IDENTIFYING IMMUNOLOGICAL SIGNATURES IN BLOOD PREDICTIVE OF HOST RESPONSE TO PLASMODIUM FALCIPARUM VACCINES AND INFECTIONS USING COMPUTATIONAL METHODS

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DEDICATION

To My Love. I cannot express how much your love and encouragement have meant to me throughout this experience. To say I could not have done this without you is an understatement.

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Malaria infects more than 240 million people every year, causing more than 640,000 deaths in 2021 alone. The complex interactions between the *Plasmodium* parasites that cause malaria and host immune system have made it difficult to identify specific mechanisms of vaccine-induced and naturally acquired immunity. After more than half a century of research into potential immunization methods, reliable immune correlates of malaria protection still have yet to be identified, and questions underlying the reduced protective efficacy of malaria vaccines in field studies of endemic populations relative to non-endemic populations still remain.

In this thesis, I use computational methods to identify biological determinants of whole-parasite vaccine-induced immunity and immune correlates of protection from clinical malaria. Our systems analysis of a PfSPZ Vaccine clinical trial revealed that innate signatures were predictive of increased antibody response but also a decrease in the cytotoxic response required for sterilizing immunity. Conversely, these myeloid signatures predicted protection against parasitemia for subjects receiving a saline placebo, suggesting a role for myeloid-lineage cells in clearing pre-erythrocytic parasite stages. Based on these findings, I created a structural equation model to examine the interactions between cellular, humoral, and transcriptomic responses and the effects these have on protection outcome. This revealed a direct positive effect of CD11+ monocyte-derived cells on parasitemia outcome post-vaccination that was mediated by the presence

of *P. falciparum*-specific antibodies at pre-vaccination baseline. Additionally, this model illustrates an indirect role of CD14+ monocyte activation in restricting immune priming by the PfSPZ Vaccine. Together, this data supports our hypothesis that innate immune activation and antigen presentation are uncoupled from cytotoxic cell-dependent immunity from the PfSPZ Vaccine and that this effect may be antibody-dependent.

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LIST OF ABBREVIATIONS

WHO: World Health Organization
CSP: Circumsporozoite Protein
PV: Parasitophorous Vacuole
PfEMP1: *Plasmodium falciparum* Erythrocyte Membrane Protein 1
DARC: Duffy Antigen Receptor for Chemokines
HLA: Human Leukocyte Antigen
MHC: Major Histocompatibility Complex
AMA1: Apical Membrane Antigen 1
MSP1: Merozoite Surface Protein 1
NHEJ: Non-homologous End Joining
CM: Cerebral Malaria
SMA: Severe Malarial Anemia
RDS: Respiratory Distress Syndrome
KC: Kupffer Cells
NK: Natural Killer (cells)
DC: Dendritic Cells
IgG: Immunoglobulin G
BSV: Blood-stage Vaccine
TBV: Transmission-blocking Vaccine
RAS: Radiation-attenuated Sporozoites
GAP: Genetically attenuated Parasites
ACT: Artemisinin-based Combination Therapy
PfSPZ: *Plasmodium falciparum* Sporozoites
TLR: Toll-like Receptor
LPS: Lipopolysaccharide
VE: Vaccine Efficacy
CHMI: Controlled Human Malaria Infection
BTM: Blood Transcription Modules
GSEA: Gene Set Enrichment Analysis
FACS: Flow-Assisted Cell Sorting
auROC: Area Under the ROC Curve
SEM: Structural Equation Modeling
CFA: Confirmatory Factor Analysis
BCG: Bacillus Calmette-Guérin
APC: Antigen-Presenting Cell

Chapter 1: Introduction

1.1 Malaria Significance and Life Cycle

The impact of malaria on humans is far-reaching: more than 40% of the human global population currently lives in areas where malaria is endemic and malaria causes hundreds of thousands of deaths each year[1]. In the 20th century malaria contributed to an estimated 5% of all deaths, equating to between 150 and 300 million lives lost[2]. By the 1950s, several nations had implemented malaria control plans, which led to calls for a coordinated global program dedicated to eradication of malaria. This first global collaborative effort began in 1955 when the World Health Organization (WHO) launched the Global Malaria Eradication Program (GMEP). At that time, the main reduction method employed by the program was use of insecticides such as Dichlorodiphenyltrichloroethane (DDT) to kill the mosquito vectors responsible for malaria transmission. During the 15 years this program was active, among countries with endemic malaria transmission in 1955, 15 countries and two territories were declared malaria-free[3]. Despite this success, the withdrawal of US financial support and banning of DDT weakened the program. The 22nd World Health Assembly re-evaluated the feasibility of malaria eradication goals, ultimately deciding to end the GMEP and shift malaria intervention efforts with control as the primary short-term focus[3].

After the termination of the GMEP, resistance to the most widely used anti-malarials was documented. Areas that were on the brink of eradication during GMEP experienced a rebound in malaria infection rates. In the 1990s, some countries undertook a recommitment to eradication, culminating in the establishment of the Roll Back Malaria

Partnership in 1998. This global partnership aimed to utilize multiple interventions to control malaria, including use of bed nets in addition to spraying with more targeted insecticides, to meet the outlined Millennium Development Goals of incidence reduction.

By 2015, many of these goals had been reached. Nine additional countries were certified as malaria-free, and implementation of artemisinin therapy contributed greatly to a reduction in case mortality. Between 2000 and 2014, global total cases decreased by 37% and incidence per thousand at risk decreased from 81.6 to 57. Most importantly, mortality due to malaria declined by 60% worldwide and by 71% in the most at-risk population: children in sub-Saharan Africa under the age of five[4]. Unfortunately, reductions in malaria incidence began stagnating after 2014, with both malaria cases and deaths increasing between 2019 and 2020 (**Figure 1**). In 2021 more than 240 million cases were reported, resulting in over 619,000 deaths – more than two-thirds of which were children under the age of five[1]. The increases may be partially attributable to disruptions in prevention and treatment efforts because of the global SARS-CoV-2 pandemic[1]. However, the stagnation in cases and mortality reduction since 2015 highlights the limitations of current methods of control.

Since 2015, efforts to address malaria transmission and infections have been led by the WHO's World Malaria Program, which coordinates the WHO's malaria control and eradication efforts[4]. Alongside the WHO, the PATH Malaria Vaccine Initiative (MVI) works with pharmaceutical companies and governments with a primary goal of developing a vaccine to complement control efforts and aid in the fight against malaria[5]. As evidenced by a lack of progress in recent years, eradication is unlikely without a highly effective and durably protective vaccine: defined in the 2015 WHO *Global*

Technical Strategy for Malaria as >75% efficacy[4] or another form of reliable and long-acting prophylaxis.

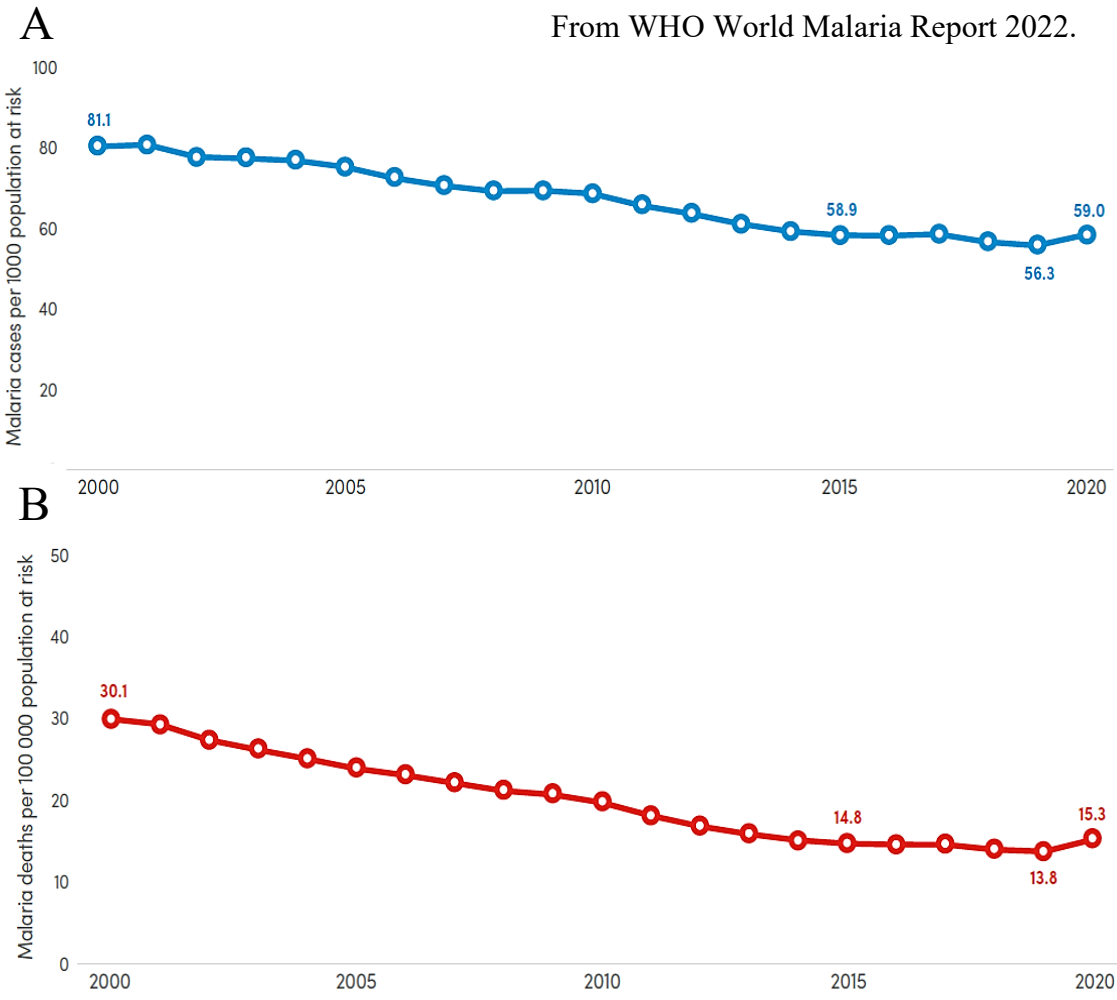


Figure 1. World Malaria Report 2022: Malaria Morbidity and Mortality.
A) Global malaria case incidence per 1000 at risk between 2000-2020.
B) Global malaria mortality rate per 100,000 at risk between 2000-2020.

1.1.1 The Discovery of *Plasmodium*

Malaria has been a substantial cause of morbidity and mortality in humans for millennia. The earliest records of periodic fevers thought to be caused by malaria infection date to ancient China in 2700 BC. Accounts of suspected malaria disease have been found throughout the world—on Mesopotamian clay tablets and in the works of Homer and Hippocrates[6,7]. The validity of these records is supported by archeological evidence of malaria infection identified in ancient remains dating back to at least 1300 BC in Europe and Africa[8,9].

Despite our extensive shared history, not much was known about the causative agent or mode of transmission before the late 19th century. Malaria is derived from "mal'aria" which translates to "bad air" in Italian; foul air was thought to be the cause[10]. It is now known to be caused by protozoan parasites in the genus *Plasmodium* and phylum Apicomplexa after a French physician, Charles Laveran, isolated these from the blood of malaria patients in 1880[7]. Once the parasites were accepted as the causative agent of malaria, Italian scientist Camillo Golgi defined the erythrocytic stages of the parasite life cycle from his observations of their asexual replication in blood[11,12]. Contemporaries Ronald Ross and Giovanni Grassi independently proved that transmission and sexual reproduction of *Plasmodium* requires an *Anopheles* mosquito vector in 1897 and 1898 respectvely[7,11]. Finally, 50 years later, the pre-erythrocytic liver stage was described by Shortt and Garnham[13].

1.1.2 Life Cycle of *Plasmodium*

The life cycle of the parasites takes place across the mosquito vector and the vertebrate host. As my thesis topic is immunology of malaria infection in humans, the life stages of *Plasmodium* inside the mosquito vector are beyond the scope and will not be covered in detail. The reader may refer to Barillas-Mury and Kumar's review "*Plasmodium*–mosquito interactions: a tale of dangerous liaisons" to gain detailed insight into tthe life stages of *Plasmodium* inside the mosquito vector and the interactions of the parasite with its invertebrate host[14].

In humans, the life cycle begins with the female *Anopheles* mosquito taking a blood meal while infected with *Plasmodium*[15]. Infectious sporozoites in the mosquito salivary glands are inoculated in the epidermis of the human host during feeding, thus beginning a complex series of life stages inside the human host (**Figure 2A**). Within minutes, viable sporozoites reach the liver[16] and—using tissue-resident macrophages, Kupffer cells (KCs) as a point of entry[17]—bind to and invade hepatic cells[18] through interactions between the sporozoite surface protein—circumsporozoite protein (CSP)—and hepatocyte surface proteoglycans[19,20] (**Figure 2B**). Once inside the hepatocytes, the sporozoites undergo schizogony, one cycle of asexual replication that results in a liver schizont containing tens of thousands of merozoites[11].

Following the liver stage, the malaria blood stage begins when schizonts burst and release merozoites into the bloodstream (**Figure 2C**). Within seconds of their release, merozoites specifically attach to and invade erythrocytes[19,21]. During invasion, the parasite is encapsulated in part of the cell membrane, forming a parasitophorous vacuole (PV), a compartment that the parasite uses to hide from the host immune response and

establish a niche for nutrient acquisition and waste removal[22,23]. Inside the PV, merozoites differentiate and multiply: going through the ring and trophozoite stages before forming a schizont containing up to 32 merozoites[24] (**Figure 2D**). To continue the cycle mature schizonts eventually burst, releasing daughter merozoites into circulation; each merozoite can rapidly invade an uninfected erythrocyte and begin asexual reproduction once more. It is during this blood stage that clinical malaria symptoms may become apparent: fevers, chills, and aches coincide with eruption of schizonts every 48 hours in *P. falciparum*.

Alternatively, a fraction of the parasites can become committed to sexual stage development prior to schizogony. These parasites do not develop into merozoites; they instead differentiate into male or female gametocytes before their release into the bloodstream. This stage acts as the mechanism of transmission from human host to mosquito vector (**Figure 2E**). Thus, another Anopheline mosquito ingests the gametocytes during a blood meal and sexual reproduction of *Plasmodium* can occur[25]. Inside the mosquito midgut, the male and female gametocytes mature into gametes which subsequently fuse to form a zygote (**Figure 2F**). This zygote differentiates into an ookinete which develops into an oocyst as it travels through the mosquito midgut. Oocysts rupture, releasing sporozoites into the mosquito's salivary gland. During this mosquito's next bloodmeal the sporozoites can be injected into another human host, potentially infecting them[26]. If this individual develops parasitemia, the transmission cycle can continue.

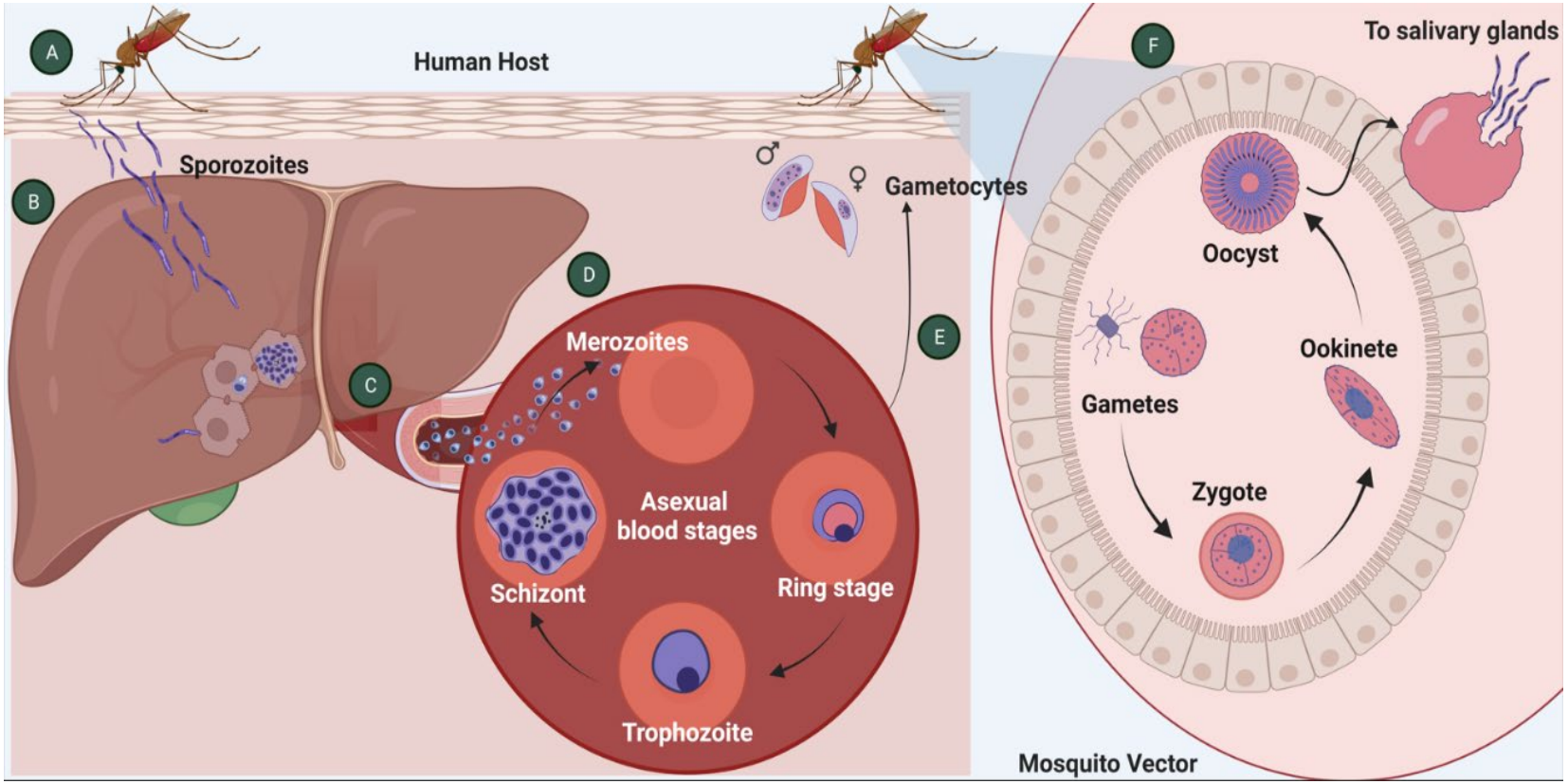


Figure 2. Life Cycle of Malaria. A) An infected female *Anopheles* mosquito infecting a human host with *Plasmodium* sporozoites. B) Sporozoites invade hepatocytes and replicate into a schizont during the liver stage. C) The schizont bursts, and the blood stage begins when tens of thousands of merozoites are released. D) Asexual replication of merozoites inside erythrocytes during the blood stage. E) Male and female gametocytes are transmitted to a new *Anopheles* mosquito vector during a blood meal. F) Sexual reproduction of the parasites in the mosquito gut. Made with Biorender.

1.1.3 *Plasmodium* Species

Five *Plasmodium* species can cause malaria in humans: *P. falciparum, P. vivax,* are the most common species while *P. malariae, P. ovale,* and *P. knowlesi* combined account for an estimated <5% of cases globally[1,27]. *Plasmodium vivax* causes the majority of cases in the American, Southeast Asian, and Eastern Mediterranean regions[1].

The species responsible for more than 90% of the cases and deaths due to malaria –*P. falciparum*– is the dominant species in the African region and is responsible for nearly all cases in that region as well as the highest mortality rate due to severe disease. Severe forms of malaria include severe malarial anemia (SMA; defined as malaria infection with hemoglobin < 5 gm/dL) , cerebral malaria (CM; coma for at least one hour that is attributable to malaria parasitemia), and respiratory distress syndrome (RDS; rapid, labored breathing due to pulmonary inflammation) *P. falciparum* is responsible for the majority of severe cases[1] though *P. vivax* is also known to cause severe disease.

1.2 Evolution and Interactions

1.2.1 Co-evolution of *Plasmodium* Parasites and Human Hosts

The specific genetic features of different *Plasmodium* species affect the pathogenesis of malaria and are the result of evolutionary adaptations which make the parasites successful pathogens. In direct contrast, humans have adapted to reduce the impact of *Plasmodium* on health and mortality. The special evolutionary relationship between competing enemies – predators and prey or in this case, parasites and their hosts – is referred to as the Red Queen hypothesis, based on the Lewis Carroll quote from

"Through the Looking Glass" when the red queen tells Alice "Now, here, you see, it takes all the running you can do, to keep in the same place"[28]. Indeed, the co-evolution of parasites and hosts reflects this imagery, with each organism continuously in reciprocal response to the other's adaptions[28,29].

Fluctuations in the frequencies of polymorphisms arise due to balancing selection between the parasite and the host. These polymorphic alleles may increase and decrease in a cyclical manner as both host and parasite attempt to gain the upper hand; this process is reflected in the strains of parasite species that circulate in certain areas. Alternatively, advantageous genotypes may become fixed in a population, permanently altering the dynamics between the two organisms[28,30].

1.2.2 Human host evolution

Remnants of an arms race between *Plasmodium* spp. and humans within the human genome illustrate how separate human populations evolved mechanisms of resistance to infection based on the species to which they were frequently exposed. Allelic variation in both components of hemoglobin, α-globin and β-globin, may be an evolutionary response to high malaria transmission. While protective phenotypes vary, structural and functional changes to globin genes can reduce parasite invasion and prevent severe disease caused by *P. falciparum* by impeding PfEMP1-mediated processes[29], increasing parasite exposure to reactive oxygen species[31], and arresting parasite growth[32,33].

Perhaps the most well-known of these malaria-resistance adaptions is the increased distribution of specific β-globin mutations (HbS or HbC) in areas where

malaria is endemic. Homozygosity of one of these mutated alleles causes a life-threatening hemoglobinopathy, sickle cell disease. However, a single HbS or HbC allele is associated with a 10-fold reduction in risk of severe malarial disease[29] and a 30% reduction in mild malaria episodes[32]. It is likely sickle trait arose in independent, random mutation events but has been maintained in the genome due to the selective pressure of malaria; this is evidenced by sickle trait mutations in geographically distant populations with different associated haplotypes[32,34,35]. Mutations in globin genes also cause hemoglobinopathies known as thalassemia that are associated with lower chance of severe disease. Interestingly, though protective against severe symptoms, α-thalassemia may be a risk factor for mild malaria episodes[29,36,37].

Erythrocyte polymorphisms that are not associated with hemoglobinopathies are further evidence of human evolution to prevent death due to malaria. The Duffy antigen receptor for chemokines (DARC) protein is required for invasion of erythrocytes by *P. vivax*[38–41]. Thus, *P. vivax* is mostly epidemiologically absent in West and Central Africa where a majority of the population are protected against *P. vivax* due to a lack of DARC expression on their erythroid cells. Since the discovery of this protective trait, there have been some reports of *P. vivax* infection in Duffy antigen-negative individuals[40,42,43], however, possibly an example of parasite evolution to overcome this host advantage. In a similar manner, erythrocyte Glycophorins A, B, and C, crucial proteins for *P. falciparum* invasion of erythrocytes, have a higher frequency of mutations in endemic areas[29,44–46].

1.2.3 Malaria parasite evolution

Human influences on *Plasmodium* population dynamics, as an example of the Red Queen hypothesis, are the result both of coevolution and, more recently, malaria control measures. Parasite survival is enhanced by changes to the host immune response – coupled with immune evasion strategies. Throughout infection, *P. falciparum* inhibits inflammatory cytokine production while upregulating anti-inflammatory cytokines, impairs activity of phagocytes in the liver and blood stages, and causes an increase in regulatory T cells, leading to increased immune tolerance of parasitemia[47–50]. A reduction in inflammation can increase circulation time and contribute to parasite transmission, however a causal relationship has not been identified and host mechanisms of reducing inflammation as well as acquired immunity to specific strains post-infection likely also play a role in reduced immune response with repeated clinical malaria episodes[49–52].

Evading the immune response is critical to parasite survival in its hosts, and, indeed, *Plasmodium* have evolved a number of mechanisms to do so. These mechanisms change throughout the life cycle of the parasite, depending on the compartment they inhabit. To survive inside a human host, *P. falciparum* evolved several evasion strategies, including the avoidance of antigen presentation by infected cells to prevent immune recognition, impairing the development of effective immunological memory that would threaten the next generation of parasites, and escaping splenic clearance of infected erythrocytes through sequestration.

Cell tropism and localization within host cells each contribute to immune evasion. Both the hepatocytes invaded by sporozoites[53] and erythrocytes containing the asexual blood stages are immunologically privileged, and the replicating parasites are protected

from a strong host immune response[47,55]. Inhabiting only erythrocytes multi-beneficial for asexual stage parasites: the erythrocyte provides a niche where the parasites can gain nutrients from host hemoglobin and lacks a nucleus and HLAs[56]. As they do not express MHC-I molecules, erythrocytes infected with *P. falciparum* are not recognized by cytotoxic CD8+ T cells. This is not true for all *Plasmodium* species: vivax preferentially infects reticulocytes, immature erythrocytes which express MHC-1 and thus can be recognized by CD8+ cells[57].

Selective invasion of these cell types has contributed to the survival and success of *Plasmodium* spp. and also influences other mechanisms of immune evasion utilized by different species. Active immune evasion is largely influenced by *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) proteins expressed on the surface of infected erythrocytes. A complex process of *var* gene silencing and switching between 60 *var* genes within a multigene family impairs host acquisition of immunity against PfEMP1 variants that have already been expressed[44,47,53,58]. In addition, PfEMP1 mediates additional mechanisms of immune evasion by *P. falciparum*: sequestration and rosetting. Protrusions on the infected erythrocyte surface allow the cell to bind endothelium and sequester in small capillaries to avoid splenic clearance through circulation[53,54,59,60]. Binding of infected erythrocytes with uninfected erythrocytes creates clusters called rosettes, uninfected cells hide parasite antigens from immune detection[47,61,62]. Notably, sequestration and rosetting contribute to severity of disease: microvasculature obstruction in the brain due to sequestration in capillaries or large rosettes blocking blood flow contributes to cerebral malaria[63–65]; rosetting leads to increased apoptosis of uninfected

erythrocytes as schizonts rupture to release merozoites or infected erythrocytes within the rosette are targeted by phagocytic cells[61,62].

Along with PfEMP1, additional multi-gene families and broad allelic diversity in *P. falciparum* proteins contribute to genetic diversity both within and between clonal populations. Surface proteins and proteins involved in erythrocyte attachment and invasion have long been targets for induction of immunity as they are more likely to be directly exposed to the immune system and may be critical for parasite growth and survival, respectively. These include CSP, a pre-erythrocytic stage antigen, and two heavily studied blood stage antigens: apical membrane antigen 1 (AMA1), and merozoite surface protein 1 (MSP1) each of which contain variable regions that are not conserved across strains of *P. falciparum.* The diversity of these surface proteins, in addition to variation and allelic switching in PfEMP1, have been implicated in the slow development of clinical immunity. Immunity only develops after repeated malaria episodes caused by infection with the same species when immune cells have been exposed to a variety of these variable alleles[66].

This diversity within the species may be party attributed to evolutionary loss of proteins necessary for DNA repair by non-homologous end joining (NHEJ)[44,67]. The lack of NHEJ increases parasite genome plasticity and allows for rapid adoption of new alleles through mutations; this contributes to development of drug resistance as well as difficulties in vaccine development as heterologous protection is likely required for durable, sterilizing immunity[68].

