CSP antibody titers that correlate with protection against sporozoite infection [5-8]. Clinical trials have also shown that RTS,S can elicit antigen-specific CD4+ T cells, although an association with protection has been
difficult to establish [9, 10]. Yet, several of these studies have failed to detect cytotoxic CD8+ T cell responses among vaccinated individuals [9-12]. This is a significant shortcoming of RTS,S as numerous studies have shown that CD8+ T cells are essential for eliminating malaria parasites that invade and replicate within hepatocytes. To address this limitation, prime-boost studies incorporating viral vectors or DNA constructs have been evaluated as possible ways to enhance RTS,S-induced cellular responses and protective capacity. However, these approaches have not resulted in considerable improvements in overall protective immunity and vaccine efficacy in humans [13-15]. It is unclear why this approach has so far failed to show significant efficacy. However, it is conceivable that some of the vectors used such as recombinant vaccinia virus, adenovirus and others are rendered poorly immunogenic in humans due to pre-existing immunity [16]. On the other hand, the design of these trials is deficient in that it is unknown whether the Class I Major Histocompatibility Complex (MHC) epitopes included in such vaccines can actually be recognized by protective human CD8+ T cells and, perhaps more critically, the Human Leukocyte Antigen (HLA) restriction of these putative epitopes remains mostly unknown. A fresh start for these studies would require, firstly, to define the MHC restriction of epitopes and, secondly, to perform vaccine trials with volunteers that express the appropriate HLA Class I molecule.

The induction of CD8+ T cells by soluble protein in adjuvant systems has remained a difficult task [17, 18]. However, a newly developed cationic liposomal adjuvant (CAF09) has recently demonstrated remarkable capacity to induce antigen-specific cytotoxic responses when tested in combination with different proteins and peptides [19]. Notably, CAF09 induces cross-priming by dendritic cells and CD8+ T cells elicited by immunizations with this CAF09 in combination with the E7 antigen from HPV16 are capable of conferring complete protection in a skin tumor model [19].

In this study, we characterized the protective capacity of immune responses induced by CAF09 in combination with full-length recombinant *P. falciparum* CSP. We demonstrate that immunizations with this protein-in-adjuvant formulation are capable of inducing potent anti-CSP antibody responses as well as antigen-specific CD8+ T cells. In addition, using newly developed chimeric rodent parasites,
we show that these immune responses are capable of strongly inhibiting sporozoite infection and conferring long-lasting sterile immunity.

6.3 Materials and methods

6.3.1 Mice

Five- to 8-week old female C3H/HeNCr MTV- or C57BL/6 mice were purchased from Charles River (Frederick, MD). All experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee of The Johns Hopkins University.

6.3.2 Recombinant *P. falciparum* CSP and CAF09

The full-length recombinant *P. falciparum* CSP (Pf rCSP) used for all immunizations was manufactured by Gennova Biopharmaceuticals (Pune, India) and kindly provided for this study by PATH-MVI. This protein is derived from the *P. falciparum* Indian strain IND637HDD1 and its use has been previously described [20]. The cationic adjuvant formulation CAF09 was developed by the Statens Serum Institut (Copenhagen, Denmark) and it consists of methylidioctadecylammonium (DDA)-liposomes stabilized with monomycoloyl glycerol (MMG)-1 in combination with Poly(I:C). The use of this formulation for animal immunizations has been previously described [19].

6.3.3 Immunizations

C3H/HeNCr MTV- mice (H-2Kk) were immunized with Pf rCSP in combination with CAF09. Briefly, for each immunization, 20 µg of Pf rCSP were mixed with CAF09 (DDA, 250 µg/dose, MMG-1, 50 µg/dose and Poly(I:C)an 0.5 mg/ml ) and administered by intraperitoneal (i.p.) injection on study days 0 and 14 (2-dose regimen) or days 0, 14 and 28 (3-dose regimen). The injection volume for each dose was 0.2 ml.

6.3.4 Assessment of anti-CSP antibody titers

Antibody titers in polyclonal sera from immunized mice were evaluated 10 days after the last dose of each immunization regimen. We used an enzyme-linked immunosorbent assay (ELISA) against synthetic peptides representing the repeat, N-terminal and C-terminal regions and of the *P. falciparum*
CSP. Briefly, MaxiSorp® ELISA plates (Thermo Scientific Nunc, Rochester, NY) were coated with 100 microliters of synthetic peptide (1 µg/ml) and incubated overnight at room temperature. The plates were then washed and incubated with a 1% (w/v) bovine serum albumin (BSA) (Sigma-Aldrich, Saint Louis, MO)/1X phosphate buffered saline (PBS) solution (1% BSA-PBS) for an hour at room temperature. After another washing step, wells were incubated for one hour at room temperature with serial dilutions of polyclonal sera. Plates were then washed and incubated for an hour at room temperature with a peroxidase-labeled goat anti-mouse (IgG H+L) secondary antibody (KPL, Gaithersburg, MD) at 0.5 µg/ml in 1% BSA-PBS. The assay was developed using a horseradish peroxidase substrate kit (KPL, Gaithersburg, MD), according to the manufacturer’s specifications.

6.3.5 Immunofluorescence assays

Indirect immunofluorescence assays (IFA) were used to assess anti-CSP titers in P. falciparum or transgenic sporozoites. In brief, poly-L-lysine-treated slides (Tekdon Inc., Myakka City, FL) were coated with a sporozoite suspension (4-6 x 10^5 parasites/ml) and allowed to air dry at room temperature. Polyclonal serum samples were then diluted in 1% BSA-PBS and incubated on slides for 30 min at room temperature in a humidity chamber. Slides were then washed with PBS-1% BSA and a secondary-antibody solution [AlexaFluor 488 F(ab')2 fragment of goat anti-mouse IgG(H+L); 2 mg/ml; Invitrogen] was added for 30 min at room temperature. Fluorescent sporozoites were visualized using an upright fluorescence microscope (Nikon Eclipse 90i).

6.3.6 Evaluation of CD8+ T-cell responses

CD8+ T-cell responses in spleens of individual mice were assessed 2-3 weeks after the last immunization. Single-cell suspensions (2 x 10^6 cells/well) were incubated with LM1 (H-2K^b) cells (5 x 10^5 cells/well) [21], pulsed with or without the synthetic peptide DYENDIEKKI (8 µM). Incubations were performed for 5-6 hours at 37°C in the presence of Brefeldin A and Monensin (BD Biosciences, San Diego, CA). Cells were surface stained with APC-anti-CD8 (eBioscience, San Diego, CA) and then treated with the Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences). FITC-anti-IFN-γ (eBioscience, San Diego, CA) was then used for intracellular staining.
6.3.7 Transgenic parasites

The protective effect of immune responses against the *P. falciparum* CSP *in vivo*, was tested using 2 new chimeric rodent parasite. In these chimeric lines the wild-type (WT) *P. berghei* csp gene was replaced with a *P. falciparum* csp construct.

