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Vaccine Mediated Immunity To Malaria

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Abstract

Malaria infects millions of people every year, and despite recent advances in controlling disease spread, it remains a global health concern. Decades of research into both naturally acquired and vaccine mediated immunity have given a broad range of correlates of protection. RTS,S, the only licensed anti-malarial vaccine, has implicated antibodies against the circumsporozoite protein (CSP) as a key correlate. Not to be discounted, CD8+ T cells targeting liver-stage (LS) antigens were associated with protection in attenuated sporozoite vaccination. Clearly there is no panacea for malarial immunity, and a broad range of responses against multiple antigens is crucial. In this work we develop novel synthetic DNA vaccines targeting antigens in multiple Plasmodium pre-erythrocytic life cycle stages, and evaluate the immunity elicited by each in the context of murine models of malaria. To further evaluate protection mediated by Liver stage antigens, we focused on the exported pre-erythrocytic proteins EXP1, PFN, EXP2, ICP, TMP21, and UIS3. SynDNA antigen cocktails were tested with and without the molecular adjuvant plasmid IL-33. Immunized animals developed robust T cell responses including induction of antigen-specific liver-localized CD8+ T cells, which were enhanced by the co-delivery of plasmid IL-33. In total, 100% of mice in adjuvanted groups and 71%–88% in non-adjuvanted groups were protected from disease following Plasmodium yoelii challenge. To further evaluate protection mediated by sporozoite antigens, five synDNA vaccines encoding variations of CSP were designed and studied: 3D7, GPI1, ΔGPI, TM, and DD2. ΔGPI generated the most robust immunity, and was the most efficacious in an IV sporozoite challenge. We then compared the immunity generated by ΔGPI vs synDNA mimics for two leading malaria vaccine candidates (RTS,S and R21). They demonstrated similar anti-CSP antibody responses, however ΔGPI induced a more focused T cell response. In an infectious mosquito challenge all three of these constructs generated potent inhibition of liver stage infection, with ΔGPI appearing to also provide the best sterilizing immunity from blood stage parasitemia. Together these studies demonstrated that synDNA vaccines encoding malaria immunogens can provide substantial protection from disease, and highlighted the importance of targeting the pre-erythrocytic life cycle stages to combat malaria.

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Dedication

To my mother, who taught me the joy of discovery.

To my father, who taught me the serenity of faith.

Thank you for your unconditional support and belief in me.

Bird by bird.

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ABSTRACT

VACCINE MEDIATED IMMUNITY TO MALARIA

Sophia M. Reeder

David B. Weiner

Malaria infects millions of people every year, and despite recent advances in controlling disease spread, it remains a global health concern. Decades of research into both naturally acquired and vaccine mediated immunity have given a broad range of correlates of protection. RTS,S, the only licensed anti-malarial vaccine, has implicated antibodies against the circumsporozoite protein (CSP) as a key correlate. Not to be discounted, CD8+ T cells targeting liver-stage (LS) antigens were associated with protection in attenuated sporozoite vaccination. Clearly there is no panacea for malarial immunity, and a broad range of responses against multiple antigens is crucial. In this work we develop novel synthetic DNA vaccines targeting antigens in multiple *Plasmodium* pre-erythrocytic life cycle stages, and evaluate the immunity elicited by each in the context of murine models of malaria. To further evaluate protection mediated by Liver stage antigens, we focused on the exported pre-erythrocytic proteins EXP1, PFN, EXP2, ICP, TMP21, and UIS3. SynDNA antigen cocktails were tested with and without the molecular adjuvant plasmid IL-33. Immunized animals developed robust T cell responses including induction of antigen-specific liver-localized CD8+ T cells, which were enhanced by the co-delivery of plasmid IL-33. In total, 100% of mice in adjuvanted groups and 71%–88% in non-adjuvanted groups were protected from disease following *Plasmodium yoelii* challenge. To further evaluate protection mediated by sporozoite antigens, five synDNA vaccines encoding variations of CSP were designed and studied: 3D7, GPI1, ΔGPI, TM, and DD2. ΔGPI generated the most robust immunity, and was the most efficacious in an IV sporozoite challenge. We then compared the immunity generated by ΔGPI vs synDNA mimics for two leading malaria vaccine candidates (RTS,S and R21). They demonstrated similar anti-CSP antibody responses, however ΔGPI

induced a more focused T cell response. In an infectious mosquito challenge all three of these constructs generated potent inhibition of liver stage infection, with ΔGPI appearing to also provide the best sterilizing immunity from blood stage parasitemia. Together these studies demonstrated that synDNA vaccines encoding malaria immunogens can provide substantial protection from disease, and highlighted the importance of targeting the pre-erythrocytic life cycle stages to combat malaria.

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CHAPTER 1: INTRODUCTION

1.1: A brief history of malaria

Malaria has haunted humanity for nearly as long as we have been keeping records. References to a malarial illness in ancient Chinese documents as far back as 2700 BC[1]. A similar malady is described in clay tablets from Mesopotamia in 2000 BC, in Egyptian papyri from 1570 BC, and in Hindu texts from as far back as the 6th century BC[1,2]. Of course, these early and somewhat vague accounts should be viewed with some level of skepticism. We find ourselves on more trustworthy ground with the Early Greeks (including Homer in 850 BC, Empedocles of Agrigentum in 550 BC, and Hippocrates in 400 BC) who were familiar with the distinctive malarial fevers and enlarged spleens seen in patients living on swampy, mosquito ridden land[1,3].

For well over 2500 years, it was believed that malarial fevers were caused by miasmas rising from swamps. This concept was so pervasive that it is widely believed that the term ‘malaria’ comes from the Italian “mal’aria” which translates to ‘bad air’. It was not until the discovery of bacteria by Antonie van Leeuwenhoek in 1676, and the subsequent development of the germ theory of disease by Louis Pasteur and Robert Koch in 1878-79 that the field turned to searching for a microorganism as an explanation for malaria. However, it would be many years before the true cause and mechanism of infection was uncovered.

By 1879, in congruence with the discoveries of Pasteur and Koch, the miasma theory was falling out of favor. The two prevailing theories which replaced it, both based on the assumption a microorganism was the culprit, were whether the microorganisms

were transmitted 1) by air and inhalation, or 2) by water and ingestion. It should be noted that at this time the field was still searching for a bacterial cause rather than a protozoan one, even so far as the discovery and naming of a novel bacterium, *Bacillus malariae*, which was implicated as the causative agent of malaria by noted German scientist Theodor Albrecht Edwin Klebs, who had also been the first to observe the bacterial agents for typhoid and diphtheria[4].

An unexpected challenger rose to dispute the bacterial hypothesis. The French Army officer Charles Louis Alphonse Laveran was posted in Algeria when he began his studies on malaria. Having noted that his malarial patients often exhibited strange pigmentation in their enlarged spleens, he adopted the strategy of 'follow the pigment' and began to search for pigment in the blood of his malarial patients. He observed several different forms of erythrocytic organisms in the blood, with varying levels of pigmentation. Based on his observations he suggested a course of events for the development of these organisms, which culminated in the bursting of red blood cells, coinciding with the cyclical fevers characteristic of malaria. He further noted that quinine, an anti-malarial commonly used by the colonialist European military officers in Africa, removed these organisms from the blood. Laveran named this parasitic protozoan *Oscillaria malariae* and presented his findings to the French Academy of Medical Sciences in 1880[5]. He was largely scoffed at by the eminent scientists of the day, who did not believe he was observing anything more than disintegrating red blood cells. Despite this inhospitable reception, Laveran continued with his research and over time convinced key members of the field of the verity of his observations[6], and was awarded with the Nobel Prize for Medicine in 1907, twenty seven years after his initial, derided presentation[1,7].

Laveran's work demonstrated that the parasitic protozoan was the microorganism responsible for malaria, however the mystery of how humans came to be infected with the *Plasmodium* parasites continued. Centuries of circumstantial evidence accumulated, and in 1883 the American physician Albert King assembled and published the evidence that would come to be known as the mosquito-malaria doctrine[8]. Patrick Manson, who had previously demonstrated the transmission via mosquitoes of another parasitic organism, the filarial worms which cause lymphatic filariasis[9], counseled his student Ronald Ross to search for a mosquito vector for malaria[10]. While Ross was posted in India, he followed Laveran's dictate to "follow the pigment" and examined several thousand mosquitoes from endemic areas. Eventually he observed the formation of pigmented bodies (which he named 'spores') on the stomach wall of a mosquito which had been experimentally fed on an infected patient. Further, he noted that after feeding on an infectious patient, the far more common 'grey' culicine mosquitoes never contained pigmented bodies, whereas the 'dapple-winged' anopheline mosquitoes contained pigmented bodies which ruptured releasing 'rods' which invaded mosquito salivary glands[11]. This was a crucial breakthrough demonstrating the presence of human malaria parasites in mosquitoes[12].

It had previously been hypothesized by William MacCallum that *Plasmodium* has a sexual stage of development, after he observed the combination of male and female gametes to form a zygote in the blood of crows infected with *Haemoproeus columbae*, a hematozoan closely related to malaria parasites[13]. Knowing of MacCallum's work in birds, combined with his own work, Ross concluded that *Anopheles* mosquitoes fed on infected mammals, took up the male and female gametocytes of the *Plasmodium* parasite in their blood meal, which then fertilized within the mosquitoes gut, and

developed into the ‘spores’ which he had observed, within which rod-like structures were produced, which invaded the mosquitoes salivary gland and were subsequently injected into a new mammalian host when the infected mosquito fed[11]. Further evidence implicating mosquitoes as the vector for malaria transmission came from a seminal study by Italian malariologist Giovanni Battista Grassi. Grassi sent volunteers to the Capaccio plains, a malarious region in Italy, and protected a fraction of them from mosquito bites from dawn to dusk. Of the 112 volunteers who were protected from mosquito bites, only 5 developed malaria. In contrast, all 415 of the unprotected volunteers contracted the disease[14]. Not only did this study cement the role of mosquitoes as the vector for *Plasmodium* scientifically, but it also set the precedent for controlling malaria by reducing contact with infected mosquitoes, through methods such as screening and mosquito-proofing homes which are still commonly employed today.

Scientists now understood that malaria was caused by a species of the parasitic protozoa *Plasmodium*, that it infected red blood cells, had a sexual stage of development in the blood, and was transmitted by mosquitoes. However, the understanding of the lifecycle of malaria in humans remained incomplete until the 1930s. The parasites could not be observed in the blood during the first ~10 days after infection, and it was unknown where in the body the parasites developed. Grassi and colleagues had begun to investigate the possibility of development in other cells types, but these endeavors were abandoned following a study by well-respected German scientist Fritz Schaudinn in 1903 which mistakenly described the direct invasion of red blood cells by the infective sporozoites of *Plasmodium vivax*[15]. Despite the field’s inability to confirm these observations, Schaudinn’s hypothesis dominated scientific opinion for over forty years, and is now referred to as Schaudinn’s fallacy. The question of exoerythrocytic parasite

development was first properly addressed in avian rather than mammalian malaria[16]. MacCallum, a noted avian malariologist who had also been the first to postulate a sexual stage of development for *Plasmodium*, observed developmental phases of *Plasmodium relictum* in the livers and spleens of infected birds[13]. However it was not until 1937 that Sydney James and Parr Tate conclusively demonstrated that in *Plasmodium gallinaceum* infections in chickens there was a phase of multiplication between the injection of sporozoites, and the appearance of parasites in the blood, and that this occurred in cells of the reticuloendothelial system[17]. There was a delay of another ten years until the same conclusion could be reached in primate *Plasmodium* infections; in 1947 Henry Shortt and Cyril Garnham showed that a phase of division in the liver preceded the development of parasites in the blood[18–20]. Shortly after, Shortt, Garnham, and colleagues found exoerythrocytic forms of *Plasmodium vivax*[21], *Plasmodium falciparum*[22], and *Plasmodium ovale*[23] in human volunteers. The only remaining mystery in the life cycle of *Plasmodium* parasites was the source of the long pre-patent periods and the appearance and reappearance of parasites in blood seen in some strains of *Plasmodium vivax*. This question was answered by Wojciech Krotoski in 1982 while he was working with Garnham's team, and discovered the dormant hypnozoite stage of *Plasmodium vivax*[24].

After thousands of years of living with malaria, an unknown French scientist with a rudimentary microscope noticed organisms in his malaria patient samples, and 122 years later the complete genome for *Plasmodium falciparum* was published in 2002[25,26]. There remain many questions to be answered concerning the basic biology of *Plasmodium*, and malaria remains an immense global health burden. New technologies

will allow us to address these questions in innovative and integrative ways as we move forward into the future from the history of malaria.

1.2: The basic biology of malaria parasites

Malaria continues to cause disease in 229 million humans annually with a death toll of approximately 409,000 per year[27]. Malaria infections are caused by the plasmodial species of parasite; six species of *Plasmodium* are infectious to humans: *Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae, Plasmodium ovale curtisi, Plasmodium ovale wallikeri, and Plasmodium knowlesi*. *Plasmodium falciparum* is responsible for the majority of deaths caused by malaria, and thus is often considered the most important for human health. Although the focus on *Plasmodium falciparum* is certainly appropriate, *Plasmodium vivax* should not be discounted, as it has recently been shown to be a comparable cause of severe disease in Southeast Asia, and it has been increasingly argued that *Plasmodium vivax* is underestimated[28]. *Plasmodium malariae, Plasmodium ovale curtisi,* and *Plasmodium ovale wallikeri* are much less common causes of severe disease[29]. *Plasmodium knowlesi* was previously considered to be a primate-infecting parasite, however it is now known that *Plasmodium knowlesi* is a zoonosis involving macaque and leaf monkeys as reservoir hosts with *Anopheles* mosquitos as the vectors, in Malaysia and elsewhere in Southeast Asia[30,31]. Further, advances in PCR identification have revealed that many infections previously attributed to *Plasmodium malariae* were in fact caused by *Plasmodium knowlesi*[31].

Like many other apicomplexan parasites, *Plasmodium* parasites have a complex life cycle, with stages in both vertebrate and invertebrate hosts (Figure 1.1). As Ronald

Ross concluded many years ago, *Plasmodium* **sporozoites** are injected into the dermis during a blood meal by a female *Anopheles* mosquito[12]. The sporozoites then use gliding motility to reach and penetrate a blood vessel, allowing them to enter the blood stream[32]. Many sporozoites are destroyed in the skin and drained to the lymphatics[29]. Those that successfully exit the dermis and access the blood stream quickly access the liver via traversal. The sporozoites cross the sinusoidal barrier comprising endothelial cells and macrophage-like kupffer cells[33], their traversal of these cells is mediated by the formation of a transient vacuole[34]. When sporozoites are injected into the dermis they are in 'migratory mode' and upon interaction with hepatocytes convert to 'invasive mode'. One molecular signal for this transition is sporozoite recognition of hepatocytes through binding highly sulfated proteoglycans, activating calcium dependent protein kinase 6 (CPK6)[35], which in turn activates the processing of the circumsporozoite surface protein (CSP) which coats the surface of the parasite and is crucial for invasion[36] (see chapter 3 for CSP specific background).

Once the sporozoite has successfully invaded the hepatocyte, the sporozoite transforms to a liver stage or **exo-erythrocytic form (EEF)**. The liver stage (LS) parasite is separated from the infected hepatocyte by a selective parasitophorous vacuolar membrane (PVM) of host hepatocyte plasma membrane origin[32,37]. The growing LS parasite acquires nutrients from its host hepatocyte and at the same time prevents its apoptosis[38–41]. This stage of development culminates in the release of up to 40,000 merozoites/hepatocyte into the blood stream by budding of parasite filled vesicles called **merosomes**[42]. See chapter 2 for in-depth liver stage background.

Free **merozoites** then invade erythrocytes in a multistep process including 1) pre-invasion, 2) active invasion, and 3) echinocytosis, all of which is complete within two

minutes[43]. The pre-invasion step involves receptor-ligand interaction between the merozoite and the erythrocyte, which results in parasite actomyosin motor driven deformation of the host cell[43]. Following this, the irreversible attachment of merozoites to erythrocytes occurs via formation of a tight junction formed between parasite derived proteins: AMA1 and the RON complex. The RON complex is deposited in the erythrocyte, with RON2 spanning the host membrane and binding to AMA1 on the merozoite surface[44]. Lipid-rich rhoptry contents form the parasitophorous vacuole membrane (PVM) as the merozoite is propelled into the erythrocyte using force generated by the parasite actomyosin motor[45]. Finally, after the active invasion phase is complete, fusion of membranes at the posterior end of the merozoite seals the parasite within the PVM within the erythrocyte. Echinocytosis follows, and causes the erythrocyte to shrink and form spiky protrusions[43].

Over the next two days, **schizogony** results in 16-32 new merozoites, which egress when developed, destroying the host erythrocyte in the process as they are released to access new host cells for invasion[46–48]. This explosive release of merozoites coupled with the rupture of host erythrocytes are responsible for the characteristic fevers and pathophysiology associated with malaria infections. Infected erythrocytes undergo extensive modification, which allows the intracellular parasite to grow and hide from host immune responses[49]. During these rounds of schizogony, a number of parasites transition to sexual development, to form male and female gametocytes. The gametocytes are taken up by a female *Anopheles* mosquito during a blood meal, receive activation signals including change in temperature and pH, and rapidly convert to fertile gametes[50,51]. After fertilization, the zygote further converts into the motile and invasive ookinete stage. Once the ookinetes are formed, they exit the gut lumen by traversing

through the mosquito midgut epithelial cell layer, before depositing on the basal site of the epithelium and converting once more into sessile oocysts, which undergo sporogonic replication[37]. Over the course of the next two weeks, this results in infectious sporozoites which migrate to the mosquito salivary glands and are subsequently injected into the human dermis during the next blood meal, and the cycle begins anew[52,53].

1.3: Naturally acquired immunity to malaria

The nature of malaria infections has made the task of identifying correlates of protection a difficult one. Many cohort studies have attempted to address this question, however small effect sizes and the polymorphic nature of immunogenic parasite proteins[54], combined with the inherent difficulty of addressing heterogeneity of malaria exposure (age, local transmission pressure, maintenance of baseline immune responses throughout the study or lack thereof)[55] have made the identification of true correlates of protection ambiguous at best. Further complicating this search, unlike many pathogens which elicit a highly potent, long-lived immune response, human naturally acquired immunity to malaria is less potent and is short lived[56]; true immunity to infection is rare, though immunity to disease does develop naturally over time and repeated exposure[57]. This difference between immunity to disease vs immunity to parasites is an important consideration. There is huge variability in parasite strains, and immunity is acquired to each isolate that is survived, giving ‘strain-specific immunity’. Over time, immunity builds, due to the acquisition of a repertoire of responses to many different isolates, and/or the development of a cross-protective response to shared antigenic targets[29].

Much of naturally acquired immunity targets the asexual blood stage, and is antibody mediated. Blood stage parasitemia is associated with the clinical symptoms of

malaria infection, as the cycles of rupturing erythrocytes causes an upregulation of pro-inflammatory cytokines. Antibodies to blood stage antigens are associated with clinical immunity in endemic areas[58–60]. Antibodies are generated either against the free merozoite, or against the infected erythrocyte. Antibodies against the free merozoite prior to invasion could block the ability of the parasite to enter the erythrocyte, or lead to cell-mediated destruction of the parasite via mechanisms including opsonophagocytosis, antibody dependent cellular inhibition (ADCI), or neutrophil mediated killing[29]. Antibodies against the infected erythrocyte may also recognize the highly modified host cell surface, triggering cell mediated ingestion or killing, or blocking cyto-adhesion of the infected erythrocyte to endothelial cells, making it susceptible to splenic removal[61].

Antibodies against parasite proteins from developmental stages other than the asexual blood stage have also been identified as being important. The Glutamate-rich protein (GLURP) is expressed in all the developmental stages of *Plasmodium falciparum* in humans[62]. The presence of antibodies to GLURP is associated with protection from clinical disease[63,64], and antibody-dependent cellular inhibition is hypothesized to be an important element in GLURP-specific protective immunity[64]. Antibodies against Liver-stage antigen 1 (LSA-1) are also associated with protection against clinical malaria[63]. LSA-1 is produced shortly after hepatocyte invasion and accumulates within the PVM surrounding the mass of developing merozoites[65]. LSA-1 plays a critical role during late-stage liver schizogony, and is thus important in the parasite transition from liver to blood stages[66]. Antibodies against the circumsporozoite protein (CSP), a key surface protein of the sporozoite stage of the parasite, have long been identified as being of importance for protection from clinical malaria (see chapter 3 for further information on CSP).