1.3 Clinical response to *P. falciparum* infection

While it is the deadliest of the malaria-causing parasites, *P. falciparum* can result in symptoms with a wide spectrum of severity: infection with a lack of clinical presentation or asymptomatic infection, uncomplicated clinical malaria, and severe malarial disease – the latter including CM and SMA, as well as RDS[64]. Years of repeated malaria episodes can eventually lead to acquisition of clinical immunity[69]. The very first malaria episodes in naïve individuals, mostly infants and young children, are the most likely to progress to severe malaria. After a child survives these initial episodes of severe disease and has acquired effective antibody responses to the parasites with the most virulent PfEMP1 variants[70], subsequent episodes manifest as uncomplicated malaria[71]. Continued, long-term exposure resulting in repeated malaria episodes confers protection against clinical malaria, allowing individuals to become asymptomatic carriers of *Plasmodium* parasites[48,51].

1.3.1 Asymptomatic Infections

While a lack of severe malaria symptoms is beneficial to the infected host, asymptomatic carriers of the parasites, by definition, do not present with clinical symptoms, thus they are unaware of the infection and are therefore not likely to seek treatment. These individuals, though asymptomatic, are still able to transmit the parasites to mosquito hosts, thereby contributing to continuing circulation of malaria in their communities. The mechanisms behind asymptomatic disease are not fully understood but are associated with host age, previous exposure to the same parasite strain, and other parasite and host factors[51,52,72].

1.3.2 Symptomatic Malaria

Plasmodium falciparum infection will typically manifest as uncomplicated disease in malaria-naïve or semi-immune individuals. These uncomplicated malaria cases are symptomatic episodes characterized by a robust inflammatory response. Clinical presentations of uncomplicated malaria include flu-like symptoms such as headache, muscle aches, nausea, and most characteristically, fever that eventually becomes cyclical as parasites synchronize erythrocyte egress during asexual reproduction[73]. With treatment symptoms usually resolve within three days though infected persons may transmit gametocytes to mosquitos for weeks after cure[74]. However, if left untreated, uncomplicated malaria can rapidly progress to severe malaria, especially in malaria-naïve individuals—typically in endemic areas these are very young children[64].

Most malaria deaths occur in patients with a severe form of malaria due to *P. falciparum* infection, with RDS and CM being more fatal than SMA[65]. The mechanisms that produce severe disease symptoms have been more often studied than asymptomatic infection; these can at least partially be attributed to species-specific characteristics of *P. falciparum* such as rosetting and sequestration[75,76].

1.3.3 Responses to Pre-erythrocytic Infection Stages

Cohort studies of natural malaria exposure have not indicated that sterile immunity which can prevent parasitemia is acquired, even after several years of intense malaria exposure[77]. Even so, experimental models of pre-erythrocytic infection in animal models and humans can provide valuable insight into the mechanisms involved in an effective pre-erythrocytic immune response that prevents parasites progressing to blood-stage infection. Although pre-erythrocytic stages of malaria do not elicit a symptomatic

response in the host and thus are clinically silent, the parasite still induces innate and adaptive immune responses during these stages. Pre-erythrocytic stages of infection can be divided into the early sporozoite stage—the time between inoculation and hepatocyte invasion (**Figure 2A-B**)—and liver stages (**Figure 2C**).

After sporozoites are injected by a mosquito during a blood meal, they quickly begin to mobilize to enter the vasculature. Macrophages amass quickly at the inoculation site and parasites may be phagocytosed in the skin as they traverse epithelial cells before entering the bloodstream[20]. As they make their way to the liver, sporozoites are vulnerable to the host immune responses, both non-specific innate immune responses and, if they have previously been infected, antibody responses against CSP.

Multiple protective mechanisms of anti-CSP antibodies have been identified in recent decades. The primary site of antibody-mediated protection may be the dermal site of sporozoite inoculation[78], likely through inhibition of inhibited sporozoite gliding motility[79]. Antibody-mediated phagocytosis of sporozoites may limit the number of sporozoites that reach the liver stage and in peripheral blood this is primarily through neutrophils, with limited evidence of monocyte phagocytosis of opsonized sporozoites[80]. During this phase of cell traversal, antibodies may also activate complement for direct lysis of sporozoites[81,82] however the primary role of antibodies against sporozoite surface antigens is likely neutralization.

Despite evidence of these mechanisms, anti-CSP titers are only partially correlated with protection in experimental models and clinical vaccine trials. It is important to note that naturally acquired anti-CSP antibodies have been associated with increased time to parasitemia and lower morbidity[79,83] but have not been correlated with

sterilizing immunity[84,85]. Only vaccine induced anti-CSP antibodies have been correlated with sterile protection, possibly because these are targeted against specific immunogenic epitopes and high titers of antibodies are required for complete neutralization of parasites during the short time between inoculation and hepatocyte invasion[86].

Prior to hepatocyte invasion, sporozoites must pass through liver sinusoids and can traverse tissue-resident macrophages called Kupffer cells (KCs). These KCs can phagocytose sporozoites in an antibody-dependent manner[87] however in the absence of a very robust antibody response, *P. falciparum* suppresses KC antigen-presentation processes and direct mechanisms of neutralization, and a strong immune response cannot be mounted before the sporozoites invade hepatocytes[20,47,88,89]. The prevention of hepatocyte invasion by neutralizing antibodies has also been repeatedly demonstrated[90–92] and antibodies against certain CSP epitopes are more effective than at prevention of liver-stage infection[92,93], through cytotoxic activity and restriction of egress from sinusoids. Intracellular sporozoites activate the innate immune response through the toll-like receptors (TLRs) TLR2, TLR7, and TLR9 and prompt production of type I IFNs[94,95]. Cytokine and chemokine production induced by type I IFNs recruit IFN- γ secreting lymphocytes, NK cells and T cells, to clear infected hepatocytes[96,97]. In murine models, the type I IFN results in recruitment of neutrophils to the infected liver, however this has not been demonstrated in humans. Additionally, there is evidence that malaria infection actually reduces recruitment of neutrophils during the asymptomatic liver stage[98].

Despite this complex immune response to the pre-erythrocytic stages, *Plasmodium* parasites directly interact with host immune cells to prevent or reduce these

responses and infection will generally progress to the symptomatic blood stage without generating protective immunity against future pre-erythrocytic infections[77,97,99].

Experimentally induced sterile protection against malaria requires complete immune clearance of liver stage parasite burden to prevent parasites progressing to the blood stage[100]. This complete pre-erythrocytic immunity is not naturally acquired through infection and relies on priming of a pre-erythrocytic antigen-specific CD8+ T cell response[90,100–104]. To generate a protective memory CD8+ T cell response, pre-erythrocytic antigens from infected hepatocytes and lysed cells presented to naïve CD8+ T cells by professional antigen presenting dendritic cells (DCs) or infected hepatocytes[101,105]. Another T cell subset, γδT cells, are required for sporozoite vaccine-induced immunity. Although an exact role for these cells is unknown, they may contribute to liver parasite clearance through production of IFN- γ and during vaccination these cells were required for a robust effector CD8+ T cell response[106]. Depletion of these cells decreased accumulation of dendritic cells, ultimately resulting in decrease in the CD8+ T cell priming required for protective immunity[106].

1.3.4 Immune response to blood stage infection

After liver stage replication, each infected hepatocyte contains thousands of merozoites that are released from schizonts into the bloodstream. The erythrocytic stage of malaria induces a robust inflammatory response when merozoites egress and re-invade erythrocytes, consequently fever and flu-like symptoms are cyclical and coincide with synchronous schizont rupture[73]. In response to free heme and parasite antigens, type I IFNs, pro-inflammatory cytokines, and chemokines are produced by activated DCs. Cytokines including IL-6, IL-12, and TNFα activate NK cell production of IFN-γ and

trigger further lymphocyte responses[88,95,107]. Another mechanism of parasite clearance by NK cells requires binding to antibodies on the surface of infected erythrocytes. Activated NK cells degranulate and release perforin, granzyme B, and granulysin; these cytolytic molecules act directly on the infected erythrocytes, inducing apoptosis[108].

Plasmodium falciparum-specific B and T cell responses have both been associated with lower parasitemia, as is passive transfer of IgG from immune adults in malaria-endemic areas to infected children[109,110]. This reduction in parasitemia and partial negative correlation between antibody titers and malaria episodes indicates a role for neutralizing antibodies in protective immunity[88,111–114]. However, for both pre-erythrocytic and blood stages, neither the generation of memory T cells nor an antibody response seem to be sufficient for protection after infection. Allelic diversity of target antigens and switching of var genes prevents heterologous protection after exposure, and naturally acquired antibody responses are not durable[58,115,116]. Sterilizing immunity must target pre-erythrocytic stages, and any surviving sporozoites will progress to blood stage—eventually resulting in patent parasitemia. This complicates the search for a highly effective vaccine or prophylactic that is consistently protective. Extremely robust responses that result in high antibody titers or large memory cell populations are required for sterile protection and natural infection does not induce a strong enough response to produce effective immunity[51,88,99,103].

1.3.5 Immune Modulations after Infection

Plasmodium falciparum modulates the immune response of the host during each stage of infection[48,50]. Though sterilizing immunity is not acquired from repeated malaria

episodes, tolerance to parasitemia and a reduction in clinical symptoms after multiple infections is common in areas where malaria is endemic. This clinical immunity is associated with lower parasite density and characterized by a lack of inflammatory response, including a shift toward anti-inflammatory Tregs and a regulatory monocyte phenotype[49,52,59,117,118]. The mechanisms underlying the interactions between a reduction in clinical symptoms, host control of parasitemia, and chronic asymptomatic infections are not fully understood; but there is evidence of multiple simultaneous processes. Increased control of parasitemia and a parallel observed reduction severity of malaria symptoms are hallmarks of clinical malaria immunity which results in asymptomatic infections[52]. While parasitemia is controlled by IFN-dependent pro-inflammatory cytokine responses and memory B cell production of diverse neutralizing antibodies, anti-inflammatory cytokine production and reduced B cell repertoire are associated with reduction in symptom severity[71,72,112,119]. Moreover, multiple candidate malaria vaccines that were protective against challenge in malaria naïve subjects had reduced efficacy in malaria-endemic areas. One explanation for this reduction in efficacy could be the vast antigenic diversity of naturally occurring parasite strains[68]; some trials that used heterologous strains for CHMI found that candidate vaccines[120,121] had lower efficacy against these than against homologous parasite challenge. Thus, genetic diversity of naturally occurring parasites likely plays a role in reduced efficacy. However, both protection against natural infection[122,123] and protection against CHMI with homologous parasites[124] have been measured during vaccine trials in malaria endemic areas[125] and demonstrated lower efficacy—highlighting the possibility that previous exposure to malaria may affect vaccine immunogenicity independently of parasite strain. As vaccine

development efforts continue, it will be necessary to tease out the impact of immune modifications and the interactions between symptomatic disease, parasitemia, modifications of the host immune system during infection, and differential vaccine efficacy.

1.4 Malaria Vaccines

Reducing morbidity and mortality due to malaria, through vaccine development or other interventions, has been active since the first GMEP was launched in 1955[3]. Since then, malaria research has reached several planned milestones and goals set forth by the WHO. Between 2000 and 2014 malaria was eliminated in 21 countries and the incidence of malaria decreased by 20% through distribution of interventions such as bed nets and anti-malarials and targeted pesticides against the disease vector, *Anopheles* mosquitoes[1,4]. However, progress began to slow in 2015– the number of yearly cases and deaths plateaued from then to 2020 when incidence and mortality rates increased, attributable to disruptions in prevention and treatment efforts because of the global SARS-CoV-2 pandemic[1]. The slowing of progress in the fight against malaria demonstrates the limitations of current control strategies and indicates eradication is unlikely without a highly effective and durably protective vaccine or another form of reliable and long-acting prophylaxis.

1.4.1 The First WHO Recommended Vaccine

In 2022 the first malaria vaccine received a WHO recommendation for widespread use[126]. The RTS,S vaccine fuses hepatitis B surface antigen (HBsAg) with B

and T cell epitopes of the immunodominant protein on sporozoites – PfCSP—to form virus-like particles (VLPs). Protection involves production of effective, high-titer anti-CSP antibodies and CD4+-dependent IFN-γ response[127,128]. Though RTS,S is a promising step toward malaria eradication, it does not meet WHO target efficacy of 75% over two years and protection from RTS,S is not durable[129]; more research is needed into the impact of RTS,S distribution on malaria incidence rates[126,130,131]. As the search for a highly effective malaria vaccine continues innovation in research methods and communication between researchers will remain essential for progress. Systems biology approaches can be used to provide key insight into immune processes and effective vaccine mechanisms.

The results of the first successful immunization against malaria were published in 1967 after mice exposed to X-irradiated mosquitos and demonstrated immunity to challenge with *P. berghei* sporozoites[132]. Successful immunization of human volunteers against both *P. falciparum* and *P. vivax* followed soon after, via exposure to hundreds of bites from irradiated mosquitos in the 1970s[133,134]. The promising results from these experiments launched decades of research with the goal of producing a highly effective malaria vaccine suitable for global distribution. The contemporaneous advent of recombinant technologies along with the led to identification of pre-erythrocytic immune target antigens and, eventually, identification of the immunodominant circumsporozoite protein (CSP) first in *P. knowlesi* followed by *P. falciparum*[135–138], led to the development of the first subunit vaccines against pre-erythrocytic malaria.

In *Plasmodium* parasites, CSP (PfCSP in *P. falciparum*) coats the exterior of the infectious sporozoites and plays a role in hepatocyte invasion. As mutations may affect

this crucial step in infection, the protein is largely conserved to maintain fitness of the parasite, making it an attractive target for pre-erythrocytic vaccination. High titers of antibodies produced against *P. berhei* CSP were found to be associated with protecting in mice[139], and anti-PfCSP inhibits invasion of hepatocytes and can induce the type I IFN response associated with clearance of liver parasite burden in humans[135,140]. PfCSP was an obvious target for vaccination and combined with the contemporary advances in recombinant DNA technologies led to the development of RTS,S[141]. The RTS,S vaccine consists of a the immunodominant B-cell epitope, the NANP central repeats, and T-cell epitopes from the C-terminal region of PfCSP fused to the hepatitis B surface antigen (HBsAg). This fused peptide is combined with excess HBsAg and the monomers self-assemble into virus-like particles that can be delivered with an adjuvant to increase the magnitude of the immune response[130].

1.4.2 Pre-erythrocytic Vaccines

Concurrently with the development of RTS,S, research into other vaccine candidates has continued since the initial WSV experiments. Along with pre-erythrocytic stages, immunization strategies include targeting blood stage vaccines (BSVs) and transmission-blocking vaccines (TBVs) which target proteins associated with erythrocyte invasion and sexual replication respectively.

While pre-erythrocytic vaccines aim to induce sterilizing immunity, preventing both symptomatic malaria and onward transmission by preventing the blood-stage altogether—blood-stage vaccines aim to control parasitemia to reduce symptomatic

disease and, potentially, transmission as reduction in overall parasitemia may result in fewer gametocytes[142].

Contrarily, transmission blocking vaccines prevent sexual reproduction and motility within the mosquito vector. This precludes vector transmission of sporozoites to humans; however, the vaccine would not prevent infection or clinical symptoms in the immunized individual.

The intricacies of developing vaccines against any of these stages of malaria infection requires more understanding of the complex host-parasite interactions. New methods of evaluation are likely required to bridge from current malaria vaccine candidates, such as the PfSPZ Vaccine which uses RAS to induce protection against sporozoite infection, to the development of a highly effective vaccine.

1.5 Systems Immunology

Systems biology describes research methods that use high-throughput analyses to combine data representing multiple levels of biological mechanisms – including genetic, transcriptomic, proteomic, molecular, and cellular data – to answer complex biological questions through integration of these data. Systems immunology and more specifically, systems vaccinology, seeks to apply these methods to create integrated models of immunity to produce more successful vaccine candidates by identifying biological determinants of differential response to immunization, molecular signatures of immunogenicity, and reliable correlates of vaccine-induced immunity.

Examination of the prior systems vaccinology studies can provide a guidance for future vaccine development. Notably, a systems study was employed to establish early

signatures associated with immunogenicity of the yellow fever vaccine, YF-17D[143]. In addition to evaluation of current vaccine candidates, systems studies can increase speed and precision of discovery of new vaccine target antigens. Such methods have been used in the pursuit of effective influenza vaccines[144,145]. Indeed, systems methods have already been applied to malaria to establish signatures of immunogenicity and protective responses associated with the RTS,S vaccine[146–149] as well as to identify immune signatures of malaria infection that could elucidate mechanisms of pathogenesis, and acquired protection against clinical malaria[48] and parasitemia[77].

As various "omics" fields continue to evolve, more information about immune responses at different levels of biological organization become available. In addition to current systems techniques, there is a need for methods of analysis that can accurately integrate data of different types and keep up with the computational demands of big data.

In search of a highly effective malaria vaccine, research into possible vaccine platforms and immunological targets has continued for more than 70 years. However, the genetic diversity of *P. falciparum* and complexity of host-parasite interactions complicated development and promising candidate vaccines often demonstrate differential efficacy without clear explanation[150]. Additionally, the effects that of alterations to the host immune system after exposure to the parasite have on immune response to vaccines are not well understood[49].

Decades of research on malaria immunology have demonstrated the need for innovation in malaria vaccinology research to answer lingering mechanistic questions. Systems immunology is an ideal approach to make sense of the complicated host-parasite

interactions and the interplay between immune responses to malaria vaccines, previous malaria infections, and future protection against parasitemia.

Research Aims

Using systems immunology, and by answering the following research questions, I intend to establish an integrated model of vaccine-induced immunity to *P. falciparum*. As computational tools have improved exponentially over the last century, the types of data available to researchers and our ability to combine data sets with computational methods has increased dramatically. The ability to generate data from various levels of organization of immune system components creates an opportunity to tease out the contributions of individual immune players as well as quantify the importance of their interactions on health outcomes. Applying systems vaccinology principles to malaria immunology, I aim to identify important determinants of vaccine-induced sterilizing immunity. After an initial hypothesis-generating analysis of global transcriptomic signatures I directed my work based on three research questions:

Research Question 1:

a. What factors at pre-vaccination baseline are predictive of *P. falciparum* infection after vaccination with the PfSPZ Vaccine?
b. What factors two weeks post-vaccination are predictive of future immunity?

Research Question 2:

How does innate immune activation at baseline affect infection outcomes post-vaccination with the PfSPZ Vaccine?

Research Question 3:

How do interactions between immunological signatures associated with vaccine-induced protection affect malaria immunity?

Chapter 2: Systems Analysis of PfSPZ Vaccine Immunogenicity and Efficacy in Kenyan Infants[151]

2.1 Introduction

In 2022 the WHO officially recommended the first malaria vaccine for widespread use—RTS,S—representing the culmination of more than 75 years of research and eradication efforts. However, RTS,S is only partially protective—36.3% with 4 doses—and immunity wanes quickly without additional doses[152]. Consequently, the search for a highly effective vaccine to induce sterilizing immunity against *P. falciparum,* and other malaria-causing parasites, continues and recent advances have been promising. In a Phase II trial, R21, another vaccine targeting PfCSP, demonstrated VE of up to 80%--depending on adjuvant dose—over two years following a primary regimen and booster dose one year post-vaccination[153,154].

Whole-sporozoite immunization has been a successful pre-erythrocyte vaccination approach as sterile protection has been induced in mice[132], non-human primates[155,156], and malaria-naïve humans with the delivery of attenuated sporozoites that invade hepatocytes but do not progress to symptomatic blood-stage infection.

Attenuated sporozoites prime an immune response to multiple pre-erythrocytic antigens and, in addition to inducing a sporozoite-specific antibody response, can confer sterile immunity by inducing a robust CD8+ T cell response[102,156,157]. The use of the entire sporozoite increases the likelihood of heterologous protection and does not require the addition of an adjuvant. WSVs can be separated into three categories: Radiation-attenuated sporozoites (RAS)[132], genetically attenuated parasites (GAP)[158], including genetically attenuated *P. falciparum* sporozoites and genetically modified *P. berghei* that

express PfCSP, and chemoattenuated sporozoites[159]. One candidate vaccine that contains *P. falciparum* RAS (PfRAS) is the PfSPZ Vaccine.

2.1.1 PfSPZ Vaccine

In 2003 Sanaria Inc. announced plans to produce and test the PfSPZ Vaccine: aseptic, cryopreserved, metabolically active, non-replicating PfRAS that must invade hepatocytes and begin replication before arrest during the early hepatic stages of infection to produce immunity[160]. The PfSPZ Vaccine induces sterile protection against *P. falciparum* through specific T cell and anti-CSP IgG responses[102,161,162]. Specifically, liver resident memory CD8+ T cells are induced by the vaccine which is a crucial mechanism of sterilizing protection[90,102,104]. Additionally, the PfSPZ Vaccine can confer ~50% heterologous protection[121,163], an important consideration as the genetic diversity of *P. falciparum* complicates immunization efforts.

In malaria-naïve volunteers the PfSPZ Vaccine demonstrated ~60-100% vaccine efficacy (VE) against challenge with homologous controlled human malaria infection (CHMI), this protection was durable for more than a year post-vaccination in subjects that were protected against initial challenge[164]. Field trials conducted to determine the efficacy of the PfSPZ Vaccine in malaria-endemic areas showed reduced efficacy in previously malaria-exposed adults when compared with North American controls. In Mali, VE was 48% against natural infection during the 6 month transmission season[123] while a small field trial in Tanzania demonstrated 20% VE although this protection was durable through two challenges with CHMI[124]. Another study revealed the PfSPZ Vaccine had reduced immunogenicity in malaria-exposed subjects, including less robust

P. falciparum-specific CD4+ T cell responses and reduced antibody responses to PfCSP[165]. These studies indicated that previous exposure to malaria may inhibit a PfSPZ Vaccine induced immune response and decrease efficacy. Additionally, the population most at risk for severe disease is young children who are not yet malaria-experienced and do not have acquired immunity to clinical symptoms.

2.1.2 KSPZV1 Trial of PfSPZ Vaccine in Kenyan Infants

A field trial to determine the safety, tolerability, and efficacy of the PfSPZ Vaccine in Kenyan children was conducted to test the hypothesis that infants may have enhanced immune responses to vaccination due to being relatively malaria inexperienced when compared to older children and adults in the same area [166,167]. The first arm of the study was a dose-escalation, age de-escalation design in children ages 6 months to 9 years old[166]. Once safety and tolerability were established, a Phase IIb double-blind, randomized, controlled study was conducted in infants between six and twelve months to determine the efficacy of three doses of PfSPZ against a saline placebo: $4.5x10^5$ PfSPZ, $9.0x10^5$ PfSPZ, or $1.8x10^6$ PfSPZ [167].

Though there was no significant VE for any dose at the primary endpoint of 6 months post-vaccination, the highest dose of $1.8x10^6$ PfSPZ conferred 41% VE ($p = 0.031$) against parasitemia at 3 months post-vaccination when compared with the saline placebo[167]. The PfSPZ Vaccine was immunogenic, generating strong CSP-specific antibody responses, which correlated with protection[167]. Phenotype distribution of infants by dose group and protection can be found in Table 1.

Infected (at or before 3 months)						
Dose	Age	Sex (%F)	Site (%Wagai)	Hemoglobin	Platelets	Weight Z-score
4.5x 10^5 PfSPZ	8	57%	51%	9.97	381.21	-0.29
9.0 x 10^5 PfSPZ	8	40%	40%	10.60	374.47	-0.22
1.8 x 10^6 PfSPZ	8	35%	42%	10.31	452.14	-0.09
Placebo	9	45%	42%	10.05	391.62	-0.15
Never Infected						
Dose	Age	Sex (%F)	Site (% Wagai)	Hemoglobin	Platelets	Weight Z-score
4.5x 10^5 PfSPZ	7.0	54%	38%	10.61	445.94	-0.34
9.0 x 10^5 PfSPZ	8.0	48%	44%	10.69	446.45	-0.15
1.8 x 10^6 PfSPZ	9.0	30%	53%	10.23	434.95	-0.18
Placebo	8.5	50%	76%	10.55	437.04	0.23

Table 1: Phenotypes of KSPZV1 Study Participants. Characteristics of study participants separated by infection outcome at three months post-vaccination. Age is reported in months at time of enrollment. Hemoglobin reported as mean g/dl. Platelets reported as mean platelets/ul. Weight Z-score included as an indicator of malnourishment.

2.2 Materials and Methods

2.2.1 Use of Human Subject Data

The work in this thesis used clinical samples and data from the KSPZV1 study, a clinical Phase IIa/b trial of the PfSPZ Vaccine (NCT02687373). This was a randomized, placebo-controlled study conducted by our collaborators at two study sites in Kenya. Details of the study can be found in the work published by the clinical trial research team[166,167] and methods have been summarized here for clarity. Use of de-identified human subject metadata and human samples from the original KSPZV1 clinical trial was approved as exempt human subjects research by the Indiana University IRB: protocol #1805696572.

2.2.2 Clinical Trial Methods

The KSPZV1 trial was conducted from January 2017 to August 2018 in Siaya County, Kenya. In this county malaria transmission is highly intense and occurs year-round. Transmission reaches peaks during two rainy seasons—a long season from April-July and a short season through October-November. After completion of the Phase IIa safety and tolerability study, the Phase IIb study began with randomization of infants aged 5 to 12 months at enrollment into 4 groups (**Figure 3A**), each receiving three intravenous injections administered eight weeks apart of $4.5x10^5$ PfSPZ Vaccine, $9.0x10^5$ PfSPZ Vaccine, $1.8x10^6$ PfSPZ Vaccine, or saline placebo control (**Figure 3B**). Artemisinin-based combination therapy (ACT), primarily artemether-lumefantrine, was administered to all study participants at least 11 days prior to the last vaccination to clear parasitemia at the beginning of active and passive surveillance for clinical malaria and *P. falciparum* infection. Subjects were actively screened for parasitemia at scheduled

monthly visits and passively by relying on self-reporting of apparent clinical malaria. Parasitemia was determined either by rapid diagnostic test or examination of contemporaneous blood smear. Only blood smears of symptomatic children were read in real time. Children determined to be infected with malaria due to fever and positive parasitemia test were treated. For this thesis, the primary outcome used was parasitemia (Susceptible) or absence of parasitemia (Protected) through 3 months of post-vaccination active and passive surveillance.

2.2.3 Sample Collection

Blood samples used in this thesis were collected by KSPZV1 study staff at Siaya County Referral Hospital and Wagai Health Centre in Kenya. Blood was collected by venipuncture and stored and shipped in PAXgene Blood RNA (BD Diagnostics), Vacutainer serum separator (Becton-Dickinson), and Vacutainer K2EDTA (Becton-Dickinson) tubes, in line with good clinical and laboratory practice principles. For thick blood smears and dried blood spots on filter paper, drops of capillary blood were used.

2.2.4 ELISA of CSP-specific IgG

To determine immunogenicity of the PfSPZ Vaccine, anti-CSP IgG in serum or plasma was measured by our collaborators using ELISA as described in previous PfSPZ Vaccine studies[166,167].

2.2.5 Plasma Cytokines

Plasma cytokines were quantified by our lab from baseline plasma samples using the 15-plex human Luminex discovery assay (R&D Systems) at a 1:2 dilution and acquired on a Bio-Plex 200 (Bio-Rad).

2.2.6 Flow Cytometry

Multi-parameter flow cytometry of PBMCs was performed by Dr. Phil Swanson as described for the clinical trial[167]. Intracellular cytokine stimulation assays were previously performed for the KSPZV1 clinical trial and T cell responses induced by PfSPZ vaccination were established with immunophenotyping data from these assays using previously described methods[164]. Cryopreserved PBMCs were thawed in tubes using "thawsome"[168] tube adaptors. After resting for 8h cells were stimulated for 17h with $1.5x10^5$ viable,irradiated, aseptic, cryopreserved PfSPZ or media control. Cells were stained and analyzed as previously described by Jongo et al[169]. To briefly summarize, cells were washed before staining with viability dye followed by surface stain. Cells were fixed and permeabilized then stained with intracellular stain. Surface staining for B cells and monocytes was performed on freshly thawed PBMCs as described for the parent clinical trial[167]. Antibodies used can be found in Table 5. Stained cells were collected using a BD FACSymphony flow cytometer (BD Biosciences). Samples were analyzed using FlowJo 10.6.1(TreeStar) and anomalous events were removed using FlowAI[170]. Remaining "good" events were used for all downstream gating as previously reported[167]. B cell analysis used CD27+IgD+ gating to identify memory B cells. This population was further divided using CD38+CD20- to separate plasmablasts and a labelled CSP-probe to identify PfCSP-specific memory B cells.