To develop the chimeric line P.b.-P.f. CSP-FL CD8CT, a 784-bp restriction fragment encompassing base pairs 246 to 1029 of the *P. berghei* *P. falciparum* CSP NT csp chimeric gene was excised from the plasmid pIC-CSPPfNT [22] using restriction enzymes BbsI and PacI (New England Biolabs, Ipswich, MA). This portion was then replaced with a 943-bp fragment, which was released using the same restriction enzymes from the plasmid pHZ-PfCSP. Thus, the csp gene (1188 bp) in the resulting plasmid, pIC-CSPPfFL-CD8CT, consists of a full-length *P. falciparum* 3D7 CSP in which the signal sequence has been replaced with the one of the *P. berghei* CSP (base pairs 1 to 69). In addition, a single base-pair in the csp gene of plasmid pIC-CSPPfFL-CD8CT was replaced to incorporate a cytotoxic epitope that is not present in the *P. falciparum* 3D7 strain. This change was introduced in base pair 1079 by site-directed mutagenesis using a QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, La Jolla, CA). We then excised the csp gene from pIC-CSPPfFL-CD8CT as a KpnI-Pael fragment and inserted it into the transfection plasmid, pR-CSPPfFL-CD8CT. Lastly, XhoI and KasI were used to linearize pR-CSPPfFL-CD8CT prior to transfection of GFP-Luciferase *P. berghei* ANKA parasites [23], as previously described [24].

P.b.-P.f. CSP-FL CD8CT transgenic parasites were selected in Swiss Webster mice by treatment with pyrimethamine (MP Biomedicals, Solon, OH) in drinking water (0.07 mg/ml). Drug-resistant parasites were cloned by limiting-dilution. The successful recombination at the 5’ and 3’ ends of the modified locus was verified by PCR. The primers used to confirm 5’ integration were PbCS5’-F (TGCCCTATTCTCATATTTACCAC) and hDHFR5’UTR-R (CACCATTTTTGAAAAGATTAATTTGA); the primers to verify integration at the 3’ end were PfCSP-F (TGAATGGTCCCCATGTAGTG) and PbCS3’UTR-R (CGCCCTAATGTGCATTGCT). Finally, DNA isolated from chimeric parasites was sequenced to verify the correctness of the replaced gene.
To generate the P.b.-P.f. CSP-FL chimeric *P. berghei* parasite we excised a 784-bp restriction fragment from the csp gene of the previously described plasmid pIC-CSPfNT [22]. This segment was then replaced with a 943-bp fragment obtained from plasmid pHZ-PfCSP upon digestion with BbsI and PacI. Therefore, the csp gene (1188 bp) in the resulting plasmid, pIC-CSPfFULL, incorporates a full-length *P. falciparum* 3D7 CSP in which the signal sequence has been replaced with the one of the *P. berghei* CSP (base pairs 1 to 69). Then, we excised the csp gene from pIC-CSPfFULL as a KpnI-XhoI fragment and inserted it into the transfection plasmid, pR-CSPfFULL. Lastly, KpnI and SacI were used to linearize the recombinant fragment from pR-CSPfFULL prior to transfection of *P. berghei* ANKA parasites.

P.b.-P.f. CSP-FL chimeric parasites were selected and cloned as described above. Recombination of the transfected DNA fragment at the 5' and 3' ends from the modified locus was verified by PCR. The primers used to confirm 5' integration were 5'-F (TCACCCTCAAGTGGGTTAAAA) and PbPfNT-R (TTATATAATTAGTGCTGCATTATCA); the primers to verify integration at the 3' end were 3'-F (TGTTAAAAATGTGTATGTTGTGTTGC) and 3'-R (GTGCCCATTACGACTTTGGT). DNA isolated from chimeric parasites was sequenced to verify the sequence of the replaced CSP.

### 6.3.8 Sporozoite challenge

Two weeks after the last immunization, mice were challenged against chimeric sporozoites. To quantitatively assess the liver parasite burden, mice were challenged intravenously (i.v.) with $2 \times 10^3$ chimeric P.b.-P.f. CSP-FL CD8CT or P.b.-P.f. CSP-FL parasites. Forty-two hours after sporozoite injection, liver parasite loads were measured by reverse transcription followed by quantitative real-time PCR (RT-qPCR) [25].

To evaluate sterile protection, mice were challenged by mosquito bites using 5 infected *Anopheles stephensi* mosquitoes. Starting on day 4 after challenge, daily blood smears were taken and observed under a microscope. Smears were fixed with methanol (for 30 s) before being stained with a 10% Giemsa stain solution (Sigma-Aldrich, St. Louis, MO) for 15 min. Challenge experiments were performed 2 weeks after the last immunization.
6.3.9 Statistics

Data were plotted using Graph Pad Prism 4 software. Experimental groups were compared for significance using a Mann-Whitney test unless otherwise stated. Analysis of flow cytometry data was performed using FlowJo software.

6.4 Results

6.4.1 Pf rCSP-CAF09 induces significant antibody and CD8+ T-cell responses

Previous research demonstrated that immunizations with cationic liposomes are capable of inducing strong cell-mediated immunity and antibody responses [26]. To determine if vaccination with Pf rCSP-CAF09 could elicit antibody responses against the *P. falciparum* CSP, we analyzed the sera of immunized mice and performed ELISAs using synthetic peptides representing the repeat, N-terminal and C-terminal regions of this protein. Mice immunized either 2 or 3 times with Pf rCSP-CAF09 developed comparable antibody responses against the repeat region peptide (~50,000 endpoint IgG titers) (Fig 6.1B). In addition, similar antibody levels were also found by IFA using *P. falciparum* sporozoites, as well as the 2 newly developed chimeric lines, in both immunization groups (Table 6.1 and Supplementary Fig 6.1). However, mice immunized 3 times developed significantly higher antibody titers against the N-terminal and C-terminal regions of the *P. falciparum* CSP than those that received only 2 immunizations (Fig 6.1A and 6.1C).

In a recent study CAF09 demonstrated remarkable capacity to induce cytotoxic responses against a variety of model antigens [19]. To test whether immunizations with Pf rCSP-CAF09 could induce CD8+ T cells specific against the *P. falciparum* CSP, we performed *ex vivo* stimulation assays using the cytotoxic epitope DYENDIEKKI [27]. For these experiments, we measured the production of IFN-γ as a surrogate of the induction of CD8+ T-cell responses against this *P. falciparum* CSP epitope. We found that 3 immunizations with Pf rCSP-CAF09 were capable of inducing a high frequency of antigen-specific CD8+ T cells; similar results were obtained in mice that received 2 immunizations (Fig 6.2).
6.4.2 CD8+ T cells against the *P. falciparum* CSP can specifically target P.b.-P.f. FULL CSP CD8 CT sporozoite infection

Numerous studies have shown that CD8+ T cells are important for eliminating malaria parasites that successfully invade and replicate within hepatocytes. To investigate the *in vivo* protective effect of immune responses against the *P. falciparum* CSP, we developed chimeric rodent parasites expressing this protein. The newly developed transgenic strain P.b.-P.f. CSP-FL CD8CT expresses the full-length *P. falciparum* 3D7 CSP in which a single amino acid has been replaced to encode the cytotoxic epitope \(359\text{DY}\text{ENDIEKKI}^{368}\) (the original sequence in the 3D7 strain is \(359\text{DY}\text{A}\text{NDIEKKI}^{368}\), which is not recognized as a CD8+ T-cell epitope). To develop this parasite line, we preserved the signal sequence of the *P. berghei* CSP (amino acids 1 to 23). Thus, in the chimeric CSP, amino acids 24 to 340 of *P. berghei* have been replaced with residues 25 to 397 from the *P. falciparum* 3D7 CSP (Fig. 2.4). We also developed a chimera in which the CSP gene has the same amino acid sequence as the P.b.-P.f. CSP-FL CD8CT line. However, this chimeric line (P.b.-P.f. CSP-FL) does not incorporate the cytotoxic epitope at the CSP’s C-terminal region, maintaining the original 3D7 sequence \((359\text{DY}\text{ANDIEKKI}^{368})\) (Fig. 2.3). Importantly, these parasite chimeras develop normally in *A. stephensi* mosquitoes and can efficiently infect naïve C57BL/6 mice through mosquito bites (Supplementary Table 6.1).