A key caveat when considering our knowledge concerning the naturally acquired correlates of protection is the limitations on the types of samples that can be collected from human patients. If the immunological information cannot be gleaned from a blood sample, either through sera analysis or through analysis of cryo-preserved peripheral blood mononuclear cells (PBMCs), it will be difficult to assess in a human population. This leaves out tissue resident populations of cells, which vaccine studies have indicated are of importance (see below). Though the study of cohorts of patience in endemic areas for naturally acquired immunity has been enlightening, many lessons about anti-malarial immunity have also been learned from decades of vaccine work.

1.4: Anti-malaria vaccines- lessons learned

There are three main avenues that have been explored for anti-malarial vaccines. These are 1) pre-erythrocytic vaccines, which aim to prevent malaria by stopping infection before it reaches the symptomatic blood stage, 2) asexual blood stage vaccines, which will be important for controlling morbidity and mortality, and 3) sexual blood stage vaccines, which have the potential to interrupt the transmission cycle, but do not have direct effect on an already established infection in the vaccinee.

The goal of pre-erythrocytic vaccines is to inhibit hepatocyte infections and hepatic parasite development, which will in turn limit erythrocyte invasion and symptomatic infection. Mechanisms of protection for vaccines targeting these developmental stages include antibody responses which prevent sporozoites from invading hepatocytes, and cytotoxic T cells which destroy infected liver cells[57]. By far the most notable anti-sporozoite vaccine is RTS,S/AS01. As for anti-liver stage vaccines,

attenuated whole sporozoite vaccines take center stage, though alternative vaccines including those using a prime and trap strategy have also been making headway.

RTS,S/AS01 (RTS,S), one of the longest studied candidate vaccines (developed by GSK over decades) is focused on generating immunity to CSP, the circumsporozoite protein of *Plasmodium falciparum* (*Pf*). RTS,S is a recombinant protein-based vaccine comprised of a fragment of CSP containing a section of the repeat region, and the T cell epitopes of the c-terminus attached to the Hepatitis B surface antigen protein, and delivered with additional HbsAg to encourage the formation of virus-like-particles in yeast, which are then harvested and administered with an adjuvant to humans to generate a T cell and antibody response. This is the only vaccine to show significant reduction in malaria in young children living in endemic regions. A phase 3 trial which spanned 5 years demonstrated that children aged 5-17 months who received 4 doses of RTS,S had a 39% reduction in malaria cases, and a 29% reduction in severe malaria cases over 4 years of follow-up[67]. After positive recommendations by WHO advisory boards, three countries, Ghana, Kenya, and Malawi began introducing RTS,S in 2019[27]. In the coming years, the Malaria Vaccine Implementation Programme (MVIP) will assess the feasibility of administering the recommended 4 doses of the vaccine in children, the potential role in reducing childhood death, and the safety of RTS,S in the context of routine use[68] as well as in specific important subpopulations.

An important recent advance in pre-erythrocytic malaria vaccines is the development of a novel polyvalent immunogen R21, a recent anti-malaria vaccine candidate, which builds on the knowledge gained from RTS,S. In contrast to RTS,S, R21 delivers a higher ratio of CSP to the Hepatitis B surface antigen (HBVsAg), which is in a 1 to 1 ratio as opposed to the 1 to 4 ratio of RTS,S[5]. Thus far R21 has proven to be well

tolerated and immunogenic in early trials, as well as protective in a controlled human malaria infection study (CHMI)[69,70]. The correlates of immunity which have emerged from these malaria vaccines support the importance of anti-CSP antibodies primarily but also suggest a role for T cells for imparting protection from *Plasmodium* infection[67,69–74]. The role of anti-*Plasmodium* T cells in vaccination has been highlighted by attenuated sporozoite vaccines[75].

The only vaccination method that has led to long lasting complete sterile protection against malaria parasite challenges in animals and in controlled human malaria infection (CHMI) is immunization with live irradiation-attenuated sporozoites[76,77]. Field studies in Burkina Faso, Mali, Kenya, Gabon, and Tanzania are currently following up on this approach[78]. While these studies are important for the efforts to develop an effective malaria vaccine, attenuation by irradiation is not easily standardized for human use. Over-irradiated sporozoites confer little protection while under-irradiation provides risk for breakthrough infections. More recently, attenuation of sporozoites was conducted by targeted deletion of genes that encode LS essential proteins in the mouse model, or by delivery of whole-sporozoites concurrently with chemoprophylaxis. In all attenuation methods, *Plasmodium* sporozoites invade hepatocytes within vacuoles, then cease growth and do not cause *Plasmodium* infection of the blood[32,37,79,80]. The protection conferred by attenuated sporozoites was confirmed to be mainly mediated by CD8+ T cells targeting LS antigens and not by antigens presented on the surface of migrating sporozoites[81–85]. Recent studies pointed out that when compared to attenuated strains that cease their LS development early, attenuated strains that grow longer in hepatocytes before ceasing growth led to more significant protective immune responses[81]. This indicated that significant exposure to LS antigens can enhance

vaccine effectiveness. Further, liver-associated T cells have been implicated in anti-malarial immunity following irradiated sporozoite vaccination[86–88]. When T cells lack CXCR6, a cell surface marker highly expressed by liver-infiltrating CD8 T cells, there is a reduction of liver-associated[89] memory and sporozoite immunity[86]. It has been established that CD8 tissue resident memory T cells are essential for protection against LS malaria following vaccination[87]. Therefore, LS proteins are also important candidates for inducing protective CD8+ T cell responses in the attenuated sporozoite model. Despite the evident potential of live attenuated parasite models as vaccines, the feasibility and large-scale application of live attenuated sporozoites that must be produced aseptically in mosquitoes in high amounts is still in development[90].

In contrast to pre-erythrocytic vaccines, the objective for anti-asexual blood stage vaccines is to mimic the observed naturally acquired immunity that people living with repeated malaria exposure in endemic regions develop. These patients achieve a state in which their immune systems control erythrocyte invasion to some extent, resulting in fewer disease symptoms or entirely asymptomatic infections[91,92]. This class of vaccine is designed to elicit immune responses that will block/limit merozoite invasion of erythrocytes, and prevent rapid replication of merozoites by targeting merozoite surface protein (MSP) family members, apical membrane antigen 1 (AMA-1), and the reticulocyte homolog (Rh) proteins[93–96]. Alternatively, blood stage vaccines may target parasite antigens which are embedded in the infected erythrocyte membranes, such as *Pf*EMP1[97]. A common challenge for blood stage antigens, is that while they are usually highly immunogenic, they are also highly polymorphic, so elicit antigen and strain specific responses[93,94]. In contrast, antigens such as Rh which are highly conserved[95,96] tend to be less immunogenic[98].

The final class of anti-malaria vaccines are anti-sexual blood stage vaccines, also known as transmission blocking vaccines, or TBVs. TBVs are designed to interrupt the transmission of parasites between humans and the mosquito vector, by exploiting host immune responses to parasite proteins, including both pre-fertilization and post-fertilization antigens. These host immune responses against sexual stage parasites are able to reduce the infectivity of the parasite, thus decreasing malaria transmission[99,100]. However, a key caveat for TBVs, which are known as the 'altruistic vaccine' is that they provide no immunity from disease for the vaccinee. For this reason, TBVs will likely be best utilized as a component of a multi-antigen vaccine targeting multiple stages of parasite development.

By themselves, none of the vaccination methods laid out above have been able to achieve high-level, durable, cross-strain protection from malaria. In order to pursue the goal of malaria elimination, radical new tools must be developed.

1.5: Advances in Vaccinology and their potential for combatting complex pathogens

Since the work of Edward Jenner in 1796, the field of vaccinology has continually evolved. Jenner noticed that inoculation with pus from cowpox lesions conferred protection from smallpox infections[101], and this landmark discovery paved the way for vaccines, eventually resulting in the eradication of smallpox in 1979 following a global vaccine administration program[102]. Jenner's work was later refined by Louis Pasteur, who was the first to attenuate viruses for use as vaccines. Although Jenner is often lauded as the founder of vaccinology, it was Pasteur who established the basis of vaccinology, i.e., the principle of inoculation, inactivation, and administration of disease-causing pathogens. Pasteur's principles allowed for the development of so-called "1st

generation" vaccines, based on whole microorganisms which had been killed or otherwise attenuated; this family of vaccines includes those against the plague, pertussis, and smallpox, and the BCG vaccine for tuberculosis[103,104]. In the second half of the twentieth century, improvements in mammalian cell culture technology enabled the development of live attenuated "2nd generation" vaccines such as those for polio, measles, rubella, mumps, and varicella. As a result, many of humanity's greatest infectious disease scourges have been greatly reduced, and polio has been nearly eradicated[105]. It is without question that traditionally developed vaccines have changed the landscape of public health, having prevented more than 700 million incidences of disease and more than 150 million deaths in the past century[106]. That said, conventional methods of vaccine design and development have limitations. First and second generation vaccines have been unable to provide protection from pathogens with antigenic hypervariability, such as HIV or HCV, or from pathogens with an intracellular phase which cause infections that are partially or primarily controlled by T cells, such as tuberculosis or malaria[107]. Antigenic polysaccharides, which primarily produce a B cell dependent immune response, are covalently linked to carrier proteins, thereby providing helper T cell activation. These glycoconjugate vaccines induced a stronger antibody response, and thus increased their protective efficacy. Progress in molecular biology led to the further development of this class of vaccines, in which purified recombinant protein antigens are made to form a virus-like-particle (VLP)[108].

Decades of research on vaccine candidates for malaria, as well as countless studies interrogating the parasite's basic biology and epidemiology have gleaned valuable information. However, leveraging recent advances in bioinformatics, systems biology, and non-traditional vaccinology has the potential to transform the field of malaria

vaccines. Reverse vaccinology allows teams to have access to the entire repertoire of pathogen proteins by sequencing and analysis, which can enable comparisons of conserved sequences shared among pathogen strains within the same species[109]. Advances in structural vaccinology have given us an improved understanding of the native structures of biological macromolecules, and how changes in their structure affect their function. This can in turn assist the identification of suitable epitopes.

Traditional vaccine design has relied on strategies like protein subunit vaccines and weakened or killed versions of the pathogen in question. These techniques, while valuable, have not yielded a high-efficacy malaria vaccine with durable cross-strain protection. Advances in vaccinology, notably in the vaccine platforms of nucleic acid vaccines and nanoparticle vaccines could be a solution. Synthetic nucleic acid vaccines combine the advantages and high immunogenicity of *in situ* expression with the high safety of subunit vaccines. Additionally, nucleic acid vaccines do not have potential complications from pre-existing vector immunity as in the case of virally vectored vaccines. Of key importance for infectious diseases of global health relevance like malaria, nucleic-acid vaccines also have the potential to be produced simply and inexpensively[108]. Among nucleic acid vaccines, both RNA and DNA vaccines must be discussed.

RNA vaccines, which are based on mRNA or RNA replicons, have some advantages and some disadvantages over DNA. RNA vaccines are active in the cytoplasm and do not require delivery to the nucleus, however RNA vaccines are also more susceptible to degradation and thus require additional stabilizing technology[110]. mRNA vaccines are enjoying a rise in popularity; while the proof of concept for RNA vaccines in humans was achieved in cancer vaccines years ago[111–113], the advent of

SARS-CoV-2 and the rapid response of Moderna and Pfizer/BioNTech resulting in the first FDA approved SARS-CoV-2 vaccines both being mRNA vaccines[114,115] will surely result in a surge in mRNA vaccines in the years to come.

DNA vaccines held much of the focus in the nucleic acid vaccine field for many years, and have been shown to be potent and efficacious in a wide variety of animal species; this work has resulted in several licensed veterinary products[116–119].

Initial studies of DNA vaccines in humans showed less than impressive immunogenicity[120], however recent advances in improved delivery through the use of electroporation[121], or through coadministration of immunostimulatory cytokines as molecular adjuvants are overcoming these limitations[122,123]. Of note the low initial immunogenicity was likely tied to lower levels of DNA expression in the early years of DNA vaccine development, however innovative delivery methods such as jet delivery, gene gun delivery, nanoparticle delivery, and others have demonstrated increased DNA uptake in vivo[124]. With these innovations has come pre-clinical and clinical success stories for DNA vaccines; multiple studies have now reported that synDNA vaccines generate robust cellular and humoral immune responses against pathogens, with impact in challenge model systems, as well as demonstrating a remarkable safety profile in the clinic[125–128].

Adaptive electroporation (EP), a technique which controls the energy delivered during in vivo EP, increases the initial uptake pf plasmid by local cells approximately 500x over classical needle and syringe delivery[129] . Following injection, plasmid DNA is taken up by local cells at the site of injection, where the DNA is transcribed into mRNA and translated into the antigen of interest intracellularly. In addition to the local myocytes

which have become antigen factories, locally transfected antigen presenting cells (APCs) can directly traffic to the regional lymph node, which is critical to initiating the immune response[130,131]. Translated antigen can also be shed exogenously, and picked up by APCs for subsequent cross presentation. Shed exogenous soluble antigen can also drain to the regional lymph node, allowing for the engagement of B-cell immunity[132]. Thus, local tissue at the site of injection becomes a sustained source of antigen, allowing for presentation of antigen on major histocompatibility complex I (MHC-I) or MHC-II molecules for re-expansion of lymph node primed CD8+ or CD4+ cells respectively[133]. DNA vaccines also have potential as part of a heterologous prime-boost routine, whereby DNA vaccines can effectively prime B- and T-cell responses (Figure 1.2). Heterologous prime-boost vaccine strategies generally use either a viral vector or a DNA vaccine for priming, followed by a protein-based vaccine boost[134]. This immunization schedule results in the induction of a strong cellular immune response, as well as a higher and more specific antibody response as compared to homologous immunization[135].

I hypothesize that DNA vaccines are an ideal vaccine platform for a next-generation anti-malaria vaccine, due to their ease of production and cost-effectiveness, and their ability to induce robust cellular *and* humoral immunity, which is of key importance for a pathogen with both extracellular and intracellular phases.

1.6: Summary

While the global incidence of malaria has decreased in the past decade, the infection remains a significant global health concern. In 2019 there were approximately 229 million cases worldwide, of which 93% were in the WHO African Region[136]. Those

cases resulted in 409,000 fatalities, with a high burden in younger populations, as 67% of cases occur in children under 5 years of age[136]. Nearly half of the world's population is at risk for malaria infection[27]. Malaria is caused by infection by *Plasmodium* parasites, and while six species can cause disease in humans, *Plasmodium falciparum* (*Pf*) remains the most prevalent malaria parasite, accounting for 99.7% of all cases in the WHO African region[136]. Symptoms can arise 10-15 days after the infective mosquito bite, and include fever, headache, and chills, which can progress to severe illness and potentially death[27].

The WHO Global Technical Strategy for Malaria 2016-2030 established a number of goals to be achieved globally by 2030, including 1) reducing malaria case incidence by 90% by 2030, 2) reducing malaria mortality by at least 90%, and 3) eliminating malaria in at least 35 countries[27]. Malaria elimination is defined as the interruption of local transmission of a specified malaria parasite species in a defined geographical area as a result of deliberate actions[27]. Achieving these goals will require varied approaches, including vector control approaches such as insecticide treated bed nets and indoor spraying with residual pesticides. The inclusion of a vaccine into these techniques has always been of paramount importance, and this importance continues to grow as drug resistant strains of the parasite emerge[27]. The following chapters describe the development of and immune mechanisms behind multiple synthetic DNA vaccines targeting pre-erythrocytic stages of malaria infection. Understanding malaria infection, the immune response elicited by vaccination, and the continued development of novel vaccine strategies remain crucial elements in the world-wide battle against malaria.

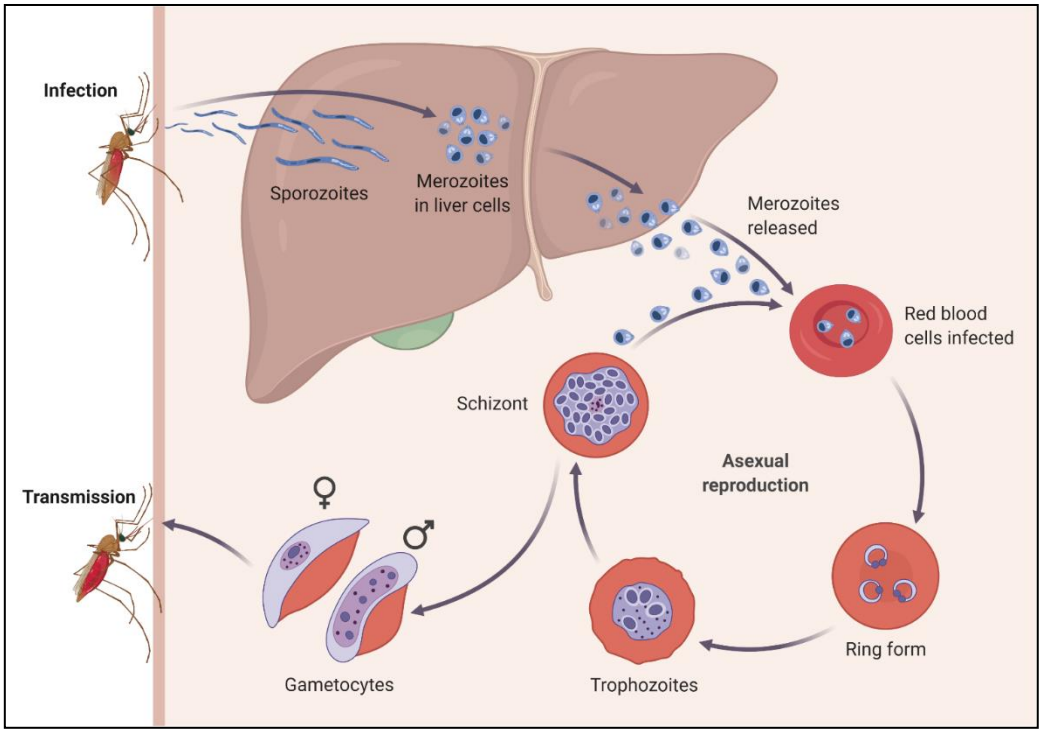

Figure 1.1: *Plasmodium* Life Cycle. Figure generated with BioRender©

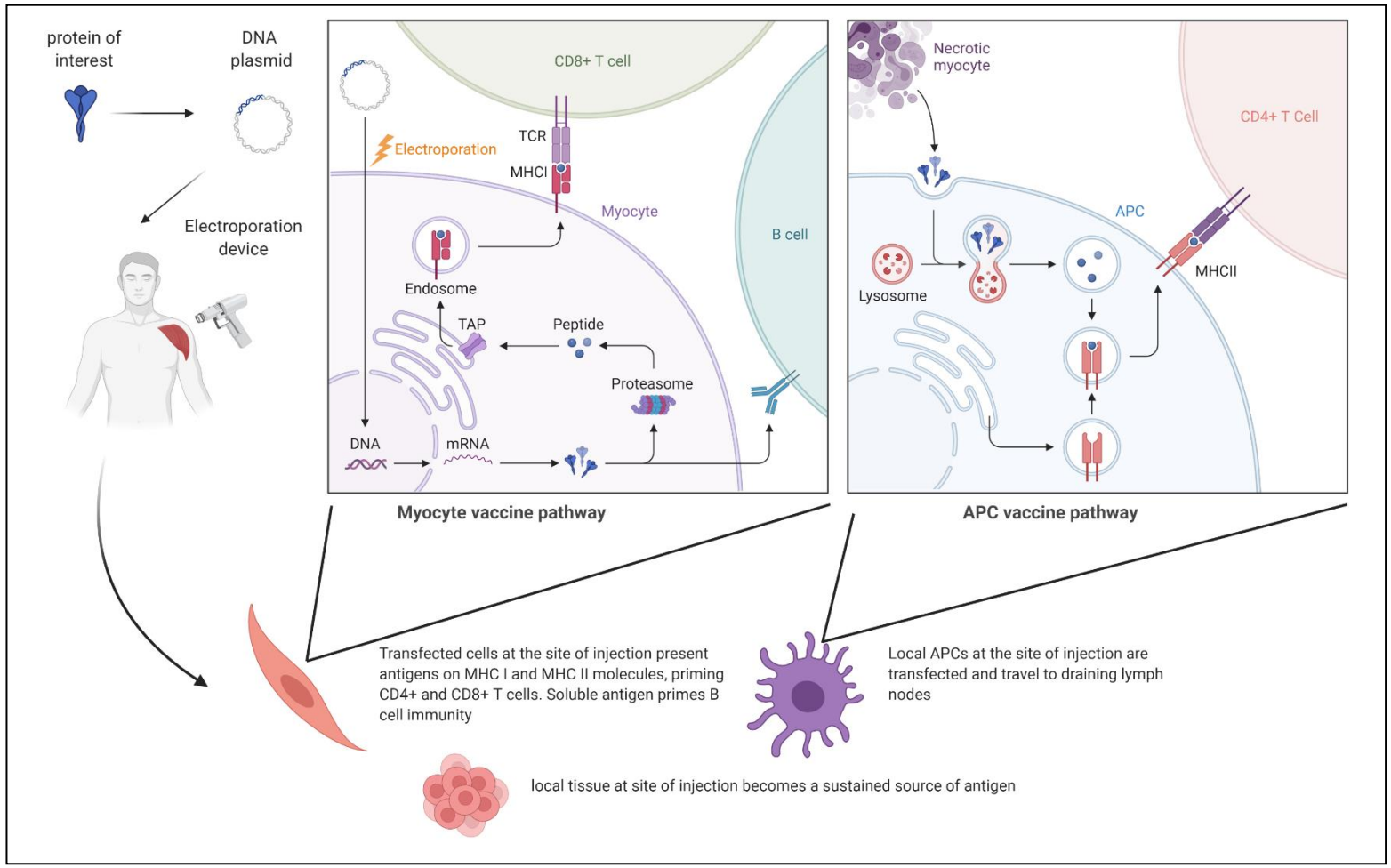

Figure 1.2: Mechanisms of DNA vaccine induction of immunity. Figure generated with BioRender©

CHAPTER 2: The importance of liver localized T cells in protection from malaria

Parts of this chapter were previously published in *Vaccines*:

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Introduction

As summarized above, infection in humans begins when the human is bitten by an infected female Anopheles mosquito and inoculated with sporozoites, which then travel through the blood or lymphatics, and invade hepatocytes. This chapter will focus on the development of a pre-erythrocytic vaccine targeting liver stage antigens, and the potential mechanisms of protection for the immunity it drives.