2.2.7 RNA Processing and Sequencing

Two separate batches of RNA extraction and sequencing were performed in 2019 by myself and former lab members Christina Salgado and Michael Macklin. Extraction and sequencing were performed on 96-well plates. Subjects were randomized to plates with pre-vaccination and post-vaccination samples from the same subject on the same plate. Total RNA was extracted from PAXgene tubes using the PAXgene 96 Blood RNA kit (Qiagen) and treated with RNase-Free DNase Set (Qiagen). RNA quality was assessed on a Fragment Analyzer (Advanced Analytical/Agilent). The average RQN was 8.4. For each sample, 100 ng of total RNA was used for library preparation. Ribosomal and globin mRNA were removed using QIAseq FastSelect rRNA and QIAseq FastSelect GlobinRNA removal kit, respectively (Qiagen). RNA was fragmented, converted to cDNA, ligated to index adaptors, and amplified using the KAPA RNA HyperPrep Kit (Roche). Quantification and quality were re-assessed. Libraries were pooled with QIAgility (Qiagen). RNA sequencing of 150 bp paired-end reads were performed on NovaSeq 6000 (Illumina). Illumina sequences were trimmed of contaminating adapters and bases. After assessing sequencing quality using FastQC (v0.11.5, Babraham Bioinformatics, Cambridge, UK), paired-end reads meeting a Phred quality score (Q score) > Q30 were mapped to reference human genome GRCh38 (version 16, Ensembl 99) using STAR RENA-seq aligner (v2.5). The following parameter was used for mapping: "Se--outSAMmapqUnique 60". Assessment of reads distribution was performed using bamutils (ngsutils v0.5.9). Uniquely mapped reads were assigned to hg38 refGene genes using featureCounts (subread v1.5.1) with parameters "-s 2 –p –Q 10". After sequencing genomic DNA (gDNA) contamination was suspected due

to high intergenic read percentages for some samples. The SeqMonk RNA-Seq quantitation pipeline was used to correct for gDNA contamination using the FASTQ results from illumina sequencing (Babraham Bioinformatics, Cambridge, UK). Expression of 88 genes encoding select lineage markers or relevant to immune responses was validated using nCounter PlexSet (nanoString), with 57 genes (65%) exhibiting strong correlation (Spearman $\rho \geq 0.6$) between RNA-seq and nCounter expression values.

2.2.8 Differential Gene Expression Analysis

Differential gene expression analysis was performed using edgeR[171] comparing parasitemia (NP or not protected) or absence of parasitemia (P or protected) through 3 months post-vaccination with the PfSPZ Vaccine. The filterByExpr function was used to remove genes with very low expression. Normalization was performed with weighted trimmed mean of M-values method[172] and a library size filter of 7.5×10^6 was applied. Expression data for these genes were converted to log count-per-million (logCPM) for unsupervised hierarchical clustering, presented as a heatmap. For differential gene expression, protection was used as the comparison and dose groups were analyzed separately. Data visualization in PCA plots revealed a batch effect between the two sequencing batches. The model matrix includes adjustments for these variables. The glmQLFtest function was used to identify differential gene expression between 3-month outcomes. Model matrix and contrast are listed below.

Model matrix: ~0 + Batch + Outcome

Contrast: P – NP

2.2.9 Gene Set Enrichment Analysis

Gene set enrichment analysis was performed in R using the fgsea package[173]. A list of all genes included in the Differential Gene Expression Analysis was ranked using the p-value and $\log_2$fold-change determined in that analysis. To create a list with the most significant genes on the top and bottom $\log_{10}(\text{pvalue})*\text{sign}(\log_2\text{FC})$ was applied as the ranking metric. Blood transcription modules were used as gene sets[174] with minimum gene set size of 20. High-level annotations of the blood transcription modules[146] were applied in a separate gene set enrichment analysis with the same metrics and parameters.

2.2.10 Upstream Regulator Analysis

Upstream Regulator Analysis was performed using QIAGEN IPA (QIAGEN Inc., https://digitalinsights.qiagen.com/IPA)[175]. Genes with absolute value of $\log_2$ fold-change over 1.2 and $p<0.25$ were included in this analysis.

2.2.11 Data Management

RNA-seq data and metadata for the KSPZV1 is available on the database of Genotypes and Phenotypes (dbGaP) under accession number phs002196.v1.p1. Sequence-level data has not been published on dbGaP due to a lack of consent from KSPZV1 participants and guardians for public storage of their data. Data management was performed by myself and Aditi Upadhye.

2.2.12 Survival Analysis

Survival analysis was performed by using the survival package[176] in R. Kaplan-Meier curve was created to estimate the effect of anti-CSP IgG response as a categorical variable on the probability of remaining parasitemia-free for 3 months post-vaccination.

2.2.13 Sporozoite Preparation

Sporozoites were isolated from infected mosquitos or obtained already purified from Sanaria. *Anopheles stephensi* mosquitoes infected with *P. yoelli* (Py) were purchased from the Insectary at Seattle Children's Research Institute. Salivary glands were dissected into RPMI 1640 Medium (Roswell Park Memorial Institute, Gibco, Cat. 11875-093) and sporozoites were isolated using the Ozaki protocol[177]. Sporozoites were collected by myself and other members of our lab and Dr. Nathan Schmidt's lab. Purified, cryopreserved, fully infectious *P. falciparum* and *P. yoelii* sporozoites (PfSPZ and PySPZ) were obtained from Sanaria. For analysis of RAS, irradiation was performed using the following parameters: 200 Gy; ~519 cGy/min for 38.5 min.

2.2.14 Mouse Studies

Approval for the animal studies was obtained from IUSM Institutional Animal Care and Use Committee (IACUC) under protocol 19024 in compliance with all applicable federal regulations and accredited by AAALAC, International.

2.2.15 Mice

Seven-week-old female C57BL/6 mice were obtained from Charles River Labs. Mice were housed in the Indiana University School of Medicine Laboratory Animal Research Center (LARC), an American Association for Accreditation of Laboratory Animal Care (AAALAC) approved facility. Mice were intravenously injected with saline (0.9%; Teknova, Cat. S5825), flagellin (10 μg; Adipogen, Cat. AG-40B-0095-C100), poly(I:C) (200 μg; Tocris, Cat. 4287), or lipopolysaccharide (LPS; 10 μg; Sigma, Cat. L3024-5MG) or treated via intraperitoneal injection with endotoxin-free phosphate - buffered saline (PBS; Corning, Cat. 21-040-CV) or β-glucan (1 mg; Sigma, Cat. G5011-25 mg) 24h prior to intravenous injection with *P. yoelli* RAS or fully infectious, metabolically active sporozoites. All sporozoite injections were done using a final volume of 200μL. CD8+ T cell responses were measured after injection with$2.5x10^3$ or $1x10^4$ RAS. Parasite liver burden and pre-patent parasitemia were measured following injection of $1x10^3$ fully infectious *P. yoelii* sporozoites. Mouse experiments were designed by Dr. Nathan Schmidt, Dr. Tuan Tran, and Dr. Rafael Polidoro and performed by myself, Elizabeth Fusco, and Morgan Little.

2.2.16 CD8+ T Cell Quantification

RAS-injected mice were anesthetized before collection of peripheral blood at -48h from RAS injection and on days 5, 6, 7, 14, 28, and 55 post-RAS. Peripheral blood from anesthetized mice was collected 24 hours prior to RAS injection, and on days 5, 6, 7, 14, 28, and 55 post-RAS-injection. Red blood cells were lysed, and leukocytes were stained with Zombie Aqua (Biolegend, Cat. 423102), washed, resuspended in FACS buffer (PBS with 1% heat inactivated FBS; Atlanta Biologicals, Cat. S11550, 0.02%

sodium azide; VWR, Cat. BDH7465-2) containing FC block (anti-CD16/32; clone 2.4G2) for 10 minutes and stained for 20 minutes with antibodies listed in the Table 5. Cells were washed, fixed for 20 minutes (Fixation Buffer; Biolegend, Cat. 420801), and then washed again. Labelled cells were acquired on the BD LSRFortessa X-20 cytometer or Attune NxT cytometer and data was analyzed using FlowJo software (v.10.7.1) (TreeStar).

2.2.17 Liver Parasite Burden

Mice receiving viable sporozoites were anesthetized ~42h post-injection using 3.5% isoflurane, 1.5L/min O2 prior to euthanasia by cervical dislocation. Livers were removed using aseptic technique and placed in RNAlater Solution (Invitrogen, Cat. AM7020). The left median lobe was dissected and weighed prior to bead mill homogenization in RLT buffer (Rneasy Plus Mini Kit, Qiagen, Cat.74134). Liver homogenates were placed on ice prior to proceeding with RNA extraction using the Rneasy Plus Mini Kit (Qiagen) according to the manufacturer's protocol, optimized for liver tissue. Purity of the RNA samples was assessed followed by cDNA synthesis (ProtoScript II Reverse Transcriptase, New England Biolabs, Cat.M0368L) using manufacturer's quick protocol for random primer mix. The cDNA was amplified using *P. yoelii* 18S primers. Real-time PCR was used to quantify relative transcript abundance in the samples using a standard curve for the 18S PCR generated with *P. yoelii* stabilite reference cDNA as standards. Express PrimeTime 5' 6-FAM™/ZEN™/3' IB®FQ chemistry was used for the 18S PCR (Integrated DNA Technologies) and SYBR chemistry was used for GAPDH PCR (Luna, New England Biolabs, Cat. M3003S). PCR

was performed on Applied Biosystems QuantStudio 6 Flex Real-Time PCR System (ThermoFisher). All primers and chemistries are reported in Table 5.

2.2.18 Parasitemia in Mice

Blood samples were taken through tail snips at regular intervals ranging from days 5-17 post infection. Blood samples for parasitemia quantification through flow cytometry were sampled directly into 96-well plates and confirmatory blood smears and dried blood spots on filter paper were taken on days 5,7,10, and 13 post-infection with fully infectious *P. yoelii* sporozoites. Parasitemia was quantified by flow cytometry by defining parasitized erythrocytes as CD45.2-Terr119+Dihydroethidium+Hoechst+ as previously described by our collaborators[178] and by real-time PCR as described above but using genomic DNA extracted from whole blood as template. The rtPCR was performed by Erik Gaskin and Morgan Little. For flow cytometry, labelled cells were acquired on the Attune NxT cytometer and analyzed using FlowJo v.10.7.1 by myself and Elizabeth Fusco.

2.3 Results

2.3.1 KSPZV1 Clinical Trial

The primary outcome in the clinical trial was presence or absence of *P. falciparum* parasitemia through 6 months of surveillance after the third and final vaccine dose. Though there was no significant protection induced by the PfSPZ Vaccine at this time point, we used the secondary endpoint of protection through 3 months of surveillance after the vaccination period because at this time point, the highest dose of PfSPZ Vaccine—$1.8x10^6$ PfSPZ—demonstrated significantly significant vaccine efficacy of 41.1% , with 95% CI 4.7, 63.6 ($p = 0.031$) [167]. The significant but approximately balanced protection outcomes within the $1.8x10^6$ PfSPZ dose group created an opportunity to potentially identify molecular mechanisms of reduced efficacy. We began our interrogation of the data with transcriptomic analysis to generate hypotheses about the mechanisms underlying differential protection induced by the PfSPZ Vaccine.

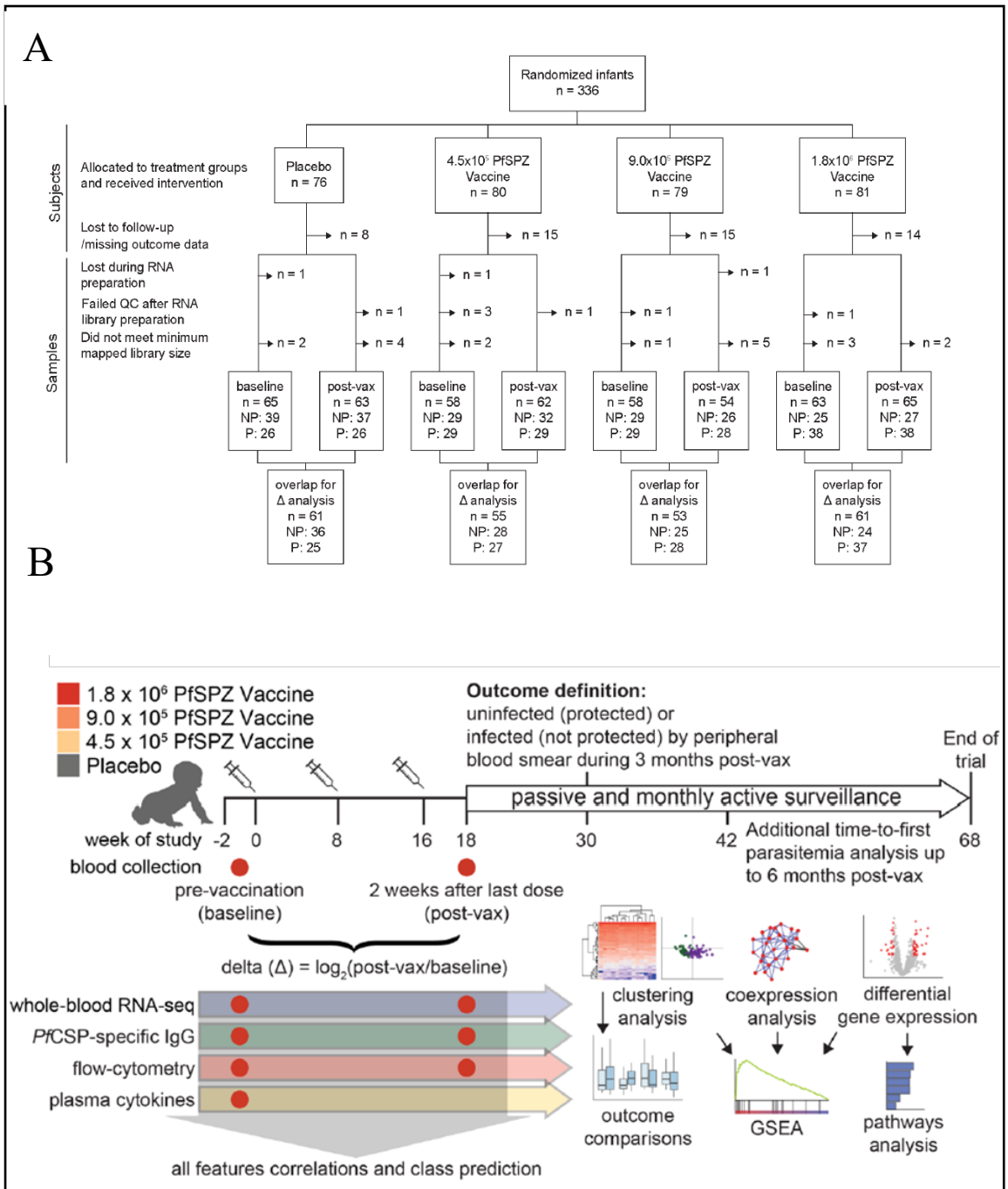


Figure adapted from Senkpeil L, Bhardwaj J, Little M, et al. In Revision.

Figure 3. KSPZV1 Phase IIb Clinical Trial. A) Flow diagram of inclusion of study participants. Participants were identified as Protected (P) or Not Protected (NP) based clinical malaria at the 3-month post-vaccination follow up appointment (Study week 30).B) Immunization and blood draw schedule, monthly surveillance to end of trial and systems study workflow. Sample types and the analyses performed are listed with corresponding blood draw time points.

2.3.2 Baseline Transcriptomic Signatures Associated with PfSPZ Vaccine Efficacy

The trial included 336 total participants and of these, 258 had RNA of sufficient quality to be used in the transcriptomic analysis for at least one time point (**Figure 3A**). Hierarchical clustering analysis of baseline transcriptomes was used to explore the possibility that pre-vaccination gene expression may cluster children based on pertinent malaria relevant variables, demographics, or protective outcome at 3 months (**Figure 4**). Within dose groups, clusters were not differentiated by age, sex, positive blood smear at the first vaccination, study site, or CSP-specific IgG response or at baseline. For one cluster within the group that is the main focus of this thesis, the $1.8x10^6$ dose group, the protected outcome was significantly overrepresented, with 82.6% of the cluster in the protected group and only 55% of the remaining $1.8x10^6$ were protected at 3 months. This represents about half of the protected group that are clustered by global gene expression alone.

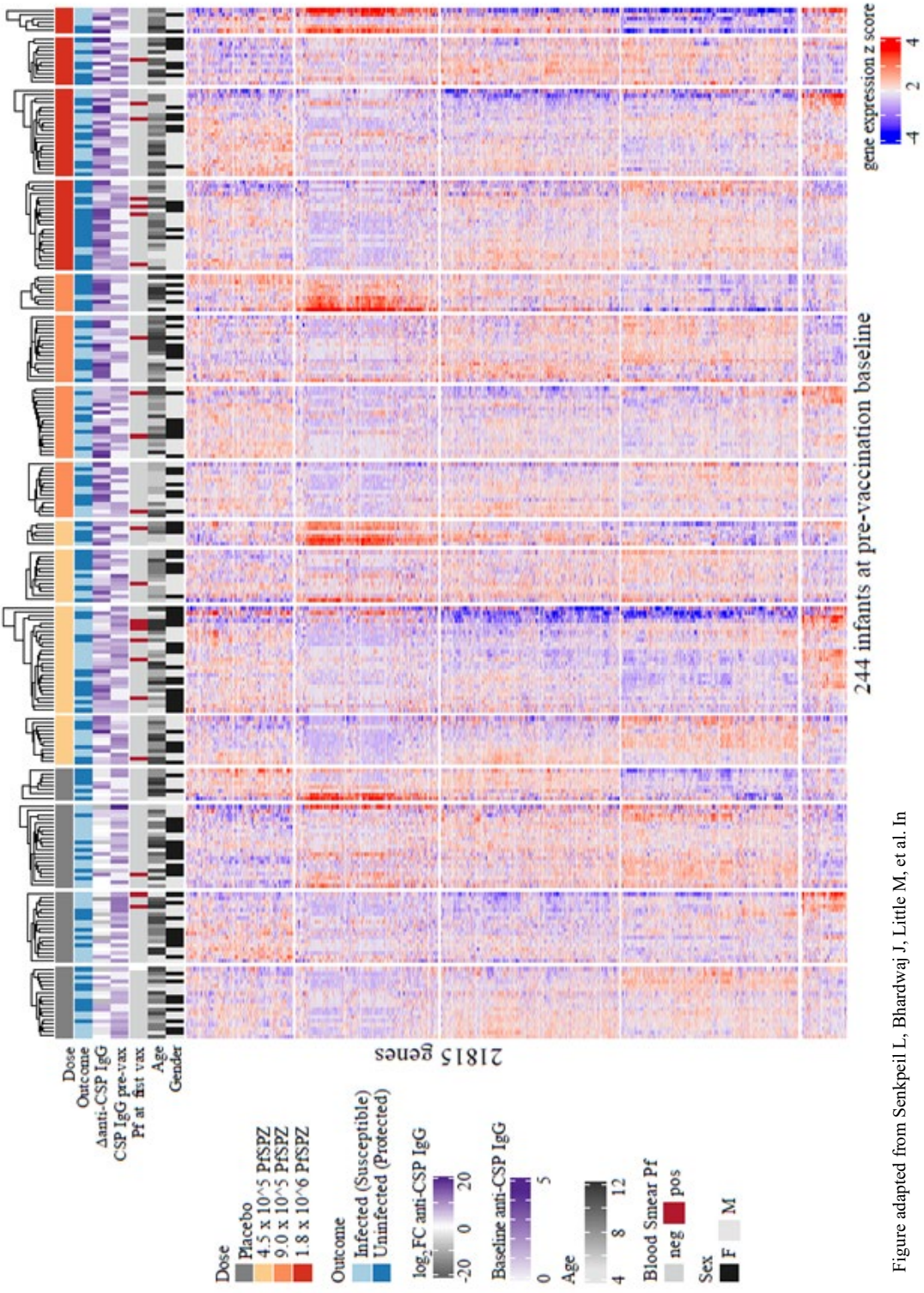


Figure adapted from Senkpeil L, Bhardwaj J, Little M, et al. In

Figure 4. Unsupervised Hierarchical Clustering of Global Transcription. Columns are subjects and rows are z-scores of gene expression values of the top 21,815 most variable genes. Samples were split by dose group after clustering to examine patterns within and between groups. Annotations of phenotypes and non-transcriptomic independent variables correspond with sample columns.

To determine whether any individual genes may contribute to protection status at the 3-month post-vaccination time point, differential gene expression analysis was performed using edgeR followed by gene set enrichment analysis. While differential gene expression analysis measures the difference between individual genes between comparison groups, GSEA identifies enrichment or depletion of sets of genes that have previously defined biological or functional annotations. Using a false discovery rate (FDR) <20% as a measure of significance, there were no differences at the gene level between the Protected and Not Protected infants in any dose group. However, gene set enrichment analysis using *a priori* defined Blood Transcription Modules(BTMs)[174] revealed differential transcriptomic signatures between the protected and not protected infants in all dose groups. Using low-annotation BTMs as gene sets, there were no consistently enriched gene sets for either protected or not protected across all four groups. As the only dose with vaccine efficacy, the $1.8x10^6$ PfSPZ dose group is of particular interest. In this group, NK and T cell signatures were enriched in Protected infants when compared with infants that were Not Protected. Additionally enriched in the Protected group were several cell cycle signatures that, when the genes from similar low-annotation BTMs are organized into high-annotation BTMs[146] corresponded with mitochondrial activation as well as the cell cycle (**Figure 5A**). Signatures that were enriched in Not Protected relative to protected in the $1.8x10^6$ PfSPZ group were largely related to innate immune activation. These included activation and surface signatures of monocytes and

dendritic cells (DCs) as well as TLR and inflammatory signaling. Intriguingly, these modules were enriched in Protected for the placebo group. Overall, using high-level annotations, four modules were inversely enriched in relation to protection between the placebo and $1.8x10^6$ PfSPZ groups: Monocytes, DC Activation, Inflammatory/TLR/Chemokines, and ECM and Migration (**Figure 5A**). This discovery led to our hypothesizing a role for innate immune activation in natural protection which may prevent infection in the placebo group who should have no induction of protection during the vaccination period, while also being a mechanism of interference with the efficacy of the PfSPZ Vaccine.

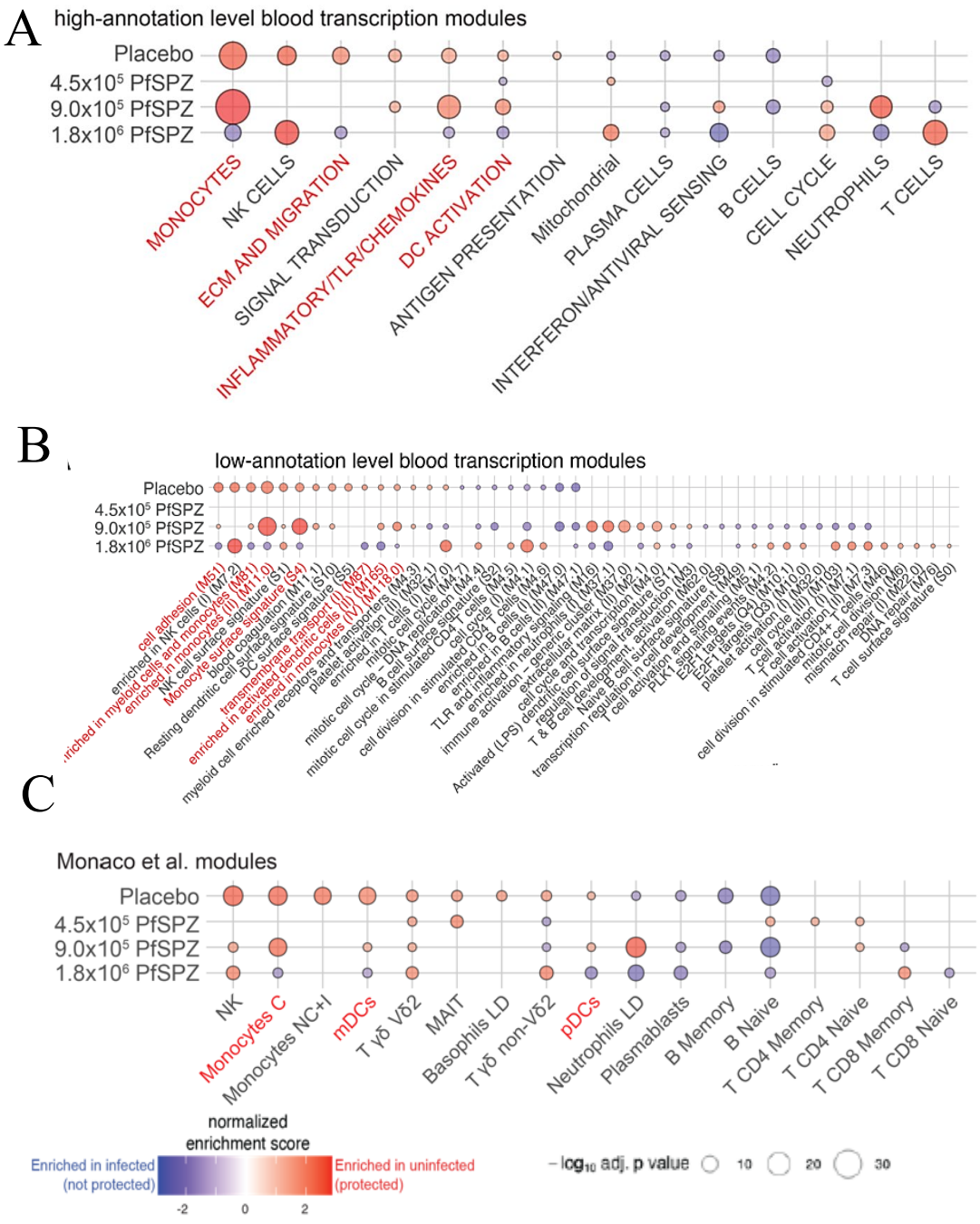


Figure adapted from Senkpeil L, Bhardwaj J, Little M, et al. In

Figure 5. Gene Set Enrichment Analysis. A) High-level annotation blood transcription modules B) Low-level annotation blood transcription modules. C) Monaco et al gene sets. For gene set enrichment analysis, pre-ranked differential gene expression data was used with sign(logFC)*$\log_{10}$(p-value) was used as a metric. Modules that have inverse association with Protected phenotype between Placebo and 1.8 x 10^6 PfSPZ are in red. Bubble size indicates significance as $\log_{10}$(p-value). Bubble color indicates association of the gene set with Protected or Not Protected groups.

To confirm these signatures and further define possible mechanisms of innate immune activation, differentially expressed genes meeting a threshold of $|\log_2$ fold-change$| > 0.263$ and $p<0.25$ were applied to Ingenuity Upstream Regulator Analysis. This overrepresentation analysis can be used to predict the activation status of upstream signaling molecules or transcription factors based on the most differentially expressed genes between comparison groups[179]. IPA uses a comprehensive proprietary database of known regulatory networks and relationships of activation and inhibition with user supplied data to predict specific regulators that explain observed patterns of gene expression. This analysis predicted innate immune stimuli as regulators of baseline gene expression in the Protected placebo group while the same regulators were predicted as activated in Not Protected for infants who received $1.8x10^6$ PfSPZ. The Protected placebo group was predicted to be regulated by TLR4 agonist lipopolysaccharide (LPS) as well as IL-1β, a downstream product of the TLR4 signaling pathway. These same regulators as well as type I and III IFNs and IFN regulatory factors (IRFs) were predicted to be activated and regulating baseline gene expression for Not Protected infants when compared to Protected infants from the $1.8x10^6$ group (**Figure 6**).