Using the described rodent chimeric lines we can directly assess the anti-parasite effect of CD8+ T cells directed against the cytotoxic epitope DYENDIEKKI in the *P. falciparum* CSP. This is demonstrated in mice immunized with recombinant influenza and vaccinia viruses capable of inducing strong CD8+ T-cell responses against the DYENDIEKKI epitope [28, 29]. We found that immunized mice have a significantly lower liver parasite burdens when challenged against the P.b.-P.f. CSP-FL CD8CT chimera than when challenged with P.b.-P.f. CSP-FL sporozoites (Supplementary Fig. 6.2).

6.4.3 Immunizations with Pf rCSP-CAF09 strongly inhibit chimeric sporozoite infection

Given the robust antibody and CD8+ T-cell responses induced by Pf rCSP-CAF09, we sought to determine whether these immunization regimens could inhibit chimeric sporozoite infection. Mice
immunized with 2 (Fig. 6.3A) or 3 doses (Fig 6.3B) of Pf rCSP-CAF09 showed a highly significant reduction (~2 logs) in liver parasite burden compared to mice immunized with adjuvant alone or naïve controls. In the case of mice immunized with 2 doses of Pf rCSP-CAF09, there were no statistically significant differences in liver parasite burdens upon challenge with P.b.-P.f. CSP-FL CD8CT or P.b.-P.f. CSP-FL sporozoites (Fig. 6.3A). However, mice that received 3 doses of Pf rCSP-CAF09 had lower liver parasite loads when challenged against P.b.-P.f. CSP-FL CD8CT sporozoites than when challenged with P.b.-P.f. CSP-FL parasites (Fig. 6.3B). This suggests that immune responses incorporating CD8+ T cells may be more efficient at inhibiting chimeric sporozoite infection. Further ongoing studies will be useful to confirm the role of CD8+ T cells.

6.4.4 Pf rCSP-CAF09 induces long-lasting sterilizing immunity against natural sporozoite infection

To determine whether Pf rCSP-CAF09 could confer sterilizing immunity, we challenged immunized against sporozoites delivered by infectious mosquito bites. This challenge route is more physiologically relevant than i.v. sporozoite injection and arguably a better model for testing vaccine efficacy.

Mice immunized 3X with Pf rCSP-CAF09 were challenged by 5 infectious mosquito bites for a 10 min period. Four days later, daily blood smears were taken to determine the presence of blood-stage parasites. Notably, we found that 90% (9 out of 10) of Pf rCSP-CAF09 immunized mice did not develop blood-stage parasitemia during the 14-day follow up period. All mice immunized with adjuvant-only and naïve controls became positive by day 5 after challenge (Fig 6.4A).

To establish if the observed sterile protection could persist over time, the 9 mice that did not develop blood-stage parasitemia upon infectious mosquito bites were re-challenged again 5 weeks later (7 weeks after the last immunization). Remarkably, none of the Pf rCSP-CAF09 immunized mice developed blood-stage infection. All of the age-matched naïve controls became positive by day 5 after challenge (Fig 6.4B).
6.4.5 Anti-CSP repeat antibody titers significantly increase in steriley protected mice after sporozoite challenge

The availability of serum samples from mice that developed sterile immunity allowed us to perform a paired-wise analysis of Pf rCSP-CAF09-induced anti-CSP titers before and after sporozoite challenge (corresponding to 1 and 6 weeks after the 3rd Pf rCSP-CAF09 immunization). Interestingly, we found different antibody kinetics depending on the *P. falciparum* CSP region under evaluation. Thus, while IgG titers against the CSP N-terminal region significantly decreased between 1 and 6 weeks after immunization, we found a highly significant increase in anti-CSP repeat antibody levels (Fig 6.5A-B). However, there was no significant change in antibody titers against the C-terminal region of the *P. falciparum* CSP (Fig 6.5C).

6.5 Discussion

The development of an entirely protective vaccine is a top priority in the fight against malaria. Over the years, the RTS,S vaccine candidate has demonstrated that a protein-in-adjuvant formulation is capable of inducing protective immune responses that can effectively prevent malaria infection. However, improvements in vaccine efficacy are still needed. In this study, we demonstrate that immunizations with full-length recombinant *P. falciparum* CSP in combination with the novel adjuvant system CAF09 induce significant humoral and cellular responses and are capable of conferring long-lasting sterile immunity against sporozoite infection.

Mice immunized with Pf rCSP-CAF09 developed high antibody titers capable of binding different regions of the *P. falciparum* CSP as well as *P. falciparum* sporozoites and chimeric *P. berghei* sporozoites expressing the *P. falciparum* CSP. Interestingly, mice immunized 2 or 3 times with Pf rCSP-CAF09 had comparable IgG titers against the CSP repeat region and *P. falciparum* or chimeric sporozoites, as determined by ELISA and IFA, respectively. This is in contrast with a previous study that used the same Pf rCSP antigen, but different adjuvant formulations, and found that 3 immunizations induced higher anti-CSP antibody titers [20]. Nonetheless, as determined by ELISA,
mice immunized 3 times with Pf rCSP-CAF09 developed significantly higher IgG titers against the N-terminal and C-terminal regions of the *P. falciparum* CSP than mice that received 2 immunizations. And while the potentially protective effect of antibodies against the C-terminal region of CSP is yet to be determined, we have recently demonstrated that antibodies against the N-terminal region of this protein can significantly inhibit sporozoite infection [22]. Additional studies should help determine whether the antibody affinity and/or IgG isotypes represented in each of the two immunized groups are different.

In addition to the significant anti-CSP antibody responses, immunizations with Pf rCSP-CAF09 were also capable of inducing robust antigen-specific CD8+ T cells. Mice that were immunized 3 times with Pf rCSP-CAF09 had a higher frequency of *P. falciparum* CSP-specific CD8+ T cells. Importantly, a previous study with CAF09 showed that 2 immunizations were capable of inducing CD8+ T cells that completely protected mice against skin tumors [19].

Further studies will be needed to define if the antibody and cellular responses elicited Pf rCSP-CAF09 depend on immunization route. However, previous research indicated that i.p. immunizations are required to achieve maximum CD8+ T-cell responses [19]. Nonetheless, it is important to note that subcutaneous and intramuscular immunizations with Pf rCSP-CAF09 were capable of inducing similar anti-CSP titers to those elicited by i.p injections (unpublished observations).

Over the last 15 years, transgenic rodent malaria parasites have emerged as useful tools for the pre-clinical evaluation vaccine candidates. For this study, we developed a new chimeric line expressing the full-length *P. falciparum* CSP incorporating the cytotoxic epitope DYENDIEKKI in its C-terminal region. Importantly, this parasite develops normally in mosquitoes, produces highly infectious sporozoites and can be specifically targeted by protective CD8+ T cells. Mice immunized with either 2 or 3 doses of Pf rCSP-CAF09 were able to significantly inhibit sporozoite infection with comparable efficiency, underscoring the robustness of the immune responses elicited by this antigen-adjuvant formulation. Ongoing T-cell depletion studies in mice immunized with Pf rCSP-CAF09 should help determine the extent to which these CD8+ T-cell responses contribute to protection.
To establish if Pf rCSP-CAF09 could confer sterilizing immunity against sporozoite infection, immunized mice were challenged by infectious mosquito bites. In the first of these experiments, 90% of vaccinated mice did not develop blood-stage parasitemia. However, all of these mice were steriley protected against a second mosquito bite challenge 5 weeks later. Given the stringency of this challenge model, these findings emphasize the remarkable protective capacity of Pf rCSP-CAF09-induced immune responses. Critically, similar studies assessing the protective capacity of full-length *P. falciparum* CSP immunizations in combination with different adjuvants did not achieve the same degree of protection and long-lasting immunity as Pf rCSP-CAF09 [20, 30].