I hypothesize that LS proteins delivered by a synthetic optimized DNA plasmid vaccine should induce protective immunity similar to that observed with live attenuated parasite models (See Chapter 1.4). While there are a plethora of liver stage antigens, I focused on the following antigens, which are expressed across *Plasmodium* spp.*:* EXP1, profilin (PFN), EXP2, ICP, TMP21, and UIS3. EXP1 (exported protein 1) is a glutathione transferase, located at the parasite-host interface, which efficiently degrades cytotoxic hematin, and is associated with the metabolism of and susceptibility to artesunate, a frontline anti-malarial drug[137,138]. EXP1 has been previously shown to be immunogenic in a mouse model of malaria[139]. There is evidence in humans that EXP1 may be an

important anti-malarial target, as a positive antibody response to EXP1 correlated with a statistically significant decrease in malarial infection in children in Burkina Faso[140]. Profilin (PFN) has been detected in all life cycle stages, including sporozoites and merozoites, and abundant PFN expression suggests PFN is important for *Plasmodium* life cycle progression[141,142]. Like all apicomplexans, *Plasmodium* utilizes a highly specialized microfilament system for motility and host cell invasion, and profilin plays a key role as an actin-sequestering protein[143,144]. It has been shown that disturbing expression of PFN results in complete life cycle arrest[145]. Similarly to EXP1, exported protein 2 (EXP2) is an integral vacuolar protein[146]. EXP2 resides primarily on the vacuolar face of the PVM, and likely constitutes the membrane pore[147]. In *Plasmodium*, inhibitor of cysteine proteases (ICP) has been shown to be necessary for malaria transmission from mosquitos to mammals, sporozoite motility, erythrocyte invasion[148], and liver stage development[149]. While little is known about transmembrane protein 21 (TMP21), vaccination with TMP21 reduced liver stage parasite load in a mouse model and has been shown to contribute to the protective immunity elicited by whole parasite vaccinations[150]. The antigen UIS3 (upregulated in infective sporozoites-3) is a membrane protein that is localized to the PVM in infected hepatocytes[151] and interacts directly with host liver-fatty acid binding protein (L-FABP)[152]. UIS3 is essential for early liver stage development[153] and vaccination with a ChAd63-MAV vaccine containing UIS3 has been shown to be partially protective in a mouse model[154].

One of the strengths of the synthetic DNA vaccine platform is the ease with which antigens can be co-formulated with each other and with molecular adjuvants in the clinic[155–158]. Given the focus on induction of cellular immunity, we explored using synthetic DNA-encoded plasmid IL-33 (pIL-33) as a molecular adjuvant. pIL-33 is a

member of the IL-1 family, which although originally associated with Th2 immunity, has been shown to facilitate the generation of protective Th1 and CD8 T cell immunity[156]. pIL-33 has immunoadjuvant effects in an HPV-associated model for cancer immunotherapy in which cell-mediated immunity is critical for protection and has been shown to enhance potent antigen specific effector and memory T-cell immunity in a DNA vaccine setting[158]. The ability to induce potent cell mediated immunity is important for a vaccine against LS malaria, as is the fact that IL-33 is predominantly expressed at the epithelial barrier as the first line of defense against pathogenic threats, activating a variety of immune cells[159], which may be relevant to the necessity for *Plasmodium* sporozoites to transverse through the dermis before LS infection. Consequently, IL-33 was chosen as a molecular adjuvant for this study. Another strength of the DNA vaccine platform is its ability to drive functional, localized cell mediated immunity (CMI). Prior work studying the immunity from a hepatitis B DNA vaccine showed the ability of this platform to drive vaccine specific CTLs to traffic to the liver, an organ that is known to be tolerogenic and suppress T cell responses[160]. The ability of this platform to drive vaccine specific CTLs in this location highlights the potential to drive relevant, functional CMI in the context of a liver infection.

Results

synDNA vaccine construct design and *in vitro* expression.

Previously identified LS proteins were optimized and encoded into a modified pVax plasmid. Plasmid 1 contains an IgE leader sequence, the EXP1 gene sequence, a linker sequence, and the PFN gene sequence. Plasmids 2, 3, 4, and 5 each contain an IgE leader sequence, the gene sequence for EXP2, ICP, TMP21, or UIS3 respectively, and an HA tag (Figure 2.1A). While all LS proteins selected are expressed across *Plasmodium* spp., the synthetic optimized DNA vaccine constructs were matched for *Plasmodium yoelii* (*Py*), to reflect the planned challenge model.

In vitro expression of *Py* constructs in transfected 293T lysates was detected by western blot. Expression was confirmed for plasmids 2-5 by detection of the HA tag. Expression for plasmid 1, which lacked an HA tag, was confirmed by probing with post-immune sera from mice immunized with the construct. GFP transfection was used as a negative control (Figure 2.1B).

Py LS vaccine constructs delivered individually elicit a robust and polyfunctional T cell response

To assess the cellular immune response to LS antigen vaccination, groups of 5 mice were immunized 4 times at 3-week intervals with one of the five constructs individually, or the empty vector pVax as a negative control. One week after final immunization splenocytes were collected for immune analysis and antigen specific cytokine production was assessed by IFNγ ELISPOT and flow cytometry. All 5 constructs induced detectable IFNγ cellular responses with EXP1_PFN and ICP being the highest inducers. EXP2 and UIS3 induced lower, but still robust levels of IFNγ secreting cells, and TMP21 induced

readily detectable, but the lowest, levels of IFNγ secreting cells (Figure 2.2A). The functional profile of antigen specific CD4+ (Figure 2.2B) and CD8+ (Figure 2C) T cells was analyzed by flow cytometry. Mono-, double-, and triple-positive CD4+ and CD8+ T cells releasing the cytokines IFNγ, TNFα, and IL-2 are shown. CD4+ T cells responded most highly to EXP1, EXP2, ICP, and UIS3. In contrast, CD8+ T cells responded most highly to PFN and EXP2. As expected, ELISPOT and flow cytometry responses in mice immunized with the empty vector control, pVax, were negligible (Figure 2.2).

Co-formulated *Py* LS vaccines delivered with and without plasmid IL-33 adjuvant elicit a robust and polyfunctional T cell response

To investigate the immunogenicity of co-formulated vaccine constructs groups of 5 mice were immunized 4 times at 3-week intervals with combinations of constructs (EXP1_PFN alone, EXP2 and ICP, TMP21 and UIS3, and all constructs together) with and without the molecular adjuvant pIL-33 all delivered in a single injection site. Splenocytes were collected for analysis of the cellular immune response using IFNγ ELISPOT and flow cytometry. All immunization groups saw an upwards trend in IFNγ ELISPOT responses with the addition of pIL-33 (Figure 2.3A). The functional profile of CD4+ (Figure 2.3B, D, E, and F) and CD8+ (Figure 2.3C, G, H, and I) T cells was analyzed by flow cytometry. In the mice immunized with EXP1_PFN alone, CD4+ T cells respond more highly to EXP1 and this phenotype is enhanced in the adjuvanted group (Figure 2.3D). By contrast, as seen in Figure 2.3G, CD8+ T cells respond more highly to PFN, and this phenotype is also enhanced in the adjuvanted group. In mice immunized with EXP2 + ICP, CD4+ T cells responded quite similarly to EXP2 and ICP; however, in the adjuvanted group the EXP2 response was preferentially increased (Figure 2.3E). CD8+ T cells responded more highly to EXP2; however, this phenotype was not recapitulated in

the adjuvant group (Figure 2.3H). In the mice immunized with TMP21 + UIS3, CD4+ T cells responded more highly to UIS3 (Figure 2.3F), a phenotype that did not change with the addition of pIL-33, whereas CD8+ T cells responded more highly to TMP21 (Figure 2.3I). As expected, ELISPOT and flow cytometry responses in mice immunized with the empty vector control, pVax, or pIL-33 alone were negligible (Figure 2.3A, B, and C).

Py LS antigen vaccination elicits a robust and polyfunctional antigen specific T cell response in the liver which is enhanced with the addition of plasmid IL-33

The functionality of liver localized antigen specific T cells was investigated using the EXP1_PFN construct, as EXP1_PFN consistently produced the most robust T cell response. Mice were immunized 3 times at 3-week intervals with the EXP1_PFN construct with and without pIL-33. One week after the final immunization lymphocytes were isolated from both liver and spleen of immunized mice and their phenotype and functional capacity was measured using flow cytometry and ELISPOT assays. The percentage of CD8+ cells in the liver expressing CXCR6, a chemokine receptor important for trafficking to the liver[87], was significantly increased from 40% without co-delivery of pIL-33 to 60% with the addition of pIL-33 (Figure 2.4A) while the expression of CXCR6 on CD8+ cells in the spleen was 10-fold lower (Figure 2.4B) than that on CD8+ cells in the liver. This suggests an improvement of trafficking to the liver with the addition of pIL-33. As seen previously, the addition of pIL-33 increased antigen specific IFNγ ELISPOT responses in the spleen (Figure 2.4D) and interestingly, also in the liver (Figure 2.4C). Further investigation of the functionality of the liver localized T cells using flow cytometry revealed that pIL-33 not only increased the percentage of IFNγ+TNFα+IL-2+ (triple positive), IFNγ+, and IL-2+ CD4+ cells (Figure 4G) and IFNγ+ CD8+ (Figure 2.4H) cells from the spleen, but also increased the percentage of IFNγ+ CD4+ (Figure 2.4E) and CD8+ (Figure 2.4F) cells from the liver.

Vaccine delivered with and without adjuvant elicits antigen-specific antibody responses

Vaccine induced antibody production was assayed by immunofluorescence. Hepa1-6 cells, were transfected with each DNA vaccine construct. Two days post transfection the cells were probed with post-immune sera from mice immunized with the respective DNA construct (with or without pIL-33) and probed with a fluorescently tagged secondary antibody. Mice immunized with EXP1_PFN showed the most positive staining indicating the highest antibody response. EXP2, ICP, and UIS3 showed intermediate antibody levels and TMP21 showed the lowest levels of antibody induction (Figure 2.5). The addition of pIL -33 did not appear to alter the amount of detectable antibody binding (Figure 2.5). However, we cannot conclude whether or not this antibody response is important for the observed immunity, as it is unknown whether these *Plasmodium* proteins would be available for antibody binding in a physiologically relevant context. It is important to note that while this assay shows the presence of antibodies elicited by vaccination, it does not provide quantitative information on antibody level or titer.

***Plasmodium* LS synDNA vaccine is protective against malaria infection in a mouse model**

To assess the ability of these DNA vaccine constructs expressing LS malaria antigens to protect against malaria, groups of 7-8 mice were immunized 4 times at 3-week intervals with combinations of constructs (EXP1_PFN alone, EXP2 and ICP, TMP21 and UIS3, and all constructs together) with and without the molecular adjuvant pIL-33, and then challenged 9 weeks later with intravenous delivery of 250 infectious *P. yoelii* sporozoites. Blood was collected daily following sporozoite injection for blood smears to check for the

presence of blood stage parasites, i.e. patency (Figure 2.6A). All empty vector and pIL-33 alone immunized animals had visible blood stage parasites 4 days after challenge. 71-88% of animals immunized with DNA vaccine delivered without adjuvant were completely protected from patency, with parasitemia in the few animals who were not protected being delayed 1.5-2 full days. 100% of animals immunized with any combination of constructs in addition to pIL-33 were completely protected from blood stage parasitemia indicating sterile protection in these groups (Figure 2.6B). Examples of the blood smears from each group showing the presence of blood stage parasites only in the pVax and pIL-33 alone groups are shown in Figure 2.6C.

Discussion

Synthetic DNA delivered by adaptive electroporation (EP) is a particularly attractive vaccine platform for a LS antigen malaria vaccine because of its ability to induce robust CD8+ T cell responses. In clinical studies, synDNA vaccines delivered with EP have proven highly effective in small and large animal models of infectious disease and cancer, and have demonstrated the ability to drive a tissue infiltrating population of antigen specific CD8 T cells[157]. In recent years, DNA vaccines for HPV[157], HIV[161,162], Zika[155], and Ebola[163] among others have moved into human clinical trials. Here, I present a novel approach using synthetic DNA vaccination with LS antigens in conjunction with a plasmid encoded molecular adjuvant IL-33 which together drive antigen specific liver associated CD8 T cells and achieve complete protection from blood stage disease in a virulent *Plasmodium* sporozoite challenge in the mouse model.

This study demonstrates that a synDNA vaccine targeting liver stage *Plasmodium* antigens drives an antigen specific liver localized T cell population. Further, this study illustrates the potential efficacy of a synDNA vaccine platform targeting liver stage proteins in providing protection from malaria infection in this model. I show that a synDNA vaccine targeting EXP1, PFN, EXP2, ICP, UIS3, and TMP21, in combination or alone, elicits a robust T cell response, as well as the production of antibodies against liver stage malaria. The use of the molecular adjuvant pIL-33 increases the immune response to vaccine and results in 100% protection from blood stage disease after sporozoite challenge, where non adjuvanted vaccine results in 70-88% protection from blood stage disease. A potential contributor to this increased protection in the adjuvanted groups is the increase in antigen responsive liver associated T cells, as well as their polyfunctionality as demonstrated by enhanced cytokine poly-positivity in

adjuvanted groups. Additional focus on LS antigens as a component of a malaria vaccine is warranted, potentially in combination with sporozoite antigens such as CSP. RTS,S and R21 combinations might be particularly interesting[164–166]. Altogether, the data suggest cell-mediated immunity as well as antibodies should be considered when designing anti-malarial vaccines and support the continued examination of liver stage antigens as components of a prophylactic vaccine.

Materials and Methods

Construct design

Protein sequences for selected *Plasmodium* antigens were accessed on PlasmoDB, the *Plasmodium* genomics resource (EXP1: PY04421, PFN: PY03207, EXP2: PY05892, ICP: PY17X_0816300, TMP21: PY06414, UIS3: PY03011). While all antigens selected are expressed across *Plasmodium* spp., the vaccine constructs were matched to *Plasmodium yoelii* 17X (*Py*), to reflect the planned challenge model, which was *P.yoelii* 17X-NL (non-lethal) strain. The synthetic DNA vaccine constructs were codon optimized for mice and humans and were incorporated into a modified pVax vector with an IgE leader sequence, and an HA tag where indicated in Figure 2.1.

Western blot

In order to assess *in-vitro* expression of the vaccine constructs, 293T cells (ATCC® CRL-3216™) were plated in 6-well plates at 0.5-0.7x10^6 cells per well in 2 mL DMEM (Gibco) + 10% FBS and incubated overnight at 37 °C in 5% CO_2. Cells were transfected with 5 µg DNA using TurboFectin 8.0 transfection kit and incubated for 48 hr. 48 hours after transfection, supernatants and lysates were collected for western blot analysis. Samples were prepared with NuPAGE LDS Sample Buffer, 10X Reducing Agent, and deionized water. Sample mixture was incubated at 70 °C for 10 minutes. 25 µl of sample was loaded per well onto an Invitrogen NuPAGE Bis-Tris Gel in 1X NuPage MOPS running buffer. The gel was run for 150 V for 50 min. Protein was transferred to a methanol activated PVDF membrane using the iBlot 2 Dry Blotting System (Life Technologies). The membrane was blocked for 1 hr at room temperature with Li-Cor Odyssey Blocking Buffer followed by overnight incubation at 4 °C with primary antibody.

Primary antibody was either an anti-HA antibody (ThermoFisher) to detect expression of the constructs which contain an HA tag, or post-immune sera collected from mice immunized with the construct to detect expression of the EXP1_PFN construct which did not have an HA tag. After washing, the membrane was incubated for 1 hr at room temperature with IRDye labeled secondary antibody (Li-Cor) and then imaged using an Odyssey CLx imager.

Immunization and CELLECTRA electroporation

Female BALB/C mice were ordered from the Jackson Laboratory aged 6-8 weeks and were housed in the Wistar Institute Animal Facility. Mice were immunized with 25 μg DNA vaccine construct with or without 30 μg of plasmid pIL-33 delivered intramuscularly using the CELLECTRA 3P adaptive constant current electroporator[167]. When mice were immunized with a cocktail of antigens, 25 μg of each DNA vaccine construct was used. Depending on the experiment, mice were immunized 4 or 3 times at 3-week intervals. Blood was collected 1 week post each vaccination for sera isolation. One week post final immunization mice were euthanized and splenocytes and hepatocytes were collected for immune analysis.

Immune Cell Isolation

After euthanasia, liver and spleen tissue were removed for immune cell isolation. Before liver excision, the hepatic portal vein exiting the liver was cut and 5-10 ml of cold PBS solution was administered through the left ventricle to profuse the liver until blanched. Once perfused the liver was removed and placed in cold complete media (DMEM + 10% FBS + 20 mM HEPES + 1X Pen/Strep). Livers were quickly homogenized for 1 min at normal speed using a Stomacher 80 (Seward). Homogenates

were transferred to 6-well plates with 10 ml of digest media (DMEM + 20 mM HEPES + 0.1 mg/ml Collagenase 4 + 0.02 mg/ml DNase) added and plates were incubated at 37 °C for 30 min. Cells were filtered through a 100 µm mesh strainer, rinsed with PBS, and then centrifuged at 300 rpm for 1 min. The supernatant containing hepatocytes was transferred to a new tube and spun down for 5 min at 1500 rpm. The cell pellet was resuspended in 40% Percoll (Sigma-Aldrich), underlaid with 80% Percoll, and spun at 3000 rpm for 20 min with slow acceleration and deceleration. Hepatocytes at the interface of the two Percoll layers were removed and diluted in complete media, then counted using a COUNTESS II (Invitrogen) and trypan blue (Gibco).