Other predicted activators that were associated with protection in infants who received the placebo while also being associated with the Not Protected phenotype in the $1.8x10^6$ PfSPZ group included S1P, a signaling lipid that is necessary for movement of activated CD8+ T cells to the liver during pre-erythrocytic stages of malaria[101]. Receptors for S1P are also expressed on activated monocytes, DCs, and neutrophils during inflammatory responses[180]. Other regulators of note with inverse expression in Protected

classes of placebo and $1.8x10^6$ PfSPZ include erythroid and mast cell transcriptional regulators GATA1 and GATA2[181] as well as HIF1A which is highly expressed in eosinophils and other innate immune cell types and activates cellular stress pathways[182].

Our lab validated these findings and the results of the GSEA using transcriptomic data from two previous PfSPZ Vaccine trials in malaria-naïve adults: VRC 312[161] and VRC 314[164]. For these trials, VE was tested by measuring parasitemia outcomes after CHMI, with absence of parasitemia through 4-week post-challenge considered to be Protected. Analysis of these data sets revealed enrichment of myeloid and innate immune signatures at baseline in individuals who would eventually be classified as Not Protected after post-vaccination CHMI. Additionally, upstream regulators LPS, IL-1β, and other products of the LPS/TLR4 signaling pathway TNF and IL-6 were predicted to be activated at baseline in the VRC 312 Not Protected group[151]. Together, these findings suggest monocyte and DC activation, possibly due to TLR agonists, may be protective against natural infections in non-vaccinated individuals but may also reduce the immune response to and protective efficacy of the PfSPZ Vaccine when present at baseline.

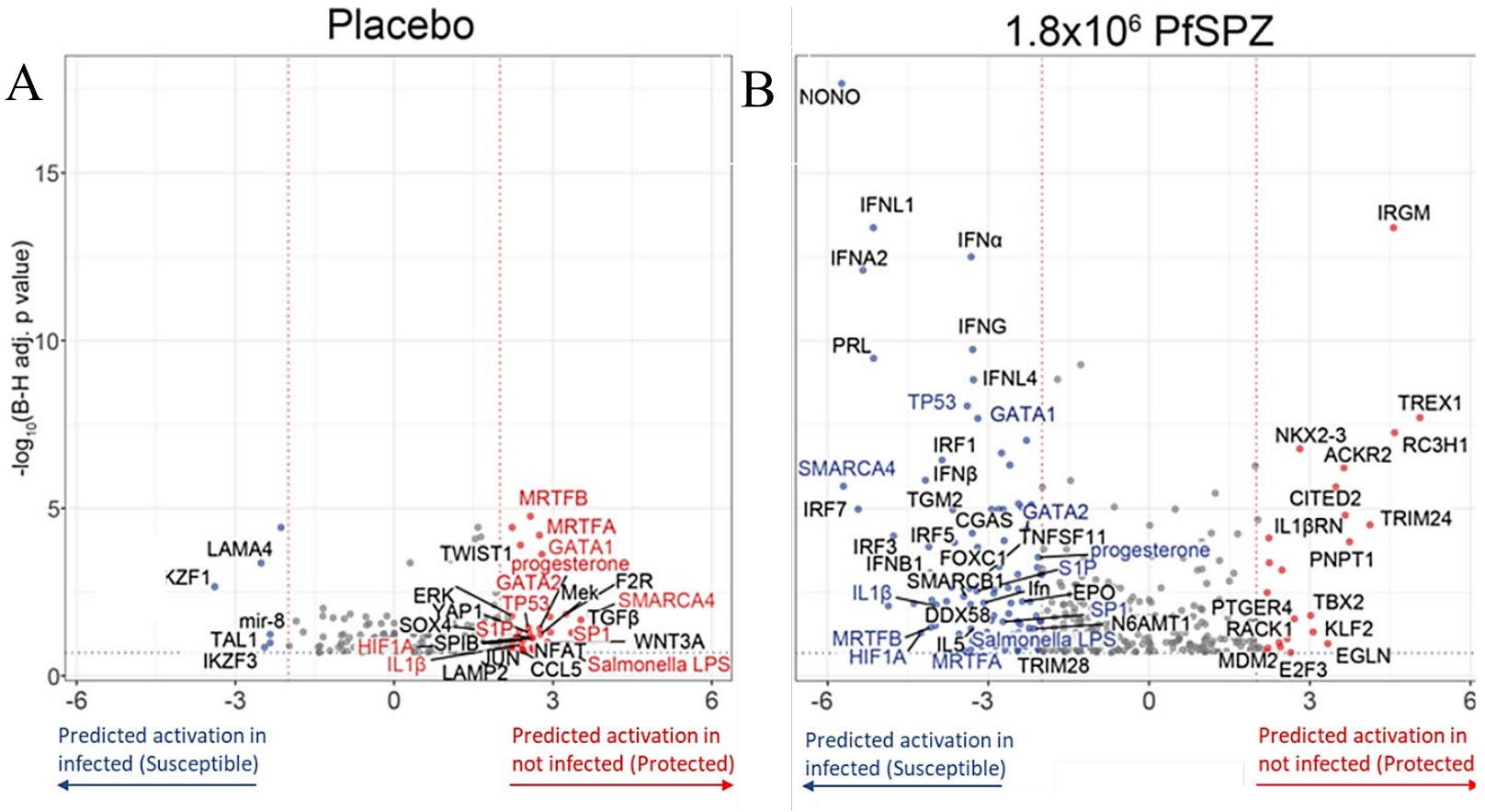


Figure adapted from Senkpeil L, Bhardwaj J, Little M, et al. In Revision

Figure 6. IPA Upstream Regulator Analysis A) Predicted upstream regulators for Placebo. B) Predicted upstream regulators for 1.8x10^6 PfSPZ dose group. Signatures significantly activated in Protected are shown in red and signatures activated in Not Protected are shown in blue.

2.3.3 Correlations of Humoral, Cellular, and Gene Expression at Multiple Timepoints with PfSPZ-induced Protection Post-Vaccination

Previous malaria exposure and infections are associated with reduced PfSPZ Vaccine VE, this has been demonstrated in multiple clinical trials in malaria-endemic areas. Because CSP is the immunodominant protein on the infectious sporozoites, anti-PfCSP IgG can reasonably be used as a proxy measure for recent infection with *P. falciparum*[183]. With this in mind and due to the positive correlation between IgG reactivity after vaccination protection in the KSPZV1 trial[167], I hypothesized that anti-CSP IgG at baseline would reduce immunogenicity and protective efficacy of the PfSPZ Vaccine.

To test this hypothesis, I first examined the relationship between baseline anti-CSP IgG and protection for both the placebo and high dose groups. Though there was no significant association in the placebo group, baseline anti-CSP IgG was significantly higher in Not Protected infants in comparison to Protected infants. To account for baseline antibody titers, I determined the $\log_2$ fold-change ($\log_2$FC) in IgG between baseline and two weeks post-vaccination as the measure of antibody response during the vaccination period and thus PfSPZ immunogenicity. The difference between the two time points without transformation was used as a secondary measure (Δ).

Baseline and post-vaccination anti-CSP IgG were negatively correlated, with the highest post-vaccination IgG titers associated with no anti-CSP IgG at baseline (**Figure 7A**). Furthermore, when I converted baseline IgG to a categorical measure of baseline antibodies, with two comparison groups: infants with anti-CSP IgG titers of 0 and infants with any positive value for baseline anti-CSP IgG titers. The infants with any detectable

anti-CSP IgG prior to vaccination had lower IgG responses, when measured either as a raw difference Δ or as log_2FC (**Figure 7B-C**).

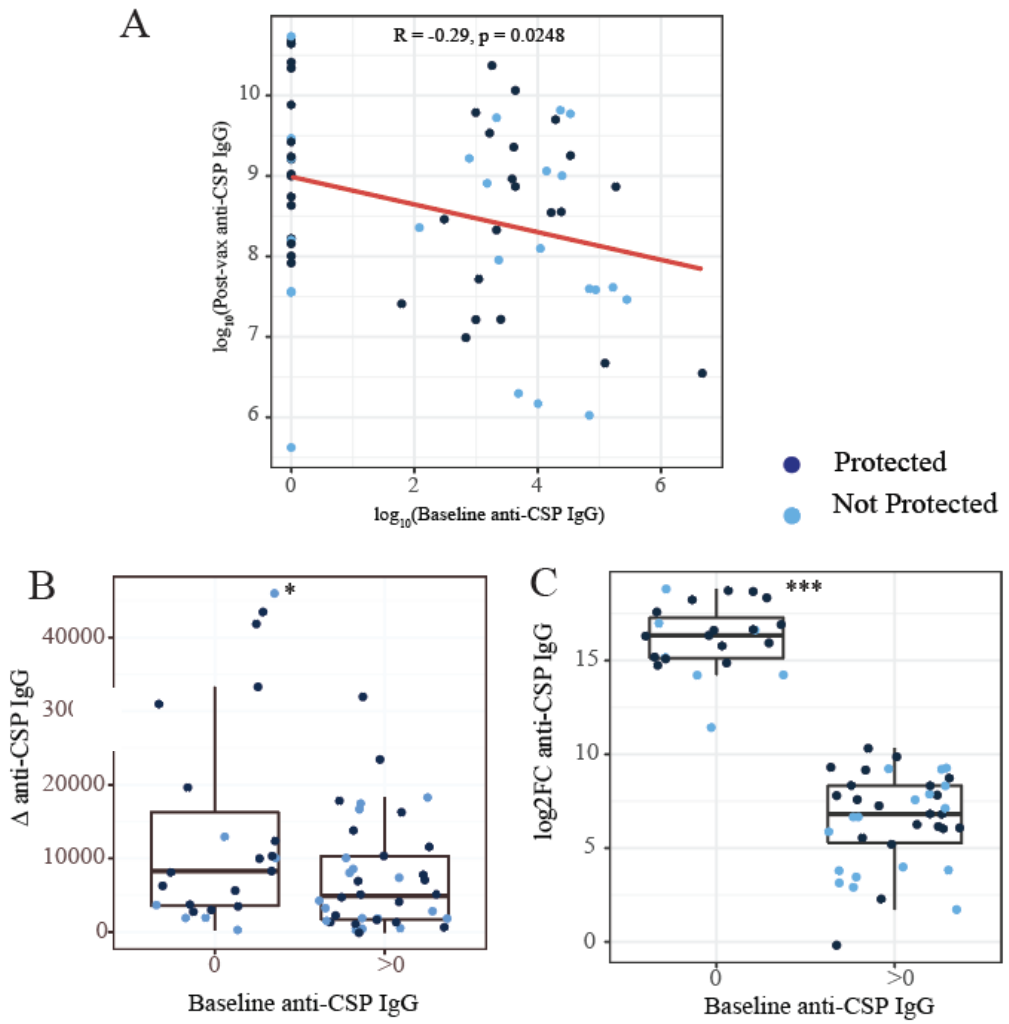


Figure 7. Detectable anti-CSP IgG at Pre-Vaccination Baseline for $1.8x10^6$ PfSPZ Dose Group. A) Correlation between pre-vaccination and post-vaccination anti-CSP IgG. B) Change in anti-CSP IgG during the vaccination period by absence or presence of detectable anti-CSP IgG at baseline. C) log_2 fold-change in anti-CSP IgG during the vaccination period by absence or presence of detectable anti-CSP IgG at baseline.

Strikingly, there were high (logFC > 12.5) and low (logFC < 12.5) anti-CSP antibody responses in both Protected and Not Protected infants receiving the $1.8x10^6$ PfSPZ Vaccine, however high responders were overrepresented in the Protected group when compared with Not Protected infants (**Figure 8A)**. As expected with no treatment, the infants receiving the saline placebo had no difference in anti-CSP IgG responses between Protected and Not Protected classes during the vaccination period (**Figure 8A)**. High antibody response was associated with longer time-to-parasitemia for the infants receiving $1.8x10^6$ PfSPZ (**Figure 8B).** Interestingly, when separated by outcome at 3 months post-vaccination, the correlation between baseline anti-CSP IgG and post-vaccination anti-CSP IgG remained significant for the Protected infants ($p = 0.036$), but not for the Not Protected infants ($p = 0.52$) (**Figure 8C).**

Also associated with protection in the originally published data[167] was the log_2FC of CSP-specific memory B cells during the vaccination period. To further verify the relationship between baseline anti-CSP IgG and a reduced humoral response, I evaluated the memory B cell response as a potential mechanism of this reduction using the same baseline anti-CSP IgG groups as above. Both this log_2FC and the post-vaccination percent CSP-specific memory B cells of live lymphocytes were significantly reduced in the group of infants with detectable anti-CSP at baseline by Wilcoxon test (**Figure 9**). Taken together with the anti-CSP IgG response, this suggests the presence of anti-CSP IgG at baseline may restrict humoral responses to the PfSPZ Vaccine through reduced expansion of PfSPZ-specific memory B cells.

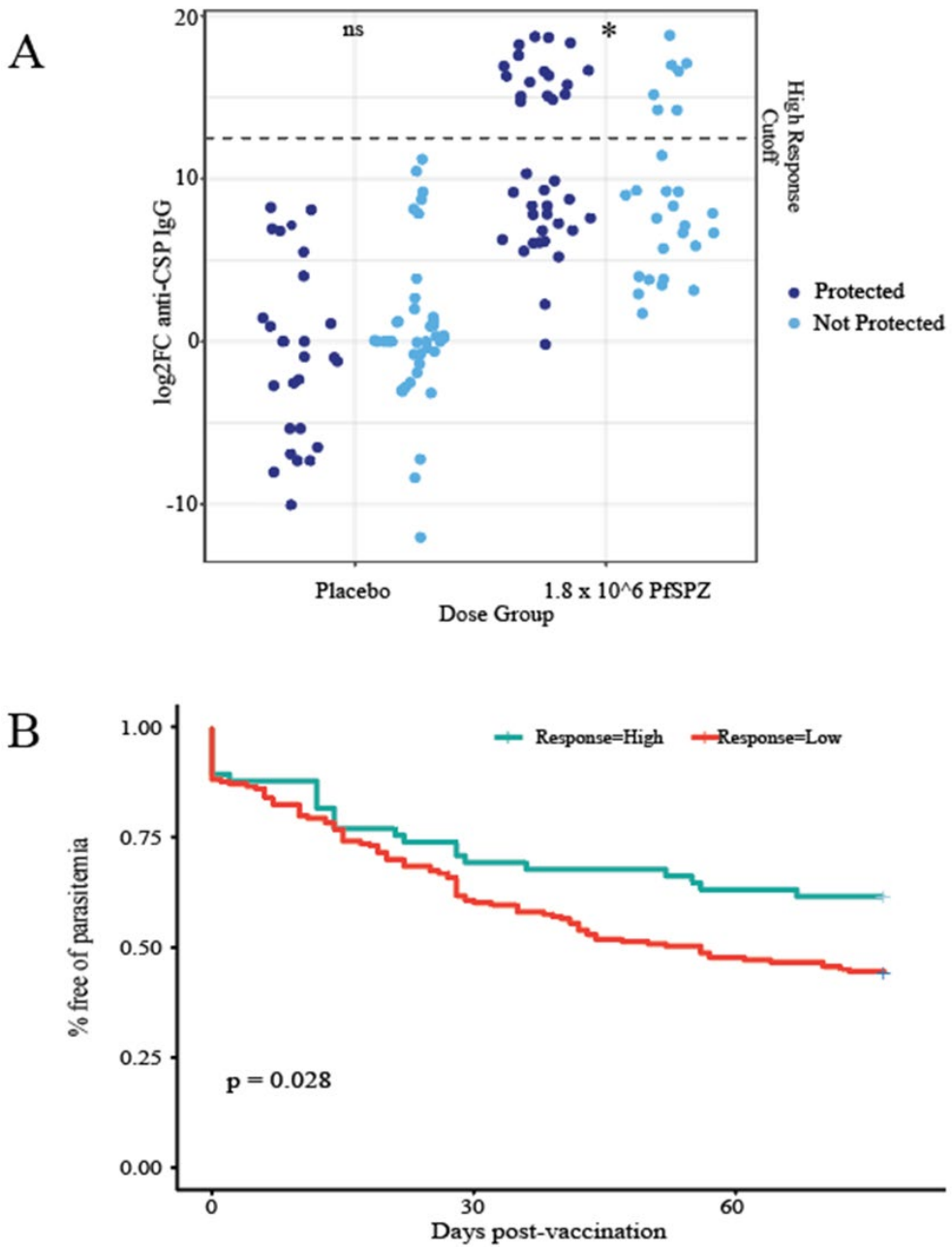


Figure adapted from Senkpeil L, Bhardwaj J, Little M, et al. In Revision.

Figure 8. Both Protected and Not Protected Groups had High and Low anti-CSP IgG Responders and High Responders had Longer Time to Parasitemia. A) Scatter plot of log_2FC anti-CSP IgG by treatment and outcome at 3 months post-vaccination. Placebo and 1.8 x 10^6 PfSPZ dose group are included. B)Kaplan-Meier curve of time to parasitemia by high or low anti-CSP IgG response up to 90 days post-vaccination within the 1.8 x 10^6 PfSPZ Vaccine group

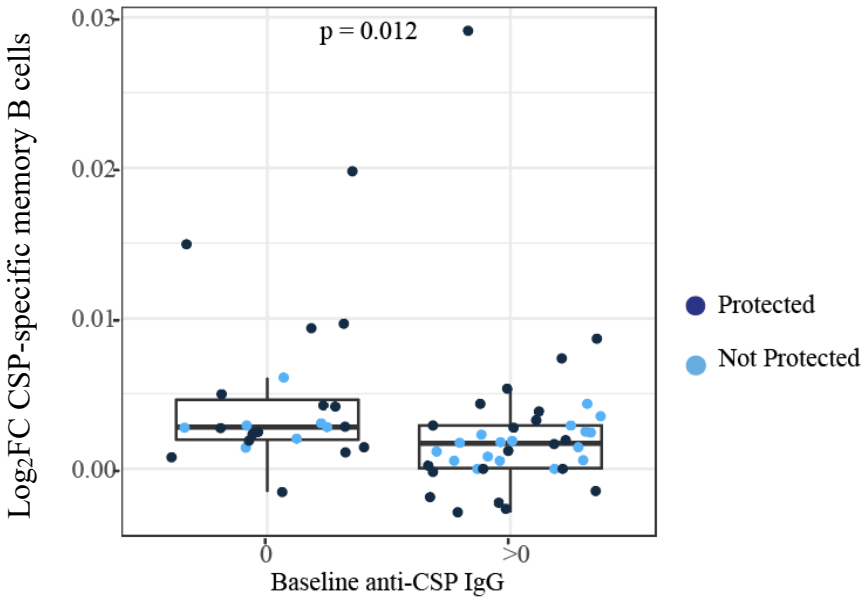


Figure 9. CSP-specific Memory B Cell Expansion by detectable anti-CSP IgG at Pre-Vaccination Baseline. Percent of lymphocytes that were CSP-specific memory B cells between infants with no detectable anti-CSP IgG at baseline and any detectable anti-CSP IgG. Specificity of memory B cells to CSP antigens was determined using a CSP tetramer probe.

Following this analysis, I examined the FACS data to determine other cell populations at baseline, post-vaccination, or cellular response during the vaccination period that were correlated with protection from parasitemia through 3 months post-vaccination. At baseline, only one cell population was significantly different between Protected and Not Protected in the $1.8x10^6$ PfSPZ group. The percentage of monocytes that were CD11c+ was higher in Protected infants at pre-vaccination baseline (**Figure 10**). These are monocyte-derived DCs that are known to be the APCs responsible for presenting sporozoite and liver-stage antigens to prime memory CD8+ T cells[184]. This memory CD8+ T cell response is essential for the generation of PfSPZ Vaccine-induced protection[102] and in the $1.8x10^6$ PfSPZ dose group the log_2FC of PfSPZ-specific CD8+ T cells was lower in Protected than in Not Protected infants (**Figure 11**).

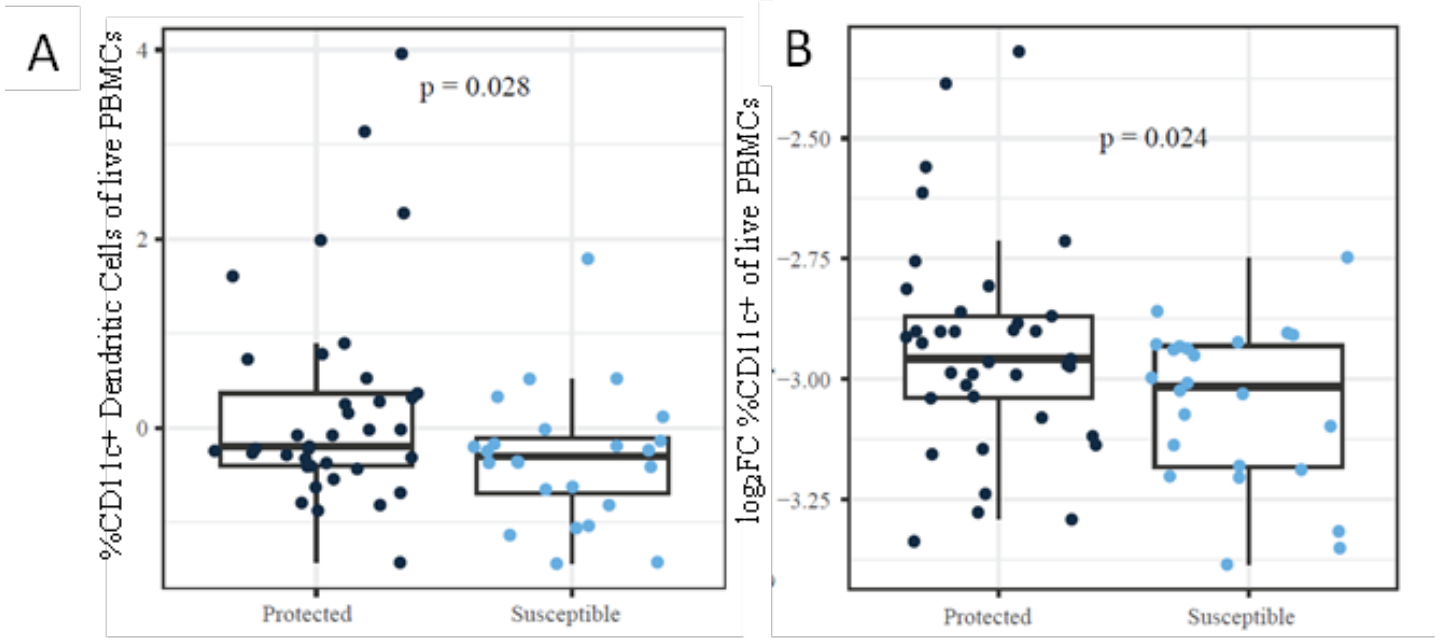


Figure 10. Monocyte-derived CD11c+ Dendritic Cells are associated with protection in 1.8 x 10^6 PfSPZ Vaccine group. A) Boxplot of % CD11c+ at pre-vaccination baseline for 1.8 x 10^6 PfSPZ group by protection status at 3-month post-vaccination. B) Boxplot of log_2FC of % CD11c+ during vaccination period for 1.8 x 10^6 PfSPZ group by protection status at 3-month post-vaccination

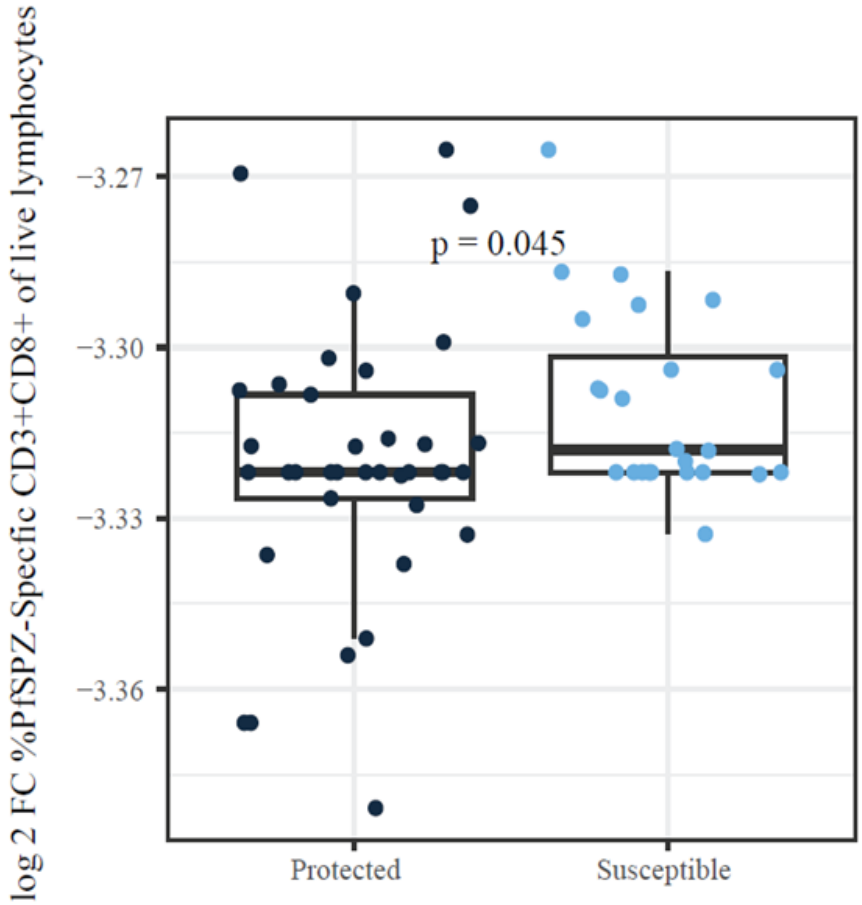
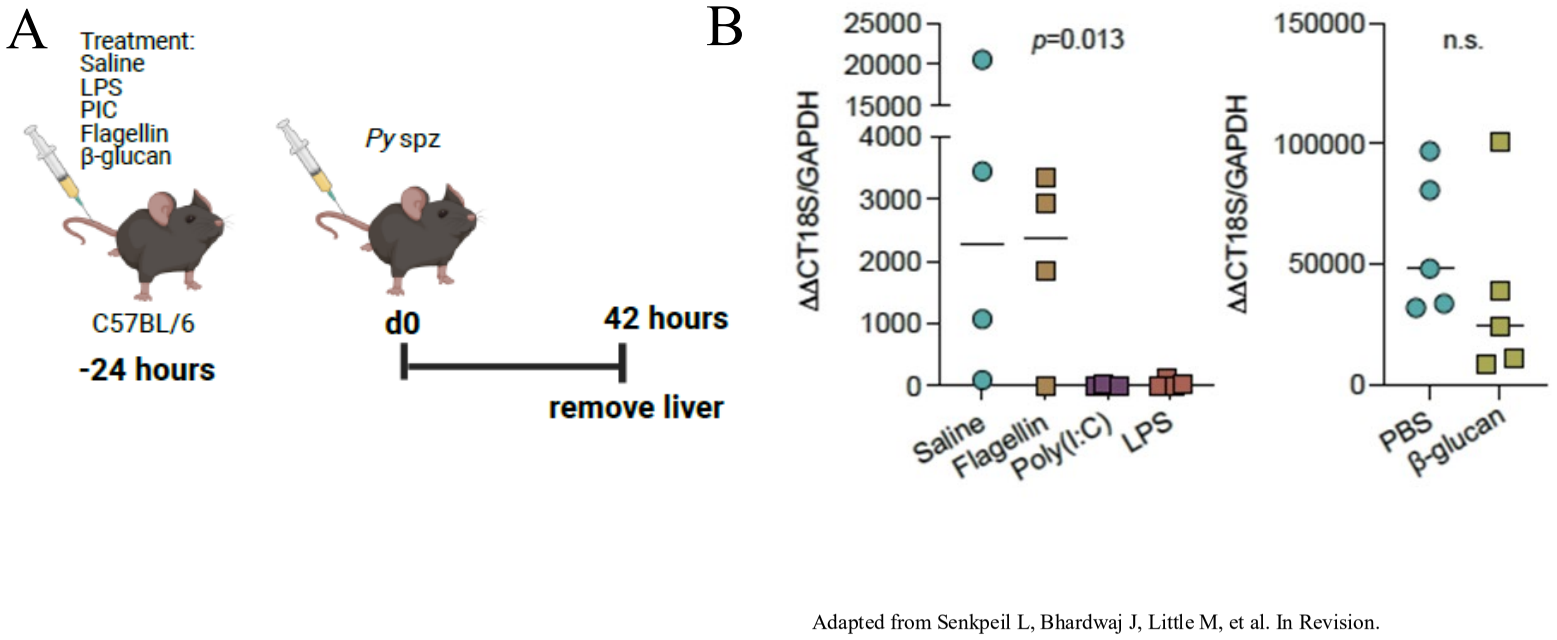


Figure 11. Reduction in circulating PfSPZ-specific CD8+ T cells is associated with protection in 1.8 x 10^6 PfSPZ Vaccine group. The Protected 1.8 x 10^6 PfSPZ infants had a larger decrease in circulating memory CD8+ T cells during the vaccine period when compared to the Not Protected 1.8 x 10^6 PfSPZ infants.