Antibody responses in Pf rCSP-CAF09-immunized mice, evaluated before and after sporozoite challenge, behaved differently against the CSP regions under evaluation. Notably, anti-CSP repeat antibody titers significantly increased in steriley protected mice, which also developed sterile immunity against a second sporozoite challenge. These results underscore the well-known protective capacity of antibody responses against the repeat region of CSP. The fact that IgG responses against the CSP N-terminal region waned over time and could not be boosted by sporozoite infection suggest that immunizations with full-length recombinant CSP are not effective at inducing long-lasting humoral responses against this domain. A similar waning trend was observed against the C-terminal domain of CSP. And while the likely protective role of antibodies against the CSP C-terminus has not been yet described, we recently demonstrated that antibody responses against the N-terminal region of CSP can significantly inhibit sporozoite infection [22]. Taken together, our data suggest that other immunization strategies should be considered in order to induce long-lasting humoral responses against non-repeat CSP regions.

In conclusion, the most prominent finding of this study is that Pf rCSP-CAF09 was capable of inducing robust antibodies and T-cell responses. In general, this is not observed upon immunizations with recombinant proteins alone, which are known to mainly induce antibodies or CD4+ T-cell responses, or recombinant viruses, which preferentially induce T-cell responses. These findings highlight the potential of this antigen-adjuvant formulation for further vaccine development.
Figure 6.1 Endpoint IgG titers against the N-terminal, repeat and C-terminal regions of the P. falciparum CSP. Mice immunized 3 times with Pf rCSP-CAF09 developed significantly higher antibody titers against the P. falciparum CSP N-terminal (YGSSNTRVLNEIYNAGTNYNELEMNYYGKQENWYSLKKSRSLSGENDDGNNEDNKEKLKPKHKKQLKQAPADHHHHH) (A) and C-terminal (SDKHIKEYLNKIQNSLSTEWSPCVNGQTVRKPSPANSNPDELDYENDIEKKCKMEKHHHHH) (C) peptides than mice that received 2 immunizations. However, mice immunized with either 2 or 3 doses of Pf rCSP-CAF09 had comparable antibody titers against the synthetic peptide [NANP]; (B). Data points are plotted as endpoint titers. n = 12. Abbreviations: ns, not significant; rCSP, recombinant circumsporozoite protein; OD, optical density.
Table 6.1 Antibodies from *Pf* rCSP-CAF09 immunized mice bind *P. falciparum* sporozoites as well as the *P.b*-P.f. CSP-FL CD8CT and *P.b*-P.f. CSP-FL chimeras. Pooled serum samples from mice immunized 2 or 3 times with *Pf* rCSP-CAF09 were incubated on slides coated with fixed sporozoites and stained with fluorescently-labeled anti-mouse secondary antibody. Slides were analyzed under a microscope. ++++, very good binding; ++, good binding; +, weak binding; −, no binding. Abbreviations: *Pf* rCSP-CAF09, *P. falciparum* recombinant circumsporozoite protein in adjuvant CAF09; *P.b*-P.f. CSP-FL CD8CT, *P. berghei*-P. *falciparum* CSP full-length CD8 epitope C-terminus; *P.b*-P.f. CSP-FL, *P. berghei*-P. *falciparum* CSP full-length.

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Figure 6.2 Pf rCSP-CAF09 immunizations induce antigen-specific CD8$^+$ T cells. CD8$^+$ T-cell responses were assessed in spleens of mice 10 days after the last immunization with either 2 or 3 doses of Pf rCSP-CAF09. The production of IFN-$\gamma$ by CD8$^+$ T cells was assessed in \textit{ex vivo} stimulation assays using LM1 cells pulsed with the synthetic peptide DYENDIEKKI. Bars represent means ± SEM (n = 3). Abbreviations: ns, not significant; IFN-$\gamma$, interferon-gamma; SEM, standard error of the mean.
Figure 6.3 *Immunizations with Pf rCSP-CAF09 strongly inhibit chimeric sporozoite infection.* Immunized mice were challenged with 2 x 10^3 P.b.-P.f. CSP-FL CD8CT or P.b.-P.f. CSP-FL chimeric sporozoites. Forty hours later, livers were harvested, RNA extracted, and liver parasite burden was determined by RT-qPCR. Mice immunized either 2 (A) or 3 (B) times with Pf rCSP-CAF09 significantly inhibited sporozoite infection. Mean ± SEM; n = 6, *P < .05; **P < .01. Abbreviations: Pf rCSP-CAF09, *P. falciparum* recombinant circumsporozoite protein in adjuvant CAF09; ns, not significant; CSP-FL CD8 CT, P.b.-P.f. CSP-FL CD8CT chimeric line; CSP-FL, P.b.-P.f. CSP-FL chimeric line; rRNA, ribosomal RNA; RT-qPCR reverse-transcription quantitative real-time polymerase chain reaction; SEM, standard error of the mean.
Figure 6.4 Pf rCSP-CAF09 immunizations confer long-lasting sterile protection. Mice immunized 3 times with Pf rCSP-CAF09 were challenged against P.b.-P.f. CSP-FL CD8CT chimeric sporozoites delivered by 5 infectious mosquito bites. Four days after challenge, daily blood smears were taken and analyzed under a microscope. (A) Nine out of 10 mice (90%) developed sterile immunity when challenged 2 weeks after the last immunization (n = 10). (B) Five weeks later, the 9 mice that were sterilely protected (in (A)) were challenged again, with none of the animals developing blood-stage parasitemia. Kaplan-Meier plots show the time to detection of parasites in the blood for each group after the challenge. **P < .01; ***P < .001. Abbreviations: Pf rCSP-CAF09, *P. falciparum* recombinant circumsporozoite protein in adjuvant CAF09; P.b.-P.f. CSP-FL CD8CT, *P. berghei*–*P. falciparum* CSP full-length CD8 epitope C-terminus; OD, optical density.
Figure 6.5 Endpoint IgG titers against the N-terminal, repeat and C-terminal regions of the P. falciparum CSP at different time points. Antibody titers against different regions of CSP were evaluated in mice immunized 3 times with Pf rCSP-CAF09 before and after sporozoite challenge (1 and 6 weeks after last immunization, respectively). IgG levels against the N-terminal region of CSP significantly decreased over time (A). However, titers against the repeat region peptide significantly increased mice that developed sterile protection against sporozoite challenge (B). Antibody titers against the C-terminal region of CSP did not change significantly (C). Data points are plotted as endpoint titers (n = 9) and compared for significance using a paired Student t-test. *P < .05; **P < .01. Abbreviations: ns, not significant; Pf rCSP-CAF09, P. falciparum recombinant circumsporozoite protein in adjuvant CAF09; OD, optical density.
6.6 References


CHAPTER 7
GENERAL DISCUSSION
More than 100 years have passed since Alphonse Laveran, Ronald Ross and Battista Grassi made the first observations that allowed identifying the malaria parasite in human blood and later establishing that mosquitoes are responsible for transmitting the disease. Since then, our understanding of the complex biology of the *Plasmodium* parasite has substantially increased. Yet, malaria remains to date a major global health problem and a leading cause of child morbidity and mortality throughout Africa.