Spleens were removed and placed in RPMI + 10% FBS + 1X Pen/Strep (R10). Spleens were homogenized for 1 min on high using a Stomacher 80. The cell solution was filtered through a 100 µm strainer and spun down for 10 mins at 1200 rpm. Cells were resuspended in 5 ml ACK lysis buffer (Gibco) and incubated for no more than 5 min. After washing with PBS, cells were spun for 10 min at 1200 rpm. Splenocytes were then resuspended in 20 ml of R10 and counted.

ELISPOT

Mouse IFN-γ ELISpot PLUS (Mabtech) plates were used as directed. Briefly, plates were washed with PBS and blocked with R10 for 30 min. Wells were seeded in triplicate with 200,000 cells in 100 µl R10. Cells were stimulated with peptide pools of 15mers overlapping by 11 amino acids spanning the entire vaccine antigen at a final concentration of 5 µg/ml per peptide. R10 and Concanavalin A were used as negative and positive controls, respectively. Plates were incubated for 18 hr at 37°C with 5% CO_2. Plates were developed as directed, scanned, and counted using a CTL ImmunoSpot S6

Universal Analyzer. Data was exported to Microsoft Excel and GraphPad Prism 8 for analysis.

Flow cytometry

Wells were seeded with 1,000,000 cells in 100 µl of R10. Cells were stimulated with peptides at a final concentration of 5 µg/ml per peptide in the presence of Protein Transport Inhibitor (eBioscience). R10 and Cell Stimulation Cocktail (eBioscience) were used as negative and positive controls, respectively. Plates were incubated for 6 hr at 37 °C with 5% CO_2. Following incubation, cells were washed with PBS, and stained with Live/Dead fixable aqua dead cell stain kit (ThermoFisher) in PBS. Cells were then stained for extracellular markers in 1% FBS in PBS (FACS buffer), fixed and permeabilized with BD Fix/Perm, stained for intracellular markers and cytokines in BD perm/wash, resuspended in FACS buffer, and run on BD LSRII flow cytometer (BD Biosciences). Splenocytes and hepatocytes were stained with following panel: LiveDead Aqua (Invitrogen, L34957), CD19-V450 (BDHorizon, 560375), CD3-AF700 (BioLegend, 100216), CD4-FITC (BD Pharmingen, 553047), CD8-BV605 (BioLegend, 100744), IFNγ-APC (BioLegend, 505810), TNFα-PE (eBioscience, 12-7321-82), IL-2-PE-Cy7 (eBioscience, 25-7021-82) and CXCR6-BV421 (Biolegenend, 151109). Gates were set using FMOs for each stain. Data were exported and analyzed in GraphPad Prism 8.1.1.

Immunofluorescence

Hepa1-6 cells (a cell line derived from mouse liver cells, ATCC® CRL-1830™) were plated on pre-coated Poly-D-Lysine (Corning) 8 chambered wells at 100,000 cells per well in 400 µl DMEM (Gibco) + 10% FBS and incubated overnight at 37 °C in 5% CO_2. Cells were transfected with 1 µg DNA using Thermofisher Lipofectamine 3000

transfection kit and incubated for 48 hr. Media was removed, and cells were washed twice with PBS (Gibco) for 5 min and then fixed with 2% PFA in PBS for 5 min. Cells were again washed twice with PBS followed by blocking in 5% goat serum + 0.05% Tween-20 for 1 hr at room temperature followed by two 5 min PBS washes. Cells were incubated with 500 µl pooled mouse sera at a 1:50 dilution in 1% BSA + 0.05% Tween-20 in PBS buffer at room temp for 1 hr. After washing three times with PBST (0.05% Tween-20 in PBS) for 5 min each, cells were incubated with 300 µl of secondary antibody diluted in 1% BSA + 0.05% Tween-20 in PBS buffer at room temp for 1 hr. Cells were washed with PBST for 5 min, then incubated with DAPI (Hoechst 33342 Fluorescent Stain, Thermo Scientific) at a 1:5,000 dilution in PBST for 5 min, followed by a final wash with PBST to remove unbound DAPI stain. Cells were imaged using a Leica TCS SP5 confocal microscope.

Mosquito feeding and sporozoite extraction

Six-to-eight-week-old female Swiss Webster (SW) mice (purchased from Envigo, Indianapolis, IN) were used for mosquito feeding experiments to generate salivary gland sporozoites for challenge studies. All animal handling was conducted according to the approved protocols of the Institutional Animal Care and Use Committee (IACUC) of Tulane University (Protocol # 4258R). Mosquito feeding experiments were conducted with *P. yoelii* 17X-NL wild-type parasites as previously described[168–170]. Briefly, SW mice, treated with phenylhydrazine, are injected intravenously with 1 million blood stage parasites, and on day 3 post infection an exflagellation assay is used to confirm the availability and formation of *P. yoelii* male microgametes. Mosquito feeding is conducted by allowing about 150 female mosquitoes or less to feed on a mouse anesthetized with ketamine/xylazine for 15 min. Salivary gland sporozoite extraction was conducted by

dissection of the salivary glands of infected female mosquitoes at day 14 or 15 post mosquito feeding (pmf) in RPMI incomplete medium, as previously described [81,171,172]. Collected salivary glands were mechanically disrupted with a pestle and the salivary gland sporozoites were counted using a hemocytometer. Doses of 250 sporozoites in 150 µl incomplete RPMI were prepared as previously described[86,87,137].

Challenge study

Six-to-eight-week-old female BALB/cJ mice (purchased from Jackson Laboratories, Bar Harbor, ME) were used for challenge experiments. Mice were put under a red heating lamp 5-10 min before injection. Each dose of 250 sporozoites was loaded in 27G insulin syringes and was injected intravenously in the tail vein of immunized mice. Giemsa-stained thin blood smears were checked every day (at least 50 whole microscopy fields at 1000x) for blood stage parasites, starting from day 3 until day 10 post infection[81,171,172].

Figures

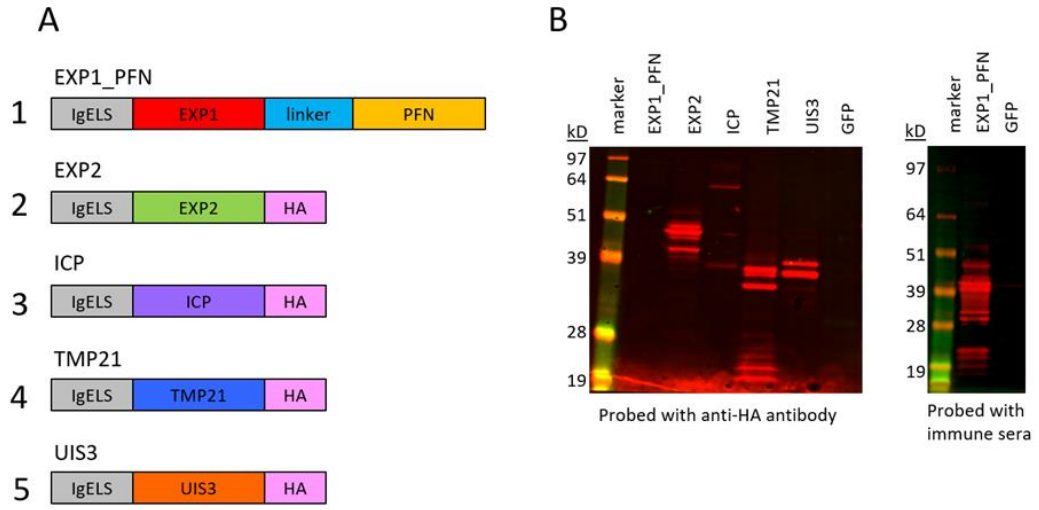


Figure 2.1 DNA vaccine construct design and in vitro expression. **(A)** Schematic diagram of Plasmodium yoelii (Py) gene inserts used to generate the codon-optimized DNA vaccine constructs. The schematic details leader sequence (IgE), gene insert, and presence or absence of HA tag. All constructs with the exception of EXP1_PFN contain an HA tag. **(B)** Expression of Py proteins detected by SDS-polyacrylamide gel electrophoresis and western blot of lysate from transfected 293T cells. Protein expression was detected by probing for the HA tag when present with an anti-HA antibody, or with immune sera from immunized mice for the EXP1_PFN plasmid. GFP is included as a negative control.

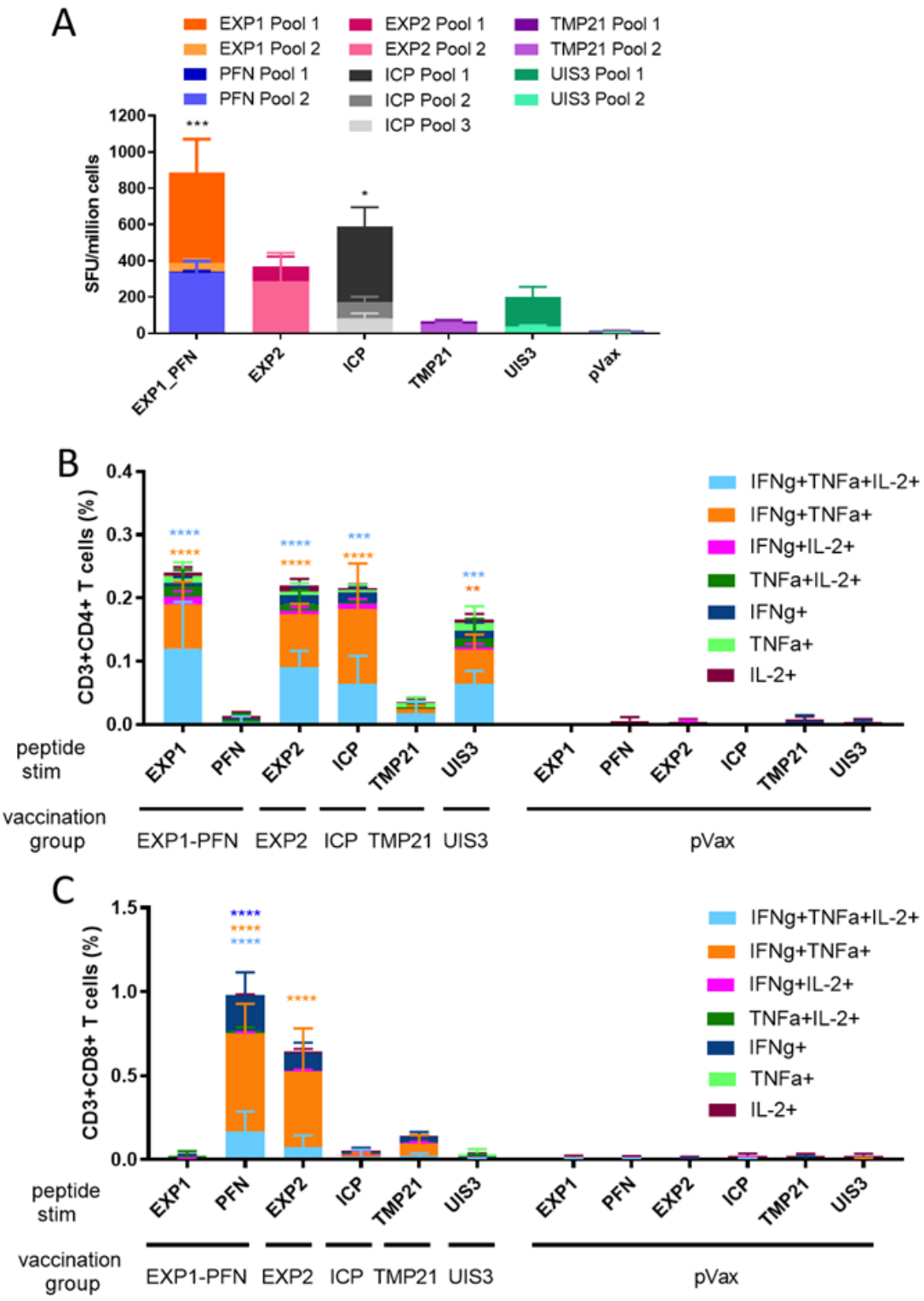

A
EXP1 Pool 1
EXP1 Pool 2
PFN Pool 1
PFN Pool 2
EXP2 Pool 1
EXP2 Pool 2
ICP Pool 1
ICP Pool 2
ICP Pool 3
TMP21 Pool 1
TMP21 Pool 2
UIS3 Pool 1
UIS3 Pool 2
SFU/million cells
EXP1_PFN
EXP2
ICP
TMP21
UIS3
pVax
B
CD3+CD4+ T cells (%)
IFNg+TNFa+IL-2+
IFNg+TNFa+
IFNg+IL-2+
TNFa+IL-2+
IFNg+
TNFa+
IL-2+
peptide stim
vaccination group
EXP1-PFN
EXP2
ICP
TMP21
UIS3
pVax
C
CD3+CD8+ T cells (%)

Figure 2.2 Functional profile of cellular immune responses elicited by individual *Py* DNA vaccines in mice. Mice were immunized 4 times at 3-week intervals with the indicated co-formulation of vaccine constructs with and without plasmid IL-33. Splenocytes were collected 1 week after the final immunization. (A) The *Py* antigen-specific cellular immune response measured by IFNγ ELISPOT of splenocytes 1 week after final immunization with the indicated *Py* DNA vaccine. Cells were stimulated for 18 hr with peptide pools encompassing the entire protein. A one-way ANOVA with Dunnet's multiple comparison test was used to compare each vaccine group to the pVax control group. (B,C) The *Py* antigen-specific cytokine production profile of CD4+ (B) and CD8+ (C) T cells from spleens 1 week after the final immunization with the indicated *Py* DNA vaccine. Cells were stimulated with pooled peptides for 6 hr, stained for intracellular production of IFNγ, TNFα, and IL-2, and then analyzed by flow cytometry. The bar graph shows subpopulations of mono-, double-, and triple-positive CD4+ and CD8+ T cells. A 2-way ANOVA with Tukey's multiple comparisons test was used to compare cytokine production between each vaccine group and the pVax control group. Asterix color indicates which cytokines were significantly different between vaccine and control. * = $p < 0.05$, ** $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$. Values represent mean responses in each group (n = 5) ± SEM.

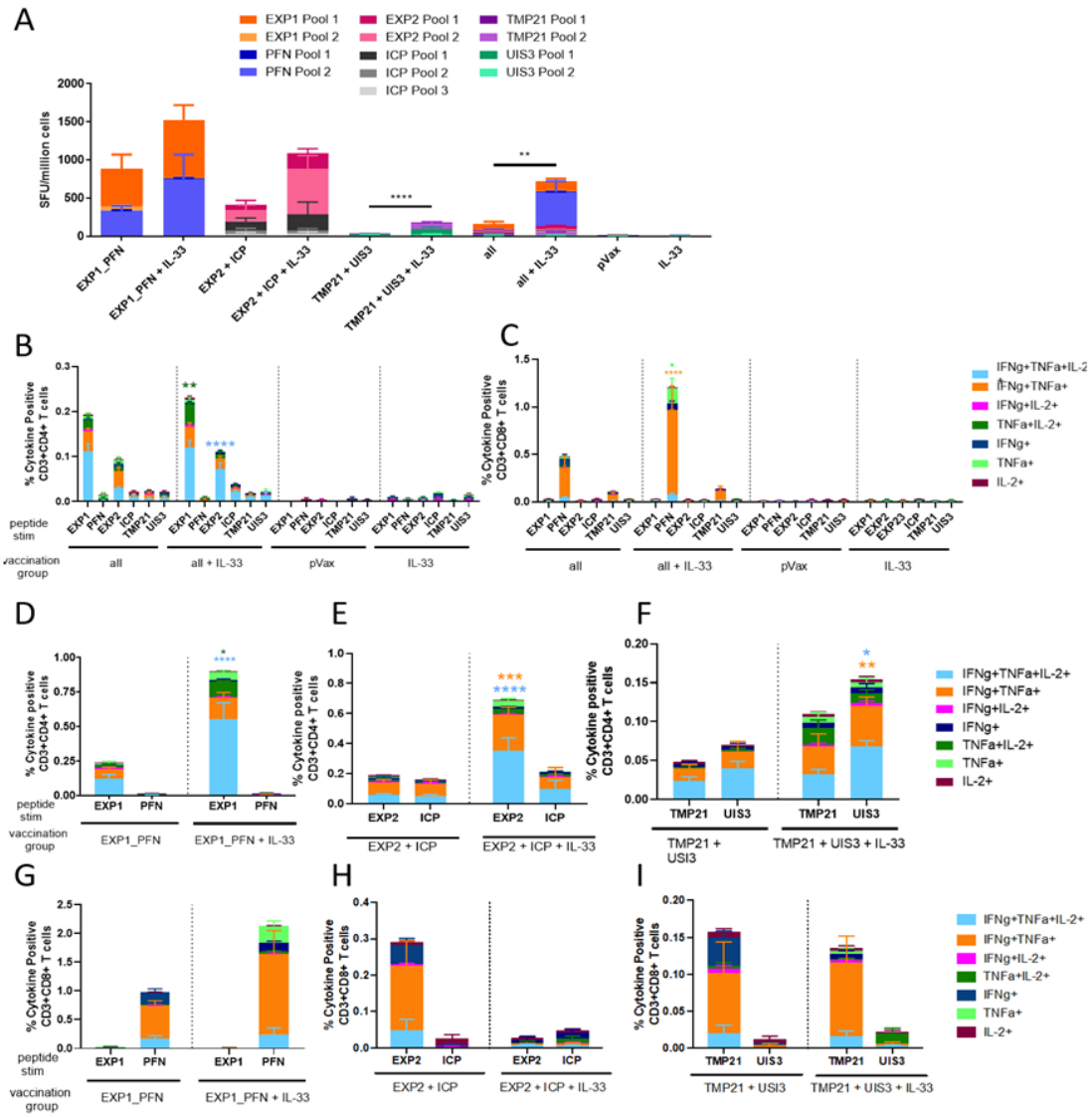

A
EXP1 Pool 1
EXP1 Pool 2
PFN Pool 1
PFN Pool 2
EXP2 Pool 1
EXP2 Pool 2
ICP Pool 1
ICP Pool 2
ICP Pool 3
TMP21 Pool 1
TMP21 Pool 2
UIS3 Pool 1
UIS3 Pool 2
SFU/million cells
EXP1_PFN
EXP1_PFN + IL-33
EXP2 + ICP
EXP2 + ICP + IL-33
TMP21 + UIS3
TMP21 + UIS3 + IL-33
all
all + IL-33
pVax
IL-33
B
C
% Cytokine Positive CD3+CD4+ T cells
% Cytokine Positive CD3+CD8+ T cells
peptide stim
vaccination group
IFNg+TNFa+IL-2+
IFNg+TNFa+
IFNg+IL-2+
TNFa+IL-2+
IFNg+
TNFa+
IL-2+
D
E
F
% Cytokine positive CD3+CD4+ T cells
EXP1_PFN
EXP1_PFN + IL-33
EXP2 + ICP
EXP2 + ICP + IL-33
TMP21 + USI3
TMP21 + UIS3 + IL-33
G
H
I
% Cytokine Positive CD3+CD8+ T cells

Figure 2.3 Functional profile of cellular immune responses elicited by co-formulated Py DNA vaccines in mice. Mice were immunized 4 times at 3-week intervals with the indicated co-formulation of vaccine constructs with and without plasmid IL-33. Splenocytes were collected 1 week after the final immunization. Immunization groups were: All vaccine constructs with or without IL-33, EXP1_PFN (exported protein 1_profilin) with or without IL-33, EXP2 (exported protein 2) and ICP (inhibitor of cysteine proteases) with or without IL-33, and TMP21 (transmembrane protein 21) and UIS3 (upregulated in infective sporozoites-3) with or without IL-33. (A) The Py antigen-specific cellular immune response induced by the indicated Py DNA vaccine co-formulation measured by IFNγ ELISPOT. Cells were stimulated for 18 h with peptide pools encompassing the entire protein. T-tests were used to compare groups with and without IL-33. (B–I) The Py antigen-specific cytokine production profile of CD4+ (D–F) and CD8+ (G–I) T cells induced by the indicated Py DNA vaccine co-formulation. Cells were stimulated with pooled peptides for 6 h, stained for intracellular production of IFNγ, TNFα, and IL-2, and then analyzed by flow cytometry. The bar graph shows subpopulations of mono-, double-, and triple-positive CD4+ and CD8+ T cells. Two-way ANOVAs with Tukey's multiple comparison test were used to compare vaccine groups against the same group adjuvanted with IL-33. Asterix color represents the corresponding cytokine groups. * = $p < 0.05$, ** $p < 0.01$, *** = $p < 0.001$, and **** = $p < 0.0001$. Values represent the mean responses in each group (n = 5) ± SEM.