2.3.4 In Vivo Effects of TLR Agonists on P. falciparum Infection and Immunizations

TLR4 agonist LPS was predicted as an upstream regulator activated for the $1.8x10^6$ PfSPZ dose in the Not Protected group and low-annotation BTM related to LPS signaling, as well as high-level BTM INFLAMMATORY/TLR/CHEMOKINES was enriched. In contrast, INFLAMMATORY/TLR/CHEMOKINES, and upstream regulator LPS were predicted to have higher activation in Protected infants that received a placebo. In the same manner as is required for sporozoites to cause parasitemia and clinical malaria symptoms, attenuated PfSPZ must reach the liver, invade hepatocytes, and begin replication before their arrest to prime memory CD8+ T cells and produce durable, sterile protection[102,104]. We hypothesized that innate immune activation may provide some protection against malaria infection without treatment (placebo), but this activation may also reduce efficacy of the PfSPZ Vaccine through a similar mechanism.

Several studies have shown innate immune activation can control malaria liver-stage burden and parasite development[185–187]. The dichotomous associations of innate immune signatures with protective outcomes in the placebo and $1.8x10^6$ PfSPZ groups may be explained by clearance of infectious sporozoites or RAS prior to the liver stage. We tested the hypothesis that innate immune stimuli reduced liver parasite burden in malaria naïve C57BL/6 mice. Mice were pretreated LPS, TLR3 agonist poly(I:C), TLR5 agonist flagellin, or an innate immune stimulus, β-glucan[188] prior to infection with non-irradiated, fully infectious *P. yoelii* (**Figure 12**). Consistent with a previous study[185], treatment with LPS and poly(I:C) 24 hours before injection with *P. yoelii* sporozoites reduced liver parasite burden at 42h post infection compared with saline control (**Figure 12B**).



Adapted from Senkpeil L, Bhardwaj J, Little M, et al. In Revision.

Figure 12. Innate Immune activators reduces liver parasite burden after infection with *Py* sporozoites. A) C57BL/6 mice were treated with saline vehicle or innate immune stimuli 24 hours prior to direct tail vein injection of *Py* 17XNL sporozoites. B) Quantification of liver parasite burden 42h post-infection by treatment. Data represented from two separate experiments.

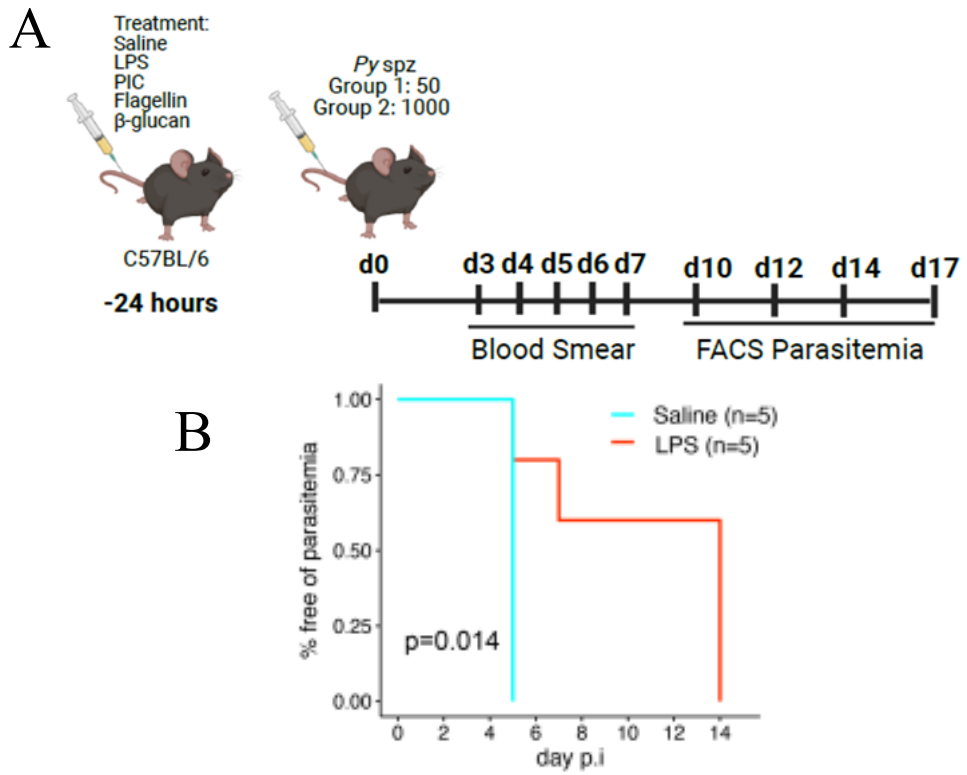


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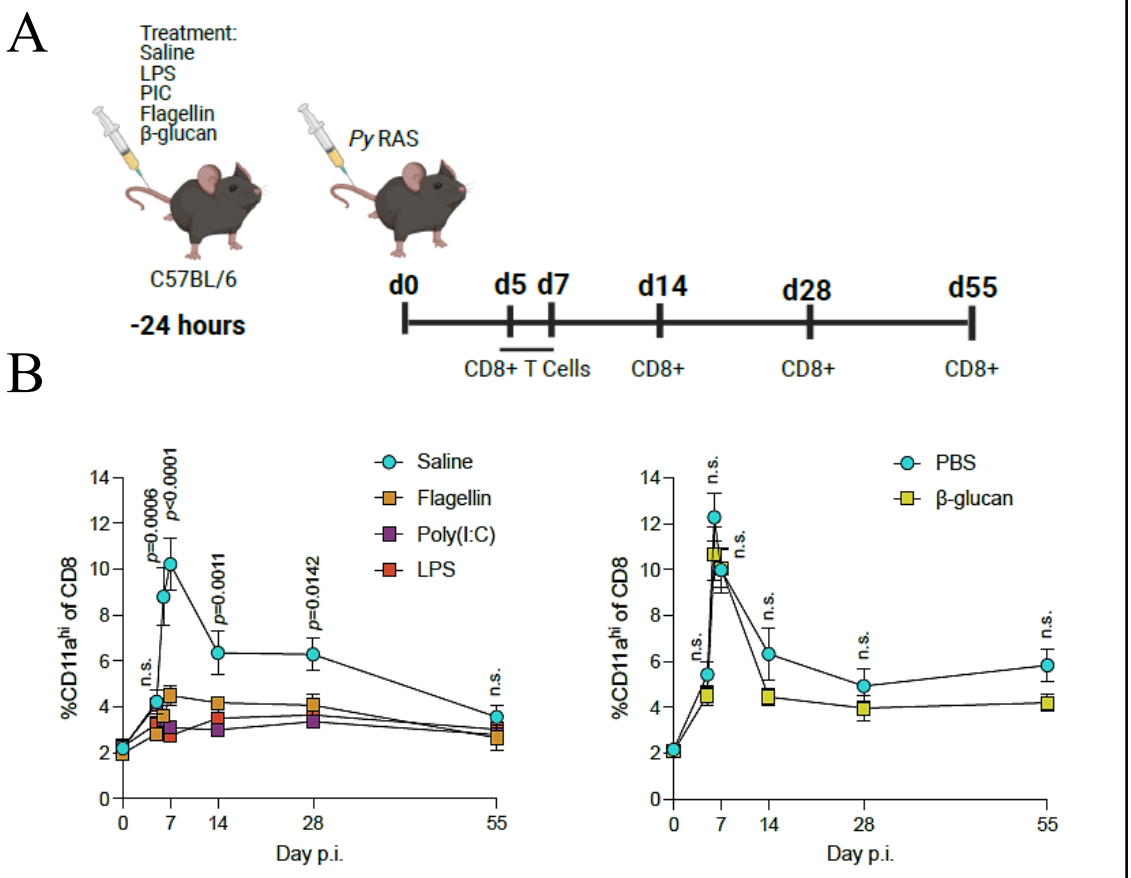
Figure 13. LPS treatment increases time to parasitemia in vivo after infection with *Py* sporozoites. A) C57BL/6 mice were treated with saline vehicle or innate immune stimuli 24 hours prior to direct tail vein injection of *Py* 17XNL sporozoites. Parasitemia was determined by flow cytometry on specified days. B) Kaplan-Meier curve of time to parasitemia by treatment with saline or LPS.

Additionally, mice receiving the LPS pre-treatment showed a significant delay in time to patent parasitemia (**Figure 13B**), though no other innate immune stimuli had this effect. The lack of T cell responses in the KSPZV1 trial, in addition to a lack of correlation between abs and CD8+ T cells led to the hypothesis that innate immune activation at baseline was mechanistically involved in the reduced T cell responses and associated lack of protection after immunization with the PfSPZ Vaccine. It has been previously established that innate immune responses can contribute to inhibition adaptive immune responses against sporozoites and liver-stage parasites[189]. To evaluate the effect of innate immune stimulation on RAS priming of CD8+ T cells, mice were given the same agonists listed above 24 hours before injection with irradiated *P. yoelii* sporozoites (**Figure 14A**). We found dampening of RAS-induced CD8+ T cell responses, measured as circulating, antigen-experienced $CD11a^{hi}CD8^{lo}$T cells between days 7 and 28 post-RAS immunization. This effect was not dependent overcome by increased RAS dose, the reduction in T cell response was significant at both 2500 PySPZ and 10000 PySPZ doses (**Figure 14B**). These data indicate innate immune activation restricts priming of CD8+ T cell response by RAS, possibly through reduction of liver parasite burden though other mechanisms have not been ruled out.



Adapted from Senkpeil L, Bhardwaj J, Little M, et al. In Revision

Figure 14. Innate Immune agonist treatment restricts RAS priming of CD8+ T cells in vivo. A) C57BL/6 mice were treated with saline vehicle or innate immune stimuli 24 hours prior to direct tail vein injection of *Py* 17XNL RAS. CD8+ T cells were measured by flow cytometry on indicated days post-injection. B) RAS-induced CD8+ T cell ($CD8^{lo}CD11a^{hi}$ % of all circulating T cells).

2.4 Discussion

2.4.1 PfSPZ Vaccine and Placebo Dose Groups Show Inverse Association of Baseline Innate Immune Signatures with Protective Outcome

In this chapter, I discuss our systems analysis, using multiple data types to gain insight into mechanisms that influence responses to the PfSPZ Vaccine. A recently published Phase IIb clinical trial found significant protection at 3 months post-vaccination for the highest vaccine dose: $1.8x10^6$ PfSPZ. We analyzed data from samples collected during the trial at pre-vaccination baseline and at two weeks after the last vaccine dose was administered. To generate hypotheses about potential mechanisms of differential immunogenicity and protective responses to the PfSPZ Vaccine, transcriptomic analysis was performed using all dose groups, including saline placebo, $4.5x10^5$ PfSPZ, $9.0x10^5$ PfSPZ, and $1.8x10^6$ PfSPZ.

Though we could not identify individual differentially expressed genes, GSEA using BTMs as *a priori* defined gene sets revealed unexpected transcriptional signatures of protection. In the $1.8x10^6$ PfSPZ dose group, expression of NK cell and T cell genes were enriched in the Protected infants compared to the Not Protected group, as well as expression of genes related to cell cycle, DNA repair, and cell division. Modules that were enriched in the Not Protected group, or genes that are associated with lower efficacy of the PfSPZ Vaccine, included signal transduction signatures and gene modules related to innate immune cells and innate immune activation via cytokines and chemokines. Intriguingly, for the placebo group, NK and T cell signatures were enriched in Protected individuals, as they were for the $1.8x10^6$ PfSPZ group, however the innate immune

activation signatures that were associated with lack of protection in the vaccine group were associated with Protection for placebo.

The inversion of these signatures between the protection classes of the two groups indicates a treatment-dependent mechanism of immunity. Transcriptional signatures of innate immune activation that were associated with protection in the absence of immunization were associated with lower VE of the PfSPZ Vaccine. I hypothesized that this innate immune activation would act on sporozoites prior to liver-stage infection due to the fact that the main mechanism of PfSPZ Vaccine-induced sterile immunity takes place during this stage[102,121,190]. Such a mechanism of innate activity may confer partial, short-term protection against natural infection with *P. falciparum* sporozoites, explaining the protective association of these signatures in the placebo group, but that this protective mechanism against infectious sporozoites may also prevent RAS in the PfSPZ Vaccine from initiating liver-stage infection. This would prevent exposure to liver-stage antigens in addition to CSP and inhibit priming of liver-resident, memory CD8+ T cells, a crucial mechanism for sterilizing, durable protection against *P. falciparum*[102,103].

These findings were supported by the results of Upstream Regulator Analysis. Regulators predicted to be activated in the Not Protected infants that received $1.8x10^6$ PfSPZ included innate immune stimuli and products of TLR signaling: LPS and Type I and III IFNs, as well as IRFs. These regulators promote activation of the innate immune system[191], particularly monocytes and DCs, which is consistent with the GSEA modules predicted to be enriched in the Not Protected group: Monocytes, DC activation, and INFLAMMATORY/TLR/CHEMOKINES. Contrastingly, these regulators were also predicted to be activated in the Protected infants that received the placebo. Again, this is

consistent with the inverse enrichment that I found between the two dose groups using GSEA. These findings are consistent with what is currently known about mechanisms of innate activation inhibiting adaptive responses while also reducing liver-stage infection and parasitemia[96,189]. Indeed, the protective effect of this innate activation resembles the concept of trained immunity. Trained immunity refers to remodeling in innate cells after exposure to an initial pathogen, resulting in long-term functional changes that can enhance immune responses, even to heterologous pathogens[191,192]. An example of immune training is the use of the Bacillus Calmette-Guérin (BCG) vaccine, a live attenuated vaccine against tuberculosis (TB) that can induce non-specific, innate protective responses including against malaria[193–195]. The BCG vaccine induces changes to innate cells, including monocytes, through pathogen-associated molecular patterns (PAMPs) that are recognized by TLRs, including TLR4. In fact, activation of monocytes with TLR4 agonist LPS as well as with CpG, a ligand for TLR9[187], can induce trained immunity in a similar manner to BCG vaccination[196]. Additionally, infection with *P. falciparum* can result in trained immunity of innate cells[197]. One study of experimental malaria infection found increased inflammatory signaling in monocytes through epigenetic reprogramming[198], while another demonstrated IFN- γ-dependent increased expression of TLRs after *P. falciparum* infection[199]. Thus, the role of malaria infection as a mediator of trained immunity and previous exposure to *P. falciparum* is one possible mechanism of reduced malaria vaccine immunogenicity in malaria-experienced individuals.

Though the mechanisms of immunity are not completely understood, vaccines containing RAS, like the PfSPZ Vaccine, consistently induce sterilizing immunity in

malaria-naïve volunteers[102,121,161–163]. Enrichment of genes associated with T cells and NK cells in the Protected class across dose groups is consistent with the known, protective role of cytotoxic cells for control of liver-stage malaria and increased time to infection with reduced parasitemia.

Specifically, NK cells produce IFN-γ, the cytokine responsible for initiation of the immune response to liver-stage malaria[97], and act as effectors to activate nitrous oxide pathways in macrophages[95] responding to sporozoite traversal of host epithelial and endothelial cells. T cells play diverse roles in malaria immunity, determined by the specific T cell subset. Both CD8+ liver-resident memory T cells[90,101,102] and ϒδ T cells[106] are required for a robust immune response resulting in durable, sterilizing protection. Some genes assigned to the NK cell BTMs are highly expressed in ϒδ T cells, and the activation of this subset is likely given the enrichment of NK and T cell signatures across low and high-annotation BTMs. To test this hypothesis, I performed GSEA using a different set of modules that included gene sets specific to ϒδ T cells[182]. These data indicated ϒδ T cell signatures, specifically, in addition to T cells, NK cells, and NKT cells, were enriched in Protected compared with Not Protected which is consistent with the current literature[106].

Though there is enrichment of these genes in the Protected infants, there was not a significant difference in the cell populations as measured with FACS[167]. This could indicate that these cell types do proliferate, in response to vaccination or infection, but are recruited elsewhere reducing the number of cells in circulation or it could be a signature that is associated with protection, but the response is not robust enough to generate protective immunity[103]. The other modules enriched in Protected according to GSEA

provide evidence of a separate mechanism of immunity, likely through inhibition of sporozoite traversal to the liver as the modules that were inversely enriched between placebo and $1.8x10^6$ PfSPZ indicate a role of innate immune activation in this process.

Innate immune activation of monocytes and neutrophils could increase the magnitude of the initial innate response as these cells react to inoculation of the parasites. This innate response occurs with the goal of controlling the number of sporozoites that can successfully invade hepatocytes and begin replication. However, this mechanism is counter-productive with regard to whole-sporozoite vaccination. Indeed, RAS do not suppress host immune responses effectively as do fully infectious sporozoites[20,200] and may be more susceptible to an innate immune response, so an infant that has this response may still get infected with malaria, though they can clear the RAS before the liver stage begins.

Monocyte and DC activation by LPS is mediated by TLR4 and CD14 as coreceptors that initiate a signaling pathway that results in activation of NF-κB to drive transcription of the genes encoding IL-6, TNF, IL-1β and IL-12[196]. This same pathway can be initiated by TLRs 1, 2, and 6 with interaction from gram-positive bacterial components[196]. Though innate immune cell subsets, including monocytes and neutrophils, are known to respond to the site of sporozoite inoculation[201], their exact role in pre-erythrocytic immunity and the immune response to the earliest stages of *P. falciparum* infection is not well understood[86].

While innate immune activation at baseline is associated with protection from natural infection but lower PfSPZ Vaccine efficacy, this may be indirect indicators of innate immune activation in tissue-resident macrophages such as Kupffer cells in the

liver. These KCs are a necessary portal for sporozoite entry into hepatocytes[17] and their activation along with the circulating innate cells from my analysis may contribute to parasite clearance and prevention of liver-stage infection independently of the circulating innate immune cells. After completion of my transcriptomic analysis, I hypothesized an innate immune mechanism that inhibits invasion of hepatocytes by fully infectious sporozoites also reduces PfSPZ Vaccine efficacy through a mechanism similar to trained immunity.

2.4.2 Baseline anti-CSP IgG is Correlated with Lower PfSPZ Vaccine Immunogenicity and Reduced Protection

While there was no evidence in the original study of PfSPZ-induced T cell responses, humoral and B-cell responses were correlated with protection in the $1.8x10^6$ PfSPZ group[167]. This is consistent with past studies that indicate anti-PfCSP IgG are associated with partial protection against malaria, though antibodies alone are not sufficient for durable, sterilizing protection[102] and anti-PfCSP antibody titers wane quickly,[116,127,131] possibly necessitating the use of frequent boosters to maintain protection[202]. Furthermore, anti-PfCSP IgG was significantly higher at baseline in the $1.8x10^6$ PfSPZ Not Protected class though there was no difference in the placebo group, suggesting these antibodies may reduce vaccine efficacy without conferring protection during exposure to fully infectious parasites. This is also consistent with previous studies of the PfSPZ Vaccine. Malaria vaccine candidates that effectively induce protection in malaria-naïve volunteers during early Phases of clinical development have shown reduced immunogenicity and efficacy in field trials[121,123,150,152,161,164,165], both against

CHMI and natural exposure. The implications of these findings are that vaccine response could be dampened by previous malaria exposure. Anti-PfCSP IgG indicative of previous exposure to *P. falciparum*[203] and, therefore may predict future exposure to the parasite. These antibodies are relatively short-lived; because of this, they are considered a marker of recent exposure[183]; this could suggest a direct role of anti-PfCSP IgG in reducing vaccine responses, or an indirect mechanism mediated by recent malaria infection that only correlates with antibody titers. To investigate the possible mechanism further, I examined how anti-PfCSP IgG at baseline may influence the immunogenicity of the PfSPZ Vaccine, hypothesizing the baseline presence of antibodies against *P. falciparum* pre-erythrocytic stages would reduce the immunogenicity of the PfSPZ Vaccine.

The baseline titer of anti-PfCSP IgG was negatively correlated with the post-vaccination titer, supporting the hypothesis that previous exposure and development of an antibody response reduces immunogenicity of the PfSPZ Vaccine. To establish a possible mechanism, I used presence of any detectable anti-PfCSP as a categorical variable. The infants with detectable anti-PfCSP prior to vaccination with PfSPZ showed lower significantly lower increase in anti-PfCSP IgG during the vaccination period. Furthermore, expansion of memory B cells was associated with protection in the original vaccine trial[167] and this expansion was significantly smaller when anti-PfCSP was detectable at baseline. Interestingly, detectable anti-PfCSP IgG at baseline had no significant relation to expansion of PfSPZ-specific CD8+ T cells during the vaccination period. Taken together, these data indicate anti-PfCSP IgG prior to inoculation with RAS reduces the induced humoral response and that this effect is uncoupled from the effector CD8+ T cell priming that is required for PfSPZ-induced protection.

2.4.3 TLR Agonist Treatment In Vivo Reduces Parasite Burden and CD8+ T Cell Response

The baseline transcriptomic results suggest a role for the innate immune system in preventing a robust PfSPZ-induced adaptive response by possibly restricting liver stage infection. This is consistent with previous work that demonstrated innate activation inhibited liver stages of *Plasmodium* development and replication[96,97,187] and this inhibition is also associated with lower protective adaptive immune responses[189]. Toll-like receptor signaling in innate immune cells also results in inflammatory cytokine production and type I IFN signaling that occurs naturally in response to liver-stage infection and has been shown to result in clearance of liver-stage parasites[97,204]. With these data in mind, we treated mice with TLR agonists prior to intravenous injection with either fully infectious or radiation-attenuated *P. yoelii* sporozoites to test the hypothesis that innate immune activation induced by these stimuli decreases liver parasite burden and reduce priming of adaptive immunity by RAS, respectively. The similar association with baseline innate immune signatures between the Protected placebo and Not Protected $1.8x10^6$ PfSPZ groups implicates a mechanism acting during pre-liver stages or liver stages of infection as the crucial mechanisms for PfSPZ Vaccine-induced immunity take place during the liver stage[102,189]. Two TLR agonists, LPS and poly(I:C), reduced liver stage burden of fully infectious *P. yoelii* sporozoites, and LPS was the only innate immune stimuli that increased time to parasitemia in the mice receiving fully infectious sporozoites. These data, in addition to the previously established role of type I IFN response in clearing liver parasitemia, imply TLR4 signaling in myeloid cells activated by LPS can reduce sporozoites that establish liver infection. This hypothetical innate

mechanism could prevent sporozoites from establishing liver-stage infection, thereby providing partial protection against natural infection with malaria but also impairing priming of adaptive CD8+ T cells by RAS.

The threshold of memory CD8+ T cells required for protective immunity is definable, and the number of RAS invading hepatocytes and expressing liver-stage antigens should correlate with the robustness of the adaptive response[103,190]. A reduction in parasites alone could reduce the immune response to infection with attenuated sporozoites, thereby reducing CD8+ T cell priming[205], possibly to a level insufficient to generate sterile immunity. Indeed, LPS and poly(I:C) treatment led to a decrease in circulating antigen-experienced CD8+ T cells after RAS immunization, in addition to reducing liver parasite burden. However, treatment with flagellin, a TLR5 agonist, also resulted in lower memory CD8+ T cell response RAS yet did not decrease *P. yoelii* infection of hepatocytes (**Figure 12**). This could suggest that reduction in liver stage infection and dampening of RAS-induced adaptive responses may in fact result from separate mechanisms of innate immune activation rather than the reduction in adaptive immunity resulting from lower parasite burden and associated lower antigen presentation.

Multiple mechanisms may explain the effects of innate immune activation on liver stage malaria infection and RAS-induced adaptive responses. First, increased innate immune activity could result direct killing of parasites through phagocytosis, either in an antibody-mediated or independent manner.

Chapter 3: Integrated Models of Malaria Immunity

3.1 Introduction

Systems vaccinology has the potential to streamline and improve the process of vaccine research and development of next-generation immunization methods. Recent strides have been made in the field of immunology with the introduction of single cell sequencing and improved data collection techniques that allow for the creation of large, comprehensive data sets. To continue this progress, analytical tools must continue to evolve to keep up with the data collection capacity. Collecting data from various levels of organization within the immune system allows for a clearer understanding of the complex "big picture" underlying important immune processes.

One complication of systems studies is determining how best to integrate the hundreds to thousands of features from various data types to facilitate understanding of biological interactions and infer causality among multiple features that may significantly correlate to varying degrees. Redundant information can add noise to analysis combining data from different levels of organization, for example combining expression of genes that are associated with monocyte activation and monocyte identification through flow cytometry may overestimate the importance of these signatures. However, biological interactions within and between system levels are difficult to quantify with correlative analysis alone. Without accounting for interactions between different systems component levels, such as cellular, gene, and protein levels, error is introduced into statistical models and actual mechanisms may be lost.

With the data collected during the KSPZV1 trial, I had a unique opportunity to create a model of vaccine-induced immunity and natural protection against malaria infection using a truly systems data set. The age-matched saline placebo can be used as a model of naturally-acquired protection and by comparing data from both groups, the effects of vaccine-induced versus naturally acquired immune signatures can readily be compared.

In this chapter I develop a predictive model using machine learning that can identify individuals who are likely to develop immunity in response to the PfSPZ Vaccine. Additionally, I used structural equation modeling (SEM) to establish directional relationships and causality between variables associated with PfSPZ-induced and natural protection (placebo).

SEM is a collection of statistical methods that can be applied in systems biology to integrate biological data and model complex relationships. SEM is especially useful with regards to modeling the complex interactions between variables and can give a more complete picture of the total immune response while minimizing error and redundancies. In general, systems biology approaches rely on correlations to identify relationships between analyses that are performed separately. This can aid in generating hypotheses and guide future directions; however, a correlation does not fully capture the complexity of interactions between independent variables, and directionality of the interactions cannot be inferred from correlations alone.

3.2 Methods

3.2.1 Class Prediction

All machine learning was performed in R (v4.0.4 – v4.2.1). The package and machine learning algorithm XGBoost[206] was used for the predictive models presented in this thesis. Two other machine learning algorithms were initially used to determine which transcriptomic features were most predictive of parasitemia at 3 months post vaccination, Random Forest[207] and Support Vector Machines[208]. Predictive accuracy of each model to classify outcome was determined using transcriptomic features from pre-vaccination baseline (baseline), two weeks post-vaccination (post), and post-pre vaccination (delta) for the $1.8x10^6$ PfSPZ group. Only a baseline model was created for the placebo group. XGBoost was the most consistently accurate across time points and dose groups (Table 2) and was chosen for all future analyses.