The public health challenge posed by malaria is formidable. It is estimated that in 2013 there were approximately 198 million cases of malaria and almost 600 000 malaria deaths globally [1]. Sadly, the majority of cases occur in low-income nations, exacting a high toll among the poorest and more neglected communities. Nonetheless, the increasing global awareness towards this disease has resulted in a significant expansion in coverage of malaria control programs as well as in funding, coming from both domestic and international agencies. And while this has allowed the sustained worldwide reduction in malaria incidence and mortality rates over the last 15 years, current financing efforts are still under the estimated US$ 5.1 billion that are required annually to achieve the global goals of malaria control and elimination [1].

Malaria can be prevented and cured by currently available interventions. Vector control approaches such as insecticide-treated bednets (ITNs) and indoor residual spraying (IRS) prevent thousands of malaria cases every year, especially among children and pregnant women [1]. Antimalarial drugs are effective for both preventing and treating malaria. Importantly, artemisinin-based combination therapies (ACTs), adopted as the first-line treatment of *P. falciparum* malaria since 2001, are effective against all five human malaria species, produce rapid parasite and fever clearance, and show fewer adverse effects [2, 3]. However, both vector control approaches and malaria chemotherapy are presently threatened by the rapid spread of resistance to insecticides and antimalarial drugs by *Anopheles* mosquitoes and *Plasmodium* parasites, respectively.

The development of a safe and low-cost malaria vaccine would allow closing the breach left by other prevention and treatment approaches. It is foreseen that even a partially effective vaccine
would prevent thousands of cases and malaria-related deaths every year. However, this does not imply that other control and prevention methods will become obsolete; the malaria parasite is a complex and adaptable organism, which means that many tools are needed to defeat this disease. Thus, while the imminent licensing of the moderately protective RTS,S vaccine candidate is perceived an important step forward in the fight against malaria, second-generation vaccines with higher efficacy are urgently needed.

For almost 5 decades rodent parasites have been useful and practical models for the assessment of immune responses against malaria. Notably, the first systematic studies showing that irradiated sporozoites could induce protective immunity against malaria were carried out with *P. berghei* parasites [4]. Over the following years, rodent models were also used for addressing basic questions in malaria biology as well as for the evaluation of vaccine candidates. However, a basic limitation murine malaria species is that rodent orthologues (i.e. functionally similar genes) are antigenically distinct from human-infecting *Plasmodium* species. For this reason, the conclusions reached in the rodent models, while important from a conceptual standpoint, cannot account for fundamental differences in the antigenic makeup of murine and human parasite antigens. To overcome this disadvantage, chimeric rodent parasites expressing proteins from other *Plasmodium* species or model antigens have emerged as practical solutions for studying malaria immunology and vaccine-induced responses. Importantly, these chimeric models have been successfully used for tracking antigen-specific T-cell responses and the functional evaluation of antibody responses (reviewed in [5]).

In 2002, the development of rodent *P. berghei* parasites expressing the repeat region of the *P. falciparum* CSP was reported [6]. Soon after, this chimeric line became a practical tool for the evaluation of vaccine-induced immune responses to the *P. falciparum* CSP and its use has been described in numerous studies. However, the applicability this chimera is restricted to the assessment of immune responses exclusively directed against the CSP repeat region, limiting the possibility of evaluating the protective effect of immune responses targeting B- and T-cell epitopes outside this domain. Thus, in view of the need for chimeric lines that could enable the assessment of vaccine-induced immunity
against other regions of the *P. falciparum* CSP, our studies began with the development of 2 new transgenic *P. berghei* parasites expressing the N- or C-terminal domains of this protein. Importantly, the work here presented demonstrated that these regions can be effectively targeted by different arms of the immune response and significantly inhibit sporozoite infection *in vivo*. Further, the developed chimeras have also allowed the pre-clinical evaluation of different malaria vaccine candidates as well as novel adjuvant formulations.

In Chapter 3, using the P.b.-P.f CSP-NT (*P. berghei*-*P. falciparum* CSP N-terminus) line, we demonstrated that the IgG1 (kappa) monoclonal antibody mAb5D5 recognizes a defined epitope in the N-terminal region of CSP as is capable of preventing this protein’s processing and significantly inhibit *in vivo* sporozoite infection. Importantly, our results were in in agreement with *in vitro* studies that showed that polyclonal antibodies against the N-terminal region of the *P. falciparum* CSP could inhibit sporozoite invasion of cultured hepatocytes [7-10]. Thus, our findings strongly suggest that the efficacy of RTS,S -as well as other CSP-based vaccine constructs- could be increased by incorporating CSP’s N-terminal region, as it is apparent that antibodies against this domain can have a major inhibitory effect on sporozoite infectivity. Notably, the amino acid sequence recognized by mAb5D5 is highly conserved among *P. falciparum* isolates, suggesting this epitope could perhaps help overcome the issue of polymorphism of parasite surface antigens. Critically, previous studies have indicated that the T-cell epitope region of RTS,S is polymorphic, imposing a significant challenge for improvements in vaccine efficacy (reviewed in [11]). Further studies should help determine how to induce effective antibody responses against the N-terminal region of the *P. falciparum* CSP thorough vaccination. In addition, the developed P.b.-P.f CSP-NT chimeric parasites could also be used to assess the protective effect of CD8+ T-cell responses against a previously described cytotoxic epitope in the *P. falciparum* CSP’s N-terminus [12].

Our studies also involved the evaluation of immune responses and protective capacity induced by new CSP-based vaccines and/or adjuvant formulations. In the published study presented in Chapter 4, we compared four different full-length *P. falciparum* CSPs expressed in bacteria (*Escherichia coli*) or
yeast (Pichia pastoris) for their capacity to induce immunity and protection against sporozoite infection when administered in combination with Poly(I:C) or glucopyranosyl lipid adjuvant-stable emulsion (GLA-SE). Importantly, the results of this study indicated that E. coli-derived CSP induced stronger antibody and CD4+ T-cell responses than proteins produced in yeast. In addition, immunizations with bacterial CSP in combination with Poly(I:C) elicited polyfunctional CD4+ T cells capable of producing the antimalarial cytokines IFN-γ, TNF-α and IL-2. And while the frequency of these cellular responses was higher when mice were immunized with Poly(I:C), immunizations with GLA-SE induced comparable levels of anti-CSP antibodies which conferred similar degrees of inhibition of sporozoite infection. Notably, we found that antibody responses against CSP, induced with either adjuvant formulation, strongly correlated with protection, as previously described in mice and human studies [13-15]. Lastly, these investigations further validated the usefulness of chimeric rodent malaria parasites for the pre-clinical evaluation of vaccine and adjuvant formulations.