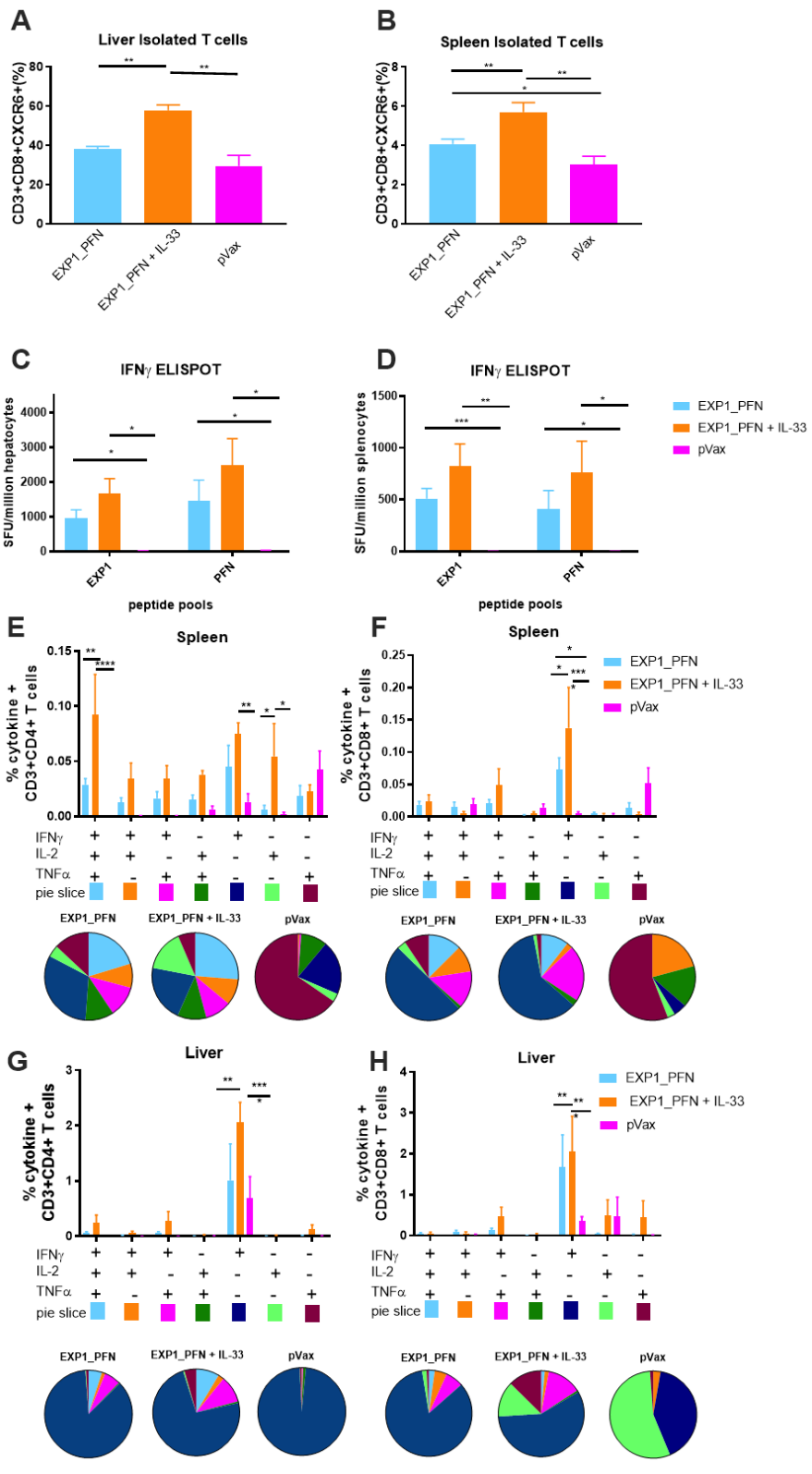

A
Liver Isolated T cells
CD3+CD8+CXCR6+(%)
EXP1_PFN
EXP1_PFN + IL-33
pVax
B
Spleen Isolated T cells
CD3+CD8+CXCR6+(%)
C
IFNγ ELISPOT
SFU/million hepatocytes
EXP1
PFN
peptide pools
D
IFNγ ELISPOT
SFU/million splenocytes
E
Spleen
% cytokine + CD3+CD4+ T cells
IFNγ
IL-2
TNFα
pie slice
F
Spleen
% cytokine + CD3+CD8+ T cells
G
Liver
% cytokine + CD3+CD4+ T cells
H
Liver
% cytokine + CD3+CD8+ T cells

Figure 2.4 Synthetic DNA vaccines drive antigen-specific liver resident T cells. Mice were immunized 3 times at 3-week intervals with the EXP1_PFN vaccine construct with and without plasmid IL-33. Lymphocytes were isolated from liver and spleen 1 week after the final immunization. (A, B) The proportion of liver resident/homing CD8+ T cells. Lymphocytes from liver (A) or spleen (B) were stained for extracellular CXCR6 and analyzed by flow cytometry. Paired t-tests were used to compare % CXCR6 positivity on CD3+CD8+ T cells across vaccine groups and against the pVax control. (C-D) The *Py* antigen-specific cellular immune response in liver (C) and spleen (D) measured by IFNγ ELISPOT. Cells were stimulated for 18 hr with peptide pools encompassing the entire protein. T-tests were performed to compare IFNγ production across vaccination groups and against the pVax control. The *Py* antigen-specific cytokine production profile of CD4+ (E,G) and CD8+ (F,H) T cells from the liver (E-F) and spleen (G-H). Cells were stimulated with pooled peptides for 6 hr, stained for intracellular production of IFNγ, TNFα, and IL-2, and then analyzed by flow cytometry. The bar graph shows subpopulations of mono-, double-, and triple-positive CD4+ and CD8+ T cells. 2-way ANOVAs with Tukey's multiple comparison test were used to compare cytokine production across vaccination groups and against the pVax control. The pie chart shows the proportion of each cytokine subpopulation. Values represent mean responses in each group (n = 5) ± SEM.

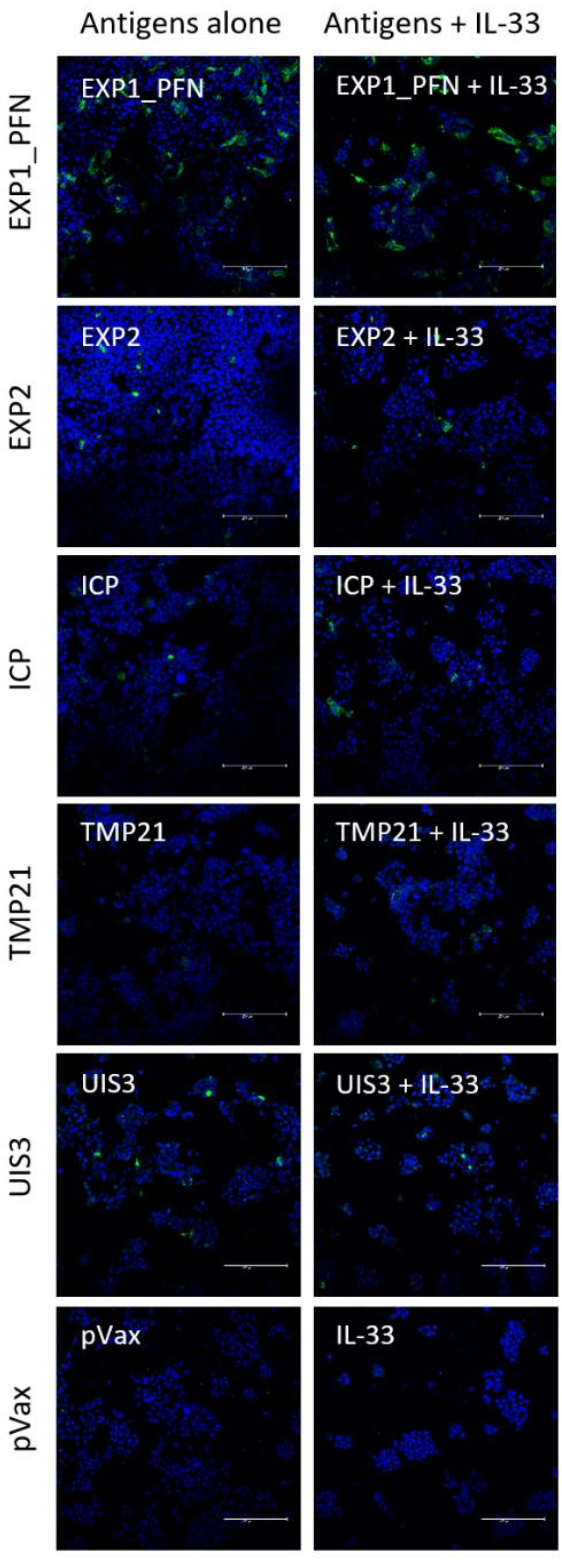
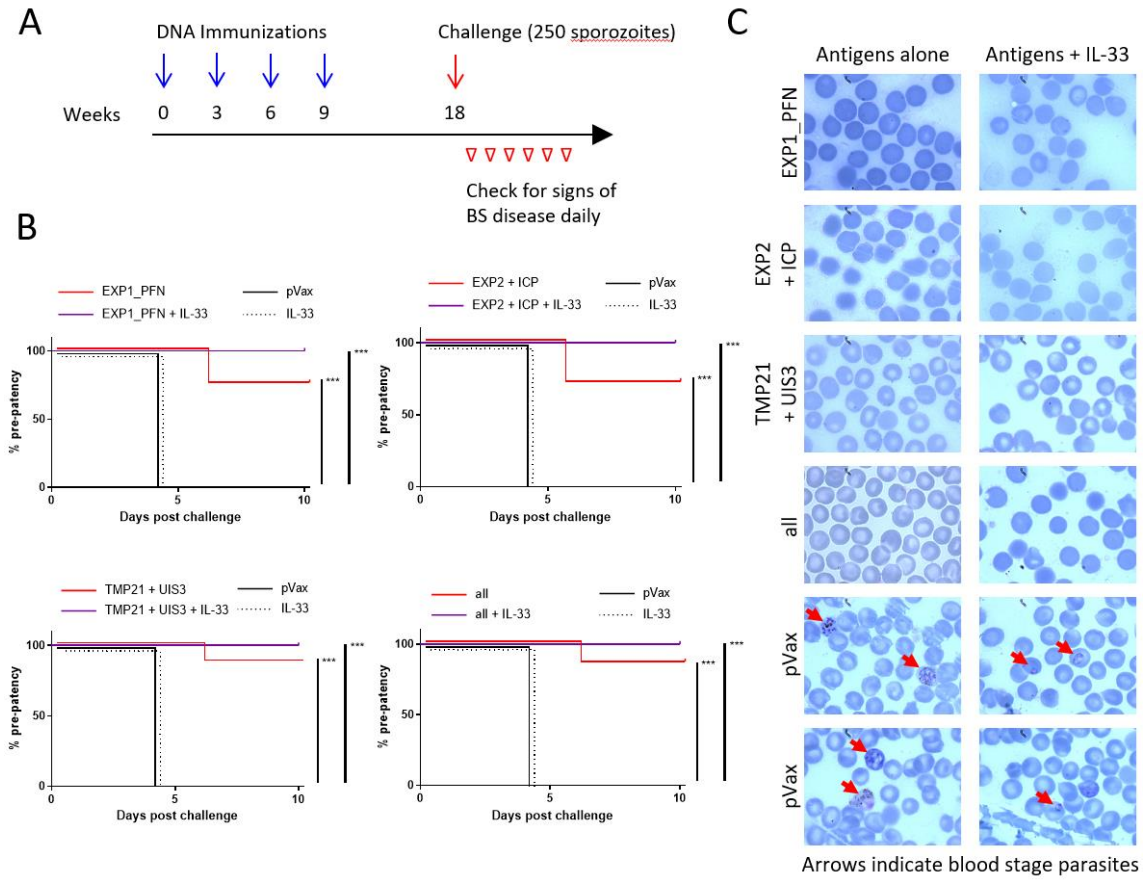


Figure 2.5 Antibodies elicited by Py DNA vaccines in mice. Hepa1-6 cells were transfected with the DNA vaccine construct listed on the left. Cells were then probed with pooled mouse post-immune sera collected 1 week after the last immunization. An anti-mouse-IgG-AF488 was used as a secondary antibody to detect the presence of anti-Py antigen antibodies. DAPI staining shows cell nuclei. White text in the top left corner of each field indicates post-immune sera vaccine group.



<u>Figure 2.6</u> DNA vaccine expressing *Py* LS antigens provides protection from blood stage disease after sporozoite challenge. (A) Vaccine and challenge timeline. Balb/c mice were immunized 4 times at 3-week intervals with the indicated vaccine co-formulations. Immunization groups were: All vaccine constructs with or without IL-33, EXP1_PFN with or without IL-33, EXP2 and ICP with or without IL-33, and TMP21 and UIS3 with or without IL-33. Mice were then challenged by injection of 250 *P. yoelii* sporozoites. Blood smears were examined daily for signs of blood stage disease. (B) Survival curves showing protection from evidence of blood stage parasites. Log-rank tests were used to compare groups and p-values less than the Bonferroni-corrected threshold are indicated. * = $p < 0.05$, ** $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$. (C) Example blood smears from each group. Red arrows indicate blood stage parasites.

CHAPTER 3: The importance of anti-CSP antibodies in protection from Malaria

Introduction

Chapter 2 addressed our efforts developing a pre-erythrocytic vaccine targeting liver stage antigens, which had the goal of eliciting cell mediated immunity targeting infected hepatocytes. This chapter will focus on our efforts developing a pre-erythrocytic vaccine to intercede before the parasite even establishes infection in hepatocytes; the studies outlined in this chapter aimed to develop an anti-sporozoite antibody response to prevent sporozoite invasion of hepatocytes, and thus prevent progression to symptomatic disease.

As discussed in chapter 1.4, RTS,S/AS01 (RTS,S) is one of the longest studied candidate vaccines, and is focused on generating immunity to CSP, the circumsporozoite protein of *Plasmodium falciparum* (*Pf*). RTS,S is a recombinant protein-based vaccine comprised of a fragment of CSP containing a section of the repeat region, and the T cell epitopes of the c-terminus attached to the Hepatitis B surface antigen protein, and delivered with additional HbsAg to encourage the formation of virus-like-particles in yeast, which are then harvested and administered with an adjuvant to humans to generate a T cell and antibody response. This is the only vaccine to show significant reduction in malaria in young children living in endemic regions. A phase 3 trial which spanned 5 years demonstrated that children aged 5-17 months who received 4 doses of RTS,S had a 39% reduction in malaria cases, and a 29% reduction in severe malaria cases over 4 years of follow-up[67]. After positive recommendations by WHO advisory boards, three countries, Ghana, Kenya, and Malawi began introducing

RTS,S in 2019[27]. In the coming years, the Malaria Vaccine Implementation Programme (MVIP) will assess the feasibility of administering the recommended 4 doses of the vaccine in children, the potential role in reducing childhood death, and the safety of RTS,S in the context of routine use[68] as well as in specific important subpopulations.

An important advance in malaria vaccines is the development of a novel polyvalent immunogen R21, a recent anti-malaria vaccine candidate, which builds on the knowledge gained from RTS,S. In contrast to RTS,S, R21 delivers a higher ratio of CSP to the Hepatitis B surface antigen (HBVsAg), which is in a 1 to 1 ratio as opposed to the 1 to 4 ratio of RTS,S[5]. Thus far R21 has proven to be well tolerated and immunogenic in early trials, as well as protective in a controlled human malaria infection study (CHMI)[69,70]. The correlates of immunity which have emerged from these malaria vaccines support the importance of anti-CSP antibodies primarily but also suggest a role for T cells for imparting protection from *Plasmodium* infection[67,69–74].

The circumsporozoite protein (CSP) has long been a vaccine candidate of interest. CSP is the major component of surface proteins on sporozoites, forming a dense coat on the parasite during this stage of the life cycle[173,174]. CSP is composed of three regions, (1) an N-terminus that binds heparin sulfate proteoglycans, (2) a four amino acid repeat region, and (3) a C-terminus that contains a thrombospondin-like type I repeat (TSR) domain. Native CSP on the surface of sporozoites appears as a glycosylphosphatidylinositol-anchored, flexible rod-like protein[175,176]. The central repeat region contains the immunodominant B cell epitope[177,178]. The C-term contains the TSR, T cell epitopes, as well as B cell epitopes[179,180]. The N-terminus region is also of importance, as it has been shown to be involved in liver attachment[181], and interacts with liver cells through heparin sulfate; antibodies against this region have been shown to be inhibitory in a sporozoite invasion assay[182]. As for overall structure, it has been shown

that CSP forms a long, flexible, rod-like superhelix composed of regular β-turns[175,176]. However, this structure undergoes conformational changes during the parasite's life cycle[36]. As referenced above, CSP interacts with heparan sulfate proteoglycans on the surface of hepatocytes during the invasion process[35,183,184]. This interaction is contingent on processing of the N-terminus and the subsequent conformation change[36]. Further, a synthetic peptide corresponding to L86 to G100 blocks salivary gland invasion, showing the biological importance of the n-terminal domain[185]. The processing of CSP by a parasite cysteine protease, and subsequent cleavage, is specifically associated with the decision between productive invasion and cell transversal[35,174], as the proteolytic cleavage of CSP regulates the switch to an open adhesive confirmation, whereas the masking of this domain maintains the sporozoite in a migratory state[173].

This knowledge of the importance of all three domains of CSP, as well as the importance of its confirmation, shaped our decisions for construct design. To that end, five synDNA constructs were designed with variations hypothesized to provide relevant expression and folding, or for testing the importance specific domains of CSP for inducing protection (Figure 3.1). The construct "3D7" contains the unadulterated full length native CSP sequence from the *Pf* 3D7 strain. The construct "GPI1" contains the CSP sequence from the *Pf* 3D7 strain, with a mammalian GPI anchor substituted in for the native protozoan GPI anchor. The construct "ΔGPI" contains the native CSP sequence from the *Pf* 3D7 strain without a GPI anchor. The construct "TM" contains the CSP sequence from the *Pf* 3D7 strain with a mammalian transmembrane domain substituted in for the native protozoan GPI anchor. The construct "DD2_3D7" contains the N-terminus region of CSP from the *Pf* DD2 strain, linked to the n-terminus region of CSP from the *Pf* 3D7 strain. These five variations on CSP tested soluble vs secreted forms, different cell membrane attachment approaches, oligomerization, and the

importance of the domains of CSP in immunization and challenge studies. Mice immunized with 3D7, dGPI, GPI1, or TM all developed robust antibody responses against both recombinant CSP (rCSP) as well as the NANP7 peptide, whereas mice immunized with DD2 had a negligible antibody response. Additionally, all 5 constructs induced IFNγ cellular responses with ΔGPI being the highest inducer. Finally, mice immunized with ΔGPI had the highest inhibition of liver stage infection in a murine challenge model. The vaccine with the highest immunogenicity and most robust protective efficacy was ΔGPI.