XGBoost models were generated independently for the saline placebo and $1.8x10^6$ PfSPZ groups. Original features and sample numbers in each prediction model for each dose group and time point are shown in Table 3. RNA-seq transcriptomic features were collapsed into module expression scores (MES) by determining median expression of all genes within each previously defined low-annotation blood transcription module (346 features)[174]. For each dose group and time point in Table 3, the following procedure was performed separately to create individual predictive models. For each time point of $1.8x10^6$ PfSPZ, two models were created: a transcriptomic model using only MES as features and a multi-modal systems model that included the features in Table 3.

Subjects missing more than two-thirds of feature data were removed from the analysis. For the remaining subjects (baseline n=63, post-vaccination n=62, Δ n=61,

placebo baseline n=64), missing values were imputed using the missMDA package[209]. Two-thirds of the individuals (baseline n=42, post-vaccination n=41, Δ n=41, placebo baseline n=42) were randomly selected as the training set and used for feature selection and model training with the remaining one-third held out for independent testing. The caret package was used to perform recursive feature elimination (rfe) on the training set until features were reduced to the top 3 to 5 most predictive features[210]. Final feature count (3, 4, or 5) for each model was based on performance using a random forest algorithm as part of the rfe function. This included 25 rounds of bootstrapping and was repeated 50 times to account for variability due to random selection. The most commonly selected features were used for developing class prediction models using a cutoff of at least 4 appearances per 100 rounds of feature elimination. Model parameters were determined using logistic regression 10-fold cross-validation of training set using XGBoost. Parameters were manually tuned if model was overfit to the training set. Seeding for each step was recorded for reproducibility. Final features were selected based on the best predictive values as determined by kappa values, or the measurement of agreement between the predicted classifier and actual classification. Final models were then evaluated for accuracy using the one-third of samples originally held back as test sets (baseline n=21, post-vaccination n=21, and Δ n=20, placebo baseline n = 22). The results of this model prediction were used to generate the confusion matrix and receiver operating characteristics curve using the prOC package[211], as well as to determine feature importance. Shapley additive explanations (SHAP) dependence plots were generated to determine the contribution of each feature to model prediction output. The $1.8x10^6$ PfSPZ Baseline multi-modal model features were applied to baseline data from the placebo

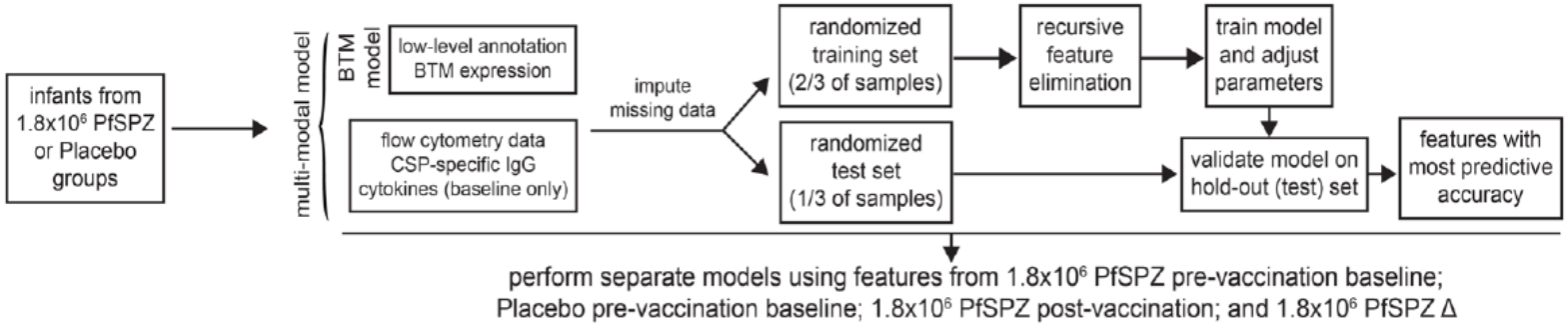
group to assess whether these features can accurately distinguish the protective outcome. After feature selection was performed independently for each model, all models were trained individually to determine ideal parameters before manual tuning and reporting final model results (**Figure 16**).

Algorithm	Predictive Accuracy	RMSE	auROC
Random Forest	60.0%	.573	.482
SVM	68.2%	35.5%	.646
Xgboost	90.5%	.361	.862

Table 2: Machine Learning Algorithms. Predictive Accuracy was determined by percent of test set that was accurately classified as P or NP. RMSE is root mean square error, a measure of error in the model. auROC refers to area under a receiver operating characteristics curve and indicates the ratio of true and false positives, plotted as sensitivity vs. 1 - specificity

Dose Group	Timepoint	(N)	RNA-seq Transcripts	Flow Cytometry	Plasma Cytokines	anti-CSP IgG	Cell Stimulation
Placebo	Baseline	65	21,815	10	15	Baseline Reactivity	11
1.8×10^6 PfSPZ	Baseline	66	21,815	10	15	Baseline Reactivity	11
1.8×10^6 PfSPZ	Post	62	21,815	10	NA	Post Reactivity	NA
1.8×10^6 PfSPZ	Delta	61	21,815	10	NA	$\log_2$FC Reactivity	NA

Table 3: Machine Learning Features. Features included in feature selection by recursive feature elimination for Placebo and 1.8×10^6 dose groups. Cytokines and cell stimulation assay data was only available for the pre-vaccination baseline timepoint. RNA-seq transcripts were collapsed into 348 low-annotation BTMs to reduce computational burden and increase information gain for each transcriptomic feature.



Adapted from Senkpeil L, Bhardwaj J, Little M, et al. In Revision.

Figure 15. Overview of Machine Learning Workflow. XGBoost was used to predict the outcome of parasitemia at 3 months post-vaccination. Note feature selection was nested within a cross-validation step and performed separately for every time point and dose group, excluding the Placebo model that was trained on features selected using the 1.8 x 10^6 PfSPZ dose group training set.

3.2.2 Structural Equation Modeling

Structural equation modeling[212] was performed in R using the lavaan package[213]. The data set created for class prediction was used, with the addition of post-vaccination data for the saline placebo dose group. Data were scaled for input to structural models using the scale function in R[214].

I decided to use the two types of SEM analyses, factor, and path analysis, to address the limitations I encountered with machine learning: module expression scores included all member genes, some of which may skew the overall score more than their actual impact on outcome and interactions between member genes, within and between modules, was ignored in my previous calculations. The first step of SEM, factor analysis, allows for estimation of non-measurable latent factors using hypothetical models constructed of known measurable component variables. Using gene expression as measured variables and BTMs as hypothetical initial models in confirmatory factor, I was able to create gene modules that when applied to my data sets accurately reflected the organization of genes and accounted for covariation between gene members.

3.2.3 Confirmatory Factor Analysis

Confirmatory factor analysis was performed using lavaan's cfa function[213] using a maximum likelihood model estimator to create a measurement model. Latent factors were defined as low-level annotation BTMs[174] and gene expression from the $1.8x10^6$ PfSPZ group was mapped on to corresponding BTMs as measured independent variables. BTMs were selected based on significantly different enrichment between protection classes as

determined by GSEA (Benjamini-Hochberg-adjusted $p < 0.2$; **Figure 5**) or ability to predict outcomes for placebo and high-dose PfSPZ groups in a machine learning model.

Each hypothetical model used for confirmatory factor analysis was initially defined as a single BTM, which consists of a list of functionally and statistically related genes. Model fit was evaluated using the Tucker-Lewis Index. TLI measures the extent to which the hypothesized model replicates the observed covariance matrix among the variables and the relationship between the measured variables and the latent constructs that they comprise. The Tucker-Lewis Index (TLI)[215] is calculated using χ^2 and degrees of freedom and compares the hypothetical model to the expected model of interactions based on the data. It is defined by the following equation:

$$\frac{\frac{\chi 2}{df(Null\ Model)} - \frac{\chi 2}{df(Hypothetical\ Model)}}{\frac{\chi 2}{df(Null\ Model)} - 1}$$

Where χ^2 is the noncentrality parameter of the chi-square distribution and df represents the degrees of freedom for the model identified in parentheses. For confirmatory factor analysis model error was evaluated using a robust Root Mean Square Error of Approximation (RMSEA):

$$\frac{\sqrt{\chi 2 - df}}{\sqrt{[df(N - 1)]}}$$

where N is the number of samples in a data set, χ^2 is the noncentrality parameter of the chi-square distribution and df represents the degrees of freedom for the hypothetical model. The robust RMSEA is specified when using the maximum likelihood estimator in lavaan[213]. RMSEA is an estimate of the average difference between the predicted relationships in the model and the actual observed data. A low RMSEA indicates good fit

and with an ideal RMSEA < 0.08 and for highly complex models, an acceptable RMSEA <0.15[212]. Confirmatory factor analysis was repeated with removal and addition of independent variables until TLI was maximized and RMSEA was minimized. Covariation between member genes was then introduced and modifications to the covariation matrix continued until TLI and RMSEA were optimized, or they reached TLI > 0.95 and RMSEA <0.08. Final model fits were reported with effect sizes of each gene mapping onto the latent BTM construct and significant covariations between genes reported. Reported CFAs included baseline gene expression values only.

3.2.4 Path Analysis

Path analysis was performed using the sem function in lavaan to create a structural model. Latent constructs previously defined with confirmatory factor analysis were included in a structural analysis of their impact on a defined outcome. For this thesis, outcome was defined as parasitemia or absence of parasitemia through 3 months post-vaccination. To create a true systems-level model non-transcriptomic independent features including anti-CSP IgG, flow cytometry data, and plasma cytokines were included as exogenous variables. The exogenous variables that were significantly different between protection classes or significantly correlated with significant predictors of protection were selected. Models were evaluated using R^2 and variables with significant impact ($p < 0.05$) or near-significant impact were included as factors in the final structural model. Covariation between exogenous and latent variables was evaluated and significant ($p<0.05$) covariation was reported. Mediation effects were modeled and included if significant ($p<0.05$).

3.3 Results

3.3.1 Predicting PfSPZ Vaccine Outcomes with Immunological Profiles

To best utilize the systems aspect of our data set I combined the different data types into a single data set for integrated machine learning analysis (**Figure 15**). Previously, we employed linear methods that relied on a single data type such as differential gene expression, enrichment methods, and correlative comparisons to distinguish protective outcomes. However, to obtain a more comprehensive understanding of the features that predict outcomes within dose groups, we have combined multiple data types into a single dataset and are now using an integrated non-linear machine learning approach. Given the associations of baseline humoral and innate signatures with protection identified above I hypothesized that a class prediction model using pre-vaccination baseline signatures would differentiate infants who would go on to have protection against parasitemia in response to the PfSPZ Vaccine. Additionally, I created models using post-vaccination and Δ immune features to identify infants that had an effective response to the vaccine and to identify predictors of PfSPZ Vaccine immunogenicity, respectively.

I first designed models including only low-annotation BTMs as features. I used XGBoost[206] to create a class prediction model that would differentiate vaccine responders and non-responders prior to vaccination. With gene expression collapsed into low-annotation BTMs as module scores of median expressions, I first applied the algorithm to the $1.8x10^6$ dose to classify Protected and Not Protected infants with transcriptomic signatures.

After identifying baseline expression of “spliceosome” (M250), “proteasome” (M226) “cytokines-receptors cluster” (M115) and “T and B cell development, activation” (M62.0) as the most likely features to accurately predict outcome using two-thirds of the data as a training set, I evaluated the resulting model with a test set to determine whether the features were truly predictive for the entire $1.8x10^6$ PfSPZ data set. The receiver operating characteristics curve—showing ratio of sensitivity or true positives (with positive defined as a prediction of parasitemia at 3 months post-vaccination) to the inverse of specificity or false positives—had an area under the curve (auROC) of 0.823. This model also correctly classified subjects as Protected or Not Protected with 81.0% predictive accuracy (**Figure 16A-C**). Both very high and very low spliceosome module expression scores predicted a Not Protected phenotype while moderate scores were predictive of protection. The remaining modules were all positively predictive of protection, meaning that higher expression scores of these modules results in a Protected classification.

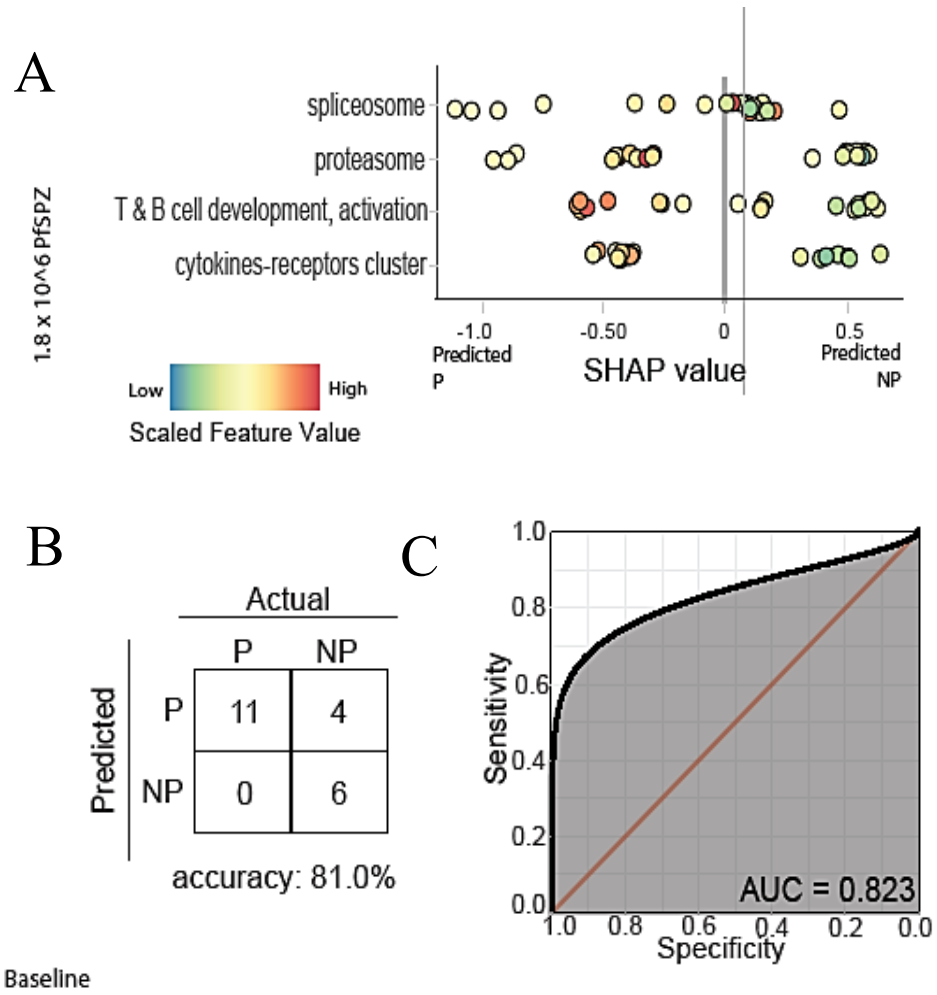


Figure 16. Predictive Model of PfSPZ-Induced Protection Using BTMs
A) SHapley Additive exPlanations (SHAP) plots for $1.8x10^6$ PfSPZ model showing marginal contribution of each feature to the predicted outcome based on feature values. Each point is a single subject in the test set (n=21). B) Confusion matrix showing the model predictions and actual outcomes in the test set for BTM model. C) Receiver operating characteristics (ROC) curve for BTM model. auROC = area under the ROC.

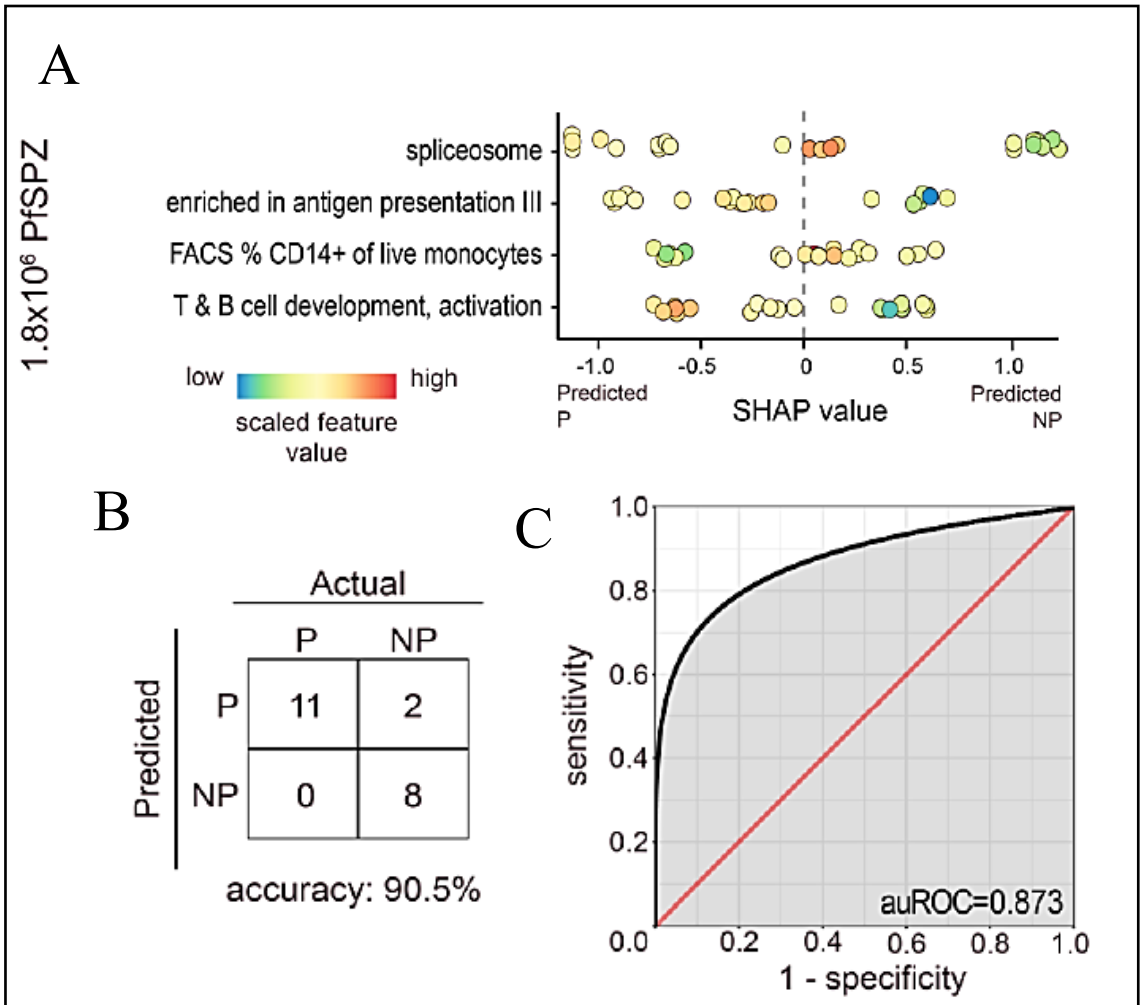


Figure 17. Predictive Multi-Modal Model of PfSPZ-Induced Protection
A) SHapley Additive exPlanations (SHAP) plots for $1.8x10^6$ PfSPZ model showing marginal contribution of each feature to the predicted outcome based on feature values. Each point is a single subject in the test set (n=21). B) Confusion matrix showing the model predictions and actual outcomes in the test set for the multi-modal model. C) Receiver operating characteristics (ROC) curve for BTM model. auROC = area under the ROC.

To gain a more complete profile of the optimal immune status at baseline to generate protective immunity in response to the PfSPZ Vaccine, I which included, in addition to the transcriptomic data, the following baseline features: PBMC immunophenotypes (determined by flow cytometry), plasma cytokines as determined by multiplex immunoassays, anti-CSP IgG titers as determined by ELISA, and *P. falciparum*-specific T-cell activation as determined by parasite stimulation assays.

The resulting systems model utilized baseline expression scores of three BTMs, "spliceosome" (M250), "T and B cell development, activation" (M62.0), and "enriched in antigen presentation (III)" (M95.1), in addition to percentage of CD14+ monocytes at baseline to classify infants as either Protected or Not Protected. This model accurately classified 90.5% of the subjects in the test set and produced auROC of 0.873 (**Figure 17A-C**). Overall, the directionality of associations between the predictive features and outcomes in this model—negative association between CD14+ monocytes and protection outcome—support the previously described hypothetical reduction in PfSPZ VE due to innate immune activation at baseline, while the role of baseline T cell gene expression implied in this model to contribute to future protection is consistent with our previous transcriptomic results.

The opposing associations of transcriptomic signatures with protection for the placebo and $1.8x10^6$ groups led me to hypothesize a natural mechanism to control parasitemia in the Protected placebo group that would inhibit effective RAS immunization using the same mechanism in the $1.8x10^6$ PfSPZ Vaccine group. To test this hypothesis, I applied the features identified in the $1.8x0^6$ multi-modal model to a randomized placebo training and test set. The model was able to predict infection

outcomes with 86.4% accuracy equal to the baseline placebo model (**Figure 18A-C**). Additionally, as I expected, increased CD14+% of monocytes in addition to high "T and B cell development, activation" increased the likelihood of a Protected classification in the placebo group.

I also wanted to determine if signatures at baseline in the placebo group were predictive of protection and whether the I could recapitulate the reversed association of protection between the two groups using independent machine learning models. A multi-modal classifier of protection in for the placebo group had auROC of 0.840 and accurately classified 86.4% of test set subjects as either Protected or Not Protected using five baseline features: FACS percentage of CSP-specific memory B cells, FACS % of NK Cells (CD56+CD16+), IL-8 serum concentration, and expression scores of "NK cell surface signature" (S1) and "cytoskeleton/actin (SRF transcription targets)" (M145.0) (**Figure 19A-C**). These features and their directional association with protection are consistent with my previous results. NK Cell transcriptomic signatures were enriched in Protected over Not Protected by GSEA. Additionally, high feature expression of "cytoskeleton/actin (SRF transcription targets)" (M145.0) increased the probability of Protected classification in this model, consistent with the upstream regulator MRTFA with predicted enrichment in Protected in the placebo group and Not Protected in the $1.8x10^6$ PfSPZ group. This gene is the co-receptor for SRF and their interactions may influence hematopoiesis and erythropoiesis[216], as well as myeloid DC cell differentiation and gene expression[217].

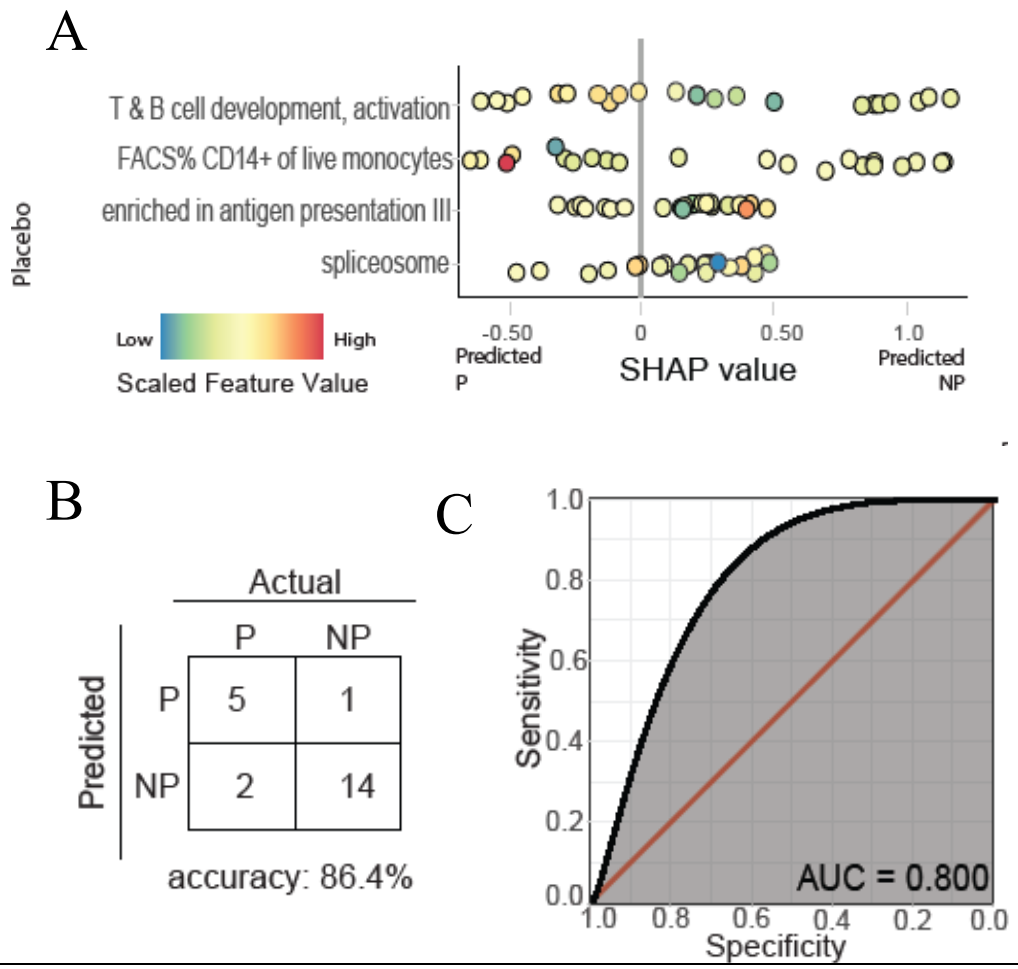


Figure 18. Predictive Model of Natural Protection using PfSPZ Features. This model trained a Placebo predictive classifier using features selected with the 1.8 x10^6 PfSPZ training set. A) SHapley Additive exPlanations (SHAP) plots for the Placebo model showing marginal contribution of each feature to the predicted outcome based on feature values. Each point is a single subject in the test set (n=21). B) Confusion matrix showing the model predictions and actual outcomes in the test set for model. C) Receiver operating characteristics (ROC) curve for BTM model. auROC = area under the ROC.

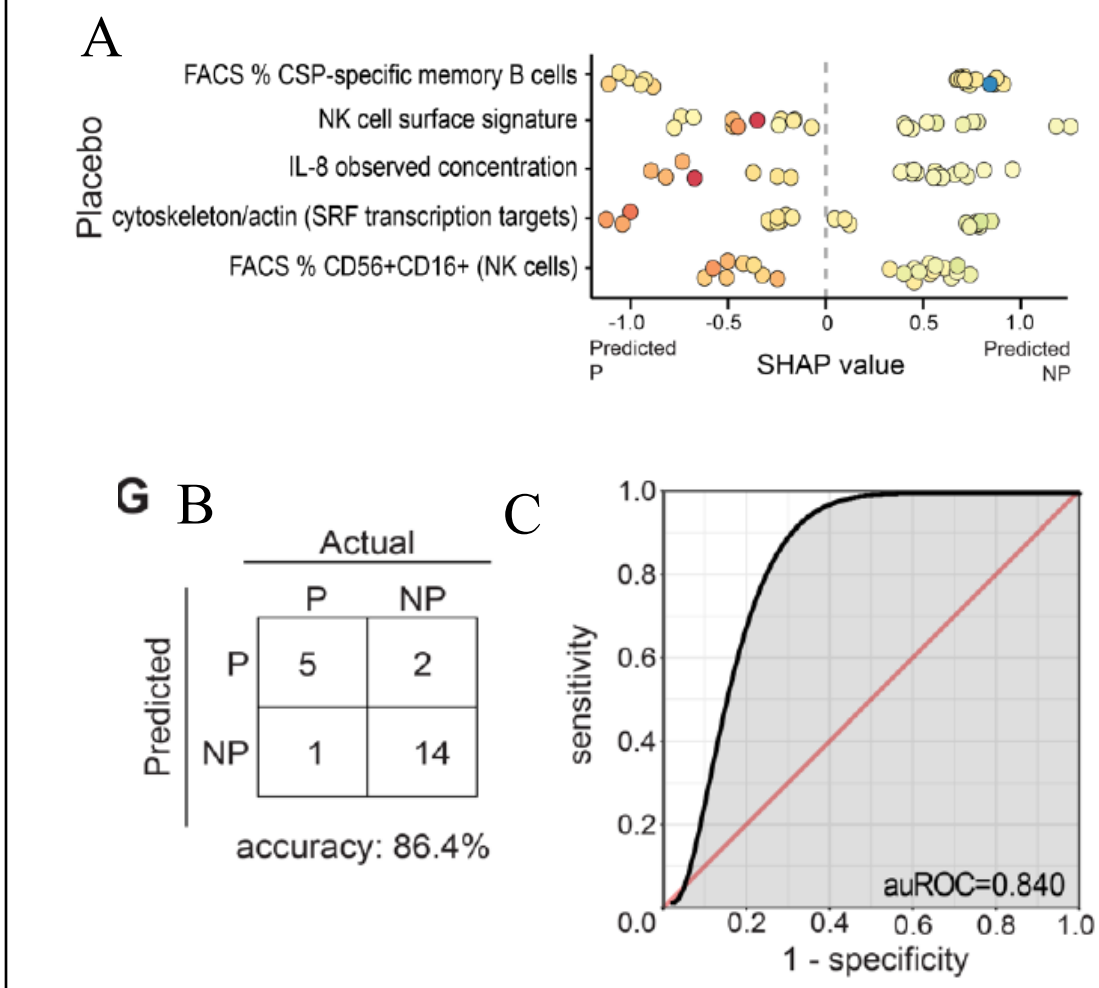


Figure 19. Predictive Modeling of Natural Protection. A) SHapley Additive exPlanations (SHAP) plots for $1.8x10^6$ PfSPZ model showing marginal contribution of each feature to the predicted outcome based on feature values. Each point is a single subject in the test set (n=21). B) Confusion matrix showing the model predictions and actual outcomes in the test set for BTM model. C) Receiver operating characteristics (ROC) curve for the model. auROC = area under the ROC.