Virus-like particles (VLPs) are an appealing approach for vaccine development as they “provide delivery systems that combine good safety profiles with strong immunogenicity and constitute a safe alternative to inactivated infectious viruses” [16]. Importantly, the RTS,S malaria vaccine candidate consists of a self-assembled VLP which incorporates the repeat and C-terminal regions of P. falciparum CSP and the hepatitis B surface antigen. In Chapter 5, we present a recently published study describing the development, immunogenicity and protective capacity of VLPs carrying different epitopes from the P. falciparum CSP. VLPs incorporating CSP repeat or non-repeat region B-cell epitopes were capable of inducing antibody responses in immunized mice. However, only VLPs that induced responses against the repeat domain of CSP were capable of inhibiting sporozoite infection. Further, immunizations with VLPs carrying only 3 CSP T-cell epitopes were unable to inhibit parasite infection, underscoring the importance of anti-CSP repeat antibodies for protection. Notably, mice developed robust sterilizing immunity upon immunization with VLPs carrying both CSP repeat B-cell epitopes as well as T-cell domains, indicating that the malaria-specific CD4+ T cells primed by these VLPs contributed to the greater efficacy of this construct. Finally, a newly developed VLP construct
carrying epitopes from the repeat region of the *P. vivax* CSP proved to be highly immunogenic and conferred sterile protection to vaccinated mice. These results demonstrate both the potential of the VLP platform for the development of novel non-*falciparum* malaria vaccines as well as the usefulness of recently generated chimeric *P. berghei* parasites [17] expressing the repeat domain of the *P. vivax* CSP to assess vaccine candidates against this widely spread malaria species.

Adjuvants are formulations that are capable of enhancing antigen-specific antibody and T-cell responses by stimulation of innate immune receptors [18, 19]. These formulations have been broadly categorized as immunostimulants, directly triggering the immune system to enhance responses to antigens, or as vehicle systems, allowing direct delivery of antigen to immune cells [20, 21]. Adjuvants are nowadays critical components in the development of subunit vaccines, which tend to be otherwise poorly immunogenic. Importantly, the development of the RTS,S vaccine candidate has been inseparably linked to the evolution of its own set of adjuvant systems [22].

The assessment of immune responses and protective capacity induced by vaccination with recombinant *P. falciparum* CSP (rCSP) in combination with a novel liposome-based adjuvant formulation, CAF09, is presented in Chapter 6. Previous investigations had demonstrated that CAF09 is capable of inducing robust CD8+ T-cell responses against different types of antigens [23]. Critically, the development of adjuvants capable of eliciting CD8+ T cells to soluble proteins had remained a challenging task [18]. Immunizations with 2 or 3 doses of rCSP in combination with CAF09 resulted in significant anti-CSP repeat antibody titers as well as CD8+ T-cell responses. In addition, these responses were able to significantly inhibit sporozoite infection and confer long-lasting sterilizing immunity. Further, the newly developed chimeric *P. berghei* line expressing the full-length *P. falciparum* CSP with a cytotoxic epitope in the C-terminal region provided us with a useful tool to directly assess the protective effect of CD8+ T cell specific against this domain. Altogether, these results underscore the remarkable degree of protection that results from targeting sporozoites with both anti-CSP antibodies and CD8+ T cells. In this regard, it should be also noted that a series of studies have failed to detect CSP-specific CD8+ T cells in individuals immunized with RTS,S [24-27]. Thus, the results of
our study suggest that further optimization of this vaccine candidate to induce CD8\(^+\) T-cell responses could translate in substantial protective gains.

In conclusion, the studies here presented show that chimeric rodent parasites are useful tools for the assessment of immune responses and protective capacity induced by novel vaccine candidates. As malaria remains a leading cause of mortality worldwide, the development of an efficient vaccine is a fundamental necessity to eradicate this disease. Our findings support that transgenic rodent parasites have significant potential to make important contributions for advancing our understanding of malaria immunology and vaccine research.
References


Supplementary Figure 3.1 Epitope specificity of anti-P. falciparum CSP mAbs. Mapping of antibody specificity by ELISA using long synthetic peptides representing the N-, Repeat or C-terminal regions of the P. falciparum CSP. The mAb5D5 strongly binds the CSP N-terminal region peptide.
Supplementary Figure 3.2 Development and characterization of chimeric P.b.-P.f. CSP-NT parasites. (A) Scheme representing the strategy used for replacing the CSP gene of P. berghei (ANKA) with a hybrid CSP containing the P. falciparum (3D7) N-terminal region. The annealing sites of the primers used to verify recombination by PCR are indicated below. Restriction sites shown are K – KpnI; P-PfMI; A-AflII; E-EagI; Xh – Xhol; S – SacI. (B) Using genomic DNA from Pb-Pf parasites, 5' and 3' integration into the CSP genomic locus was verified by PCR. Primers 5'F and PbPf NT-R yield an 850 bp product; primers 3'F and 3'R yield a 1000 bp product. (C) To verify the successful cloning of the chimeric strain, a 1400 bp PCR fragment was amplified from the CSP gene using primers 5'F and PbWT NT and then digested with AflII, which does not digest the WT CSP gene. Genomic DNA from P. berghei (ANKA) was used as control. Comparison of the in vivo infectivity of P.b.-P.f. CSP-NT sporozoites and WT P. berghei (ANKA) after i.v. injection. Mean ± SEM; n = 5, results are representative of two independent experiments. ns = not significant.
<table>
<thead>
<tr>
<th></th>
<th>% Midgut infected</th>
<th>Oocysts/midgut</th>
<th>% Salivary glands infected</th>
<th>Spz/Salivary gland (x 10^3)</th>
<th>% of infected mice after mosquito bites^b</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. b.-P. f. CSP-NT</td>
<td>78.64</td>
<td>67.11</td>
<td>80</td>
<td>10</td>
<td>100%</td>
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<tr>
<td>Pb ANKA</td>
<td>75.14</td>
<td>42.25</td>
<td>66</td>
<td>10</td>
<td>100%</td>
</tr>
</tbody>
</table>

**Supplementary Table 3.1 Developmental characteristics and infectivity of P. b.-P. f. CSP-NT parasites in A. stephensi mosquitoes^a.**

^a Mean of 3 experiments, with at least 20 mosquitoes examined in each experiment.

^b Three infected mosquitoes were allowed to feed on C57BL/6 mice (n=5) for 3 minutes. Blood stage parasitemia was assessed 5 days later.
Supplementary Figure 4.1 Cellular and antibody responses in NHP. Rhesus macaques were immunized with 100μg of CSP1 protein mixed with 1mg of poly-ICLC. A total volume of 1ml was injected s.c. in the deltoid area of the upper arm. For boosting, the opposite arm was used. NHPs were immunized at weeks 0, 5 and 16. At indicated time-points after immunizations blood was collected and PBMCs were used for stimulation for ICS. Serum was also collected at indicated time-points to determine antibody titers. Frequency of CSP-specific IFN-γ-, IL-2- or TNFα- producing CD4+ T cells in PBMCs collected at peak (two weeks) and memory (15 weeks) after two (A) or three (B) immunizations. Relative proportion of each individual combination of IFN-γ-, IL-2-, or TNFα-producing cells in PBMCs collected at peak (two weeks) and memory (15 weeks) after two (C) or three (D) immunizations. (E and F) CSP-specific IgG antibody titers.
Antibodies in serum from monkeys that received three immunizations with CSP1 and poly-ICLC adjuvant showed a strong binding capacity to sporozoites in vitro. Serum was collected at indicated time-points and IFA was performed as described in Table 4.1. +++ very good binding, ++ good binding, + weak binding, - no binding.