Additionally, based on previous work in the lab demonstrating the ability of DNA launched nanoparticles to assemble *in vivo*[186], I developed mimics of the leading CSP vaccines, RTS,S and R21 (both recombinant protein based polyvalent vaccines), and tested them side by side with our other DNA vaccines. SynDNA RTS,S (dRTS,S) and synDNA R21 (dR21) displayed similar immunogenicity and protective efficacy to their protein counterparts. Both dRTS,S and dR21 elicited anti-CSP antibodies, and only dRTS,S elicited anti-HBsAg antibodies, where dR21 did not, recapitulating what has been seen in prior studies[69]. Additionally, both dRTS,S and dR21 vaccinated mice produced IFNy in response to stimulation with CSP antigen. Consequently, dRTS,S and dR21, as well as ΔGPI, demonstrated high protective efficacy against a rigorous infectious mosquito bite murine malaria challenge.

Thus we observe that a uniquely designed synthetic DNA vaccine, ΔGPI, as well as genetically encoded RTS,S and R21 form polyvalent protein structures which elicit a robust anti-CSP antibody response, as well as a T cell response which was associated with protection from *Plasmodium* infection. Additional study of this approach appears warranted based on our demonstration of robust immunogenicity in the putative correlates of protection for malaria infection, specifically the high titer antibody response,

the high antigen specific IFNy production, and the sterilizing immunity demonstrated in challenge.

Results

synDNA CSP vaccine construct design and *in vitro* expression

Five synDNA constructs were designed with variations hypothesized to increase immunogenicity. All constructs target *Plasmodium falciparum* (*Pf*). Each construct was synthesized and then inserted into a pVax backbone, with an added IgE leader sequence as described[128]. The construct “3D7” contains the unadulterated full length native CSP sequence from the *Pf* 3D7 strain. The construct “GPI1” contains the CSP sequence from the *Pf* 3D7 strain, with a mammalian GPI anchor substituted in for the native protozoan GPI anchor. The construct “ΔGPI” contains the native CSP sequence from the *Pf* 3D7 strain without a GPI anchor. The construct “TM” contains the CSP sequence from the *Pf* 3D7 strain with a mammalian transmembrane domain substituted in for the native protozoan GPI anchor. The construct “DD2_3D7” contains the N-terminus region of CSP from the *Pf* DD2 strain, linked to the n-terminus region of CSP from the *Pf* 3D7 strain. These five variations on CSP tested soluble vs secreted forms, different cell membrane attachment approaches, and the importance of domains of CSP in immunization and challenge studies. *In vitro* expression of each construct was verified by western blot. Because the detection Ab used was directed against the repeat region, the construct DD2 would not be readily detected by this method (Figure 3.1B). The synDNA vaccine construct ΔGPI exhibited the highest expression in the supernatants collected from transfected cells (Figure 3.1C). This may indicate a superior ability to be secreted from transfected myocytes *in vivo*, and thus superior immune activation, though further study is needed.

synDNA CSP vaccine constructs elicit a robust immune response

To assess the immunogenicity of each unique construct, groups of five BALB/c mice were immunized with 25 µg of vaccine four times, three weeks apart (week 0, 3, 6, and 9) (Figure 3.2A). pVax was included as an empty vector (negative) control. Sera was collected to assess the antibody response. Previous work has shown that antibodies are of critical importance for targeting CSP, particularly the NANP region[187]. The sera from immunized mice was used as a primary antibody to probe ELISA plates which had been coated with either recombinant CSP, or the NANP peptide alone. The monoclonal antibody 2A10[188] was used as a control. Mice immunized with 3D7, ΔGPI, GPI1, or TM all developed robust antibody responses against both rCSP and the NANP peptide. Mice immunized with DD2 induced a negligible antibody response, and those immunized with the empty vector control had no antibody response (Figure 3.2B-C). In a parallel experiment, splenocytes were collected one week after final immunization for immune analysis and antigen specific cytokine production was assessed by IFNγ ELISPOT. All 5 constructs induced IFNγ cellular responses with ΔGPI being the most potent. 3D7, GPI1, and TM induced lower, but still robust levels of IFNγ secreting cells, and DD2 induced readily detectable, but the lowest, levels of IFNγ secreting cells (Figure 3.2D). The bulk of the IFNγ response is directed against the n-terminus of CSP, follow in reactivity by the c-terminus. The NANP repeat region induced a low level of IFNγ. 3D7, the only construct designed to contain a *Pf* GPI anchor, did elicit a response to the GPI anchor (Figure 3.2D).

synDNA CSP vaccine constructs are protective against IV sporozoite *Pf* challenge

Groups of five BALB/c mice were immunized with 25 µg of vaccine four times, three weeks apart (weeks 0,3,6,9) as in Figure 3.2A. Two weeks after the last boost, mice were inoculated with 250 sporozoites IV. Immunized and non-immunized BALB/c

mice were challenged with *P.berghei* sporozoites expressing both the *P.falciparum* circumsporozoite protein (CSP) and luciferase. 42 hours after intravenous injection of 250 sporozoites, mice were intraperitoneally injected with 100 µl of D-luciferin (30 mg/mL), anesthetized and liver luminescence was measured with the Perkin Elmer IVIS Spectrum Imaging System to assay liver loads. Inhibition of liver infection is expressed as a function of relative infection compared to naive mice. Mice immunized with ΔGPI have the highest inhibition of liver infection (64.17%) out of the immunized groups, while the positive control, which was 100 µg of the MAb 311[187] delivered 16 hours before challenge showed an 80% inhibition of liver stage infection (Figure 3.2E-G). ΔGPI showed significant vaccine induced protective immunity in this challenge model (Figure 3.2E-G).

DNA encoded CSP polyvalent vaccines

We were encouraged by these initial challenge results, and wanted to compare them to the important standards in the field (RTS,S and R21) which are more complex vaccine formulations, both of which form polyvalent particles. However, research acquisition of the protein forms was limited. Thus to compare such more polyvalent forms, we moved to develop genetically encoded DNA vaccines in the form of dR21 and dRTS,S. The sequences for R21[189] and RTS,S[190] were retrieved, and then modified to include changes in RNA and codon bias, as well as the addition of an efficient IgE leader sequence, to generated dR21 and dRTS,S. The final constructs were inserted into a pVax backbone (Figure 3.3A). The dR21 construct is delivered *in vivo* as is, as it is a singular antigen fusion between the Hepatitis B surface antigen (HbsAg) and CSP. To assemble a mimic of RTS,S we designed two constructs, one encoding HbsAg and a second RTS construct. The final plasmids were mixed at a 1:4 ratio of RTS to HbsAg (Figure 3.3A) to mimic its production as a final protein particle antigen[190].

Expression of each construct was studied by western blots of supernatants collected from transfected cells, using the anti-CSP monoclonal antibody 311 for detection. dR21 and dRTS,S both expressed *in-vitro* (Figure 3.3B). To elucidate whether dR21 and dRTS,S were forming nanoparticles similarly to their protein formulation counterparts, supernatants from transfected cells were run on a discontinuous sucrose gradient and separated by molecular weight via ultracentrifugation for 24 hours. The resulting gradient fractions were analyzed by western blot for CSP expression (Figure 3.3C). We observed protein bands with high molecular weight from supernatants collected from both dRTS,S and dR21 transfected cells, that may correspond to higher-order structures such as polyvalent particles. We also observed CSP in high molecular weight fractions of supernatants collected from ΔGPI transfected cells, suggesting that this vaccine construct is also capable of forming multimeric aggregates similar to dRTS,S and dR21. This phenomenon was not observed in supernatants collected from 3D7 transfected cells, and thus may be a contributing factor behind the superior protection observed in Figure 3.2. To evaluate the biophysical properties of the high molecular weight fractions which contained CSP (Figure 3.3C), we analyzed relevant fractions with negative stain electron microscopy (nsEM). Higher-order structures ranging in diameter from 20nm to 50nm were observed in the high molecular weight fractions from dRTS,S, dR21, and ΔGPI transfected cells, and not in mock transfected cells (Figure 3.3D).

Both dRTS,S and dR21 induced an potent IFNγ cellular response. Using separate peptide pools for mapping we observed that the majority of the CSP response to dRTS,S and dR21 was directed at the c-terminus region of CSP. Although the cellular response does target CSP, majority of the vaccine-induced cellular response was specific to HbsAg but not to CSP (Figure 3.4D). In contrast, immunization with ΔGPI only

elicits an IFNγ response against the CSP protein, and at a higher magnitude than dR21 and dRTS,S (Figure 3.4C-D). However, this focusing of the cellular response onto the carrier protein did not impair the antibody response, as immunization with both dRTS,S and dR21 elicited robust anti-rCSP antibody titers. Comparison of the responses demonstrated that immunization with ΔGPI resulted in slightly higher Ab responses against recombinant CSP than dR21 and dRTS,S (Figure 3.4A). Neither ΔGPI nor dR21 elicited an anti-HBsAg antibody response, whereas dRTS,S did (Figure 3.4B). The higher molar ratio of HBsAg in dRTS,S may be responsible for this observation, however further study of these differences could generate additional insight.

Polyvalent CSP constructs elicit a robust Ab response and are protective against a rigorous mosquito bite challenge model

ΔGPI, the most potent construct from the initial CSP DNA vaccine study and challenge experiment, and the construct which demonstrated high-molecular weight aggregate formation along with dRTS,S and R21, were evaluated in a more rigorous mosquito bite malaria challenge model[191]. Groups of five BALB/c mice were immunized with 25 μg of vaccine four times, three weeks apart (weeks 0,3,6,9). Three weeks after the last immunization, the mice were challenged with *Plasmodium berghei* (*Pb*) transgenic parasites which express the full-length *Plasmodium falciparum* circumsporozoite protein (*Pf*CSP), as well as luciferase as a reporter for liver parasite load. This parasite is denoted from this point on as *PbPfLuc*. A cage of *Anopheles stephensi* mosquitoes, 20 days after blood feeding on *PbPfLuc* infected mice was found to be 90% infected with *PbPfLuc*. Based on this calculation that 90% of the mosquitos in the cage were infected, 5 mosquitoes were required to challenge mice to ensure robust infection by mosquito bites, as described[192]. Mice were anesthetized with 2% Avertin prior to challenge. Mosquitoes were allowed to feed on the animals for ~10 minutes.

After feeding, the number of mosquitoes positive for a blood meal were determined (Figure 3.5A).

42 hours after challenge, mice were intraperitoneally injected with 100 µl of D-luciferin (30 mg/mL), anesthetized and liver luminescence was measured by using the Perkin Elmer IVIS Spectrum Imaging System to assay liver parasite loads. Inhibition of liver infection is expressed as a function of relative infection compared to naive mice. Mice immunized with ΔGPI have the highest inhibition of liver infection observed in vaccinated groups (84.6%), while the positive control, 100 ug of the MAb 311 demonstrates an 88.7% inhibition of liver stage infection in this model (Figure 3.5B-D). dRTS,S and dR21 demonstrated overall potent protection of 81.5% and 82.6% inhibition respectively (Figure 3.5B-D). Beginning on day 4 post-infection, blood smears were taken to evaluate protection from blood stage parasitemia. All mice immunized with the empty vector control, pVax, succumbed into blood stage infection by day 4. Of the five mice immunized with dRTS,S, two mice developed blood stage parasitemia by day 5, and a third on day 6. Of the five mice immunized with dR21, one developed blood stage parasitemia at day 4, a total of three by day 5, and 4/5 mice had developed parasitemia by day 6 post infection. Of the mice immunized with ΔGPI, one fell ill by day 4, and a second by day 5, leaving 3/5 mice with sterile protection from blood stage parasitemia. Finally, of the mice treated with 100 ug of the MAb 311 as a positive control, one developed BS parasitemia by day 4, and a second developed BS parasitemia by day 8, leaving 3/5 mice with sterile protection from blood stage parasitemia (Figure 3.5E) and serves as a robust control. This is the first demonstration that genetically encoded dRTS,S or dR21 can drive robust protection, similar to prior reports of these immunogens as protein based vaccines. Furthermore, in the side by side comparison

ΔGPI exhibited protection which was comparable to the genetically encoded dRTS,S and dR21, as well as the monoclonal antibody positive control.

Discussion

CSP has long been a focus in malaria vaccine research, and for good reason, as anti-CSP antibodies are one of the predominant correlates of protection for malaria infection[187]. CSP is composed of an N-terminal domain containing a conserved proteolytic cleavage cite, a central repeat region, and a c-terminal domain[173]. CSP undergoes significant conformational change during the parasite's migration from the mosquito salivary gland to the mammalian liver. When first entering the bloodstream, CSP is in a folded conformation on the surface of the sporozoite. As the parasite reaches the liver, CSP undergoes proteolytic cleavage, which has been shown to be a critical requirement for hepatocyte invasion[174]. In addition it is known that antibody binding to sporozoites abolish their motility[193] and induces a cytotoxic effect[194] that neutralize their infectivity. Consequently, antibodies targeting CSP have the potential to strongly inhibit sporozoite invasion of hepatocytes and can thus be protective against disease[195].

RTS,S is a recombinant protein-based vaccine comprised of a fragment of CSP containing a section of the repeat region, and the T cell epitopes of the c-terminus attached to the Hepatitis B surface antigen protein, and delivered with additional HbsAg to encourage the formation of virus-like-particles in yeast, which are then harvested and administered with an adjuvant to humans to generate a T cell and antibody response. In contrast, while the R21 vaccine is also a recombinant protein-based vaccine, containing the same NANP repeats and T cell epitopes of the C-terminus as RTS,S does, it is able to form particles in yeast without additional HbsAg, meaning that a higher proportion of antigen seen by the immune system will be *Plasmodium* antigen rather than Hepatitis B antigen. RTS,S, when formulated with adjuvant, induces antigen-specific humoral and

CD4 T cell cellular responses in BALB/c mice[196] and in humans [197–200]. R21, when formulated with adjuvant or delivered with a TRAP-based viral vector prime-boost, also demonstrated a robust humoral and cellular response[69]. Genetically encoded RTS,S and R21 surrogates were highly immunogenic and protective in a rigorous malaria challenge model and performed similarly to their protein counterparts[69,196–199,201]. However, the majority of the IFNy response elicited by dRTS,S and dR21 was directed against the HbsAg rather than against CSP. In contrast, our primary CSP candidate vaccine, ΔGPI, elicited a cellular response to only CSP, not to HbsAg, thus the response appears more focused towards the CSP antigen targeted by the vaccine.

This study demonstrates the potential for a synDNA vaccine targeting CSP to be highly immunogenic and efficacious. I show that a synthetic DNA vaccine targeting the circumsporozoite protein of *Plasmodium falciparum* can induce a high-titer anti-CSP antibody response, as well as a robust cellular response producing IFNy in response to stimulation with CSP antigen. This immunogenicity elicited by the ΔGPI CSP vaccine resulted in protection from infection in multiple models of murine malaria, including an IV sporozoite challenge, and a rigorous infected mosquito bite challenge. Additionally, this study is the first to show that synDNA mimics of the leading CSP vaccine candidates RTS,S and R21 generate strong humoral and cellular responses, resulting in protection from infection in an infected mosquito bite challenge model.

Nanoparticle vaccines have become a focus in recent years. Nanoparticles, ordered structures with dimensions in the range of 1-1000 nm, can function as both a delivery system, and/or immune potentiators[202,203]. Nanoparticles which have a comparable size to pathogens are taken up efficiently by APCs[202]. Further, the display of antigen in a repetitive array mimics the surface of a pathogen (i.e. as CSP densely coats the surface of sporozoites), and this allows for enhancement of innate immune

activation, improved drainage and retention in the lymph node, stronger engagement with B cell receptors, and consequently augmented T cell help to B cells[203]. Within the context of prophylactic malaria vaccines, nanoparticle vaccines have been shown to drive broader humoral responses, a balanced Th1/Th2 cytokine profile, and robust germinal center formulation[204]. Malaria protein targeting nanoparticles have also been shown to increase Ab titers, and increase antigen specific plasmablasts, circulating memory B cells, and plasma cells in the bone marrow, as well as inducing antigen specific circulating Tfh cells[205]. There is precedent for synDNA launched nanoparticle vaccines spontaneously self-assembling *in vivo*, and driving stronger humoral responses than monomeric DNA vaccines[186].Thus I postulate that our synDNA vaccine mimics of the nanoparticle forming RTS,S and R21 vaccines, as well as our novel synDNA vaccine "ΔGPI", all of which form high molecular weight aggreagtes which may be nanoparticles *in vitro*, may promote enhanced trafficking to lymph nodes, robust germinal center formation, and consequent increases in anti-CSP antibodies as compared to non-nanoparticle forming vaccines.

I believe this platform of *in-vivo* launched polyvalent nanoparticle vaccines targeting CSP has the potential to deliver high level protection from malaria infection, in a temperature stable and cost-effective manner which makes it particularly well suited to use in low-resource settings.

Materials and Methods

Cell lines and transfection

To probe for *in vitro* expression of the vaccine constructs, the Expi293F transfection kit (ThermoFisher) was used for all transfections. Expi293F cells were maintained in Expi293 Expression Medium for passages, and cells were incubated in 8% CO_2 conditions on an orbital shaker at 37°C. Briefly, one day prior to transfection, $2x10^5$ Expi293F cells at 95% viability or greater were plated in Expression Medium. DNA plasmids were added to Opti-MEM media separately from ExpiFectamine transfection reagent. After a 5-minute incubation period, DNA and ExpiFectamine were complexed during a 20-minute incubation period. Subsequently, the DNA plasmid complex was added to Expi293F cells in suspension. Eighteen hours after the addition of DNA, Transfection Enhancers were added according to manufacturer's instructions. After three to five days, cell supernatants and lysates were collected for further studies.

Western Blot

To detect vaccine construct expression in transfection supernatants and lysates, 12µL of sample was run on 4-12% Bis-Tris gel (ThermoFisher) in MES buffer. Samples were boiled and reduced before being run. Upon completion of the gel, contents of the gel were transferred to a nitrocellulose membrane via the iBlot 2 Transfer system (ThermoFisher). Upon transfer completion, the membrane was blocked using Intercept Blocking Buffer (LI-COR) for one hour at room temperature. After blocking, the membranes were probed with the anti-CSP human MAb 311 at a 1:1000 dilution in blocking buffer at 4°C overnight. The following day, a fluorescently labelled anti-human secondary antibody was added to the membrane, formulated in Intercept Blocking Buffer, SDS, PBS, and Tween-20 for a one-hour incubation. Following secondary

incubation, the membrane was washed with PBS + 0.1%% Tween-20 four times, and was subsequently imaged using the LI-COR Odyssey CLx.

Ultracentrifugation and gradient fraction collection

In order to assess the polyvalency and structural formation of vaccine constructs, transfectant supernatants were collected and filtered through a 0.45micron filter to remove cell debris. Then an Amicon Ultra-15mL 10k filter was used to concentrate 15 mL of sample with a 4,000x g spin for approximately 15-40 minutes. Concentrated protein (1g) was loaded onto a 5ml 10-50% discontinuous sucrose gradient (50 mM Tris, pH 8, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 5 mM sodium pyrophosphate, 10-50% sucrose) and ultracentrifuged (39,800 rpm) for 24 hours at 4°C. Fractions (250 µL) were collected and stored at -20°C.

Electron Microscopy

Fractions were collected from the density gradient analysis, and dialysed into PBS overnight using Slide-A-Lyzer mini dialysis devices (ThermoFisher), before being concentrated via an Amicon Ultra-0.5mL 10k filter. 3 µl of each sample was applied to a thin carbon grid that was glow discharged for 30 seconds at 30 mAmps current using pelco easyglow glow discharger. 3µl of freshly made solution of 2% uranyl acetate was used to stain each sample twice on the grid with 1 min of incubation time. Excess stain and sample were removed by carefully blotting the grid at the edge with a Whatman filter and the grid was allowed to dry until imaged. TEM micrographs were collected using Tecnai T12 TEM microscope operated at 100KeV and the images were recorded at 20x mag on Gatan 4K CMOS camera.