To classify whether an infant had been successfully immunized and whether they were likely to be protected against future infections I next created a multi-modal model using data from two weeks post-vaccination. The immune signatures that were most predictive of infection through 3-months of surveillance post-vaccination included 3 post-vaccination expression of 3 BTMs, "MHC-TLR7-TLR8 cluster" (M146), "cytoskeleton/actin (SRF transcription targets)" (145.0), "enriched in extracellular matrix & associated proteins" (M202) as well as logFC anti-CSP IgG as features predictive of protection. Higher logFC anti-CSP was predictive of protection, consistent with the original KSPZV1 study findings[167], while lower expression scores for all 3 BTMs increased the probability of a Protected classification (**Figure 20A-C**).

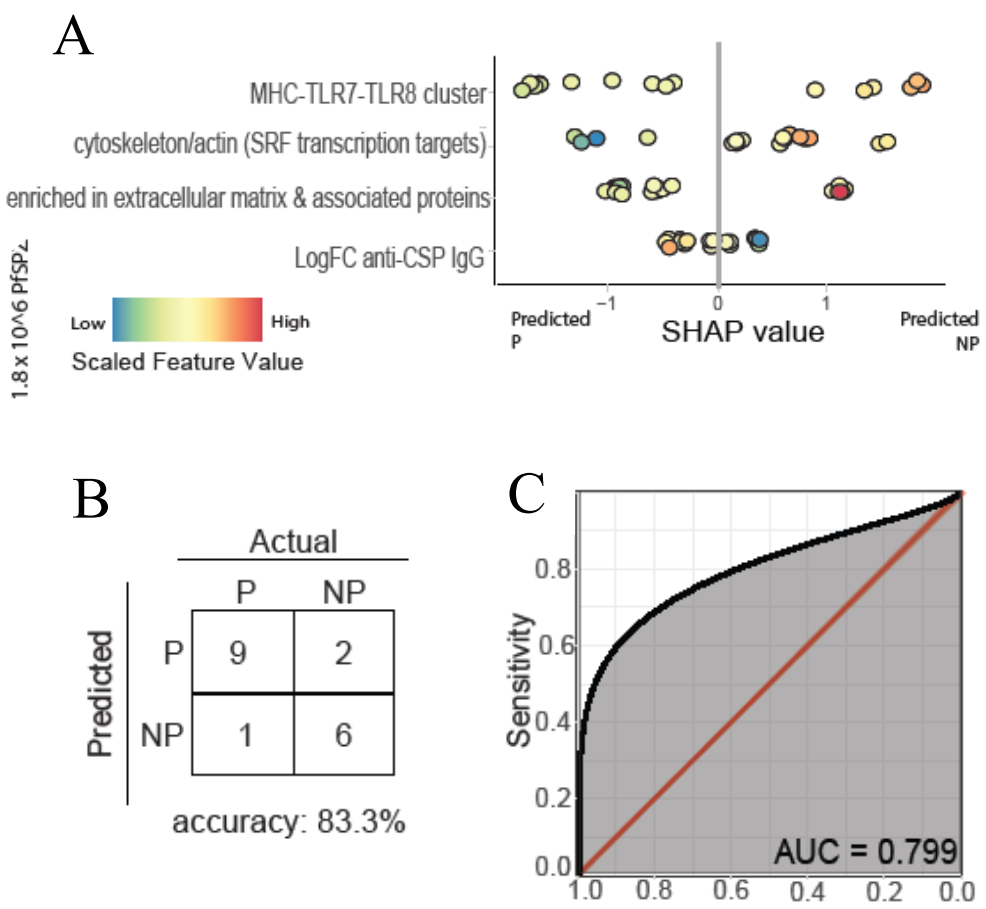


Figure 20. Predictive Modeling of Protective Immune Response. A) SHapley Additive exPlanations (SHAP) plots $1.8x10^6$ PfSPZ post-vaccination features, showing marginal contribution of each feature to the predicted outcome based on feature values. Each point is a single subject in the test set (n=21). B) Confusion matrix showing the model predictions and actual outcomes in the test set for this model.C) Receiver operating characteristics (ROC) curve for the model. auROC = area under the ROC.

3.3.2 A Structural Equation Model of PfSPZ-Induced Malaria Immunity

One limitation of my machine learning models was estimation of gene expression features. Collapsing gene expression into BTM module expression scores allowed me to increase the information gain of each gene expression feature over using individual genes as features and median expression of member genes is a common method of gene module estimation in systems immunology[218]. However, covariation between genes within modules is high, and genes assigned to multiple modules contribute to covariation between modules[219]. Moreover, the integration of different levels of organization into complex models in systems studies creates room for error when variables contribute redundant information. For example, the class prediction results of protection for the placebo group using baseline signatures included a BTM comprised of NK cell surface marker genes in addition to FACS % of NK cells. These are highly correlated—an expansion of NK cell populations will naturally lead to a proportional increase in NK cell gene signatures. Given these challenges, I wanted to find a method that would allow me to integrate gene, cell, and molecular data types while accurately accounting for overlapping information gain, a metric for effectiveness of a feature at classifying outcome variables[220].

I performed confirmatory factor analysis (CFA) to modify BTMs to include only the genes that significantly contribute to the value of the module as a latent construct to address my previous concerns about potentially inaccurate estimations of BTM module expression score. I selected low-annotation BTMs for CFA using previous GSEA results (**Figure 5**) and the top predictive machine learning features (**Figures 16-20**). The KSPZV1 data set size limited the number of genes I could accurately include in a model

to ~30 genes to conserve degrees of freedom and I performed confirmatory factor analysis to create a measurement model of each module that met the criteria for inclusion (Table 4) and met the size criteria for the data set. I completed CFA for six low-annotation BTMs – "spliceosome" (M250), "proteasome" (M226), "enriched in antigen presentation (III)" (M95.1), "NK cells (II)" (M61.0), "T and B cell development, activation" (M62.0), and "CD1 and other DC receptors" (M50). Prior to CFA the average TLI and RMSEA of the low-annotation BTMs were 0.573 and 0.212, respectively. After iterative exclusion and inclusion of member genes and accounting for covariation between highly correlated genes, the resulting amended modules had an average TLI and RMSEA of 0.938 and 0.063. The final construct for M95.1, containing genes related to antigen presentation, had the best fit model with TLI of 1 and RMSEA of 0.

CFA also identified genes that would significantly contribute to the estimation of BTMs as latent constructs which could be applied to calculation of a more accurate MES. Some of these latent constructs required inclusion of covariate genes that did not significantly contribute to the final estimation of BTM expression, these are shown in grey (**Figure 21**). For example, CD1 and other DC receptors – CD13 regulates cross-presentation[190,221], IL1R and CSF1R indicates activated state at baseline[189,222] – activated baseline DCs decrease probability of protection with effect size of .3, consistent with our hypothesis that innate immune activation is deleterious for inducing protection with the PfSPZ Vaccine.

After defining the latent BTM constructs, I performed path analysis to integrate these with other data types as exogenous variables, including flow cytometry data,

cytokine data, and anti-CSP IgG. Because SEM requires a hypothetical model for input, I narrowed my focus to the variables that were associated with protection in the original study, predictive features from my machine learning analysis, and features that were significantly correlated with parasitemia time-to-event (**Table 4)**.

Variable	Justification
Baseline CD11c+	Associated with protection in KSPZV1 Study
logFC Memory B	Associated with protection in KSPZV1 Study
logFC anti-CSP IgG	Associated with protection in KSPZV1 Study
logFC CD8 Cells	Correlated with Protection
baseline anti-CSP IgG	Correlated with Protection
IL-10	Correlated with Time-to-Parasitemia
CXCL10	Correlated with Time-to-Parasitemia
TNF	Correlated with Time-to-Parasitemia
IFNa	Correlated with Time-to-Parasitemia
CD14+CD16+ monocytes	Correlated with Time-to-Parasitemia
Spliceosome	Machine Learning Predictive Feature
Proteasome	Machine Learning Predictive Feature
T & B cell development,activation	Machine Learning Predictive Feature
cytokines-receptors cluster	Machine Learning Predictive Feature
FACS CD14+ monocytes	Machine Learning Predictive Feature
enriched in antigen presentation (III)	Machine Learning Predictive Feature
MHC-TLR7-TLR8 cluster	Machine Learning Predictive Feature
cytoskeletn/actin (SRF transcription targets)	Machine Learning Predictive Feature
enriched in extracellular matrix and associated proteins	Machine Learning Predictive Feature
NK Cells BTM	Machine Learning Predictive Feature
IL-8	Machine Learning Predictive Feature

Table 4: Features from KSPZV1 Study Used in SEM modeling. Transcriptomic features were included in CFA measurement model and structural model. All other features were considered exogenous, independent variables and were included in the structural model only. Features used are all from baseline analysis unless stated otherwise.

My final SEM model included data from both time points: baseline and post-vaccination All hypothetical relationships were modeled but only significant direct effects and covariations are shown. Values shown in SEM figures indicate standardized effect sizes (β). Each β coefficient is the resulting change in the dependent variable if the independent variable undergoes an increase of one standard deviation. Larger β coefficients indicate proportionally larger impact of the independent variable on the dependent variable. Sign of the β coefficient indicates positive or negative relationship between independent and dependent values.

For the $1.8x10^6$ PfSPZ dose group, the final structural model was chosen as the hypothetical model with the largest R^2 value ($R^2 = 0.359$) (**Figure 22**).

Five independent, directly measurable variables have a significant, causal effect on protection outcomes at the three-month post-vaccination time point for the $1.8x10^6$ PfSPZ dose group. Percentage of circulating PfSPZ-specific CD8+ T cells (β = -0.238) and percent circulating CD14+ Monocytes (β = -0.156) each have a negative standard effect on Protection outcome. Additionally, three directly measurable variables have a significant positive impact on protection. The log2 fold-change of anti-CSP IgG during the vaccine period (β = 0.341) has the single highest impact on protection and baseline IL-10 observed plasma concentration (β = 0.118) and percentage of circulating CD11c+ DCs (β = 0.319) have positive direct effects on PfSPZ-induced protection. Although only one latent BTM construct has a direct impact on protection outcome, 'CD1 and other DC Receptors' (β = -0.300), two additional latent BTM constructs have significant interactions that explain a percentage of variation in protection outcomes, 'T and B cell development, activation' and 'enriched in antigen presentation (III)' each have significant

covariations within the model, and 'enriched in antigen presentation (III)' ($\beta = 0.153$) exerts an indirect effect on protection through its impact on anti-CSP IgG response during the vaccination period. An increase in 'enriched in antigen presentation (III)' member gene expression increases IgG response to PfSPZ Vaccine, and this higher anti-CSP IgG response increases the probability of protection (**Figure 22**).

Two other indirect effects within the model were significant. In addition to the negative causal impact of CD14+ Monocytes on PfSPZ-induced protection, the percentage of CD14+ monocytes also exerts a smaller, positive effect on outcome through the effect CD11c+ DCs have on protective outcome. Because CD11c+ DCs are derived from CD14+ monocytes, a decrease in the overall percentage of CD14+ monocytes also a negatively impacts protective outcome indirectly. My previous results indicated baseline anti-CSP IgG was significantly higher in the Not Protected infants that received the $1.8x10^6$ PfSPZ. This structural model reveals that the impact of baseline anti-CSP IgG on protection outcome is indirect, through the negative causal relationship between baseline IgG and the IgG response.

The significant covariations between variables that affected the model R^2 were included in the reported structural model. 'Enriched in antigen presentation (III)' had significant covariation with the other two latent BTM constructs, 'CD1 and other DC receptors" ($\beta = 0.357$) and 'T and B cell development, activation' ($\beta = 0.340$). The 'enriched in antigen presentation (III)' latent construct also demonstrated significant covariation with the baseline percentage of CD11c+ DCs ($\beta = 0.334$).

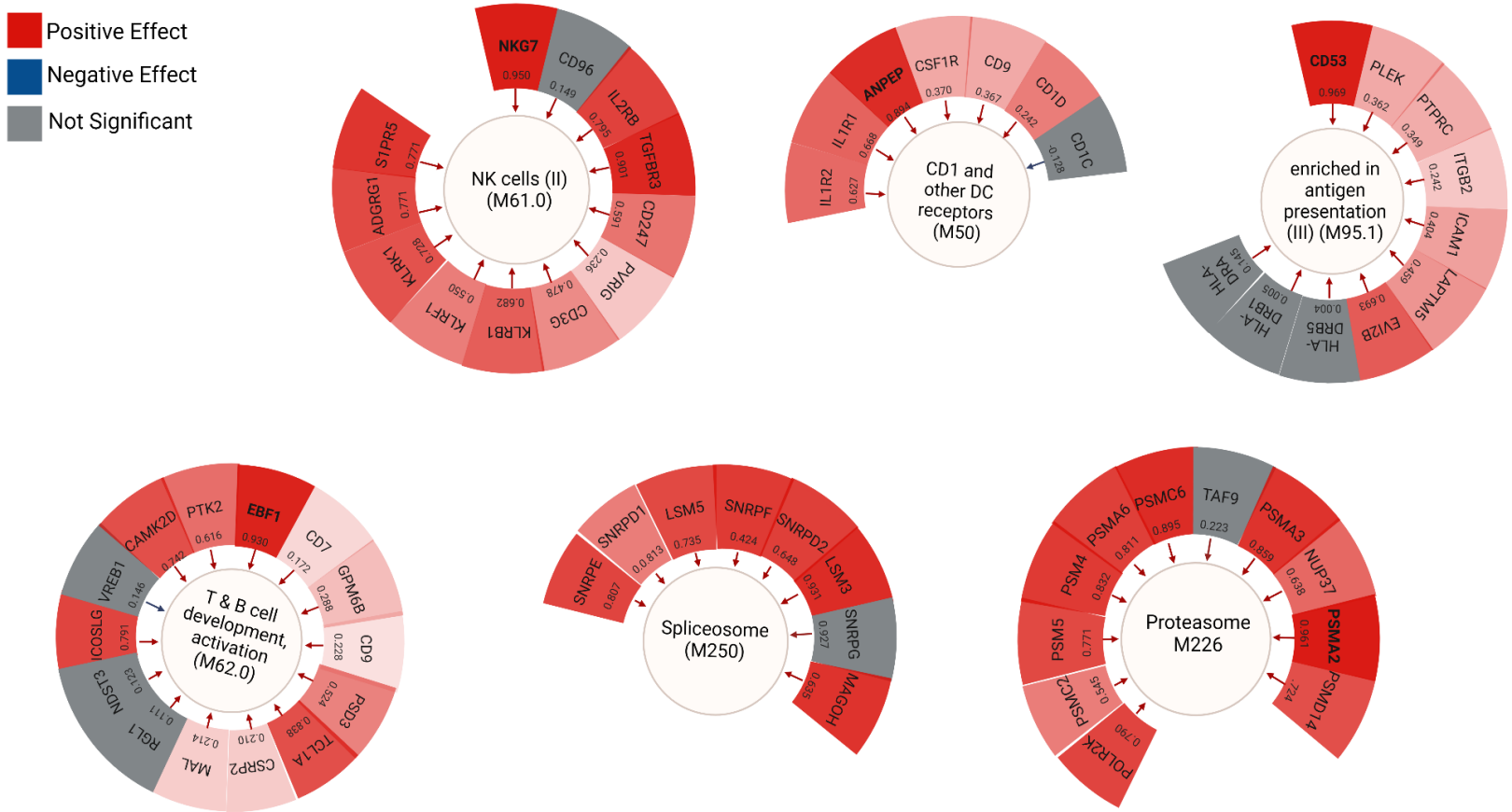


Figure 21. Measurement Models of Selected BTMs Created Using CFA. Latent factors are all low-annotation BTMs. Intensity of color indicates the value of effect size, how much a measured variable contributes to the estimate of the latent construct. Arrow color indicates direction of impact.

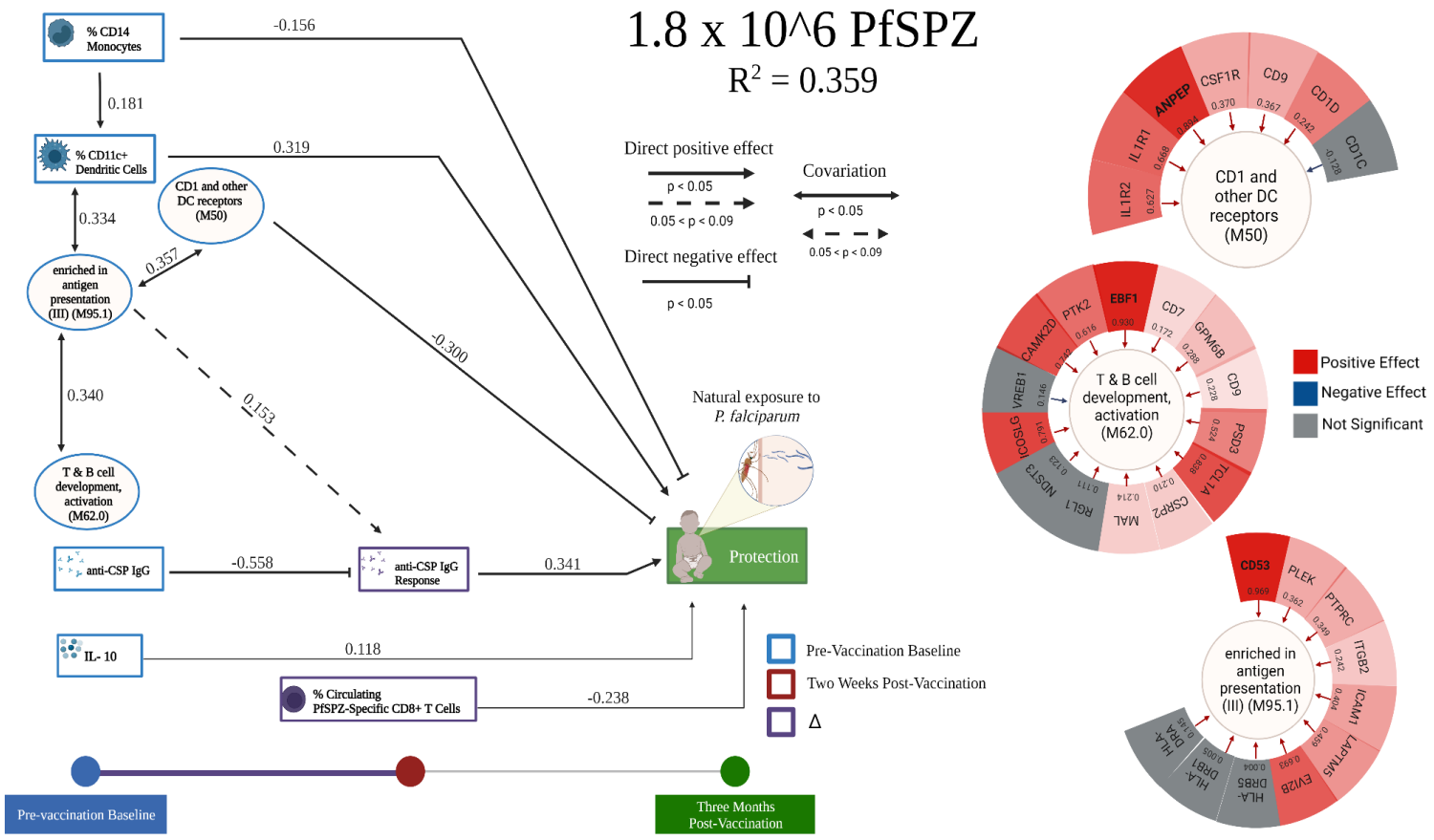


Figure 22. Structural Model of PfSPZ-induced Immunity. Latent variables from Figure 21 are shown if they have significant impact on 3 months post-vaccination outcome in the model. Latent factors are represented in circular nodes while independent variables are represented in square nodes.

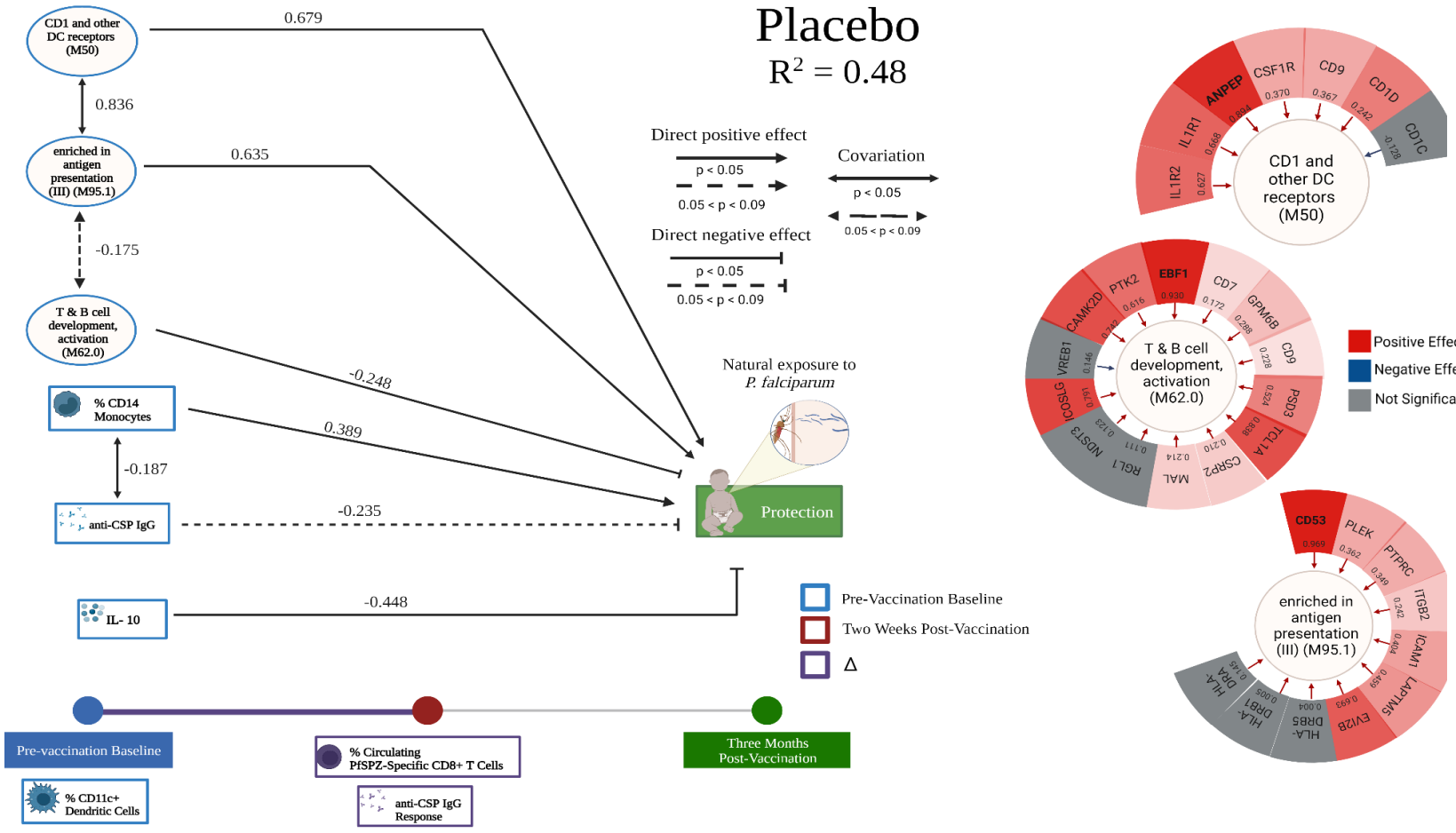


Figure 23. Structural Model of Natural Immunity. Latent variables from Figure 21 are shown if they have significant impact on 3 month post-vaccination outcome in the model. Latent factors are represented in circular nodes while independent variables are represented in square nodes.

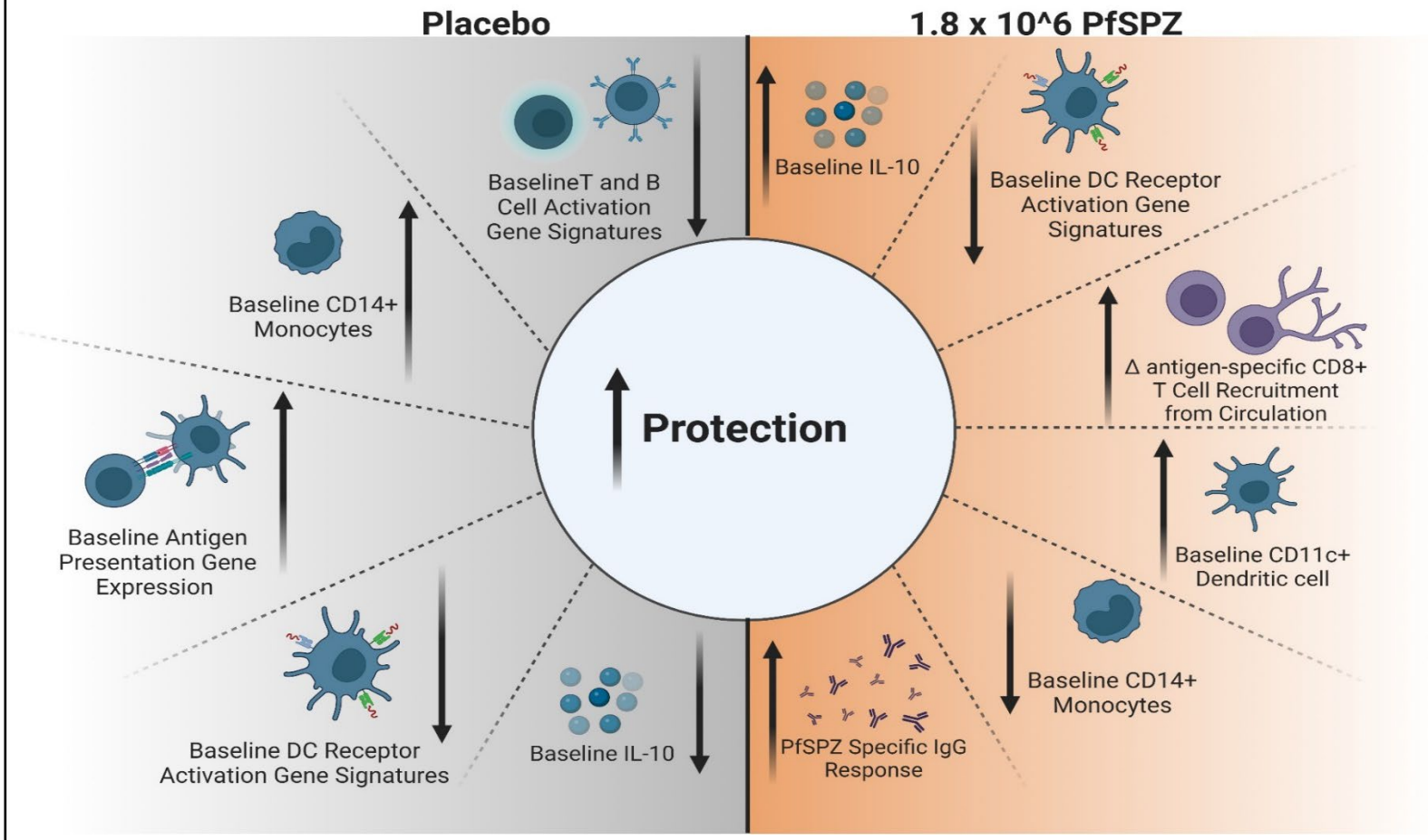


Figure 24. Contrasting Interactions of Innate and Adaptive Immune Responses in Natural and PfSPZ-Induced Immunity. Features with significant impacts on the structural models are shown. Directionality is indicated by arrows for each variable.