<table>
<thead>
<tr>
<th>TIME-POINT</th>
<th>FLUORESCENT INTENSITY BY DILUTION</th>
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<tbody>
<tr>
<td></td>
<td>1:100</td>
</tr>
<tr>
<td>PRE</td>
<td>-</td>
</tr>
<tr>
<td>2 weeks after 2 immunizations</td>
<td>++</td>
</tr>
<tr>
<td>2 weeks after 3 immunizations</td>
<td>+++</td>
</tr>
<tr>
<td>15 weeks after 2 immunizations</td>
<td>+</td>
</tr>
<tr>
<td>15 weeks after 3 immunizations</td>
<td>++</td>
</tr>
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</table>
Supplementary Figure 5.1 Development and characterization of hybrid PB/PF parasites carrying the PF C-Terminus (PB/PF-CSP-CT). (A) Scheme representing the strategy used for replacing the CSP gene of *P. berghei* (ANKA) with the *P. falciparum* (3D7) C-terminal region. The annealing sites of the primers used to verify recombination by PCR are indicated below. Restriction sites shown are K—KpnI; Se—SexAI; P—PacI; Xh—XhoI; S—SacI. (B) The resulting amino acid sequence of the hybrid CS protein. The sequence derived from the *P. falciparum* (3D7) sequence is depicted in red text.

```
1 M KKCTILVVA SLLLVVNLLP GYGQNKSIQA QRNLNELCYN EGNDKLYHV 50
51 LNSKNGKIYN RNTVNRLLAD APEGKKNEKK NEKIERNNKL KQPPPNNPN 100
101 DPPPPNPNDP PPPNPNDPPP PNNNDPPPPN ANDPPPPNAN DPAPPNDP 150
151 APPNDPPAP PNANDPAPPN ANDPPPPPFPN DAPPNDNPAP PPPNNPDPA 200
201 PQGGNNNPQFO FRQPOPOPOFO PQQPOPOPOFO PRFQPOPOPOFO GNNNKNNNN 250
251 DDSYIPSAEK ILEYNKIQN SLSTEWSPCS VTCGNGIQVR IKPGSANKPK 300
301 DELDYENDIE KKICKMEKCS SVFNVNNSI GLIMVLSFLF IN* 342
```
### A. Epitope Sequences

#### B cell epitope sequences

<table>
<thead>
<tr>
<th>Epitope</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mal</td>
<td>NANP NVDP NANP NANP NANP</td>
</tr>
<tr>
<td>Krich</td>
<td>DEDKRDGNENEDNEKLRPKHKKL</td>
</tr>
<tr>
<td>N1</td>
<td>KLQPGDGQPDP</td>
</tr>
<tr>
<td>CS(298-315)</td>
<td>HNMPNDPNVNDENANAN</td>
</tr>
<tr>
<td>Mal5</td>
<td>NANP NANP NANP NANP</td>
</tr>
<tr>
<td>Pv</td>
<td>DRADGQPAG DRADGQPAG DRAAGQPAG DRAAGQPAG D</td>
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#### T cell epitope sequences

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<thead>
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<th>Epitope</th>
<th>Sequence</th>
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<tr>
<td>UTC</td>
<td>EYNKIQNLSLSTWEWSCPVT</td>
</tr>
<tr>
<td>TH</td>
<td>EYNKIQNLSLSTWEWSCPVTDEIKKICKMEKCSSV</td>
</tr>
<tr>
<td>3T</td>
<td>EYNKIQNLSLSTWEWSCPVTSGNGIQVRIRKPGSANKPDDELDYENDIIEKICKMEKCSSV</td>
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### B. Peptide Sequences

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<th>Epitope</th>
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<tr>
<td>NANP</td>
<td>NONPNVPDNANPANPANPANP</td>
</tr>
<tr>
<td>NVDP</td>
<td>DNNANPNVNDPNANPNV</td>
</tr>
<tr>
<td>Krich</td>
<td>DKRDCGNEDNEKLRPKHKKL</td>
</tr>
<tr>
<td>N1</td>
<td>KLQPGDGQPDP</td>
</tr>
<tr>
<td>CS(298-315)</td>
<td>HNMPNDPNVND</td>
</tr>
<tr>
<td>Pv1</td>
<td>DRADGQPAGDRADGQPAGDRAAGQPAGDRAAGQPAGDRAAGQPAGD</td>
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**Supplementary Figure 5.2 CS Epitope Sequences.** (A) Amino acid sequences of each epitope engineered onto the WHc VLPs (see Fig 5.1 for list of VLPs). (B) Amino acid sequences of the synthetic peptides used for antibody titer determination by ELISA.
**Supplementary Figure 5.3** Only anti-CS repeat antibodies protect against a sporozoite challenge. Mice were immunized with rCS and sera either unadsorbed or adsorbed with NANP/NVDP-containing VLPs (ΔNANP, NVDP) were incubated with 10,000 sporozoites prior to challenge. NMS, normal mouse sera.
Supplementary Figure 5.4 Comparison of WHc-CS VLPs containing malaria-specific T cell epitopes. Groups of 3 mice were immunized (2 doses: 20 μg and 10 μg in IFAd) with the indicated WHcAg hybrid VLPs. Secondary antisera were pooled and serially diluted and analyzed by ELISA for binding to solid-phase WHcAg, NNP and NVDP. End-point titers of pooled sera are shown.
**Supplementary Figure 5.5** Conservation of T cell epitopes on *P. falciparum* and *P. berghei* CS. Alignment of the *P. berghei* UTC, TH.3R and CS.T3 T cell domains with the *P. falciparum* T cell domains incorporated into the WHc-Mal-78-3T VLP. The percentage represents homologies between the two sequences.
### Supplementary Table 5.1

**Kinetics of IgG Ab titers through primary immunization (1°) with WHc(C61S)-Mal-78-3T, at the boost (2°) and at 3 months post-challenge.** Mean endpoint dilution titers from 9–10 mice in each group are shown.

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>Formulation</th>
<th>Endpoint Titer (1/dilution)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>α-rCSP</td>
</tr>
<tr>
<td><strong>WHc(C61S)-Mal-78-3T</strong></td>
<td>Alum</td>
<td>1°</td>
</tr>
<tr>
<td></td>
<td>Alum</td>
<td>2°</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 mo post-challenge</td>
</tr>
<tr>
<td><strong>WHc(C61S)-Mal-78-3T</strong></td>
<td>Alum+QS-21</td>
<td>1°</td>
</tr>
<tr>
<td></td>
<td>Alum+QS-21</td>
<td>2°</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 mo post-challenge</td>
</tr>
<tr>
<td><strong>WHc(C61S)-Mal-78-3T</strong></td>
<td>ISA-720</td>
<td>1°</td>
</tr>
<tr>
<td></td>
<td>ISA-720</td>
<td>2°</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 mo post-challenge</td>
</tr>
<tr>
<td><strong>WHcAg</strong></td>
<td>ISA-720</td>
<td>1°</td>
</tr>
<tr>
<td></td>
<td>ISA-720</td>
<td>2°</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 mo post-challenge</td>
</tr>
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</table>
### Supplementary Table 5.2 Immunogenicity of WHc-Pv-78 VLPs

Mean endpoint dilution Ab titers shown. 1°, primary; 2°, secondary antisera. IFA assay used Pb/Pv dry sporozoites.