ELISA

For binding detection of CSP in transfection supernatants as well as quantification of serum antibody titers, MaxiSorp 96-well plates or half-area plates

(ThermoFisher) were coated overnight with 1 µg/mL of recombinant CSP (courtesy of MVI/PATH) at 4°C. The next day, each plate was washed with Phosphate Buffered Saline + 0.01% Tween-20 (PBS-T) four times (4x). Plates were then blocked with 5% milk in PBS for two hours at RT. Upon completion of blocking, plates were washed again, and samples diluted in 1% newborn calf serum (NCS) in PBST were transferred onto the plates for a two-hour incubation at RT. Following sample incubation, plates were washed, and goat anti-mouse or anti-human heavy and light chain HRP conjugated secondary was diluted to 1:10,000 and transferred onto plates for a one-hour incubation at RT. After secondary incubation, plates were washed and developed using SIGMAFAST™ OPD (Sigma-Aldrich) for 10 minutes and then stopped with sulfuric acid. The Biotek Synergy 2 plate reader was used to read plates at 450 nm. Data were exported to Microsoft Excel and analyzed using GraphPad PRISM 8.

Animal studies and immunizations

BALB/c mice were purchased from the Jackson Laboratory. Animal experiments were conducted under protocol #201236 approved by the Wistar Institutional Animal Care and Use Committee (IACUC). All animals were housed in the Wistar Institute Animal Facility, with the exception of challenge studies, which were performed at the Johns Hopkins Bloomberg School of Public Health. Mice were immunized with 25µg of DNA in sterile water intramuscularly in the tibialis anterior (TA) muscle and subsequently electroporated using the CELLECTRA 3P adaptive electroporation device (Inovio Pharmaceuticals). Mice were immunized four times, three weeks apart for all experiments, including the challenge experiments. One week following each vaccination, blood was collected via submandibular bleed to isolate sera for future experiments. In addition, one week after the final immunization, mice were euthanized for splenocyte collection for subsequent assays.

Sample processing and ELISpot

Following euthanasia, spleens were harvested and temporarily stored in R10, consisting of 10% FBS and 1% penicillin-streptomycin in RPMI 1640 (Invitrogen). Using the Stomacher 80 tissue stomacher, spleens were homogenized for one minute before filtering through a 40µm strainer. The cell mixture was centrifuged for 10 min at 1200 rpm and subsequently resuspended in ACK lysis buffer (Gibco) for a 5 minute period. Cells were washed with PBS prior to centrifugation and resuspension in 20mL R10 media for counting on a COUNTESS II (Invitrogen).

To assess antigen specific interferon gamma (IFNγ) production, mouse IFNγ ELISpot PLUS (Mabtech) plates were used according to the protocol provided by the manufacturer. Briefly, plates were washed using sterile PBS followed by blocking with 200µL per well of R10. Plates were seeded with 200,000 cells in 100µL R10 in triplicate. Cells were stimulated with peptide pools of 15mers overlapping by 11 amino acids spanning the entire vaccine antigen at a final concentration of 5 µg/mL per peptide. R10 and Concanavalin A were used as negative and positive controls, respectively. After an 18-hour incubation in 5% CO_2 conditions at 37°C, plates were developed according to the protocol provided by the manufacturer. After developing, the CTL ImmunoSpot S6 Universal Analyzer (Cellular Technology Limited) was used to scan and count plates. Data analysis was performed using GraphPad PRISM 8.

IV sporozoite challenge

Challenge study was performed at Johns Hopkins under IACUC #MO16H35. Each mouse was immunized via intramuscular injection followed by electroporation on weeks 0, 3, 6 and 9. On week 11 of the experiment, mice were challenged with *Plasmodium berghei* (*Pb*) transgenic parasites that express the full-length *Plasmodium falciparum* circumsporozoite protein (*Pf*CSP), as well as luciferase to report liver parasite

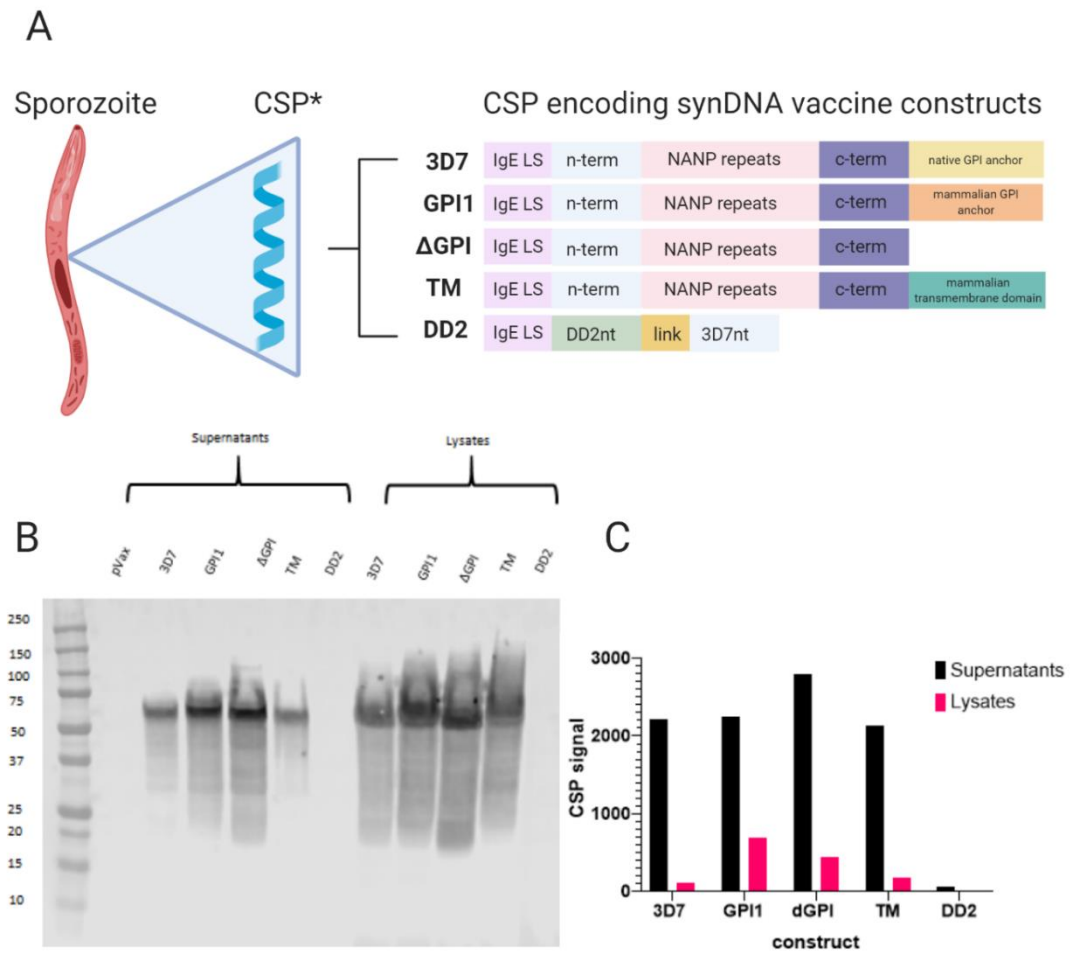
load as described[192]. This parasite is denoted from this point on as *PbPf*Luc. Briefly, the 311-100 µg cohort was injected with 100 µg of human mAb 311 16 hours prior to challenge, and age-matched all other mice; this cohort served as a control for protection. 42 hours after intravenous injection of 250 sporozoites, mice were intraperitoneally injected with 100 µl of D-luciferin (30 mg/mL), anesthetized and liver luminescence was measured with the Perkin Elmer IVIS Spectrum Imaging System to assay liver loads. Mann Whitney tests were used to compare luminescence between immunized groups and the naïve control.

Infectious mosquito bite challenge

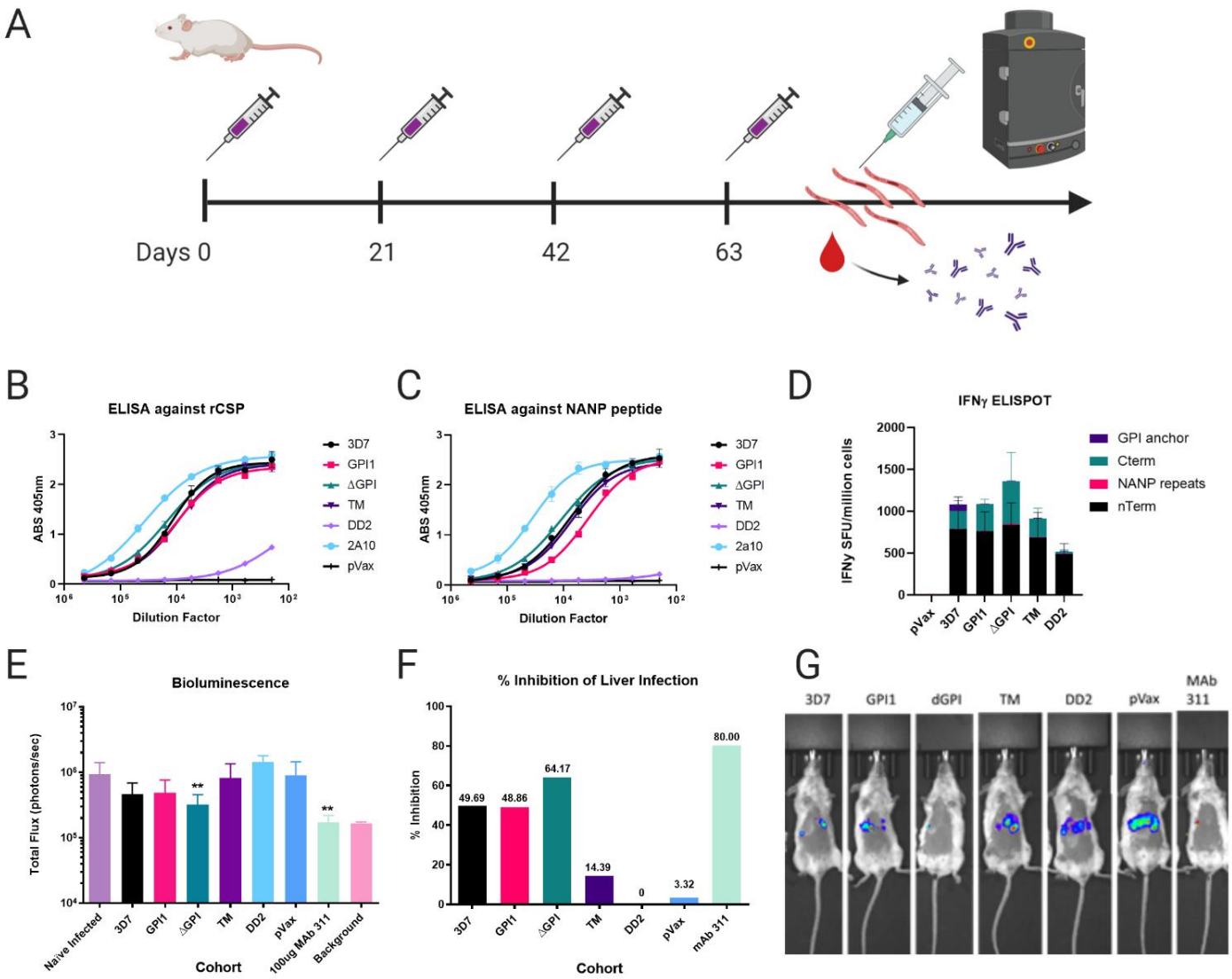
Challenge study was performed at Johns Hopkins under IACUC #MO16H35. Each mouse was immunized via intramuscular injection followed by electroporation on weeks 0, 3, 6 and 9. Serum was isolated from each mouse via retro orbital bleeding 3 days prior to challenge. The 311-100 µg cohort was injected with 100 µg of human mAb 311 16 hours prior to challenge, and age-matched all other mice; this cohort served as a control for protection. On week 12 of the experiment, mice were challenged with *PbPf*Luc via infectious mosquito bite. A cage of *Anopheles stephensi* mosquitoes, 20 days after blood feeding on *PbPf*Luc infected mice was determined to be 90% infected with *PbPf*Luc. Based on this calculation, it was determined that 5 mosquitoes were needed to challenge mice with infected mosquito bites, as described[192]. Briefly, mice were anesthetized with 2% Avertin prior to challenge. Mosquitoes were allowed to feed on mice for ~10 minutes. After feeding, the number of mosquitoes positive for a blood meal was determined. 42 hours after mosquito bite challenge, liver parasite load was measured using the Perkin Elmer IVIS Spectrum Imaging System. Mice were injected with 100µl of D-Luciferin (30mg/mL), anesthetized with isoflurane and imaged with the IVIS Spectrum to measure bioluminescence expressed by the transgenic parasites.

Mann Whitney tests were used to compare luminescence between immunized groups and the naïve control. Blood smears were taken beginning on day 4 post-infection to evaluate parasitemia. A positive result was considered an endpoint. Mice were euthanized upon confirmation of blood stage parasitemia.

Figures



<u>Figure 3.1</u> DNA vaccine construct design and *in vitro* expression. **(A)** Schematic diagram of *Plasmodium falciparum* (*Pf*) gene inserts used to generate the codon-optimized DNA vaccine constructs. The schematic details leader sequence (IgE) and gene insert. *This does not represent the true structure of CSP, and is merely a graphic **(B)** in-vitro expression of vaccine constructs in 293T cells via western blot, anti-CSP monoclonal Ab MAb 311 used as probe. **(C)** quantified CSP signal from (B)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ELISA against rCSP
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ABS 405nm
Dilution Factor
3D7
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ΔGPI
TM
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D
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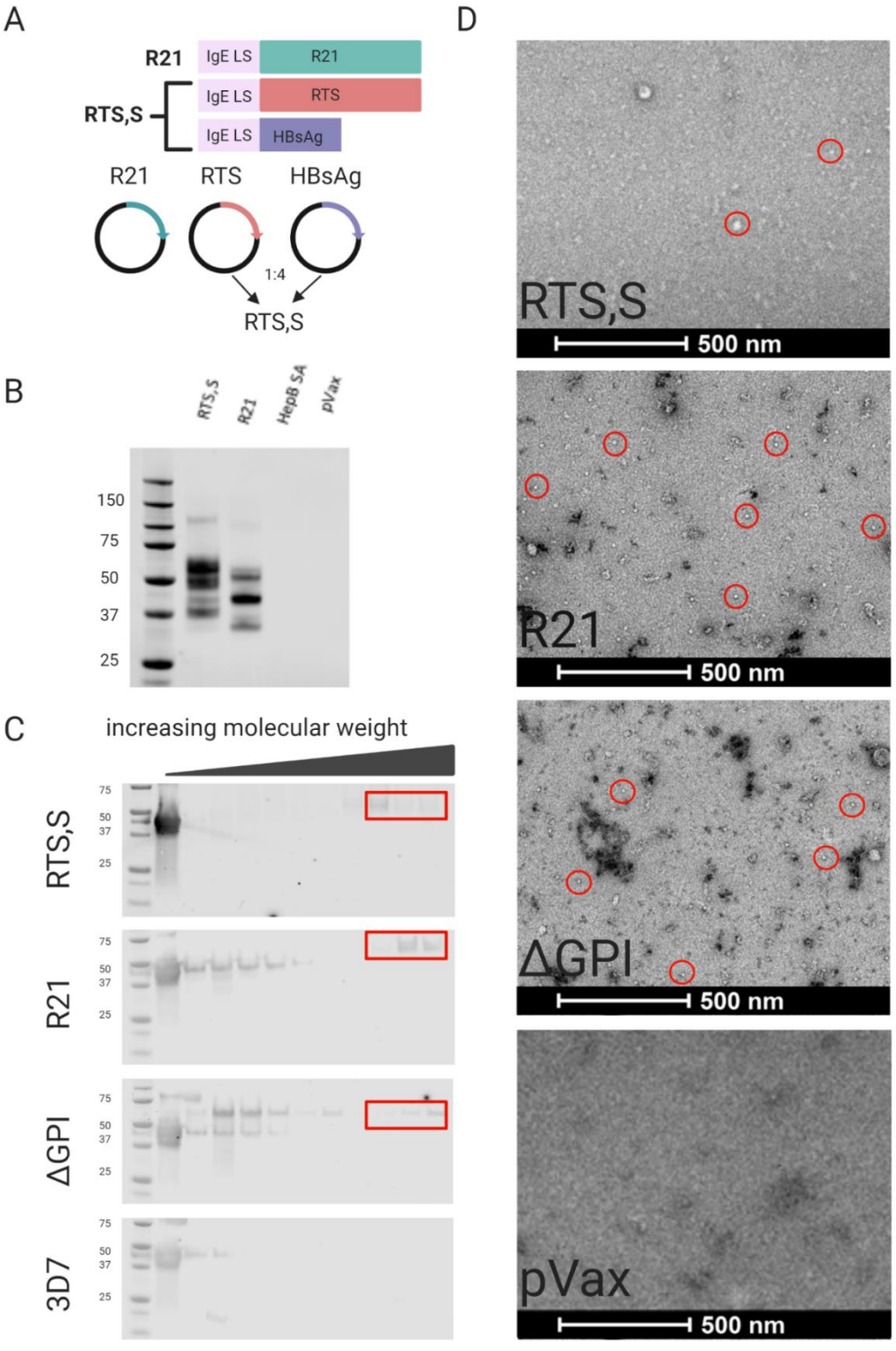
GPI anchor
Cterm
NANP repeats
nTerm
pVax
3D7
GPI1
ΔGPI
TM
DD2
E
Bioluminescence
Total Flux (photons/sec)
Naïve Infected
3D7
GPI1
ΔGPI
TM
DD2
pVax
100ug MAb 311
Background
Cohort
F
% Inhibition of Liver Infection
% Inhibition
49.69
48.86
64.17
14.39
0
3.32
80.00
3D7
GPI1
ΔGPI
TM
DD2
pVax
mAb 311
Cohort
G
3D7
GPI1
dGPI
TM
DD2
pVax
MAb 311

Figure 3.2: synDNA CSP vaccine constructs elicit a robust immune response and are protective against IV sporozoite *Pf* challenge

(A) Experiment layout. Mice were immunized four times, three weeks apart, and challenged with 250 sporozoites IV three weeks after the last immunization. Sera was collected prior to challenge for Ab analysis. Liver parasite burden was measured by IVIS. In a separate experiment mice received the same treatment, and splenocytes were harvested for immune cell analysis as in panel D. (B-C) ELISA's of pooled sera for each cohort. Panel B tested sera against recombinant CSP, while panel C tested sera against the NANP peptide. Sera was initially diluted 1:200, and 3-fold serial dilutions were made afterwards. Monoclonal antibody 2A10 was used as a positive control. (D) The Pf CSP antigen-specific cellular immune response induced by the indicated DNA vaccine measured by IFNγ ELISPOT. Cells were stimulated for 18 hr with peptide pools encompassing the entire protein. (E) Graphical representation of luminescence data. Bar graph of mean luminescence for each group, and results of Mann Whitney tests comparing groups to Naive Infected. Both AB311 and dGPI demonstrate statistically significant differences compared to Naive Infected (**:$P < 0.05$). (F) Inhibition of liver infection as expressed as a function of relative infection compared to naive mice. Mice immunized with dGPI have the highest inhibition of liver infection (64.17%), while 311 treatment demonstrates an 80% inhibition of liver stage infection. (G) Representative IVIS images for each experimental group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RTS,S
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HepB SA
pVax
150
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increasing molecular weight
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ΔGPI
3D7
75
50
37
25
D
RTS,S
500 nm
R21
500 nm
ΔGPI
500 nm
pVax
500 nm

Figure 3.3: The development of benchmark CSP control vaccines. (A) Schematic diagram of gene inserts used to generate the DNA vaccine constructs. The schematic details leader sequence (IgE) and gene insert, as well as ratio of delivery. (B) western blot of construct expression, supernatants from transfected 293T cells were probed with the MAb 311 (anti-CSP) (C) Discontinuous sucrose gradient fractions probed for CSP. The high molecular weight fractions denoted in red boxes were combined and imagined by negative staining electron microscopy as shown in (D). (D) Particle formation of vaccines *in vitro*. Examples of particles are encircled (not exhaustive, i.e. not all particles are circled).