3.4 Discussion

3.4.1 Baseline Innate and Adaptive Immune Activation and CSP-Specific IgG Predict PfSPZ Vaccine Response

The baseline multi-modal predictive model revealed innate immune signatures, consistent with our other analyses, were associated with PfSPZ Vaccine-induced protection while T and B cell genes were enriched in Protected infants regardless of treatment. Investigation into the genes comprising this BTM could generate more insight into the development of immunity to *P. falciparum*. The role of baseline innate immune activation in protective outcome is treatment-dependent based on the data. Though more study should be done to determine precise mechanisms of innate immune constraint of PfSPZ Vaccine-induced protection, screening potential vaccinees for innate immune signatures prior to vaccination could determine ideal respondents.

3.4.2 Structural Model Quantifies the Role of Baseline Immune Status and PfSPZ Immunogenicity on Protective Efficacy

One limitation of application of SEM to this data was the size of the data set and the complexity of the model. To accurately perform CFA on larger BTMs with appropriate degrees of freedom, between 5-10 samples would be required per variable included in the model[223]. By combining the placebo and $1.8x10^6$ data sets I was able to minimize the error complexity introduces into the model. The BTM measurement models are proof-of-concept that gene set constructs can be accurately estimated using CFA. These resulting measurement models can be used to calculate accurate module expression scores for downstream analysis, in place of median total expression as I used for

transcriptomic class prediction features. This method can be easily applied to very large transcriptomic data sets to create gene modules that are specific to the data prior to GSEA or other analyses that require priori-defined modules. For data sets that are large enough (>1000 subjects), Exploratory Factor Analysis can be applied to create modules without a hypothetical model input. This removes the need to use pre-defined modules and would create completely data-driven gene sets while accounting for the covariation between member genes that is not addressed by current gene set constructs.

3.4.3 Explaining PfSPZ Efficacy: Myeloid Cell Subsets and IL-10 at Baseline, IgG Response, and Circulating PfSPZ-specific CD8+ T Cells

The baseline percentage of two innate cell subsets, CD14+ monocytes and CD11c+ monocyte-derived dendritic cells, has inverse impact on protection outcome 3 months after the end of the vaccination period, although they are positively correlated with each other. This led me to conclude that the cytotoxic cellular and antibody responses to the PfSPZ Vaccine are opposing pathways, both related to antigen presentation through different mechanisms. The two cell types are highly correlated because CD14+ monocytes differentiate into CD11c+ dendritic cells[184] and are therefore necessary to all downstream mechanisms involved the DCs. However, CD14+ monocyte percentage of live PBMCs has a direct, negative impact on PfSPZ-induced immunity, while CD11c+ DCs have a direct, positive impact on immunity. CD11c+ cells influence immunity to *P. falciparum* through cross-presentation of liver-stage antigens to prime tissue-resident CD8+ memory T cells,[184,190] generating the adaptive cytotoxic response that is required for durable, sterile immunity[102]. Other innate immune cells, including

CD14+, may directly phagocytose sporozoites before the liver stage and act as APCs[224], but induce the less effective antibody response which is not required for immunity and is not highly correlated with the cytotoxic response[116]. An increased CD14+ monocyte population may generate more CD11c+ cells but the increase in phagocytic macrophages from these monocytes or direct response from the monocytes themselves could prevent the liver phase, subverting the CD11c+ - dependent mechanism for the cytotoxic memory cell response and decreasing the overall contribution of CD11c+ cells, as is seen in the model.

The placebo structural model demonstrated an inverse effect of CD14+ monocyte percentage at baseline, consistent with my previous findings, and indicative of the protective effect of innate immune activation prior to *Plasmodium* infection. As discussed in chapter 2, trained immunity of innate immune cells through TLR agonists such as LPS or through vaccination with live vaccines such as the BCG tuberculosis vaccine is associated with fewer malaria episodes and lower parasitemia. This association between trained immunity and reduced PfSPZ Vaccine efficacy introduces complications to malaria control and eradication efforts as some prophylactic interventions like BCG vaccination of newborns are beneficial while there is not a specific malaria vaccine but could prevent successful vaccination efforts with vaccine candidates that are known to induce protective immunity. This also raises questions about future vaccine efficacy after vaccination with some malaria vaccines, ie: could the anti-CSP IgG response induced by RTS,S reduce efficacy of future WSVs that would confer more durable sterile protection? The benefits of partial vaccination must be weighed against potential future detriments. According to the model, plasma IL-10 at baseline increases the probability of protection

after immunization with PfSPZ Vaccine however baseline IL-10 had a direct, negative effect on protection in the placebo group. There is conflicting data on the impact of IL-10 on parasitemia and severity of malaria symptoms. Some studies indicate that IL-10 production is associated with increased severity of malaria symptoms[225] while other data suggest a negative correlation between IL-10 and parasitemia, while IL-10 was higher in uncomplicated malaria cases than in severe malaria cases[226,227]. While a protective role in malaria may not be clear from the literature, mechanisms of IL-10 include reduction of IFN-γ signaling, inhibition of antibody production, and decreased inflammation, including decrease in macrophage activation[227,228]. A possible explanation for differential levels of IL-10 in severe malaria pathogenesis is increased production in response to inflammation. In *P. falciparum* immunopathology, IL-10 regulates inflammatory responses[229] so individuals with higher IL-10 expression prior to infection will likely have less robust immune responses while severe parasitemia would likely lead to an increase in IL-10 as protective mechanism against tissue damage due to hyperinflammation[227]. Intriguingly, production of IL-10 reduces inflammation caused by malaria without dampening antigen-specific adaptive responses[230]. As IL-10 supports germinal-center B cell responses and antibody production[231], the effects of IL-10 may help to overcome the dampening of humoral responses due to pre-existing anti-CSP IgG. This effect at baseline could also decrease the immune response to RAS or infectious sporozoites without interfering with the cellular immunogenicity of the PfSPZ Vaccine, allowing the parasites to begin replication during the liver-stage while also allowing priming of effector CD8+ T cells against liver-stage antigens.

3.4.4 Antigen Presentation Genes and Baseline anti-CSP IgG Mediate the Impact of Specific Antibody Response on Protective Efficacy

The antigen-presenting module M95.1 is comprised of innate immune genes and is correlated with innate immune activation module M50 (CD1 and DC receptors) as well as T and B cell activation module M62.0. Activation of M50 at baseline reduces the probability of protection and because the model is defined with directionality and demonstrates causality, it can be inferred that upregulation of the 7 genes that comprise the amended BTM directly influence this outcome. This is consistent with our previous transcriptomic findings as well as our theory of innate activation by TLR agonists. Activation of IL-1β by NF-κB is the outcome of TLR1, TLR2, and TLR4 signaling pathways. Additionally, DCs can be activated by innate immune agonists through IL-1R signaling, inducing migration of these DCs and an inflammatory response, including priming of circulating CD8+ T cells[222]. DCs activated at baseline that prime an immune response against RAS prior to hepatocyte invasion is a potential mechanism of innate-immune restriction of PfSPZ through a reduction in exposure and priming in the liver against liver-stage antigens.

Though expression of the genes in this module do not have a direct impact on protection, higher expression of these genes at baseline resulted in increased $\log_2$FC of anti-CSP IgG and this increased humoral response had a direct positive effect on protection outcome. In other words, as antigen presenting gene expression at baseline increases, so does the $\log_2$FC of abs and through this mechanism the probability of protection against malaria also increases. This is useful information for considerations of interventions and vaccine design. Although some signatures are important, creating a

directional model allows mechanistic understanding. As we hypothesized, antigen presentation after PfSPZ Vaccination is important for generation of an antibody response and therefore partially protective. However, the model reveals this mechanism is uncoupled from the CD8+ T cell response, supporting the theory that phagocytosis by APCs prior to hepatocyte invasion by sporozoites reduces CD8+ T cell priming. The model does give more insight into whether this may be an independent mechanism of reduced CD8+ priming or whether the reduction in liver stage infection is sufficient to decrease the immune response below the threshold for sterile protection.

The effect of baseline anti-CSP IgG on protection is mediated through the reduction in anti-CSP IgG response as I hypothesized in chapter 2. This means that having anti-CSP IgG detectable at baseline does not directly impact outcome, rather, the presence of these antibodies reduces the protective response generated by PfSPZ vaccination and the dampened response is not sufficient to confer protective immunity. Based on the available information, the most likely explanation for the reduced antibody response due to antibodies at baseline is antibody-mediated clearance of RAS through non-antigen presenting immune mechanisms. If sporozoites were controlled through antibody-dependent antigen presentation pathways alone, having antibodies at baseline would not decrease the anti-CSP response, it would likely only decrease the response to liver-stage antigens which are not measured with anti-CSP IgG titers. A known parasite control mechanism that aligns with this hypothesis is activation of the complement classical pathway, resulting in complement fixation and direct destruction of the parasites. Additionally, neutrophils are the predominant cell type implicated in antibody-mediated phagocytosis of sporozoites[80]. This opsonic phagocytosis by neutrophils could

explain the negative association between anti-CSP IgG detectable at baseline and anti-CSP IgG response to PfSPZ as neutrophils are not generally considered to be professional APCs, though they may acquire the capacity to present antigens under certain conditions[232].

The structural model demonstrates interactions between various immune components in addition to direct effects of immune mechanisms on outcome. Thus, the structural model implies a multi-factorial and sometimes self-opposing immune response to sporozoite inoculation, with the eventual consequence of controlling liver infection and, later, parasitemia. First, the innate immune response to sporozoites can prevent infection of hepatocytes through phagocytosis by macrophages and other myeloid-lineage cells. This effect may be amplified in the presence of antibodies against sporozoite antigens, as opsonization is known to increase phagocytic destruction of *Plasmodium* by neutrophils as well as professional APCs[80,90]. The presence of antibodies against pre-erythrocytic *Plasmodium* parasites also results in complement fixation and NK cell destruction of the sporozoites directly, prior to the establishment of liver stage infection. The innate immune activation is also not limited to the circulating cells we can measure, liver resident macrophages, Kupffer cells, are also activated by TLR agonists and can destroy sporozoites as they attempt to enter hepatocytes, in an antibody-enhanced process. Additionally, activation of innate immune cells at baseline may indirectly affect immunogenicity as activated monocytes are not able to differentiate into the APCs that are necessary to prime the CD8+ T cell response. Based on the available data, the most likely explanation is a combination of these processes, as reflected by the model.

Chapter 4: Conclusions and Future Directions

The work presented in this thesis suggests there are multiple independent mechanisms of PfSPZ Vaccine-induced protection against malaria infection, and that these may be influenced by differing host immune factors prior to vaccination with PfSPZ. After transcriptomic analysis, it became clear that signatures of reduced protection from the PfSPZ Vaccine were consistent with signatures of children protected from infection in the Placebo group, both of these showed enrichment of innate immune activation at the level of gene expression. These data led to a hypothesis that baseline innate immune activation mechanisms may prevent liver stage infection by sporozoites; the liver stage is a crucial step for priming the PfSPZ Vaccine-induced immune response[102] and this stage also represents a gateway in natural infection to the blood stage—only one sporozoite needs to make it through the liver stage to produce thousands of merozoites and initiate parasitemia[86].

Additionally, investigation into baseline humoral signatures indicated the presence of antibodies at pre-vaccination baseline was associated with impaired cellular and humoral adaptive responses to the PfSPZ Vaccine, though there was no relationship between baseline anti-CSP IgG and protection in the Placebo group. The impact of baseline antibodies is likely more impactful against RAS immunization than against natural infection because of the dynamic stated above. A reduction in fully infectious sporozoites will not prevent infection if any remain, conversely, a decrease in the number of RAS can have a considerable impact on vaccine efficacy, as a vigorous immune response is necessary for the generation of immunity. A minor reduction in the number of sporozoites may be sufficient to decrease the immune response below the required

threshold for sterile protection. Additionally, anti-CSP IgG response during the vaccination period demonstrated both high and low responders in the Protected infants and the Not Protected infants. This supports multiple mechanisms of protection, including an antibody-independent pathway. The final structural model of PfSPZ-induced protection did not indicate interactions between antibodies and the other immune signatures, confirming this hypothesis.

Further investigation of baseline immune cell populations indicated that while innate immune signatures at baseline were generally associated with reduced PfSPZ VE, one myeloid DC subset that was CD11c+ was positively associated with protection from the vaccine. This is likely due to the role of this APC in priming the hepatic CD8+ T cell memory response[184,190,233]. In contrast with the importance of this response, there was a decrease in PfSPZ-specific CD8+ T cells circulating during the vaccine period. Though the liver-resident cells are generally associated with protection[156], circulating PfSPZ-specific CD8+ T cells are expected to increase as they are primed by the vaccine. These circulating memory CD8+ T cells have previously been found to be a marker of protection[103] and treatment that reduced liver burden in our murine model also resulted in a decrease in circulating specific CD8+ T cells after vaccination with PyRAS. This contradicting data may be due to the timing of the post-vaccination blood draw. Antigens from RAS immunization can persist in the liver for several weeks and priming of the CD8+ T cell response in the liver and draining lymph node requires persistent antigen presentation[190]. At two weeks post-vaccination it is possible the CD8+ T cells are still being recruited and primed, or that these cells have been recruited to the liver to assist in the clearance of hepatocytes containing RAS. To test this hypothesis, I would propose

weekly monitoring of circulating PfSPZ-specific memory CD8+ T cells for up to eight weeks after the last vaccine dose was given.

In addition to discrepancies between the results of the KSPZV1 trial and our murine model, our murine model was limited to one strain of mice and one species of *Plasmodium*. To gain more insight into the inconsistencies between the mice and humans, we should first establish variations in immune responses between different mouse breeds as well as the differing responses of those mice to both infection and immunization with different parasite species. Including BALB/c mice in addition to C57BL/6 could identify immune responses we were unable to observe in our model. Additionally, *P. yoelii* is a non-lethal parasite in the mice and while this is convenient for monitoring of parasitemia, using the lethal *P. berghei* in our model may have differing results for the RAS and infectious experiments. Further investigation into these dynamics is necessary to fully understand the strengths and weakness of our model. Such an investigation can also lead to valuable insights into the future response to immunizations and pathogen protection after immunization, which can enhance vaccine outcomes and provide valuable information for the development of future vaccination platforms.

In the context of vaccine research, gene-level data has limitations in discerning vaccine responders from non-responders, as immunity is a complex process involving multiple biological systems working together. Pre-defined gene sets, often used in the analysis of gene expression data to improve the power of analysis and inject biological meaning, may not be data-driven and can obscure biological meaning, as they are highly correlated within and between sets. SEM can be employed to create data-driven gene sets, accurately accounting for covariation between genes. Unlike other data-driven

network approaches such as Weight Gene Co-Expression Network Analysis (WGCNA)[234], SEM can be used to infer causality as directionality of the relationships between variables is included in the hypothetical structural models. Using a hypothetical model can also allow exploration of mechanisms that explain the contribution of variables to the dependent or outcome variable. WGCNA reports only the strength of associations between genes and causal relationships cannot be distinguished from indirect interactions. In addition, SEM allows for the inclusion of exogenous independent variables in a structural model, allowing for combination of systems data into an integrated model, while WGCNA can only be applied to gene expression data. Methods like WGCNA are best for hypothesis generation while SEM is testing hypothetical models, allowing for interpretation of biological meaning from the significant relationships in a structural model.

It is important to note that an accurate hypothetical model is crucial for the inference of biological meaning from SEM results. For a method such as exploratory feature selection (EFA), which does not require a hypothetical model, causality and directionality cannot be inferred from the model results alone. Future investigation would be required to identify possible biological relationships. Once those have been established, however, CFA and path analysis can be applied to a hypothetical model based on the EFA results after they are adjusted according to known biological interactions. Path analysis always requires a hypothetical model as directionality is inherent in the calculation of the interactions between variables and outcome. It is the input of the hypothetical model with directionality that allows for interpretation of directionality and interactions between variables within the resulting structural model.

Regarding the predictive modeling, the relatively small sample size of each dose group limited the number of features that could be included in a model. The addition of more features could impact the importance of the features that were selected and included in the model. Additionally, the estimates for each BTM used as a feature included all member genes and did not account for covariation between these genes. This could influence the information gain attributed to each of the features. As a method, predictive modeling could be helpful for identification of potential vaccinees most likely to respond to vaccination, however validation of these models on independent data sets would be required to ensure they are generalizable and accurate in various clinical settings. The lack of data sets containing similar data and using PfSPZ as an intervention prevented this validation but future studies with similarly complex data sets would be extremely useful. Predictive modeling also only identifies statistically associated predictors of outcome and biological meaning cannot be gleaned from the results.

That the models were consistent with our previous findings is promising for their potential usefulness, but more study would be needed to test each of the features individually and together. One method to streamline this process could be using SEM to identify how each of the features interact with each other. The benefit of this method would be the ability to link each predictive feature from the classifier directly to the protective outcome, determining whether some features may be incidentally predictive due to indirect relationships. This would help to direct future experimentation toward only the features that can directly influence outcome, as well as identify areas that need future study to identify missing confounders and causal variables. Additionally, CFA could be applied to BTMs to narrow them to features that contribute significantly to

accurate estimation of the BTMs as latent constructs prior to feature selection. This would likely identify some BTMs that are predictive of protection outcomes but were not selected initially and BTMs that have spurious association with outcome because of the inclusion of genes that are not related to protection against malaria.

Characterizing immune profiles indicative of future response to immunizations or of future protection against a pathogen soon after immunization can improve vaccine outcomes as well as inform design of future vaccination platforms. New technologies, largely introduced in the 21st century, have allowed for the generation of large "omic" datasets from gene variants to differential expression of immune genes, control of this expression on an epigenetic level to measurement of the protein products of these genes. These methods have become increasingly precise with single-cell sequencing allowing for discrimination of gene expression or activation of individual cell types. To effectively utilize this data, researchers must continue to develop and implement statistical methods that can discern the subtle differences between outcomes. For vaccine design, specifically, a hypothesized "vaccine chip" would allow for screening of prospective vaccinees to predict optimal responses or to confirm induction of protection after immunization. In this thesis, I applied predictive and explanatory modeling methods to pre- and post-vaccination signatures to gain a better understanding of the complex immune interactions that determine PfSPZ Vaccine efficacy and resulting malaria disease outcomes. While my use of these tools was limited by the data set, expanding the application to other, larger vaccine cohorts could model immune interactions with and without immunization to inform research and clinical best practices for ideal immunity outcomes.

For future work, I propose using both SEM and class prediction in combination to complement and improve upon conventional bioinformatics methods such as differential gene expression and gene enrichment. By leveraging the power of SEM, researchers can gain a deeper understanding of the causal relationships between variables, which can help to identify potential targets for future interventions and guide the development of more effective vaccines. Additionally, this approach allows researchers to test and refine their hypotheses in a more systematic and rigorous way, ultimately leading to more informed and evidence-based direction for future works. Overall, the use of SEM in vaccine research has the potential to enhance our understanding of biological mechanisms, for vaccine development and drug discovery or characterization of new gene signaling pathways.

A common challenge in drug discovery and approval is the production of drugs that elicit the desired response without a known mechanism of action[235]. One example is the efficacy of ketamine in treating depression though no hypothetical mechanisms have been confirmed[236]. To evaluate hypothetical mechanisms more efficiently, SEM could be used to identify the effects of ketamine on potential targets and signaling pathways using in vitro studies. The ability to model the relationships between biological systems and clinical outcomes with directionality would optimize the process of drug discovery by identifying novel targets of intervention, in addition to evaluating existing drugs to identify potential avenues for greater efficacy and a reduction in side effects. Utilizing computational tools such as SEM can prevent failure of drug candidates at later stages of development and prevent unnecessary use of animals, time, and money on ineffective drug candidates. With the ability to determine more nuanced effects of treatments SEM

can be a powerful tool for investigating hypothetical mechanisms and possible drug targets, in addition to guiding the production of more effective therapies.

Ultimately, SEM has diverse potential applications, including estimation of module expression or pathway activity. Incorporating exogenous variables can also demonstrate interactions and covariations between data from different biological systems. For instance, cell population data and gene level data are highly correlated for the same cell type, and not including information from both levels of organization may lead to the exclusion of important information. Using confirmatory factor analysis-defined gene sets, vaccine protection outcomes can be defined, and introduction of exogenous variables with covariation can create a causal model of immunity, including directionality and mediation of variables to gain a sense of the entire system. Once the model is defined, class prediction algorithms can be applied to create clinical tools to predict vaccine responses before vaccination or to predict protective outcomes with early post-vaccination signatures. This method, when combined with resources like the Immune Signatures data resource[237], could accelerate vaccine research, and improve its accuracy, directing the field of immunology.

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-human CD3 BUV496 clone UCHT1	BD	564809
Anti-human CD4 BUV805 clone SK3	BD	564910
Anti-human CD8 BUV563 clone RPA-T8	BD	565695
Anti-human CD14 BUV805 clone M5E2	BD	565779
Anti-human CD16 BUV496 clone 3G8	BD	564653
Anti-human CD19 BV750 clone HIB19	Biolegend	302261
Anti-human CD20 PE/Dazzle 594	Biolegend	302348
Anti-human CD56 BUV563 NCAM16.2	BD	564653
Anti-human CD8 BV570 RPA-T8	Biolegend	301038
Anti-human TCR γδ PE B1	Beckman Coulter	3312210
Anti-human CD3 BUV661 UCHT1	BD	565065
Anti-human CD11c PE/Cy5.5 3.9	ThermoFisher	35-0116-42
Anti-human TCR AOC-Vio770 Clone REA591	MIltenyi	130-113-509
Anti-human TCR V9 PE/Cy5 Clone IMMU360	Beckman Coulter	A63663
Anti-human TCR V1 FITC Clone Ts8.2	Thermo Fisher	TCR2730
Anti-human TCR V2 VioGreen Clone 123R3	Miltenyi	130-106-653
Anti-mouse CD4 PE-Cy7 Clone RM4-5	Biolegend	100528
Anti-mouse CD8 APC Clone 53-6.7	Biolegend	100712
Anti-mouse CD49d PE	Biolegend	103608
Anti-mouse Ter119 APC	Biolegend	116212
Anti-mouse CD45.2 PE Cy7	Biolegend	109830
Anti-mouse CD4 PerCP Cy5.5	Biolegend	100434
Anti-mouse CD8a BV421	Biolegend	100753
Anti-mouse CD11a FITC Clone M17/4	Biolegend	101106
Mouse PyCSP monoclonal (c) antibody	Noah Sather	PMID 24478094
Goat anti-Mouse IgG (H+L), Superclonal™ Recombinant Secondary Antibody, Alexa Fluor 488	Invitrogen	A28175
CSP-probe AlexaFluor 647	Oneko et al. Nature Medicine 2021	PMID 34518679
Mouse PyCSP monoclonal (RAM-1) antibody	Noah Sather	
Mouse igG1 Isotype control	InvivoMab	BE0083
In Vivo Grade Recombinant Mouse IgG1 Isotype Control	Syd Labs	PA007126
Chemicals, Peptides, and Recombinant Proteins		
Protein Transport Inhibitor Cocktail	eBioscience/Thermo Fisher Scientific	00-4980-03
Hoechst 33342	Sigma Aldrich	Cat# 875756-97-1; RRID:AB_10626776
dihydroethidium	Sigma Aldrich	Cat# 37291
Foxp3 Transcription Buffer set	eBioscience/Thermo Fisher Scientific	00-5523-00
LIVE/DEAD™ Fixable Green Dead Cell Stain Kit	Invitrogen™/Thermo Fisher Scientific	L23101
Live/Dead Blue Viability (Amine-reactive)	Thermo Fisher	L34962
flagellin (mouse studies)	Adipogen	AG-40B-0095-C100
poly I:C (mouse studies)	Tocris	4287
lipopolysaccharide (LPS) (mouse studies)	Sigma-Aldrich	L3024-5MG
β-glucan, Saccharomyces cerevisiae (mouse studies)	Sigma-Aldrich	G5011-25MG
Critical Commercial Assays		
Human cytokine 15-plex magnetic luminex assay	R&D Systems, a Bio-Techne brand	LXSAHM-15
PAXgene 96 Blood RNA kit	Qiagen	762331
QIAseq FastSelect rRNA kit	Qiagen	334386
QIAseq FastSelect GlobinRNA removal kit	Qiagen	334376
KAPA RNA HyperPrep Kit	Roche	8098107702
RNeasy 96 Kit	Qiagen	74181
NEBNext Ultra Directional RNA Library Prep Kit for Illumina	New England BIoLabs	E7420L
NovaSeq 6000 Sequencing System S1 300 cyle kit v1.0	Illumina	20012863
NovaSeq 6000 Sequencing System S2 300 cyle kit v1.0	Illumina	20012860
NovaSeq 6000 Sequencing System S4 300 cyle kit v1.0	Illumina	20012866
Lightning-Link® Rapid DyLight 488 Antibody Labeling Kit	Novus Biologicals	322-0010
Deposited Data		
KSPZV1 RNA-seq dbGaP	ClinicalTrials.gov Identifier: NCT02687373	phs002196.v1.p1
Experimental Models: organisms/strains		
C57BL/6 mice	Charles River Laboratories	
Anopheles stephensi* mosquitoes infected with *P.yoelli	Seattle Children's Research Institute	
***Plasmodium yoelii* sporozoites**	Seattle Children's Research Institute	
Sanaria® PySPZ Attenuated Sporozoite Reagent	Sanaria	SAN-408
Sanaria® PySPZ Infective Sporozoite Reagent	Sanaria	SAN-405
Sanaria® PfSPZ Infective Sporozoite Reagent	Sanaria	SAN-303
Oligonucleotides		
***P. yoelii* 18S forward primer**	5'- GGG GAT TGG TTT TGA CGT TTT TGC G-3'	
***P. yoelii* 18S reverse primer**	5'- AAG CAT TAA ATA AAG CGA ATA CAT CCT TAT-3'	
Murine GAPDH forward primer	5'- CCT CAA CTA CAT GGT TTA CAT-3'	
Murine GAPDH reverse primer	5'- GCT CCT GGA AGA TGG TGA TG-3'	
TaqMan probe sequence	56-FAM/CA ATT GGT T/ZEN/T ACC TTT TGC TCT TT/3IABkFQ	

Table 5: Reagents Used

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Education

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Professional Experience

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Publications

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Senkpeil L, Tran TM. Malaria Vaccines: Differing approaches and progress since the 20th century. Vaccines. (Forthcoming)

Presentations

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