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>Adjuvant</th>
<th>Dose</th>
<th>1°/2°</th>
<th>Antibody Titer (1/dilution)</th>
<th></th>
<th></th>
</tr>
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<tbody>
<tr>
<td>WHc-Pv-78</td>
<td>Alum</td>
<td>100 μg</td>
<td>1°</td>
<td>80.5K</td>
<td>24.5K</td>
<td>-</td>
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<tr>
<td>n=9</td>
<td>100 μg</td>
<td>2°</td>
<td>9.5x10^5</td>
<td>2.2x10^6</td>
<td>8.1K</td>
<td></td>
</tr>
<tr>
<td>WHc-Pv-78</td>
<td>IFAd</td>
<td>20 μg</td>
<td>1°</td>
<td>250K</td>
<td>250K</td>
<td>-</td>
</tr>
<tr>
<td>n=3</td>
<td>10 μg</td>
<td>2°</td>
<td>3x10^6</td>
<td>3x10^6</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>WHc-Pv-78</td>
<td>IFAd</td>
<td>100 μg</td>
<td>1°</td>
<td>3x10^6</td>
<td>9x10^6</td>
<td>-</td>
</tr>
<tr>
<td>n=8</td>
<td>100 μg</td>
<td>2°</td>
<td>6.5x10^7</td>
<td>1.5x10^9</td>
<td>72.9K</td>
<td></td>
</tr>
<tr>
<td>WHcAg</td>
<td>IFAd</td>
<td>100 μg</td>
<td>1°</td>
<td>6x10^6</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>n=10</td>
<td>100 μg</td>
<td>2°</td>
<td>2.3x10^8</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Mab 2F2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>45x10^6</td>
<td>-</td>
</tr>
<tr>
<td>(mg/ml)</td>
<td></td>
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</tbody>
</table>
Supplementary figure 6.1 Polyclonal sera from mice immunized with Pf rCSP-CAF09 binds P. falciparum as well as P. berghei chimeric sporozoites. Sera from mice that received either 2 or 3 doses of Pf rCSP-CAF09 bind air-dried sporozoites with comparable efficiency. Images are representative of 1:16000 sera dilutions.
### Supplementary Table 6.1 Developmental characteristics and infectivity of P. b. - P. f. CSP-FL CD8CT and P. b. - P. f. CSP-FL chimeric parasites in A. stephensi mosquitoes

| Parasite | % Midgut infected | Oocysts/midgut | % Salivary glands infected | Spz/Salivary gland (x 10³) | % of infected mice after mosquito bites
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>P. b.-P. f. CSP-FL CD8CT</td>
<td>87.50</td>
<td>60.33</td>
<td>80</td>
<td>9.5</td>
<td>100%</td>
</tr>
<tr>
<td>P. b.-P. f. CSP-FL</td>
<td>70.58</td>
<td>83.25</td>
<td>85</td>
<td>10</td>
<td>100%</td>
</tr>
<tr>
<td>P. berghei ANKA</td>
<td>80.20</td>
<td>55.25</td>
<td>75</td>
<td>10</td>
<td>100%</td>
</tr>
</tbody>
</table>

A Mean of 3 experiments, with at least 20 mosquitoes examined in each experiment.

B Three infected mosquitoes were allowed to feed on C57BL/6 mice (n=5) for 3 minutes. Blood stage parasitemia was assessed 5 days later.
Supplementary figure 6.2 CD8+ T-cells specifically target chimeric P.b.-P.f. CSP-FL CD8CT parasites. (A) C3H/HeNCr MTV- mice were immunized with recombinant influenza (Pf rFlu) and vaccinia (Pf rVV) viruses expressing selected sequences of the P. falciparum CSP prior to challenge with chimeric sporozoites. (B) Immunized mice challenged with P.b.-P.f. CSP-FL CD8CT chimeric parasites, incorporating the cytotoxic epitope DYENDIEKKI, significantly inhibited sporozoite infection. However, immunizations with recombinant viruses did not inhibit infection of P.b.-P.f. CSP-FL sporozoites. Mean ± SEM; n = 5; **P < .01. Abbreviations: ns, not significant; Pf rFlu, recombinant influenza virus expressing a cytotoxic epitope of the P. falciparum CSP; Pf rVV, recombinant vaccinia virus expressing the entire P. falciparum CSP; VP, viral particles; PFU, plaque forming units; CSP-FL CD8 CT, P.b.-P.f. CSP-FL CD8CT chimeric line; CSP-FL, P.b.-P.f. CSP-FL chimeric line; rRNA, ribosomal RNA; SEM, standard error of the mean.
SUMMARY OF QUALIFICATIONS

Ph.D. candidate focused on vaccine research for pre-erythrocytic stages of malaria parasites. Developed several transgenic rodent malaria parasite lines for the pre-clinical evaluation of malaria vaccine candidates using a variety of molecular biology tools. Skilled in working with mouse models of malaria as well as with mosquito vectors. Ample experience in the assessment humoral and cellular responses through several techniques including passive/adoptive transfer assays, ex vivo T-cell stimulation, ELISA and flow cytometry.

EDUCATION

Johns Hopkins University, Baltimore, MD 2009-present
Ph.D. candidate in the Department of Molecular Microbiology and Immunology of the Johns Hopkins School of Public Health.
Current dissertation proposal describes the development and use of transgenic rodent malaria parasites as tools for the pre-clinical evaluation of vaccine candidates.

Universidad Peruana Cayetano Heredia, Lima, Perú 2002-2006
B. Sc. Biology

PROFESSIONAL EXPERIENCE

Instituto de Medicina Tropical “A. von Humboldt”, Lima, Perú 2005-2009
Research assistant in Jorge Arevalo’s group.
Primary line of research involved the development and application of PCR-based methods for diagnosis of cutaneous and mucocutaneous leishmaniasis.

Instituto de Medicina Tropical “A. von Humboldt”, Lima, Perú 2006-2008
Laboratory manager.
Daily activities included managing budgets, purchasing supplies and overseeing routine procedures.

PUBLICATIONS

*Contributed equally to this work*


*Contributed equally to this work.


*Contributed equally to this work.


*Contributed equally to this work.


AWARDS AND HONORS

Johns Hopkins Vaccine Initiative – 2014 Vaccine Day 2014
First place in the Vaccine Day poster session.

Lindau Nobel Laureate Meetings 2014
Selected to participate in the 64th Lindau Nobel Laureate Meeting (Lindau, Germany).

Johns Hopkins Vaccine Initiative – 2012 Vaccine Day 2012
Outstanding student poster award.

Department of Molecular Microbiology and Immunology – JH School of Public Health 2011
Carlton and Estelle Herman Award in Parasitology, Vector Biology and Animal-Borne Diseases.
Universidad Peruana Cayetano Heredia, Lima, Perú 2007
Contenta award to the 2006 Valedictorian of the Department of the Sciences and Philosophy.

**ORAL PRESENTATIONS**

Gordon Research Conference, Tropical Infectious Diseases 2015
“Antibodies Targeting a Functional Domain in CSP Strongly Inhibit Infectivity of Malaria Sporozoites”.

57th Meeting – American Society of Tropical Medicine and Hygiene (ASTMH) 2008
"Diagnostic Accuracy of Leishmania OligoC-TeS T for the Diagnosis of Cutaneous Leishmaniasis in Peru”.

**TEACHING EXPERIENCE (TEACHING ASSISTANT)**

Johns Hopkins School of Public Health, Baltimore, MD 2010-2015
Department of Molecular Microbiology and Immunology courses:
- Graduate Immunology (PH 260.717, Q4, 2015)
- Topics in Immunology I (PH 260.801 - Q1, 2014)
- Introduction to Biomedical Sciences (PH 260.600 - Q1, 2010)

Universidad Peruana Cayetano Heredia, Lima, Perú 2006-2009
- Basic Techniques in Molecular Biology: PCR, Real-Time PCR and Sequencing (2006-2009)
- Introduction to Biochemistry (School of Dentistry) (2007)