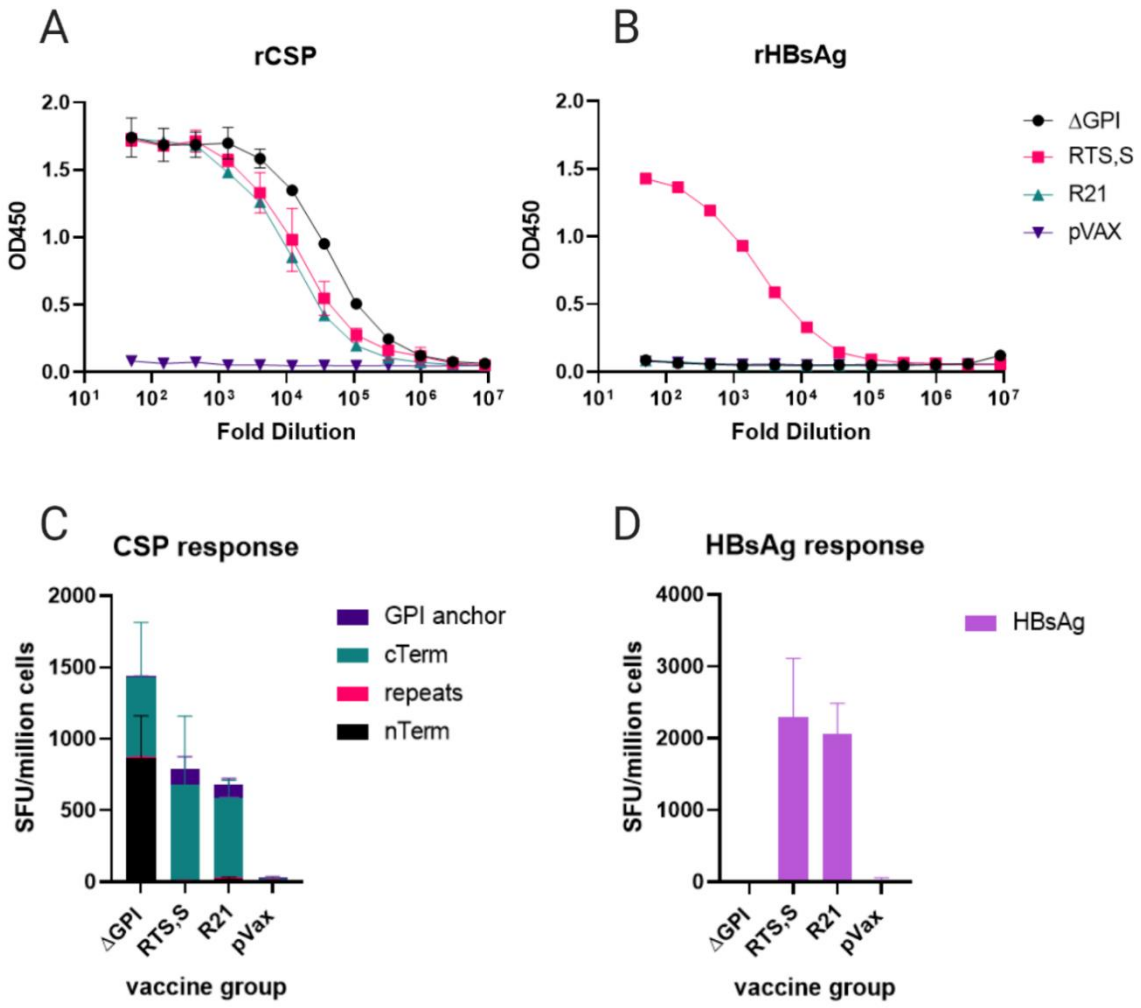

Figure 3.4: synDNA CSP constructs elicit a robust response. Mice were immunized four times, three weeks apart. Sera and splenocytes were collected three weeks after the last immunization for immune analysis. (A) ELISAs to assess rCSP binding by Ab elicited after final vaccination. (B) ELISAs to assess HBsAg binding by Ab elicited after final vaccination (C) The Pf CSP antigen-specific cellular immune response induced by the indicated DNA vaccine measured by IFNγ ELISPOT. Cells were stimulated for 18 hr with peptide pools encompassing the entire protein. (D) The HBsAg specific cellular immune response induced by the indicated DNA vaccine measured by IFNγ ELISPOT. Cells were stimulated for 18 hr with peptide pools encompassing the entire protein.

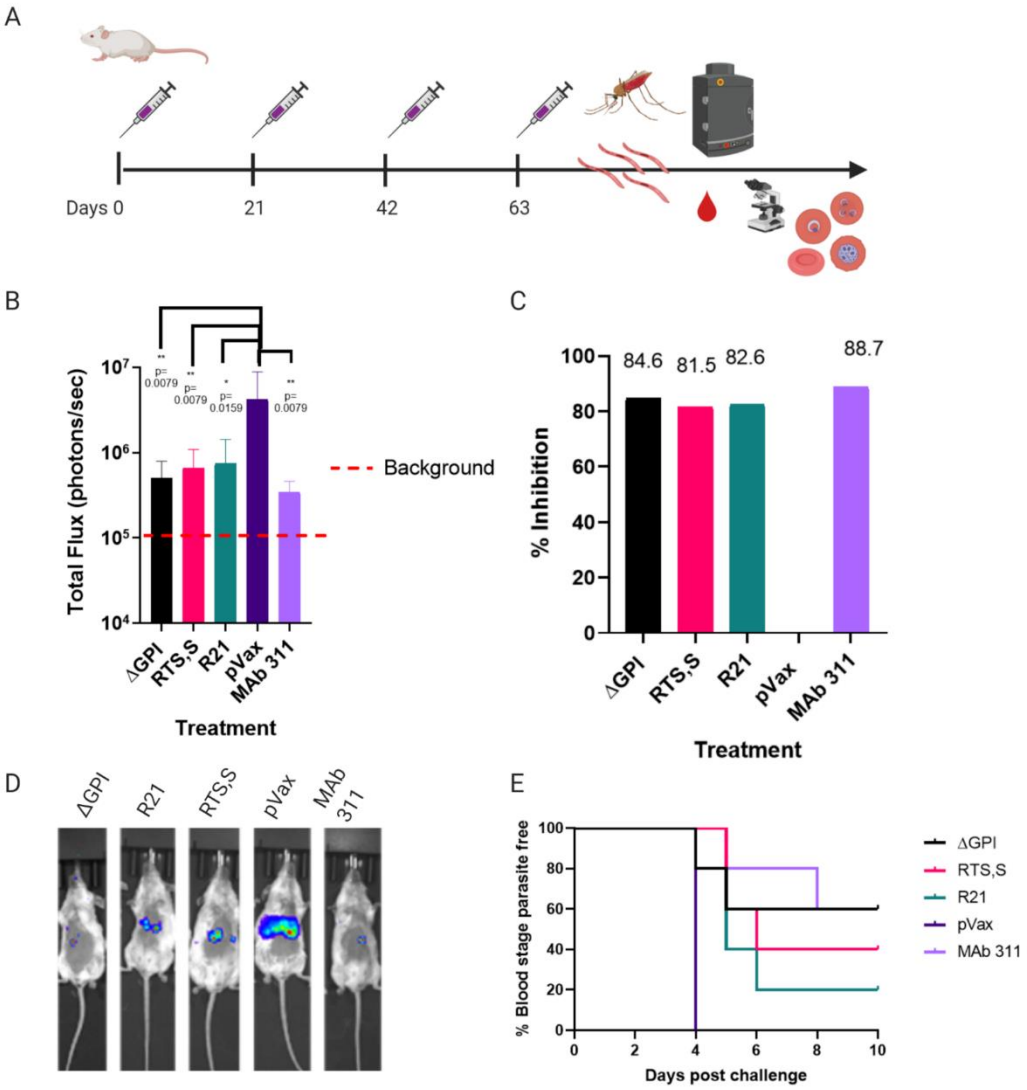

Figure 3.5: synDNA CSP constructs are protective in challenge. (A) Experimental layout: Mice were immunized four times, three weeks apart, and challenged three weeks after the last immunization by infected mosquito bite. Liver parasite burden was assessed by IVIS. Blood parasite burden was assessed by daily blood smears**.** (B) Graphical representation of luminescence data. Bars indicate the mean and standard deviations. Bar graph of means and results of Mann Whitney tests comparing groups to Naive Infected. (C) Inhibition of liver infection as expressed as a function of relative infection compared to naive mice. (D) representative IVIS images from each group (E) %blood stage parasite free mice, as assessed by blood smears each day post challenge.

CHAPTER 4: Discussion and Conclusions

4.1 Strategic interruption of the *Plasmodium* life cycle

As discussed in chapter 1, understanding the *Plasmodium* lifecycle and its points of weakness is crucial to developing effective interventions. The *Plasmodium* lifecycle has several "bottleneck" steps which make enticing vaccine targets. One of these so-called bottlenecks is the invasion of hepatocytes by sporozoites. Although many sporozoites are injected in the saliva of the mosquito vector, the majority of them die in the dermis and never migrate to the liver. While the number of sporozoites which leave the dermis and migrate to the liver is small, if even one sporozoite manages to evade immune detection and removal, schizogony in the liver will result in tens of thousands of merozoites to cause the symptomatic blood infection. If the goal of a prophylactic malaria vaccine is the same as most prophylactic vaccines- that is, to prevent symptomatic disease, then it is clear that our focus should be on a pre-erythrocytic vaccine. Two strategies of pre-erythrocytic vaccines are (1) to prevent the sporozoites from successfully invading hepatocytes and (2) to kill infected hepatocytes before merozoite development is complete (Figure 4.1).

In chapter 2 I focused on the second strategy, cell mediated immunity targeting the liver stage of infection. The studies in this chapter built off of previous work in the field of malaria vaccinology which highlighted the importance of both CD4+ and CD8+ T cells in anti-malaria immunity, namely studies by Steven Hoffman's[206–208] and Stephan Kappe's[66,84,153] groups on irradiated and genetically attenuated sporozoites, respectively. I showed that a synDNA vaccine targeting liver stage *Plasmodium* antigens can drive an antigen specific liver localized T cell population. I demonstrated through T cell assays

including ELISpots and Flow Cytometry that synDNA vaccination with EXP1, PFN, EXP2, ICP, UIS3, and TMP21, in combination or alone, elicits a robust T cell response, as well as the production of antibodies against these peptides. This T cell response was consequential, and resulted in 70-88% protection from blood-stage disease in an IV sporozoite challenge model of murine malaria infection. The use of the molecular adjuvant pIL-33 increased the immune response to vaccine and results in 100% protection from blood stage disease after sporozoite challenge. I hypothesize that this increase in protective efficacy in the adjuvanted groups is related to the increase in in antigen responsive liver associated T cells, as well as their polyfunctionality as demonstrated by enhanced cytokine poly-positivity in adjuvanted groups, though this hypothesis should be investigated further to elucidate mechanism in more detail. Altogether, the data suggest cell-mediated immunity against this obligate intracellular stage of the pathogen should be considered when designing anti-malarial vaccines, and support the continued examination of liver stage antigens as components of a prophylactic vaccine. Additional focus on LS antigens as a component of a malaria vaccine is warranted, potentially in combination with sporozoite antigens such as CSP.

In chapter 3 I focused on the first strategy of pre-erythrocytic vaccines, prevention of sporozoite invasion and establishment of liver infection. This work was largely inspired by the RTS,S vaccine studies which implicated antibodies against CSP as a key correlate of protection[180,197,200,201]. In these studies, I showed that a synDNA vaccine targeting the circumsporozoite protein of *Plasmodium falciparum* can induce a high-titer anti-CSP antibody response, as well as a robust cellular response producing IFNy in response to stimulation with CSP antigen. This immunogenicity elicited by the ΔGPI CSP vaccine resulted in protection from infection in multiple models of murine

malaria, including an IV sporozoite challenge, and a rigorous infected mosquito bite challenge. Additionally, this study is the first to show that synDNA mimics of the leading CSP vaccine candidates RTS,S and R21 generate strong humoral and cellular responses, resulting in protection from infection in an infected mosquito bite challenge model. However, the majority of the IFNy response elicited by dRTS,S and dR21 was directed against the HbsAg rather than against CSP. In contrast, our primary CSP candidate vaccine, ΔGPI, elicited a cellular response to only CSP, not to HbsAg, thus the response appears more focused towards the CSP antigen targeted by the vaccine. It would be interesting to compare the original protein and adjuvant formulations of RTS,S and R21 against the synDNA mimics we developed, as well as against our own novel anti-CSP vaccine ΔGPI. It is possible that the synDNA platform would offer a novel and potentially more immunogenic method of delivery for vaccine antigens such as CSP. I believe this platform of DNA encoded, *in-vivo* launched vaccines targeting CSP has the potential to deliver high level protection from malaria infection, in a temperature stable and cost-effective manner which makes it particularly well suited to use in low-resource settings.

In both of these series of studies, I demonstrated the efficacy of targeting the pre-erythrocytic stages of infection for prevention of disease. I found that targeting either of the pre-erythrocytic stages, sporozoites or liver stage, elicited robust immunity to malaria infection in a murine challenge model. I consequently hypothesize that a multi-valent vaccine which targets *both* pre-erythrocytic stages of infection may exhibit synergistic effects.

4.2 The next generation of malaria vaccines: emphasis on multivalency and advanced platforms

Prior vaccine studies suggest that a mono-antigen vaccine will be of limited use in preventing a disease with such a varied antigenicity and multiple strains. Mono-antigenic vaccines, notably RTS,S, have had limited success. The fact that the most robust protection is elicited by attenuated sporozoites is indicative of the need for a vaccine which targets many antigens and potentially multiple lifecycle stages. The next generation of malaria vaccines will need to be cognizant of this. The field of malaria vaccine development has been plagued by several challenges. The first is the questionable usefulness of the murine models which are widely used[209]. Even within a single species of Plasmodium, there is wide antigenic variability between strains, and the variability between species is even higher. Because of this, many groups have begun testing their novel vaccines in controlled human malaria infections (CHMI), in which naïve western volunteers are vaccinated and subsequently challenged with a single strain of *Plasmodium falciparum*[210]. However, even this model of malaria infection has significant constraints, as vaccines which perform remarkably well in CHMI trials (RTS,S[211], irradiated sporozoites[212]) struggled when deployed in areas of endemic infection. One potential cause of this dissonance is the interference of natural immunity with vaccine elicited immunity. It has been shown that non neutralizing antibodies against the sporozoite can abrogate protection from neutralizing antibodies, which may be a mechanism through which *Plasmodium* evades vaccine immunity[213]. Unfortunately, unlike immunity to many viral infections, natural immunity to malaria infection has not been a fount of wisdom to inform vaccine design. As discussed in chapter 1.3, natural immunity to malaria is strain-specific, built up slowly over time, and wanes quickly. We

must continue to toil to understand natural immunity to malaria, and if we cannot mimic it to design effective vaccines, we must understand how to work with it rather than against it, OR how to avoid the potential inhibitory effects of natural immunity on vaccine elicited immunity.

The next generation of malaria vaccines must be able to elicit durable, cross-strain protection. Some of the answer to this dilemma lies in the choice of antigen or antigens included in the vaccine, and some lies in the choice of vaccine platform. I argue that the ideal malaria vaccine will include antigens from multiple life cycle stages, both pre-erythrocytic and erythrocytic. Including pre-erythrocytic antigens will aim to prevent the infection from reaching the symptomatic blood stage, and including erythrocytic antigens will aim to mitigate the symptoms in the case of breakthrough infection. The inclusion of a transmission blocking antigen, as discussed in chapter 1.4, may also be a valuable addition to the malaria elimination arsenal. In addition to antigen selection, vaccine platform will also be of importance. As covered in chapter 1.5, the 1st and 2nd generation vaccine platforms, while formidable and hugely important in eradicating, eliminating, or greatly decreasing many pathogens of importance for human and animal health, have historically struggled to elicited the CD8+ T cell mediated immunity needed to control pathogens with an obligate intracellular stage of infection. Novel vaccine platforms, such as nucleic acid vaccines and nanoparticle vaccines, will be able to address this gap in vaccine elicited immunity in future vaccination endeavors.

4.3 Concluding remarks

Although I strongly believe in the power of vaccines to control infectious diseases, it is unlikely that any one prophylactic malaria vaccine will be a silver bullet. Rather, a prophylactic malaria vaccine like RTS,S or one of the nucleic acid vaccine

strategies described above are an important part of the global health community's arsenal in combatting one of humanity's most ancient foes. Other aspects of this arsenal include novel therapeutics, continued vigilance against drug resistant parasites and pesticide resistant mosquitoes, and the continued use of bed nets and other such avoidance techniques. The WHO Global Technical Strategy for Malaria 2016-2030 established the goals of 1) reducing malaria case incidence by 90% by 2030, 2) reducing malaria mortality by at least 90%, and 3) eliminating malaria in at least 35 countries[27]. Through a coordinated, interdisciplinary effort between immunologists, parasitologists, vaccinologists, epidemiologists and others we can look to a future where these goals have been realized. Eventually, though perhaps not by 2030, I believe the combined efforts of generations of scientists, doctors, and public health officials will culminate in a world free of malaria.

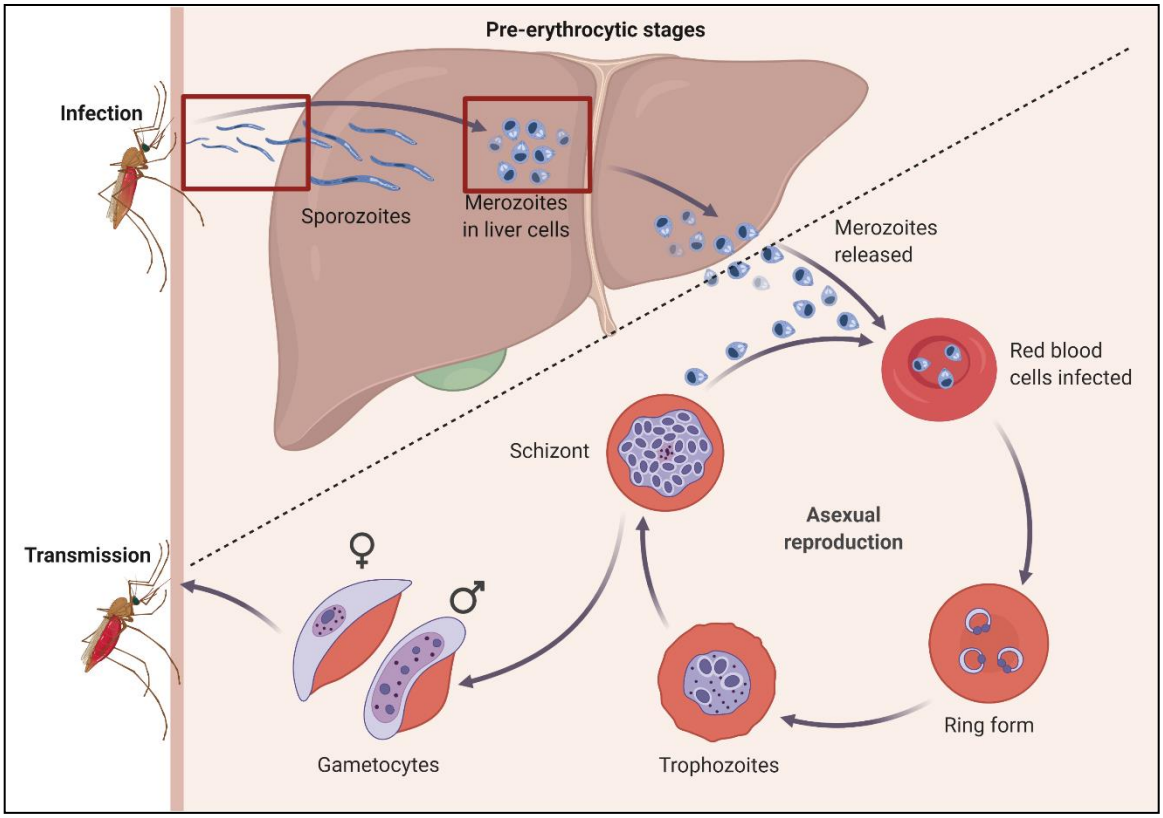

Figure 4.1: Strategic interruption of the *Plasmodium* life cycle. Life cycle stages to be targeted by vaccines to prevent symptomatic infection shown in boxes. Figure generated with BioRender©

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